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A VAPOUR DILUTION SYSTEM FOR DETECTOR CALIBRATION

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INTRODUCTION

The present emphasis in gas chromatography is being placed on accurate quantitative analysis and the need for a relatively simple but reliable method for detector calibration is becoming acute. A method is described in this paper for producing accurate relative concentrations of solute vapours in a gas that can be used for the assessment of detector linearity and the determination of the relative response factors of a detector to different substances. The system can also be applied to the determination of trace compounds in the presence of a bulk component of widely different volatility. Numerous methods for producing known relative concentrations of solute vapour in a gas have been described previously in the literature. The two methods most applicable to detector calibrations are those decribed by DESTY *et al.*[1] and LOVELOCK[2]. The method described by DESTY utilises the diffusion of a solute through a narrow glass capillary into a gas stream, to produce the required solute concentration. If employed correctly this method gives absolute instead of relative concentrations, but the apparatus is somewhat complex and can only be used for a single component in the gas stream. The method described by LOVELOCK depends on the continuous dilution of a known quantity of vapour contained in a suitable vessel by means of a gas stream, which results in the concentration of the solute vapour in the exit gas decreasing exponentially with time. If the stream of gas from the dilution vessel is passed through a detector, then, providing the response of the detector is linear, the logarithm of the signal produced will be linearly related to time. The LOVELOCK system suffers from two disadvantages. At low concentrations a considerable proportion of the solute vapour is adsorbed on the walls of the dilution vessel and the dilution rate no longer varies exponentially with time. Secondly, since different substances are adsorbed to different extents on the walls of the vessel, the system is only applicable to single substances. A modified dilution system based on that described by LOVELOCK is described in this paper in which the effect of adsorption is greatly reduced and which also simultaneously produces a mixture of different solute vapours in a gas at known relative concentrations. The theory of the method is given in detail and preliminary experimental results from the assessment of the macro-argon detector using this system are shown, together with results obtained from the analysis of a mixture containing traces of toluene and chlorobenzene in benzene.

METHOD

The apparatus used is shown in Fig. 1. The dilution vessel is made of glass and consists of a small gas wash-bottle of the form shown in Fig. 2. Dry argon passes through the sintered filter into a suitable non-volatile liquid and thence to the automatic gas sampling system. The dilution vessel is charged by a hypodermic syringe through a

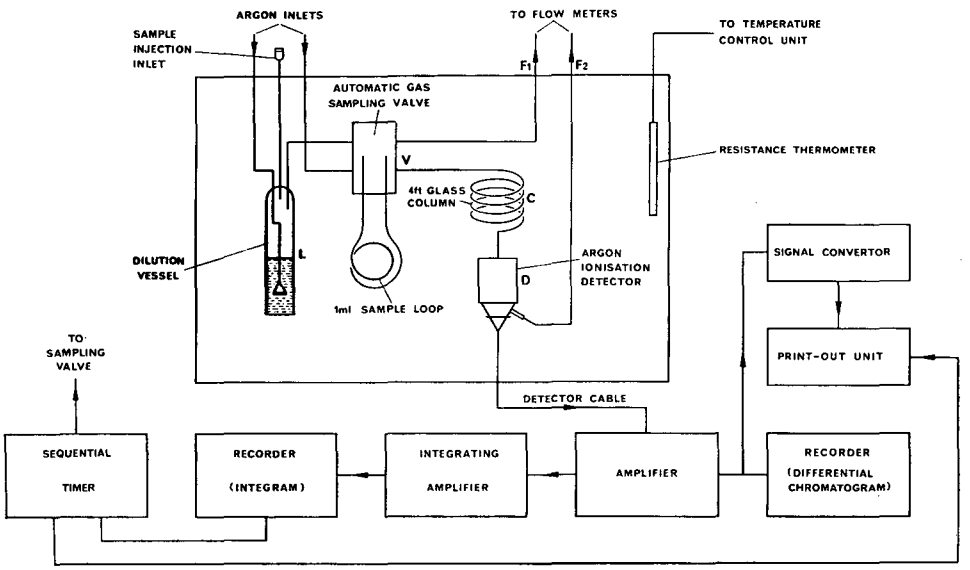

Fig. 1. Detector calibration apparatus.

serum cap. At given intervals of time a sample of the exit gas from the dilution vessel is placed in line with the argon supply to the chromatographic column and detector. The whole unit, dilution vessel, sampling system, column and detector are contained in the same thermostatically controlled oven.

Two methods of estimating peak area were employed, a digital and an electronic integrator, which were automatically reset after each peak by the timer that operated the sampling system.

THEORY

Let the volume of liquid and gas in the dilution vessel (Fig. 3) be V_l and V_g respectively and let a mass m_A of the substance A, whose partition coefficient with respect to the non-volatile liquid is K_A, be placed in the vessel. Let a volume δV of gas flow through the vessel and let the mass of solute removed change the concentrations of the solute in the gas and liquid phases from X_g^A and X_l^A by δX_g^A and δX_l^A respectively.

Then:

$$-X_g^A \delta V = V_g \delta X_g^A + V_l \delta X_l^A = -\delta m \text{ (mass removed from dilution vessel)}$$

Now if X_l^A is small

$$X_l^A = K_A X_g^A$$

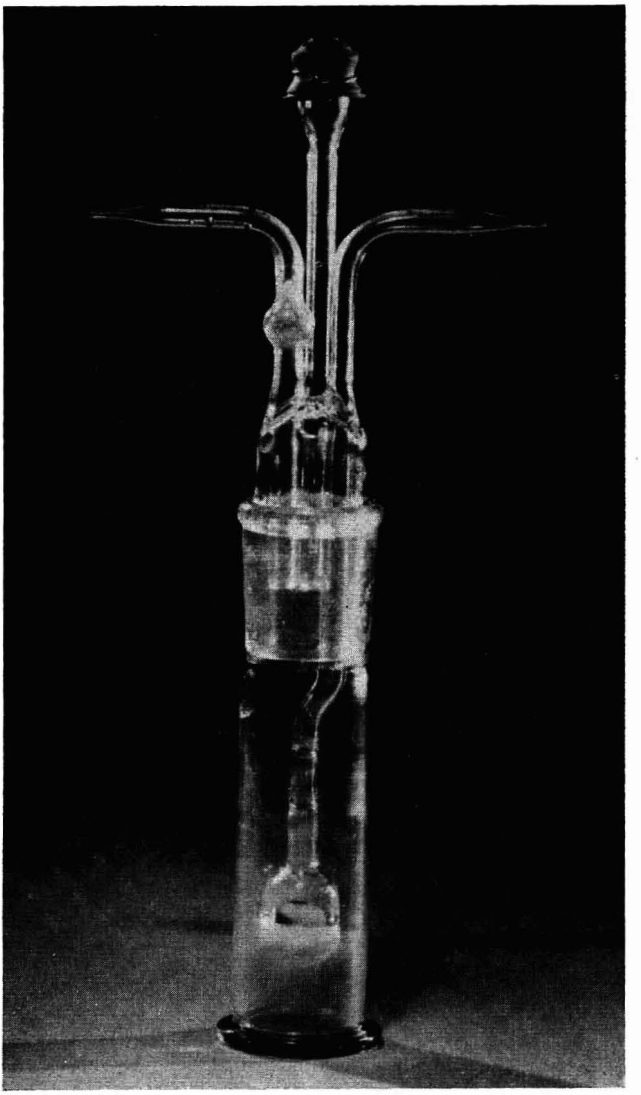

Fig. 2. Dilution vessel.

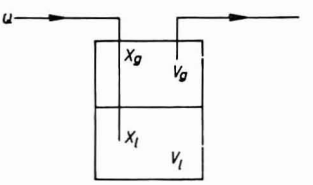

Fig. 3. Diagram of the dilution vessel.

Then:

$$X_g{}^A \delta V = -(V_g + K_A V_l)\, \delta X_g{}^A$$

Thus:

$$\frac{\delta X_g{}^A}{X_g{}^A} = \frac{-\delta V}{(V_g + K_A V_l)}$$

Integrating:

$$X_g{}^A = Y e^{-\frac{V}{V_g + K_A V_l}}$$

where Y is a constant.

Now $V = Qt$ where Q is the flow of gas through the vessel in volume/unit time and t is the time. Thus when:

$$t = 0,\ X_g{}^A = X_{g_0}{}^A \text{ and } \therefore\ Y = X_{g_0}{}^A$$

Thus:

$$X_g{}^A = X_{g_0}{}^A e^{-\frac{Qt}{V_g + K_A V_l}} \tag{1}$$

Consider the peak produced on the chromatogram resulting from a single operation of the injection device. The concentration in the detector at any point on the curve is given by the equation of the elution curve:

$$X_{g_n} = \frac{X_{g_0} e^{-\frac{w^2}{2n}}}{\sqrt{2\pi n}}$$

where X_{g0} is the initial concentration placed on the first plate, and $w = v - n$ where v is the "plate volumes" of gas passed through the column, and n is the efficiency of the column. Now let the signal given by the recorder $D\delta w = \sigma(X_{g_n})^\phi \, dw$ where σ and ϕ are constant *i.e.* the response of the detector is a function of a power of the concentration of solute contained in it.

Then:

$$\text{Peak area} = \int_{-\infty}^{+\infty} D\delta w = \int_{-\infty}^{+\infty} \sigma(X_{g_n})^\phi \, \delta w = \int_{-\infty}^{+\infty} \sigma\left(\frac{X_{g_0}}{\sqrt{2\pi n}} e^{-\frac{w^2}{2n}}\right)^\phi \delta w$$

Thus:

$$\text{Peak area} = \int_{-\infty}^{+\infty} \sigma\left(\frac{X_{g_0}}{\sqrt{2\pi n}}\right)^\phi e^{-\frac{\phi w^2}{2n}} \delta w = \sigma\left(\frac{X_{g_0}}{\sqrt{2\pi n}}\right)^\phi \frac{\sqrt{2\pi n}}{\phi} = \Lambda X_{g_0}{}^\phi \tag{2}$$

when:

$$\Lambda = \sigma\left(\frac{1}{\sqrt{2\pi n}}\right)^\phi \frac{\sqrt{2\pi n}}{\phi}$$

Now X_{g0} will be proportional to the charge placed on the column *i.e.* $X_{g0} = \gamma X_g{}^A$, where γ is a constant, thus combining eqns. (1) and (2):

$$\text{Peak area} = \Lambda\left(\gamma X_{g_0}{}^A \cdot e^{-\frac{Qt}{V_g + K_A V_l}}\right)^\phi$$

Thus:

$$\log(\text{Peak area}) = \log \Lambda + \log(\gamma X_{g_0}{}^A)^\phi - \frac{Qt\phi}{V_g + K_A V_l} = C - \frac{Qt\phi}{V_g + K_A V_l} \tag{3}$$

Thus if the detector has a linear response a plot of log peak area against time will give a straight line and:

$$\phi = \alpha \frac{(V_g + K_A V_l)}{Q} = 1$$

where α is the slope of the line.

Having ascertained that the detector is linear and $\phi = 1$ then $X_g{}^A \propto S^A$, where S^A is the area of the respective peak on the chromatogram, and the linearity of the detector can be assessed from the correlation coefficient[3] of the line obtained by plotting $\log S^A/t$. By extrapolation of this linear curve to $t = 0$, a value of $S_0{}^A$ can be obtained which will be proportional to $X_{g0}{}^A$.

Now:

$$X_{g_0}{}^A = \frac{m_A}{V_g + K_A V_l}$$

and if $V_g \ll K_A V_l$, which can be easily arranged experimentally, then:

$$X_{g_0}{}^A = \frac{m_A}{K_A V_l}$$

Now if m_A and m_B grams of two substances A and B are injected into the dilution vessel then:

$$\frac{X_{g_0}{}^A}{X_{g_0}{}^B} = \frac{m_A}{K_A} \cdot \frac{K_B}{m_B} = \frac{D_A S_0{}^A}{D_B S_0{}^B}$$

Where D_A and D_B are the detector response factors to the two substances A and B. If the nonvolatile liquid used in the dilution vessel is the same as the liquid phase on the column then $K_B/K_A = R_{B-A}$ where R_{B-A} is the retention ratio of B to A obtained from the chromatogram:

$$\frac{D_A}{D_B} = \frac{m_A}{m_B} \cdot \frac{S_0{}^B}{S_0{}^A} \cdot R_{B-A} \tag{4}$$

If circumstances arise such that V_g is not small compared with KV_l, then:

$$\frac{X_{g_0}{}^A}{X_{g_0}{}^B} = \frac{m_A}{m_B} \cdot \frac{V_g + K_B V_l}{V_g + K_A V_l}$$

However, if α and β are the slopes of the $\log S^A/t$ curves, then:

$$\frac{\alpha}{\beta} = \frac{Q}{V_g + K_A V_l} \cdot \frac{V_g + K_B V_l}{Q} = \frac{V_g + K_B V_l}{V_g + K_A V_l}$$

Thus eqn. (4) becomes:

$$\frac{D_A}{D_B} = \frac{m_A}{m_B} \cdot \frac{S_0{}^B}{S_0{}^A} \cdot \frac{\alpha}{\beta}$$

Thus if a known mixture of substances A and B is placed in the dilution vessel, the linearity and relative response factors of the detector to the substances concerned can be accurately assessed. The relevant data required are the correlation coefficient

of the line obtained by plotting log S/t (the verification that $\phi = 1$), the values of S_0^A and S_0^B by extrapolation of these lines to $t = 0$, and the relative retention ratio R_{B-A}. It should be noted that a knowledge of the absolute masses of A and B placed in the dilution vessel is not necessary. The effect of adsorption of the solute vapour on the walls of the column is insignificant at low concentration levels, as the concentration of the solute in the gas phase is dependent on the concentration of the solute in the bulk of the liquid.

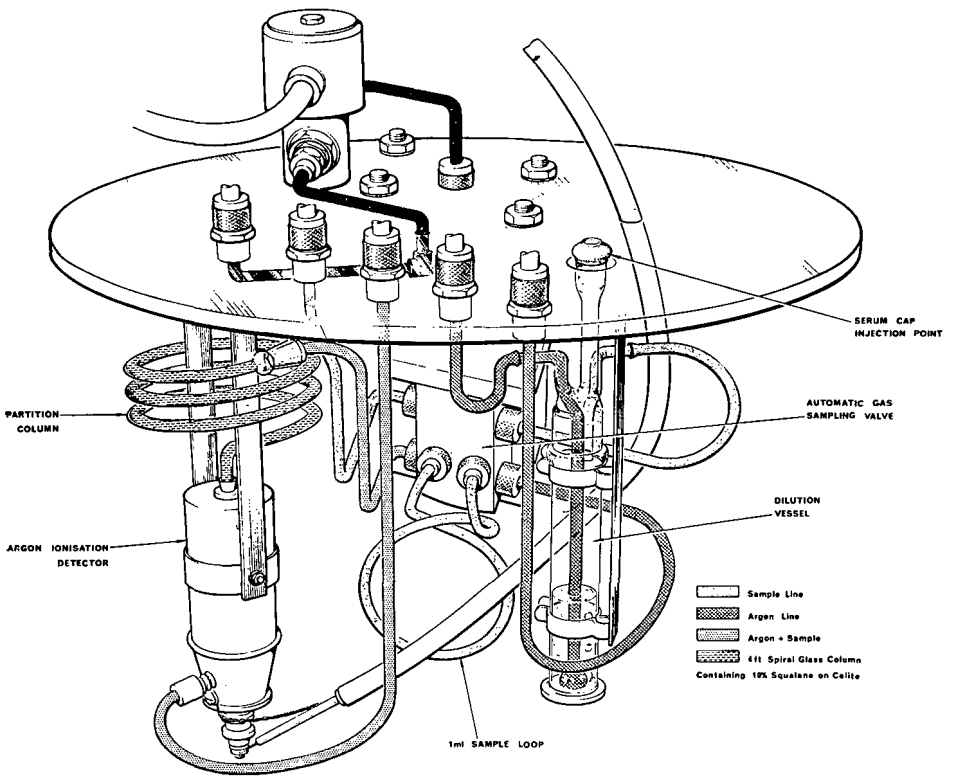

Fig. 4. Detail of dilution vessel and column system.

ANALYSIS OF MIXTURES CONTAINING COMPONENTS AT WIDELY DIFFERING CONCENTRATION LEVELS

Examination of eqn. (1) shows that, providing a sufficiently large charge is placed in the dilution vessel and a constant volume of the exit gas is placed on to the chromatographic column, there will be a period of time, during the dilution, in which each component will be shown on the chromatogram as a series of peaks over a given concentration range. If the logarithm of the peak area for each substance is plotted against time, for the period in which they are "on scale" on the chromatogram, then the initial concentration of each component present at $t = 0$ can be determined by extrapolation. Providing that the relevant response factors for each substance are known, the mass ratios of the components can be determined from the peak area

intercepts at $t = 0$ and the retention ratios of the substances concerned. Further, by this method, all components present are determined with similar accuracy independent of their original concentration level.

EXPERIMENTAL

The determination of detector linearity

Details of the construction of the dilution system are shown in Fig. 4. The argon supplies to the column and dilution vessel were carefully dried by passage through cylinders containing activated Linde molecular sieve 5A. The drying agent was reactivated every time the argon cylinder was renewed. Normally, a column flow rate of 40 ml/min was found to be quite adequate and a flow of about 60 ml/min through the dilution vessel ensured that a complete "run" could be carried out in about 8 h. In order to determine the value of ϕ accurately precise measurements of Q, K_A, V_g and V_l were required. Q was measured by means of a carefully calibrated soap film meter at room temperature and this value was corrected for the pressure and temperature existing in the dilution vessel. The temperature of the liquid in the dilution vessel was continuously measured by means of a thermocouple immersed in the liquid and the gas pressure in the vessel determined by means of a static mercury manometer. V_l was taken as the volume of liquid added to the vessel and V_g taken as the difference between the total volume of the vessel and the volume of liquid added. Values for K were taken from the results of DESTY AND GOLDUP[4] and EVERED AND POLLARD[5]. Squalane was used in the dilution vessel and samples were injected through a serum cap by means of a hypodermic syringe fitted with a 10 in. needle. The column consisted of a coiled glass tube 4 ft. in length and 4 mm in diameter, packed with 10 % w/w of squalane on 100–120 mesh celite. The detector employed was the macro-argon detector containing a strontium-90 radioactive source. The whole apparatus

TABLE I

CHARACTERISTICS OF THE MACRO ARGON DETECTOR

Compound	*Correlation coefficient*	*No. of observations*	*Detector response index* ϕ	*Concentration range*
Chloroform	>0.999	4	0.931	10^3
	<1.0	4	0.975	
Di-isopropyl ether	>0.999	4	0.889	10^3
	<1.0	4	0.808	
Toluene	>0.999	8	0.950	10^1
		8	0.906	
	<1.0	8	0.951	
Chlorobenzene	>0.999	8	0.945	10^1
		8	0.994	
	<1.0	8	1.042	
Heptane	>0.999	8	1.095	10^1
		8	1.061	
	<1.0	8	1.056	

was situated in an oven controlled at 45° ± 0.2°C. The automatic gas sampling valve was actuated by a sequential timer and sampled the exit gas from the dilution vessel every 40 min. The detector was operated at 770 V and the output fed to a Pye Argon Chromatograph amplifier and Integrating amplifier. Differential and integral curves were obtained on two Honeywell Brown recorders and the integrating amplifier was automatically reset after each peak, by use of additional channels on the timer. Graphs of log peak area/time for a mixture containing chloroform, di-isopropyl ether, toluene and chlorobenzene are shown in Fig. 5. The lines shown on this figure are the regression lines for the points obtained. The correlation coefficients and the respective values of ϕ for a series of substances are shown in Table I.

Analysis of mixtures of widely varying composition

The vapour dilution apparatus was used to provide representative vapour sample of a mixture containing 98.9 % benzene, 1.0 % toluene and 0.1 % chlorobenzene. Approxi-

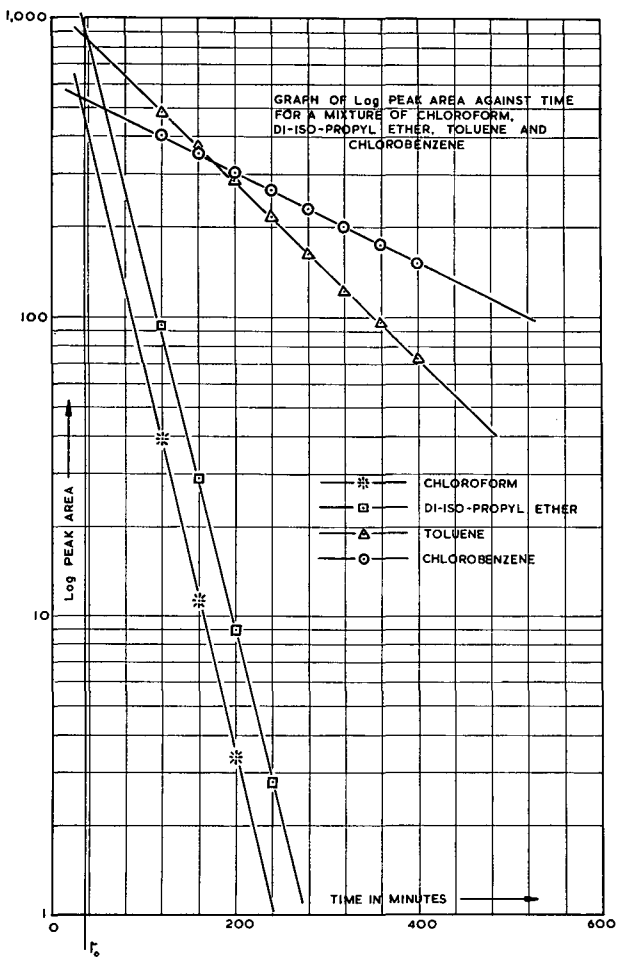

Fig. 5. Graph of log peak area against time for a mixture of chloroform, diisopropyl ether, toluene and chlorobenzene.

mately 250 μl of the mixture was injected into the dilution vessel and samples of the vapour were automatically taken every 40 min for 10 h.

A graph of the peak height against time for each component is shown in Fig. 6, and it may be seen from the intercepts at $t = 0$, that the concentration of each component can be calculated with similar precision although the range of concentrations between the components was as great as 1,000. It should be noted that the values shown in Fig. 6 are based on peak heights and do not take into account relative response factors. It may also be seen that by variation of the flows through the column

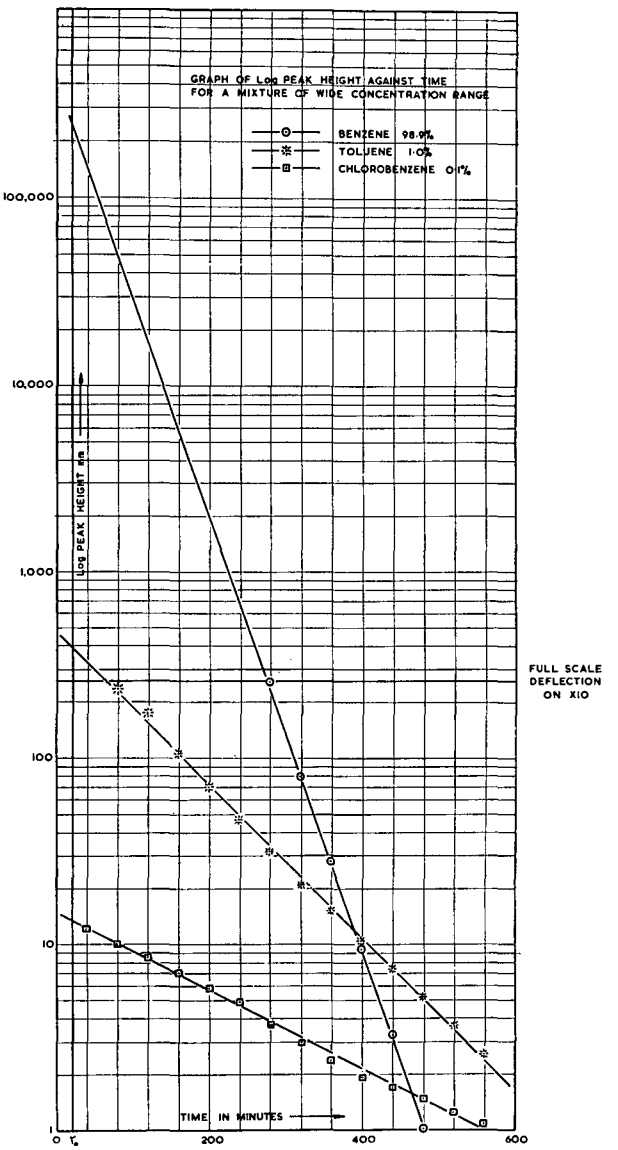

Fig. 6. Graph of log peak height against time for a mixture of wide concentration range.

or dilution vessel, or operation at different temperatures or with different liquid phases, the analysis time can be shortened and the system can be applied to the analysis of a wide range of substances present in diverse relative concentrations.

CONCLUSIONS

The vapour dilution system described in this paper provides a very precise method for determining detector linearity and relative response factors. Due to the wide limits of variation for each operating parameter the system can be made applicable to almost all substances that can be separated by a gas–liquid chromatographic technique. The apparatus can also be used to advantage for the analysis of mixtures containing substances at concentration levels that differ by several orders. By using the system described, each component of such a mixture is determined with the same precision.

The paper gives preliminary results for a macro argon detector and is the first of a series that will be concerned with the investigations of the characteristics of various ionisation detectors. The effect of the various operating parameters of each detector on the linearity and response factors will be examined.

SUMMARY

A vapour dilution apparatus, very suitable for detector calibrations, is described which provides known relative concentrations of a vapour in a gas for sampling on a partition column. The theory of the system is considered in detail, and examples given of the application of the method to the determination of detector linearity and detector response factors and to the analysis of mixtures, whose components are present at widely different concentration levels.

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A TEMPERATURE-PROGRAMMED PREPARATIVE-SCALE GAS CHROMATOGRAPH

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During an investigation into the chemistry of essential citrus oils, a need developed for a large preparative-scale gas chromatograph so that sufficient quantities of some of the minor constituents could be separated for identification and study. A number of such chromatographs have been described[1-4] which required exceptional skill for construction or which had insufficient capacity. The temperature-programmed chromatograph, herein described, is of relatively simple design and was constructed of readily available materials. The resulting chromatograph handled 25 g of terpenes with resolution comparable to that of a 250-ft., 0.020-in. capillary column containing the same substrate.

APPARATUS

The apparatus was constructed of two vertical round sheet metal ducts 8 in. and 6 in. in diameter respectively, approximately 11 ft. long connected so that hot air was circulated in a closed circuit up through the smaller duct containing electrical heaters and down through the larger duct containing the bundle of tubes. A photograph of the entire apparatus is presented in Fig. 1, a general schematic diagram is given in Fig. 2, the flash vaporizer is illustrated in Fig. 3, and the wiring diagram in Fig. 4.

Column

Eight 1½-in. o. d. × 10-ft. columns were bundled as shown in Fig. 2 section A-A and fastened to the six air baffle plates making a rigid system. The ends of the stainless steel tubes were plugged, drilled, and tapped for ½-in. pipe to facilitate replacement of the packing. The eight tubes were connected in series using ¼-in. tubing and ½-in. pipe to ¼-in. tube fittings. Tube connections could be changed for column lengths of 20, 40, or 60 ft. The entire assembly was placed in the 8-in. × 11-ft. sheet metal duct, the latter being secured to the baffle plates with sheet metal screws. A removable insulated sheet metal cap was fitted to each end of the housing. Interconnecting ducts were secured to the upper and lower housing sides to receive the blower air at the top and exhaust it into the heater chamber at the bottom.

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* One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. References to specific products of commercial manufacture are for illustration and do not constitute endorsement by the U.S. Department of Agriculture.

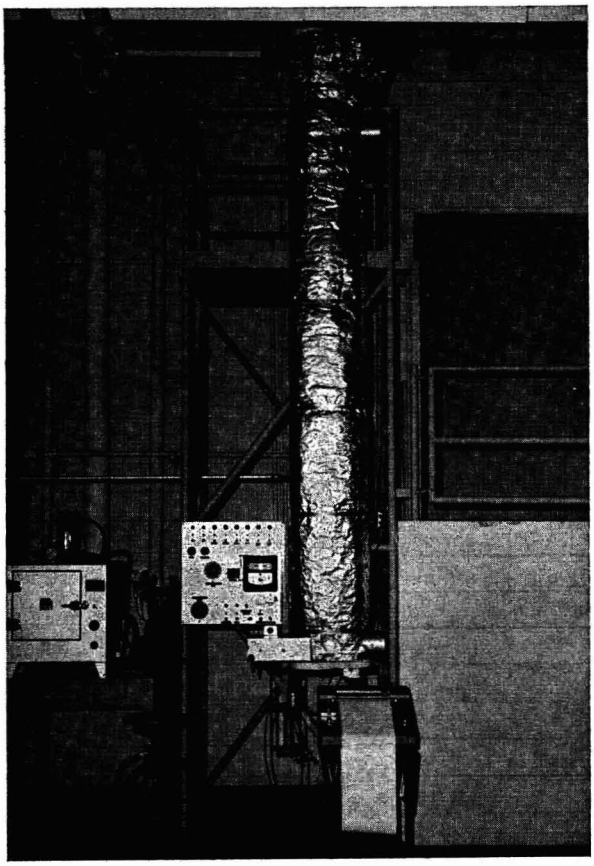

Fig. 1. Photograph of the temperature-programmed preparative-scale chromatograph.

Heaters

The heaters were made from chromel heater coils cut into lengths to form three 1000-W and three 750-W elements. The elements were suspended from transite rings which were spaced with rods and the entire assembly inserted into the lower end of the 6-in. duct as shown in Fig. 2. The heaters were wired as shown in Fig. 4. The three 1000-W heaters and one 750-W heater were provided with on-off switches and fuses. One of the other 750-W heaters was controlled with a variable transformer and the other with a temperature indicating thermocouple controller and a variable transformer. Thermocouple leads were placed in a well extending 8-in. into the packing at the top of one of the $1\frac{1}{2}$-in. tubes, and in the air stream at the top, center, and bottom of the 8-in. duct. The temperatures were read on the indicating controller through a six-point switch. Control was from the thermocouple in the air at the top of the 8-in. duct. Air gates are shown at the top and bottom of the 6-in. duct and a damper toward the top of the duct. During operation, the gates were kept closed and the damper open. Rapid cooling of the column was achieved by opening the gates, closing the damper and allowing the blower to run.

Air was circulated through the apparatus by means of a pressure blower (Sutton

PB-45-A) rated at 300 c.f.m. against a static head of 3-in. of water. An anemometer inserted in the 6-in. duct showed an air velocity of about 3000 lineal feet per minute. Since the pressure blower was not designed especially for high-temperature work, a

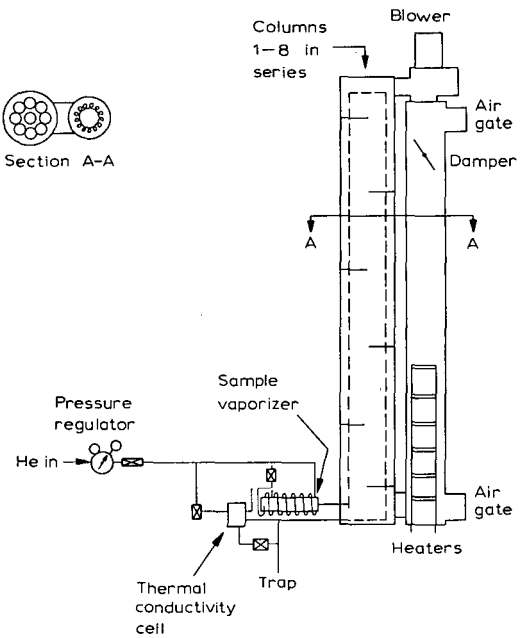

Fig. 2. Schematic flow diagram.

small air blower (40 c.f.m.) was installed and directed against the main blower motor to keep it from overheating.

The entire duct assembly was insulated with two 2-in. thicknesses of aluminum sided glass wool. The column housing caps which provide access to the column fittings were insulated with a 2-in. thickness of asbestos board.

Thermal conductivity cell

A full diffusion type hot-wire detector cell was constructed similar to the Gow-Mac TE-III. It was heated with a Chromolox 100-W Type A ring heater through a variable transformer as shown in Fig. 4. A thermocouple was inserted into the block and read on the indicating controller through the six-point selector switch. A portion of the eluting materials entered the cell through $\frac{1}{8}$-in. tubing after being split and reduced from $\frac{1}{4}$ in. at the tee. The amount of material passing through the cell was controlled by a valve in the line just after the cell. The line from the valve was connected to the original $\frac{1}{4}$-in. tube leading to the trap. The reference gas was metered through the cell and exhausted. The entire unit was placed in a compartment to the side and bottom of the main column and packed with loose asbestos.

Flash evaporator

A flash evaporator was fabricated as shown in Fig. 3. A $\frac{7}{16}$-in. hole was drilled through a 1-in. × 6-in. stainless steel rod and the ends were tapped for $\frac{1}{4}$-in. pipe

fittings. A hole was drilled and tapped for a $\frac{1}{4}$-in. pipe fitting in the side, $\frac{3}{4}$-in. from one end. A $\frac{1}{4}$-in. tube to $\frac{1}{4}$-in. tube to $\frac{1}{4}$-in. pipe fitting was inserted into this tapped hole. One end of the cylinder was plugged and into the other end was placed

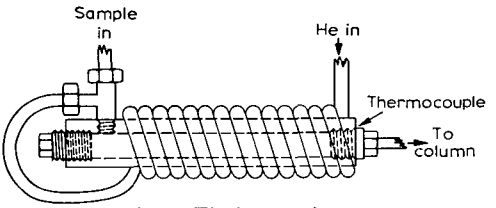

Fig. 3. Flash vaporizer.

a $\frac{1}{4}$-in. pipe to $\frac{1}{4}$-in. tube after the cavity was filled with Podbielniak stainless steel Heli-Pak 3013. The cylinder was wrapped with 22-gage asbestos insulated chromel wire and covered with a coil of $\frac{1}{4}$-in. copper tubing. A thermocouple was inserted into the cylinder end face and read on the indicating controller via the six-point selector switch. The entire unit was insulated with asbestos cloth tape, placed in the cell-flash evaporator compartment, and packed with loose asbestos.

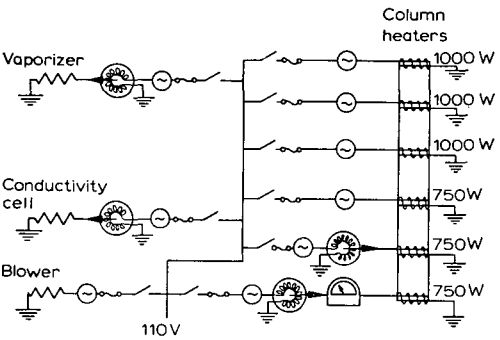

Fig. 4. Schematic wiring diagram.

A shut-off valve was placed on the injection port to which could be attached a pressurized cylinder containing the sample to be chromatogrammed.

Temperature programmer

The unit is capable of attaining 160° in 20 min, therefore, the column can be programmed at a maximum of 8° per minute. This can be accomplished by merely switching on the entire bank of heaters. For slower heating rates, the temperature indicator controller can be driven by any variable speed device. A surplus (gun fire control) ball-disc type variable speed drive, propelled by a 4 r.p.h. clock motor, was used successfully in the present apparatus. A temperature lag of 15° existed between the top and bottom of the column under these conditions; however, resolution was excellent.

PROCEDURE

The tubes were packed with 21 lb. of 30–60 mesh Chromosorb-P containing 30 % Carbowax 20M and tapped with a hammer and punch until the material ceased to

settle. Nitrogen gas was passed through the column for all purposes except when materials were separated, at which time helium was used. Peak inversion[5] resulting from use of nitrogen as a carrier gas complicates the chromatogram of these materials at the temperatures required for separation. A pressure of 70 lb. p.s.i. was used resulting in a flow rate of 4 l/min.

Samples were introduced into the vaporizer under pressure using a 30 ml valved cylinder. In use, the sample cylinder was first evacuated, the sample was drawn in, and then pressurized to 4–5 times the column pressure. It was then connected to the valve on the sample vaporizer. Opening both valves permitted the entire sample to enter the vaporizer. A 25 ml sample could be injected and vaporized in 2–3 sec.

The block temperature was regulated at 150° and the flash evaporator at 180°. Both remained heated at all times. Because only a brief period was required to heat the column, it remained unheated, except when in use. For example, the column could be brought to equilibrium at 160° in 20 min.

APPLICATION

The preparative column was compared, for efficiency of separation, with a 250-ft. 0.020-in. capillary column. The parameters of this latter column were the same for obtaining the chromatograms shown in Figs. 5 and 7. The temperature was maintained at 100° with a helium flow of 15 ml/min resulting in a pressure of 36 lb. p.s.i. The 250-ft. column was coated with Carbowax 20M. An F & M 1609 flame ionization detector was used in conjunction with the F & M 500 gas chromatographic apparatus.

The preparatory column parameters were maintained the same for both chromatograms shown in Figs. 6 and 8. The column temperature was controlled at 150° with a helium pressure of 65 lb. p.s.i.

Terpene hydrocarbons

Fig. 5 shows the chromatographic separation of a 1-μl sample of terpene hydrocarbons

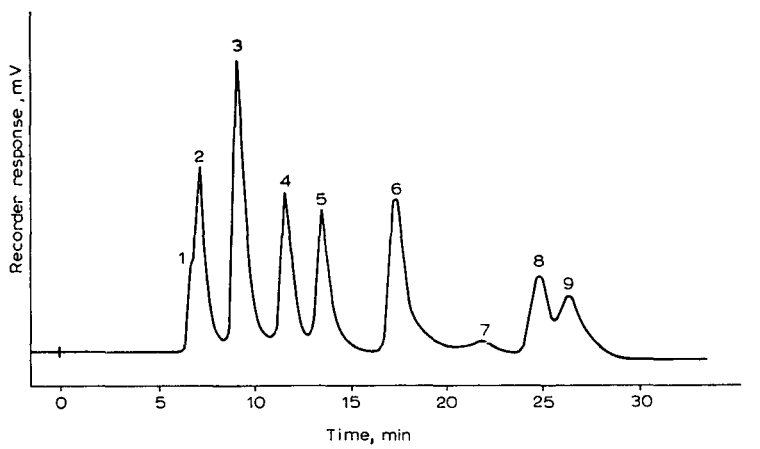

Fig. 5. Chromatogram of some hydrocarbon terpenes on a 250-ft. capillary column containing Carbowax 20 M, isothermal operation at 100°. Identified peaks: 1 = cyclofenchene; 2 = α-pinene; 3 = camphene; 4 = sabinene; 5 = myrcene; 6 = *d*-limonene; 7 = γ-terpinene; 8 = *p*-cymene; 9 = terpinolene.

on a 250-ft. capillary column. The following peaks were obtained: cyclofenchene (1), α-pinene (2), camphene (3), sabinene (4), myrcene (5), *d*-limonene (6), γ-terpinene (7), *p*-cymene (8), and terpinolene (9). The curve represents a 1-μl sample which had been split. Fig. 6 shows the separation of 20 ml of this same terpene hydrocarbon mixture on the preparatory column. The separation of sabinene (4) and myrcene (5) is incomplete, however, by trapping the leading edge and the trailing edge separately, rather pure materials can be obtained.

Commercial terpinolene

A 1-μl sample of Matheson Coleman and Bell terpinolene on the 250-ft. capillary column gave the chromatogram shown in Fig. 7. The peaks were identified by use of the preparatory column and confirmed by infrared spectroscopy as α-pinene (A), camphene (B), myrcene (C), 1,4-cineole (D), *d*-limonene (E), 1,8-cineole (F), γ-terpinene (G), *p*-cymene (H), terpinolene (I), and isoterpinolene (J). Fig. 8 shows the separation of 20 ml of this same material on the preparatory column. In addition to the above peaks, the large column resolved α-fenchene (A') and isoterpinolene (J). It should also be noted that *d*-limonene (E) and 1,8-cineole (F) have been more satisfactorily resolved.

Single peak material obtained from the preparatory column was rechromatogrammed on the 250-ft. capillary column and found to be chromatographically pure. Fig. 9 shows a temperature programmed separation of 10 ml of isoprene (b), *n*-pentane (c), *n*-hexane (d), and *n*-heptane (e) by means of the preparatory column. Nitrogen (a) was the injection gas. The parameters outlined above were maintained except the temperature was programmed at 2.5° per min from 60–120°.

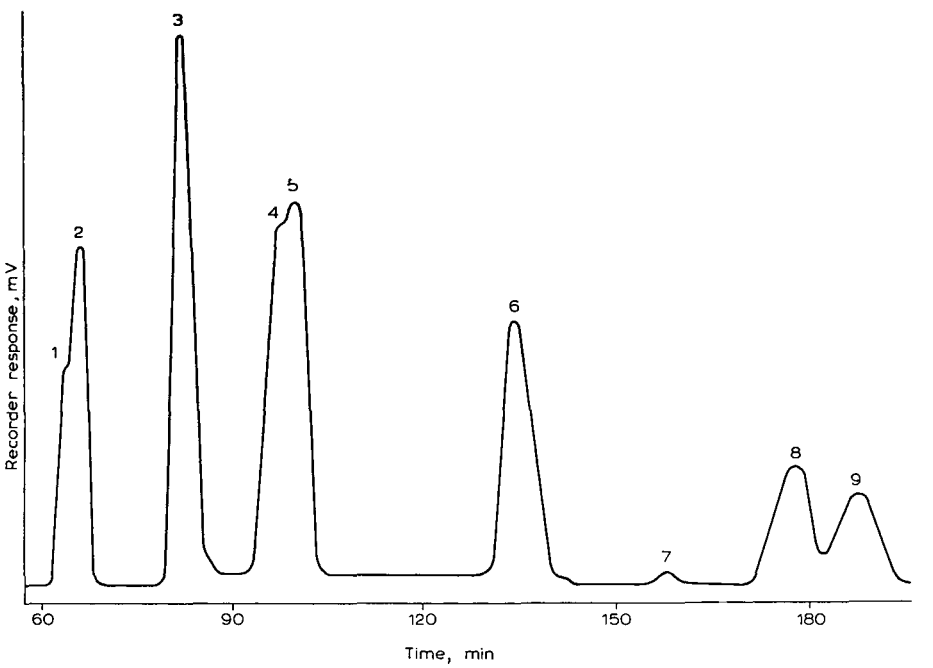

Fig. 6. Chromatogram of the hydrocarbon terpenes, shown in Fig. 5, on the preparatory column packed with 30% Carbowax 20M on 30–60 mesh Chromosorb P, isothermal operation at 150°.

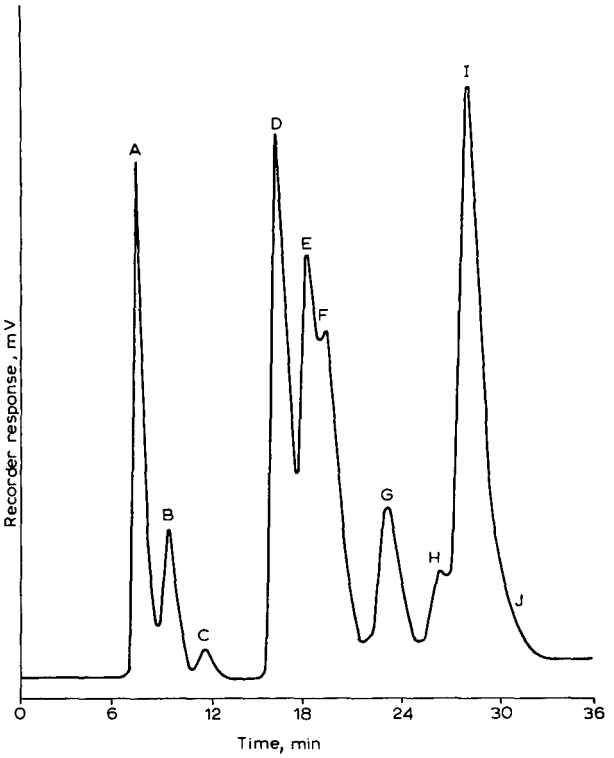

Fig. 7. Chromatogram of commercial terpinolene on a 250-ft. capillary column containing Carbowax 20M, isothermal operation at 100°. Identified peaks: A = α-pinene; B = camphene; C = myrcene; D = 1,4-cineole; E = *d*-limonene; F = 1,8-cineole; G = γ-terpinene; H = *p*-cymene; I = terpinolene; J = isoterpinolene.

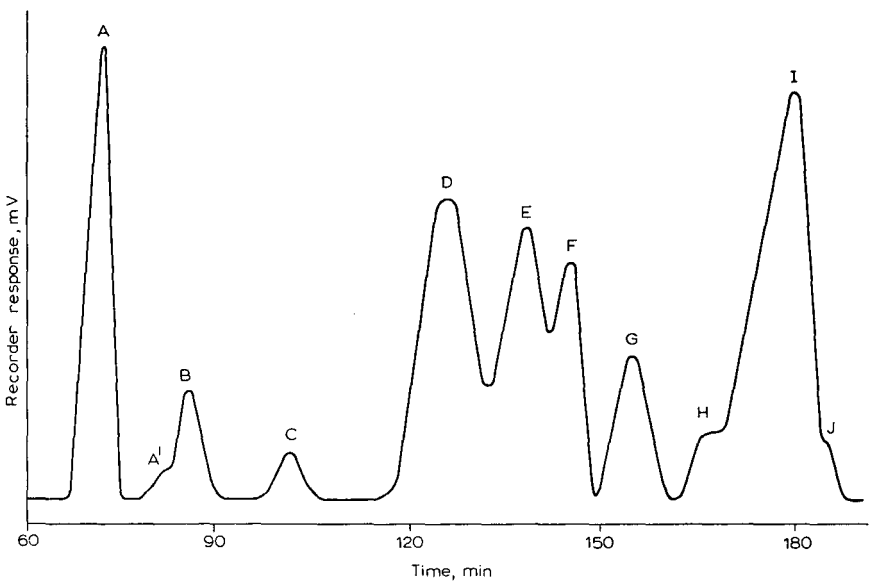

Fig. 8. Chromatogram of commercial terpinolene shown in Fig. 7 on the preparatory column at 150°, packed with 30% Carbowax 20M on 30–60 mesh Chromosorb P. In addition peak A′ (α-fenchene) appeared.

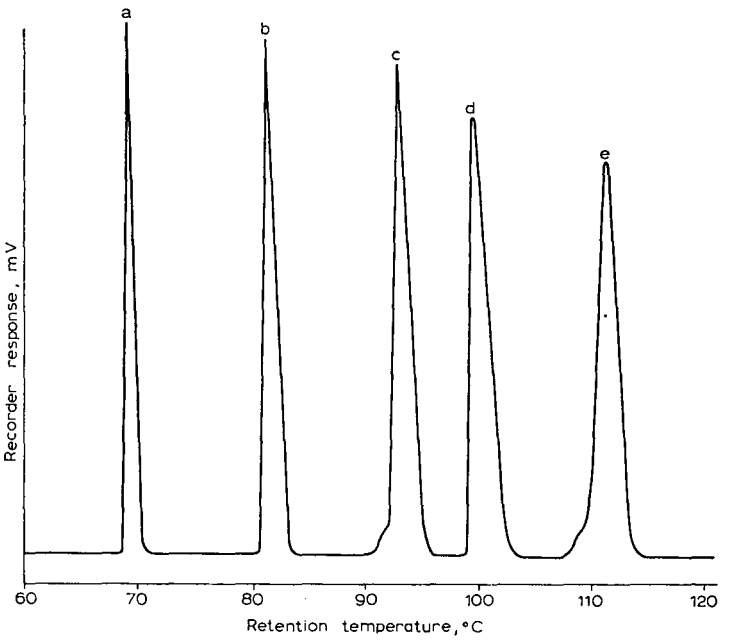

Fig. 9. Chromatogram of some hydrocarbons on the preparatory column temperature programmed at 2.5°/min from 60–120°. Peaks identified: a = nitrogen; b = isoprene; c = pentane; d = hexane; e = heptane.

ACKNOWLEDGEMENTS

The authors are indebted to W. B. BROGDEN for obtaining some of the chromatograms and in particular to C. E. KUNSMAN for its construction and many suggestions.

SUMMARY

An efficient temperature-programmed preparative-scale gas chromatography unit has been developed having $1\frac{1}{2}$ in. × 10 ft. tubes connected in series. The unit is heated by thermostatically controlled hot air making possible temperature programming. The unit is capable of attaining a temperature of 150° in 15 minutes and can be cooled to 50° in the same time limit.

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ROTATING UNIT FOR PREPARATIVE-SCALE GAS CHROMATOGRAPHY

PART II. THEORETICAL ASPECTS

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INTRODUCTION

The main problem in preparative scale gas chromatography is the separation of the greatest possible amount of substance of the desired purity in the shortest time.

As far as the rotating unit previously described[1] is concerned, the problem of maximum feed rate can be dealt within the following three ways:

(1) establish the maximum feed per column,

(2) establish the maximum number of revolutions,

(3) find out conditions under which the time required for the separation is the shortest one.

By making simplifying assumptions the problem may be solved analytically in a rather simple manner. The results, even approximate ones, can then be used to design a larger scale apparatus.

Furthermore, one of our aims was to find out whether there are any critical dimensions, *e.g.* column length, which may limit the applications of the apparatus within the limits of design. This has been achieved by studying a particular mathematical model of our apparatus. A result of some interest—limited to this particular choice of the model—is the relation existing between the retention times of the components when the model is optimized.

Furthermore, some practical hints are given that make it possible to predict some quantities useful for designing apparatus.

To find out the necessary parameters it is sufficient to carry out experiments on a pilot column (fixed) of any convenient length.

THEORY

In the case of the separation of a mixture of two components 1 and 2, let

t_1, t_2 be the retention times ($t_2 > t_1$)

q the amount of mixture injected per column

ν the number of revolutions per unit of time

N the number of columns

Q the total feed rate.

The quantity Q is defined by: $Q = q\,\nu\,N$.

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In this relation N may be neglected, Q being an increasing function of N; on the other hand the total number of columns in the set should be fixed on the basis of other considerations (feed rate, etc.); for simplicity we put $N = 1$.

It is necessary to associate the maximum value of Q with the condition of minimum residence time, that is of obtaining the separation in the shortest time; therefore the function of which one has to find the maximum is

$$f(t) = \frac{Q}{t_2}$$

Let Δ_1, and Δ_2 be the peak widths (measured between the points of intersection of the tangents with the base line), then v is expressed by the relation:

$$v = \frac{1}{t_2 + \frac{1}{2}\Delta_2 - (t_1 - \frac{1}{2}\Delta_1)} = \frac{2}{2(t_2 - t_1) + \Delta_1 + \Delta_2},$$

$t_2 + \frac{1}{2}\Delta_2$ is the total time required for the separation.

From the plate theory of chromatographic columns[2,3] it follows that for rather large amounts the peak width changes linearly with the quantity q of mixture injected per column (Fig. 1), as given by

$$\Delta_1 = K_1 q + b_1; \qquad \Delta_2 = K_2 q + b_2$$

where we suppose that K_1 and K_2 are constants independent of retention time and column length, and therefore under the same conditions we may assume:

$$K_1 \simeq K_2 \ (\mu l^{-1}.sec)$$

K may be considered as a "dilution factor" of the samples in the carrier gas, essentially depending on the evaporation rate of the sample (see also experimental part).

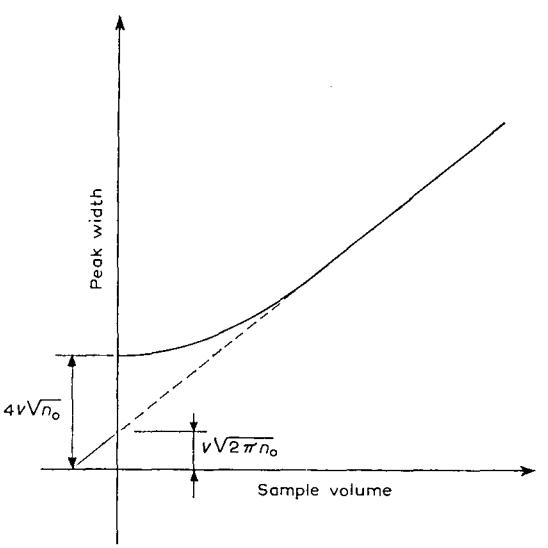

Fig. 1. Plot of peak width against amount of mixture injected.

The theoretical expression of constants b_1 and b_2 is[2]:

$$b_1 = v_1\sqrt{2\pi n_0}\,; \qquad b_2 = v_2\sqrt{2\pi n_0}$$

where

$v_1(v_2)$ is the effective volume of a theoretical plate given by: $V_{g1} + V_{l1}\,k_1$ (V_{g1} = volume of gas in one theoretical plate; V_{l1} = volume of stationary phase in one theoretical plate; k_1 = partition coefficient)

n_0 is the effective number of theoretical plates for infinitesimally small samples.

The constant b_1 may be expressed as follows:

$$b_1 = v_1 n_0\sqrt{\frac{2\pi}{n_0}} = V_{R_1}{}^\circ\sqrt{\frac{2\pi}{n_0}}$$

or

$$\bar{b}_1 = t_1\sqrt{\frac{2\pi}{n_0}} = ct_1$$

where $V_{R_1}{}^\circ = V_g + V_l\,k_1$ is the retention volume (measured at the maximum of the peak for infinitesimally small samples), and, for brevity,

$$c = \sqrt{\frac{2\pi}{n_0}}$$

Consequently the expressions for the peak widths, appropriate for our aim, are:

$$\Delta_1 = K_1 q + ct_1\,; \qquad \Delta_2 = K_2 q + ct_2$$

The total width Δ_t is (putting $K = K_1 + K_2$)

$$\Delta_t = Kq + c(t_1 + t_2)$$

From the definition[4] of "Resolution" S_{12}

$$S_{12} = 2\,\frac{t_2 - t_1}{\Delta_t}$$

it immediately follows that:

$$q = (\alpha - \eta)t_2 - (\alpha + \eta)t_1$$

putting, for brevity,

$$\alpha = \frac{2}{KS_{12}}\,; \qquad \eta = \frac{c}{K}$$

Further, the number of revolutions is given by:

$$v = \frac{S_{12}}{1 + S_{12}} \cdot \frac{1}{t_2 - t_1}$$

From the above considerations it follows that the function to study is:

$$\mathrm{f}(t) = \frac{Q}{t_2} = \frac{S_{12}}{1 + S_{12}} \cdot \frac{(\alpha - \eta)t_2 - (\alpha + \eta)t_1}{t_2(t_2 - t_1)}$$

The maximum value with respect to t_2 is reached when

$$t_2 = \frac{\alpha + \eta}{\alpha - \eta} t_1 + t_1 \sqrt{\left(\frac{\alpha + \eta}{\alpha - \eta}\right)^2 - \frac{\alpha + \eta}{\alpha - \eta}} \qquad (1)$$

With respect to t_1 the behavior of $f(t_1)$ is monotonic; a three-dimensional representation of the function $f(t_1,t_2)$ is given in Fig. 2.

As may easily be noted, among the different pairs of values of t_1 and t_2 which satisfy the relation (1) one has to choose the pair having the lowest value of t_1. This means that in order to obtain the optimum conditions, it is better to employ short columns in spite of the fact that the amount of sample to be injected is low.

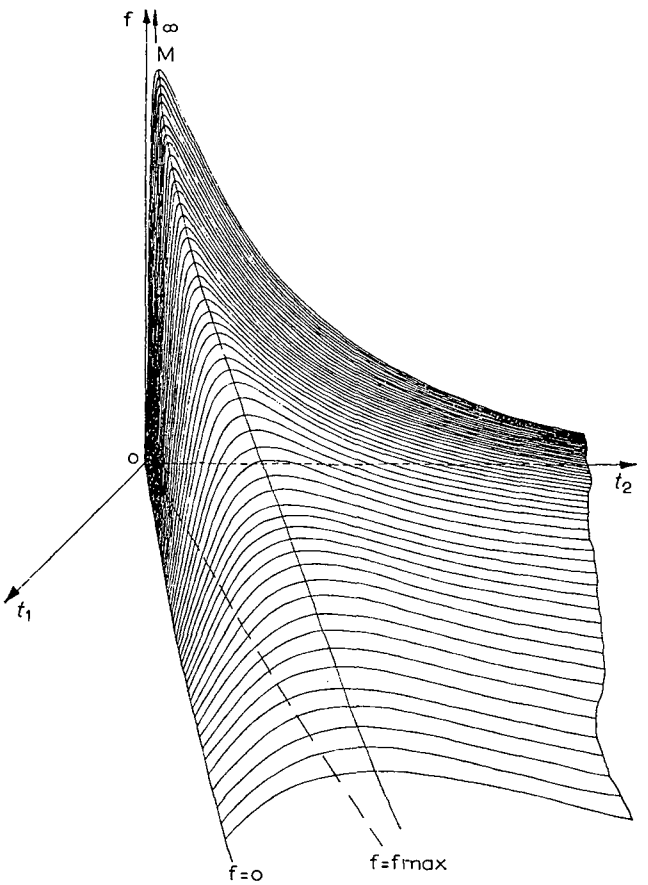

Fig. 2. Three-dimensional representation of the function $f(t_1, t_2)$.

Another interesting parameter is the number of plates n_0 corresponding to the maximum condition; according to eqn. (1), this is given by:

$$n_0 = \frac{\pi}{2}\left\{\frac{(\alpha_{12} + 1)^2 - 2}{(\alpha_{12} - 1)^2} S_{12}\right\}^2 \qquad (2)$$

having introduced the "separation factor"

$$\alpha_{12} = \frac{t_2}{t_1} \simeq \frac{K_2}{K_1}$$

This expression should be compared with the equation

$$\overline{n_0} = 4\left(\frac{\alpha_{12} + 1}{\alpha_{12} - 1} S_{12}\right)^2 \tag{3}$$

which gives the minimum number of plates required for a specific resolution S_{12}. In Fig. 3 the functions n_0 and $\overline{n_0}$ are plotted against α_{12} for $S_{12} = 1.5$ and $S_{12} = 1.2$.

The above theory is presented as a guide for the optimum performance of the rotating unit. However, for practical preparative purposes, instead of requiring

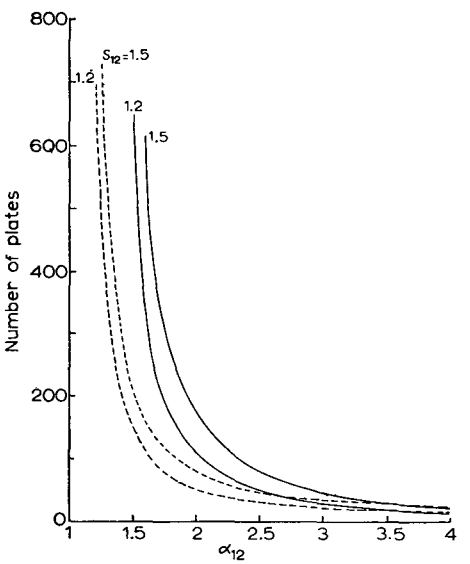

Fig. 3. Plot of the functions n_0 (———) and $\overline{n_0}$ (– – – –) against α_{12} for $S_{12} = 1.5$ and $S_{12} = 1.2$

minimum residence time in the stationary phase, it may be more profitable to examine the behaviour of feed rate when, for instance, the column length is changed. The explicit expressions of load per column q, number of revolutions ν and load per unit of time Q are respectively (putting for brevity $x = c\,S_{12}$):

$$q = (\alpha - \eta)\alpha_{12}t_1 - (\alpha + \eta)t_1 = \frac{t_1}{KS_{12}}[(2 - x)\alpha_{12} - (2 + x)] \ (\mu\text{l per column}) \tag{4}$$

$$\nu = \frac{S_{12}}{1 + S_{12}} \frac{1}{\alpha_{12} - 1} \frac{1}{t_1} \ (\text{sec}^{-1}) \tag{5}$$

$$Q = q\nu = \frac{(2 - x)\alpha_{12} - (2 + x)}{K(1 + S_{12})(\alpha_{12} - 1)} \ (\mu\text{l.sec}^{-1} \text{ per column}) \tag{6}$$

From eqn. (6), assuming that $n_0 = l/h_0$, it is found that

$$Q(l) = \frac{S_{12}}{K(1 + S_{12})}\left(\frac{2}{S_{12}} - \sqrt{2\pi h_0}\,\frac{\alpha_{12} + 1}{\alpha_{12} - 1}\,\frac{1}{\sqrt{l}}\right) \qquad (6')$$

The plot of this relation is given in Fig. 4.

This function has no maximum and for $l \to \infty$,

$$Q \to \frac{2}{(1 + S_{12})K}$$

This equation shows that larger values of α_{12} and h_0 lead to a larger value of Q.

Other authors[5] have also reached these same conclusions when dealing with high speed gas chromatography in packed columns. Of course, the conditions of temperature, pressure, etc. to obtain the best values of α_{12} and h_0 must be found experimentally.

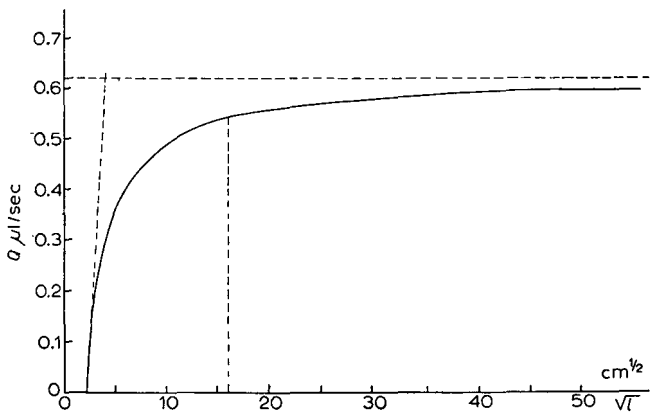

Fig. 4. Relationship between total feed rate and column length.

EXPERIMENTAL

In this section we summarize briefly the experimental work performed in order to test some of the statements made in the previous section and to work out from experimental data the parameters required for applications of relations (5) and (6').

Most experiments were performed using a cyclohexane (1)–benzene (2) (1:1 v/v) mixture injected in various amounts under the following conditions

temperature 80°
stationary phase polyethyleneglycol 400 (30 %) on firebrick
carrier gas nitrogen
column length 120 cm
300 cm

From plots of peak widths (Δ_1,Δ_2) between the tangents against the amount of mixture injected (a typical plot is given in Fig. 5), it is possible to find the constants K, angular coefficients of the straight lines; the heights h_0 can be obtained from plots of

TABLE I

Column length (cm)	Inlet pressure (atm)	Carrier gas flow rate (cm^3/min)	Data obtained from plots						Calculated values		Corrected values	
			K_1	K_2	b_1	b_2	h_{01}	h_{02}	b_1	b_2	K_1	K_2
	1.45	103	0.60	0.90	14	59	0.20	0.13	14	52	0.60	0.90
120	1.60	109	0.53	0.69	14	45	0.25	0.14	11	42	0.57	0.75
300	2.10	103	0.39	0.57	29	142	0.25	0.14	28	115	0.52	0.75

plate heights by extrapolation. Some results are summarized in Table I (K has been corrected for mean pressure inside the column[6]).

We see that, at least in the range of interest for practical applications in preparative gas chromatography, K and h_0 are independent of column length to a sufficient approximation.

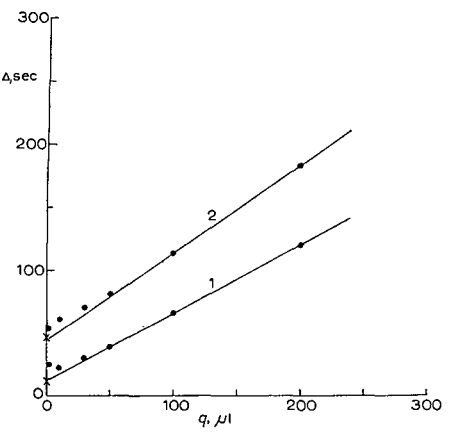

Fig. 5. Plot of peak width against the amount injected. Column length 120 cm; $\Delta p = 0.6$ atm.

To test the applications of the formulae derived in the preceding section we have chosen the same mixture working under the following conditions

column length 120 cm
inlet pressure 1.6 atm
temperature 108°
stationary phase tricresyl phosphate on firebrick
carrier gas nitrogen, 75 c.c./min

Under these conditions the relevant experimental data are

$t_1 = 160$ sec $(\alpha_{12} = 2.03)$
$t_2 = 325$ sec
$K_1 = 0.50\ \mu l^{-1}$ sec $(b_1 = 17$ sec$)$
$K_2 = 0.83\ \mu l^{-1}$ sec $(b_2 = 35$ sec$)$
$h_{01} = 0.13$ cm (mean value $h_0 = 0.145$ cm)
$h_{02} = 0.16$ cm

From these data and for $S_{12} = 1.5$ our formulae (6′) and (5) give, respectively,

$$Q = 0.502\ \mu l.sec^{-1}$$
$$v = 13 \quad h^{-1};$$

for the set of 100 columns one obtains a feed rate of 182 ml/h. Working with our rotating unit under these same conditions we were able to use a feed rate of 190 ml/h at 10 revolutions per hour.

It is thus possible to make reasonable predictions about running conditions of the unit from data obtained on a pilot column.

From the expression of Q as a function of column length l

$$Q(l) = 0.620 - 1.297 \frac{1}{\sqrt{l}} \mu\text{l.sec}^{-1} \text{ per column,}$$

the plot of which is given in Fig. 4, it appears that with a length of 200 cm one should obtain a feed rate improvement up to 200 ml/h.

SUMMARY

A two-component mathematical model for the rotating gas chromatography unit previously described is suggested.

From well-known chromatographic relations, an explicit function depending on the retention times is derived. By studying this function an equation can be obtained, which gives the retention times necessary to achieve optimal operating conditions.

Furthermore, in view of practical applications on a preparative scale, the relation between feed rate and some of the column features, such as length, H.E.T.P., etc., is examined.

Experimental results are reported which support some of the assumptions made in the theoretical part.

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INFLUENCE OF SUPPORT MODIFICANTS ON SUBSTRATE SELECTIVITY IN GAS CHROMATOGRAPHY

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INTRODUCTION

The importance of selectivity in gas chromatography is well known. POLLARD AND HARDY[1] in 1956 demonstrated the effect of selectivity in the separation of halohydrocarbons, and in 1957 TENNY[2] compared substrates of widely differing selectivities ranging from hydrocarbon oils to β,β'-oxydipropionitrile.

By proper use of substrate selectivity, difficult separations can be made using short columns which otherwise would require much longer columns. Selectivity is most useful with mixtures of compounds which enable one to take advantage of differences in polarity between closely boiling materials. Unfortunately, tailing is most severe with highly polar materials which are partially adsorbed by the solid support. This limits the range of substrates that may be used to separate polar materials. To reduce tailing, solid support modificants have come into use. JAMES AND MARTIN[3] used stearic acid. JOHNS[4] used both oleic acid and base amines. More recently METCALFE[5] used 2 % phosphoric acid to enable direct separation of carboxylic acids, and SMITH AND RADFORD[6] used various amounts of sodium hydroxide to enhance separations of diamines. These latter modificants may do more than suppress tailing; as binary or ternary mixtures with other substrates they may function as entirely new substrates.

EXPERIMENTAL

Apparatus and materials

The instrument used in this work was a dual column thermal detector instrument constructed in this laboratory. Columns were prepared from $^1/_4$ in. copper tubing coiled after packing. The solid support used was White Chromesorb (Johns Manville Products Corporation). Liquid phases were Silicone Oil 550 (Dow Corning), Polyethylene Glycol 20 M (Union Carbide Corporation) and Lac 296 (Cambridge Industries Company, Inc.). The chemicals used in the separations were Eastman Organic Chemicals Products. Liquid samples were injected with a Hamilton Microliter syringe equipped with a Chaney Adapter.

Influence of phosphoric acid on Lac 296

The influence of 2 % phosphoric acid on the selectivity of Lac 296 is illustrated in Fig. 1. The only difference in the two columns which produced these chromatograms

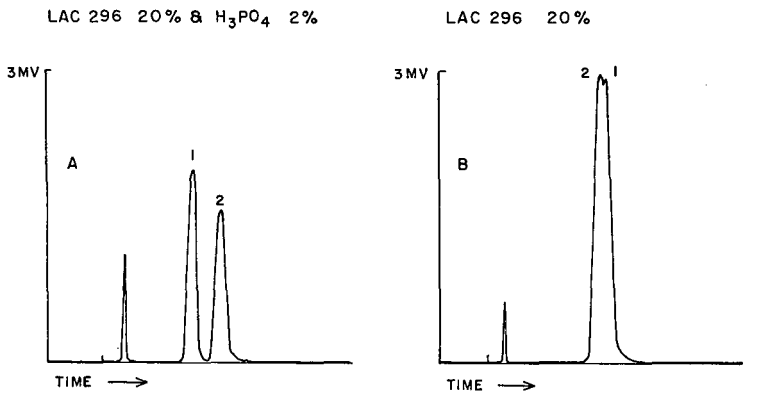

Fig. 1. Influence of H_3PO_4 on the selectivity of Lac 296. Operating conditions: flow: 55 ml/min He; temperature: 70°; column length: 2 m. (1) *tert.*-butyl acetate; (2) *tert.*-butyl alcohol.

was the presence of 2 % phosphoric acid on the one which produced the separation represented as curve A. Not only does the acid-containing column make a complete separation of *tert.*-butyl alcohol and *tert.*-butyl acetate, but it reverses the order of elution from the Lac 296 column. This column also separated methacrolein, water, and methacrylic acid. Not only did it produce well-shaped peaks for the methacrylic acid, but it resolved methacrolein and water. Without phosphoric acid, water and methacrolein were not resolved. Carbowax 20 M functioned in the same way. Without acid, it did not resolve water and methacrolein. With acid, it did.

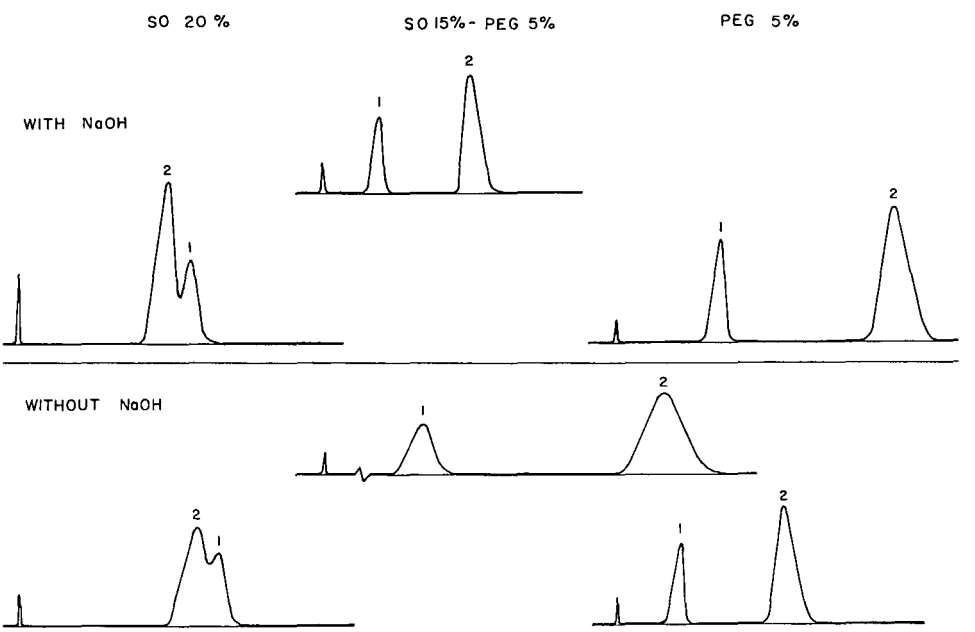

Fig. 2. Influence of 2 % NaOH on substrates. Operating conditions: flow rate: 50 ml/min; temperature: 150°; column length: 0.72 m. (1) *m*-Chloronitrobenzene; (2) *m*-Chloroaniline.

Influence of strong base on silicone oil and polyethylene glycol

During an investigation to provide a rapid, accurate method to follow the reduction of chloronitrobenzene to *m*-chloroaniline it was discovered that the addition of 2 % NaOH to a packing loaded with a 15 to 5 % mixture of Silicon Oil (SO 550) and Polyethylene Glycol (PEG 20M) produced substrate properties which could not be deduced from the effect of NaOH on the individual substrates. The effect is shown in Fig. 2. The columns were operated under virtually identical conditions. The top chromatograms in each case were from columns containing 2 % sodium hydroxide. The presence of NaOH markedly hastened the elution of both components from the mixed column yet retarded their elution from the PEG 20M.

The effect of NaOH on SO 550–PEG 20M mixtures at several different proportions was investigated. The results are shown in Table I. Ten columns of equal length were

TABLE I

SEPARATION DATA FOR *m*-CHLOROANILINE AND *m*-CHLORONITROBENZENE

	20 % Silicone		*15 % Silicone–5 % PEG*		*10 % Silicone–20 % PEG*		*20 % PEG*		*5 % PEG*	
	No NaOH	*2 % NaOH*	*No NaOH*	*2 % NaOH*	*No NaOH*	*2 % NaOH*	*No NaOH*	*2 % NaOH*	*No NaOH*	*2 % NaOH*
150°										
Separation factor	—1.12	—1.15	1.83	2.59	2.16	2.76	2.86	3.12	2.59	2.54
Plates*	148	236	570	272	460	400	350	491	248	341
Resolution	0.42	0.71	4.95	6.55	6.22	8.78	8.73	11.8	6.25	7.10
Analysis time	16	13	60	19	53	70	103	162	17	30
Retention time *m*-chloronitrobenzene**	14.5	11.6	29.3	5.9	21.3	21.7	31.5	42.2	5.3	9.8
190°										
Separation factor			1.45	2.03	1.81	2.16	2.37	2.53	2.06	1.81
Plates*			410	236	342	260	340	409	286	360
Resolution			2.22	3.95	3.75	4.68	6.30	7.61	3.29	3.86
Analysis time			20	5	15	12	27	44	5	15
Retention time *m*-chloronitrobenzene**			11.8	1.7	6.9	4.3	9.6	15.4	1.7	7.1

* Based on the *m*-chloroaniline peak.
** Corrected to 50 ml/min.

loaded as shown; all helium flow rates were between 40 and 55 ml per minute. The investigation showed that NaOH increased the retention and improved the resolution[7] of the PEG 20M at either the 5 % or the 20 % load. It decreased retention and increased resolution of the silicone column. But, the effect in the 15 SO–5 PEG–2 NaOH mixed column was most striking. With NaOH in the mixture the resolution is improved by 1/3 and the analysis time reduced to 1/3 of that without NaOH present. A comparison of the optimized mixed phase column with an equivalent length single-phase column is shown in Fig. 3.

It was observed that the separation characteristic of the 15 SO–5 PEG–2 NaOH mixed column approximated that of the 5 % PEG single phase column. Therefore, the quantitative performance of these columns was investigated to evaluate the influence of NaOH on the adsorption isotherms. The calibration curves in Fig. 4 show

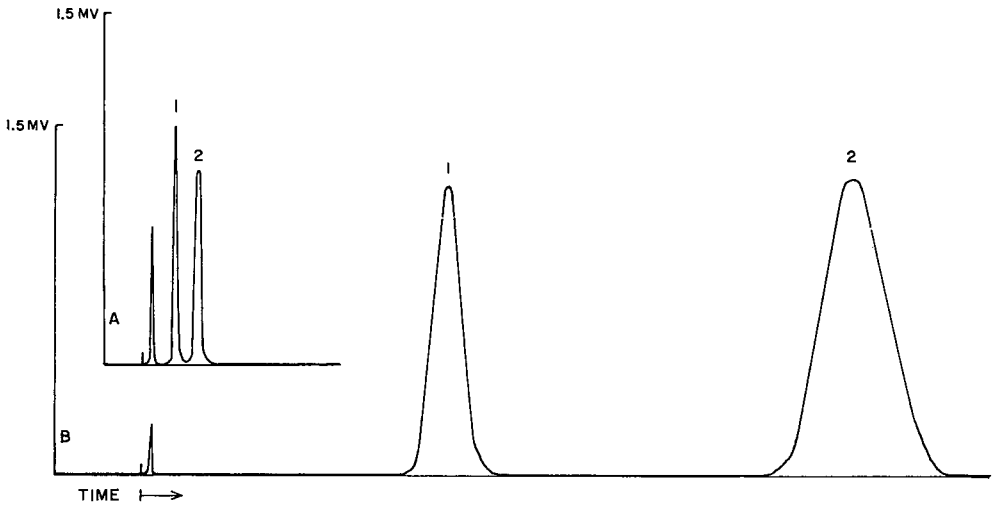

Fig. 3. Comparison of a single-phase column with an optimized mixed-phase column. Operating conditions: (A) column: 15% SO–5% PEG–2% NaOH; length: 0.77 m; temperature: 190°; flow: 97 ml/min He; (B) column: 20% PEG; length 0.77 m; temperature: 190°; flow: 39 ml/min He. Component identity same as in Fig. 2.

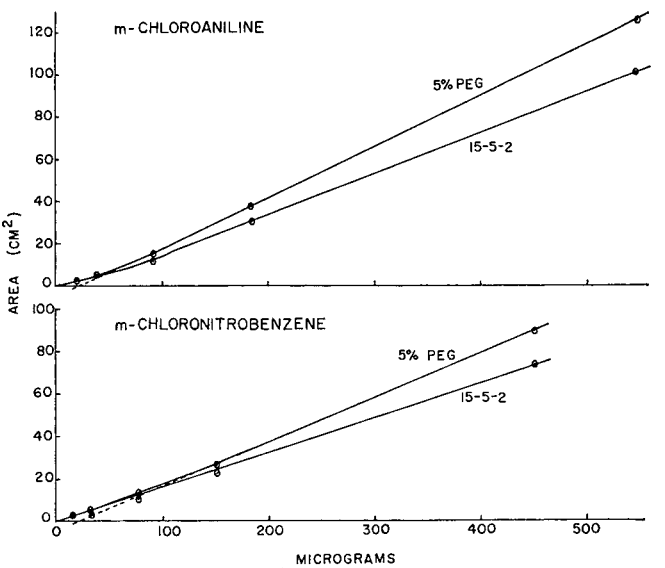

Fig. 4. Calibration curves comparing a 5% PEG column with a 15% SO–5% PEG–2% NaOH column. Operating conditions: same as Fig. 2. Top graph has a 24 μg intercept with either column. Bottom graph has a 20 μg intercept with the 5% PEG column only.

that the mixed column containing NaOH provided a linear calibration curve with a zero intercept with *m*-chloronitrobenzene while the 5 % PEG 20M column shows a slight curvature at low *m*-chloronitrobenzene levels, or an extrapolated intercept of 20 μg. Neither column produced a linear curve for *m*-chloroaniline. Both lines extrapolated through an intercept at 24 μg. This indicated that the 2 % NaOH did not suppress the low level *m*-chloroaniline adsorption beyond that afforded by the 5 % PEG.

Either column would be satisfactory for quantitative analysis of the *m*-chloronitrobenzene–*m*-chloroaniline mixture with an adequate component load. However, at low load, such as with traces of *m*-chloronitrobenzene, the mixed column would be preferred. An additional advantage afforded by the mixed column is that it can serve in a dual capacity, as both a selective column for the *m*-chloroaniline–*m*-chloronitrobenzene mixture, and as a primarily silicone column for a wide variety of hydrocarbon mixtures.

ACKNOWLEDGEMENT

I wish to thank Mr. C. E. COOK for helpful assistance in obtaining these data.

SUMMARY

It has been shown that solid support modificants as phosphoric acid and sodium hydroxide may do more than suppress peak tailing. They may alter substrate properties significantly. As binary or ternary mixtures with other substrates, they may provide a new dimension to substrate selectivity. Benefits may be achieved through the ability to alter substrate selectivity and through reduced analysis time.

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GAS CHROMATOGRAPHY WITH STATIONARY PHASES CONTAINING SILVER NITRATE

IV. THE METHYLCYCLOBUTENES, METHYLENECYCLOBUTANE, C_5 DIENES AND RELATED COMPOUNDS

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INTRODUCTION

It has been reported previously[1–3] that silver nitrate dissolved in ethylene glycol is a highly selective stationary phase for the gas chromatographic separation of cyclic olefins with a five- or a six-membered ring. In order to gain a better understanding of the factors involved in complex formation, the stability constants of olefin–silver ion complexes in glycol solution were determined subsequently[4,5].

In the present work the investigation of the relationship between structure and chromatographic behaviour has been extended to include isomeric olefins containing a four-membered ring (Table I, compounds I–III), as well as spiropentane (XV) and a series of C_5 mono- and di-olefins (IV–VIII), which are encountered in the preparation and reactions of I–III. In order to clarify structural relationships, additional compounds (IX–XIV) were included in the study.

The selectivity of the silver nitrate–glycol phase promised to be of particular advantage in a number of analytical applications:

For instance, I, IV and XV are the main products formed by debromination of pentaerythrityl tetrabromide, which is one of the key reactions leading to cyclobutane derivatives[6,7]. Elaborate and time-consuming methods have been used in the past for estimating the composition of the product formed, *e.g.* fractional distillation, infrared[8] and Raman[9] spectroscopy, electron diffraction[10], etc. More recently, APPLEQUIST, FANTA AND HENRIKSON[11] applied gas chromatography to the separation of methylenecyclobutane from spiropentane using di-2-ethylhexyl sebacate as the stationary phase.

Furthermore, in equilibrium studies[12] undertaken in this laboratory a rapid method was needed for the determination of I, II and III in their mixtures. On the other hand, the study of the thermal decomposition[13] of 1-methylcyclobutene (II) and 3-methylcyclobutene (III) required a method with which these compounds could be easily separated from the isomeric products in which the ring was split open, *i.e.* isoprene (VI) and 1,3-pentadiene (VII, VIII), respectively. It also seemed of some interest to examine the analysis of mixtures containing 1,2-propadiene (XIII) and ethene (XI), which are formed by thermal decomposition of methylenecyclobutane (I)[14].

The results obtained in the present study are summarized in Table I.

TABLE I

RETENTION VOLUMES OF THE METHYLCYCLOBUTENES, METHYLENECYCLOBUTANE, C_5 DIENES AND RELATED COMPOUNDS

Stationary phase 30 g $AgNO_3$/100 ml ethylene glycol; column length 2 m; weight ratio of solid support[1,2] to liquid phase 3:1; helium flow rate 50 ml/min.

No.	*Compound*	*B.p. (°C)/ 760 mm*	*r* at 30°*
I	Methylenecyclobutane	41.2	0.70
II	1-Methylcyclobutene	37.3	0.059
III	3-Methylcyclobutene	37	0.12
IV	2-Methyl-1-butene	31.2	0.15
V	2-Methyl-2-butene	38.6	0.058
VI	Isoprene	34.1	0.32
VII	1,3-*cis*-Pentadiene	44.1	0.63
VIII	1,3-*trans*-Pentadiene	42.0	0.46
IX	1,4-Pentadiene**	26.0	1.34
X	Cyclopentadiene	42.5	0.97
XI	Ethene	−103.7	0.042
XII	Propene	−47.7	0.057
XIII	1,2-Propadiene (Allene)	−34.5	0.036 (0.063)***
XIV	Cyclopropane	−32.8	0.008
XV	Spiropentane	39.1	0.031 (0.056)***

* Relative to toluene.
** The structurally analogous 1,5-hexadiene was retained irreversibly by the column.
*** Relative to benzene.

It can be seen that, with the exception of 1-methylcyclobutene (II) and 2-methyl-2-butene (V), the retention volumes of all compounds examined differ sufficiently to permit qualitative and quantitative analysis of mixtures of various composition, such as are formed in the reactions mentioned above. It should be noted that the selectivity of the complex-forming phase for the separation of methylenecyclobutane (I) from 1-methylcyclobutene (II) ($r_I/r_{II} \simeq 12$) is much higher than that obtained with di-*n*-propyl tetrachlorophthalate* ($r_I/r_{II} \simeq 1.2$) at the same temperature (30°). Experiments with synthetic blends, containing not less than 10 % of each component, showed that the accuracy was about $\pm$ 1 % of the total. The time required for a complete analysis does not exceed 15 minutes.

The method has been applied successfully to the analysis of the products of debromination of pentaerythrityl tetrabromide formed under different experimental conditions[6,11,15,16]. It was found that previous analytical results, though only approximative, were generally in agreement with data obtained by the present procedure. In certain cases, however, larger deviations were observed. Thus, the product formed under the conditions described by ROBERTS AND SAUER[9], which these authors considered to be practically pure methylenecyclobutane, was found in our case to contain methylenecyclobutane 81.4 %, 2-methyl-1-butene 12.6 % and spiropentane 6.0 %.

There are certain limitations to the analytical application of the stationary phase. For instance, if crude or insufficiently purified methylcyclobutane is used for the isomerization to 1-methylcyclobutene, then 2-methyl-2-butene, formed from contaminat-

* 6 m column filled with 7 % di-*n*-propyl tetrachlorophthalate on Chromosorb.

ing 2-methyl-1-butene, is also present in the product. The procedure used to correct for the proportion of V in the peak of II has been described elsewhere[12].

It is important to note that 1,5-hexadiene (see further below) is retained irreversibly by the column under our chromatographic conditions. This must be kept in mind when analysing unknown mixtures of polyenes with silver nitrate solutions. Further, it should be pointed out that the relative order of retention volumes of non-conjugated dienes as compared with the corresponding conjugated isomers ($r_{IX} > r_{VII}, r_{VIII}$) is the reverse of that found with non-complex-forming phases[17].

DISCUSSION OF RESULTS

The following conclusions can be drawn from the data given in Table I, assuming that differences in relative retention volume for close boiling isomers essentially parallel the stability of the complexes formed*:

(a) As in the series of the cyclopentenes[2] and cyclohexenes[1,3], the 1-alkyl compound in the methylcyclobutene series has a lower retention volume than the 3-alkyl isomer ($r_{II} < r_{III}$), owing to the higher substitution of the double bond in the former. On the other hand, methylenecyclobutane (I), which contains a disubstituted *semi*-cyclic double bond, has a higher retention volume than III, which has an *endo*cyclic double bond with the same degree of substitution. The relative order of the retention volumes of the methylenecyclane (I) and the isomeric 3-methylcyclene (III), parallels the sequence of the six-membered ring homologues, but is the reverse of that found in the five-membered ring series.

It has already been reported[4] that the stability constant of the 1-methylcyclobutene–silver ion complex is much lower than that of the corresponding 1-methylcyclopentene and 1-methylcyclohexene complexes, contrary to what would be expected from the higher strain in the four-membered ring compound. The observed retention volume of 3-methylcyclobutene seems to point once again to the lower tendency for complex formation of a cyclobutene as compared with a similarly substituted cyclopentene or cyclohexene system.

(b) The values of the retention volumes of compounds VI–X provide some interesting information on the relative stability of complexes formed by different types of dienes:

Complex formation differs markedly in ethylene glycol as compared with aqueous medium. WINSTEIN AND LUCAS[18], who carried out measurements in aqueous $AgNO_3$, found that conjugation may reduce the stability of the complex drastically. In ethylene glycol, however, no such effect is apparent from the chromatographic behaviour of either isoprene or the 1,3-pentadienes.

The retention volume of isoprene has a value ($r = 0.32$) approximately equal to the one expected if the contributions from the double bonds were additive, taking 2-methyl-1-butene ($r = 0.15$) and 1-pentene ($r = 0.19$)[17] as the reference monoolefins. The retention volumes of the 1,3-pentadienes are found to be even $\sim$ 30 % higher than estimated* on the basis of additivity from 1-pentene and *cis*- and *trans*-2-pentene ($r = 0.21$ and 0.07)[17], respectively.

* See ref. 4 for a short discussion of the influence of structural factors on the stability constants of olefin–silver ion complexes in ethylene glycol.

* The data for the retention volumes of the reference olefins were corrected for differences in volatility, using the plots of log retention volume *versus* boiling point for families of monoolefins, given by TENNEY[19]. The correction was 0.06–0.07 for 1-pentene and 0.02 for both *cis*- and *trans*-2-pentene.

Cyclopentadiene (X) has a higher retention volume ($r = 0.97$) compared with the conjugated open-chain dienes VI–VIII, in line with the already established[4] enhanced tendency for complex formation of the double bond in a five-membered ring. However, conjugation seems to weaken the coordination capacity in this case, since the retention volume of X is much lower than the additive value ($r = 1.8$) calculated from data of the corresponding monoolefin (cyclopentene, $r = 0.90$)[17].

Another striking difference is displayed by the non-conjugated dienes. 1,4-Pentadiene has a retention volume 3.6 times higher than calculated on the basis of additivity (using 1-pentene as the reference monoolefin), while 1,5-hexadiene forms such a stable complex that it is irreversibly retained on the silver nitrate–glycol column. The stability constant ($K_0 = 0.395$) of the latter compound in aqueous solution has been determined by WINSTEIN AND LUCAS[18] and found to be only twice as high as estimated (reference compound: 1-hexene, $K_0 = 0.095$).

The behaviour of the non-conjugated dienes in ethylene glycol points to chelate formation, with the diolefins acting as bidentate ligands. The greater stability of the 1,5-hexadiene complex as compared with the 1,4-pentadiene complex can be readily explained by lesser strain of the chelate ring in the former case. The strong 1:1 complex formed by 1,5-cyclooctadiene[20], which, in contrast to the silver ion complexes of 1,2- and 1,3-cyclooctadiene, is stable in aqueous solution even at 90–100°, has also been assigned a chelate type structure by AVRAM, MARICA AND NENITZESCU[21]. Further, X-ray analysis[22] has shown that the silver ion in the 1:1 complex of cyclooctatetraene is situated above the plane formed by carbon atoms 1, 2, 5 and 6, the distance being 2.46, 2.51, 2.78 and 2.84 Å, respectively.

Chelation does not exclude the coexistence in solution of other types of complexes, such as disilver compounds, in which each double bond coordinates with a separate silver ion.

(c) The following incidental results are also noteworthy: 1. The lower retention volume ($r = 0.036$) of allene (XIII), as compared with those of the more volatile ethene ($r = 0.042$) and propene ($r = 0.057$), indicates that a cumulative double bond system has a lower complex-forming capacity than a single double bond.

2. Spiropentane (XV) has a very low retention volume, which indicates the absence of interaction with the stationary phase. The lack of complexation in the case of saturated three-membered ring derivatives is further shown by the very low retention volume ($r = 0.008$) of cyclopropane itself, as compared with the isomeric propene ($r = 0.057$). The behaviour of the silver ion (in ethylene glycol) thus contrasts with that of the more strongly coordinating Pt, which has recently been shown to form a complex with cyclopropane[23].

It is planned to test the above conclusions, and to further clarify the behaviour of conjugated and non-conjugated dienes by measuring the stability constants of their silver ion complexes in ethylene glycol solution.

EXPERIMENTAL

Apparatus and procedure

A Perkin-Elmer Model 154 A Fractometer was used in the study. The procedure was essentially the same as described previously[1,2]. The retention volumes given in Table I represent the average value of at least three determinations; the maximum deviation

observed was about 3 %. In order to obtain reproducible values, it is best to coat the support directly with the $AgNO_3$–glycol phase, avoiding the use of a solvent.

Materials

Methylenecyclobutane (I) was prepared by debromination of pentaerythrityl tetrabromide according to the procedure of ROBERTS AND SAUER[6]. The compound was fractionally distilled on a Piros-Glover spinning band column in order to remove the lower boiling spiropentane and 2-methyl-1-butene, which are formed as side products. A sample of I of more than 99 % purity was obtained. B.p. 41.2° (760 mm); n^{20}_D 1.4212 (reported[24] 42°; n^{15}_D 1.4235).

1-Methylcyclobutene (II). Sodium-catalyzed isomerization of pure methylenecyclobutane[12] at 8–10° gave a product containing about 85 % of II and 15 % of I. A sample of II about 99 % pure was obtained by fractionation on the same column as above. B.p. 37.3° (760 mm); n^{20}_D 1.4080 (reported[24] 38°; n^{23}_D 1.4034).

3-Methylcyclobutene (III) was synthesized by detosylation of the tosyl ester of *trans*-2-methylcyclobutanol according to BROWN AND ZWEIFEL[25], using a modified procedure, which will be reported elsewhere.

2-Methyl-1-butene (IV) and *2-methyl-2-butene* (V) were research grade N.B.S. samples.

The C_5 *dienes* (VI–X) and *1,5-hexadiene* were commercial products (purity > 95 %), which, where necessary, were distilled before use.

Ethene (XI), *propene* (XII) and *cyclopropane* (XIV) were pure products (> 99 %) supplied by the Matheson Co.

1,2-Propadiene (XIII) of about 95 % purity was prepared by debrominating 2,3-dibromopropene according to the procedure of KISTIAKOWSKY and coworkers[26].

Spiropentane (XV) was prepared by debrominating pentaerythrityl tetrabromide according to the procedure of APPLEQUIST, FANTA AND HENRIKSON[11], *i.e.* using zinc in conjunction with tetrasodium ethylenediaminetetraacetate as the debrominating agent. The compound was purified to a degree of nearly 95 % by fractional distillation.

ACKNOWLEDGEMENT

Thanks are due to Mr. CH. GRÜNER for assisting in the preparation of the compounds.

SUMMARY

The retention volumes of the methylcyclobutenes, methylenecyclobutane, a series of C_5 open chain mono- and diolefins and of some related compounds have been determined using silver nitrate–glycol as the stationary liquid. As in the case of other groups of olefins, the complex-forming phase has been found to be highly selective, permitting ready separation of almost all compounds examined.

By comparing the retention volumes of close boiling isomers, correlations between structure and stability of the silver ion–olefin complexes formed have been established. The main conclusions are as follows: (1) Cyclobutenes have less tendency to complex formation than the corresponding five- and six-membered cycloolefins; (2) in contrast to findings in aqueous solution, conjugated dienes do not show reduced complex stability in ethylene glycol; (3) unconjugated dienes coordinate strongly, probably as a result of chelation.

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GAS–LIQUID CHROMATOGRAPHY OF MIXTURES CONTAINING PHENOL AND FIVE OF ITS *tert.*-BUTYL DERIVATIVES*

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INTRODUCTION

The analysis of mixtures containing phenol and *tert.*-butylphenols is of interest in connection with the study of the acid catalyzed alkylation of phenol[1,2] with isobutylene. Substitution takes place almost exclusively in the *ortho* and *para* positions. Furthermore, even when the source of isobutylene is a hydrocarbon fraction containing other butenes, the reaction is selective and the major products are *tert.*-butylphenols.

Examination of the properties of these phenols, listed in Table I, shows that analysis by distillation will yield considerable information. However, the procedure is time consuming and not very accurate when compared with instrumental methods.

TABLE I

PHYSICAL PROPERTIES OF PHENOL AND SOME *tert.*-BUTYLPHENOLS

Compound	*M.p.* (°C)*	*B.p.* (°C)/760 mm*
Phenol	41	182
o-tert.-Butylphenol	—	221
p-tert.-Butylphenol	100	237
2,6-Di-*tert.*-butylphenol	39	253**
2,4-Di-*tert.*-butylphenol	57	263
2,4,6-Tri-*tert.*-butylphenol	131	278

* According to PARDEE AND WEINRICH[3], except as otherwise indicated.
** Estimated from the data of HART AND CASSIS[4].

Infrared spectra of the phenols are sufficiently selective to permit both qualitative and quantitative analysis[5,6] of the individual components. However, since impurities sometimes cause interference at the infrared analytical wavelengths, and since gas–liquid chromatography (G.L.C.) is usually more rapid, analysis by this procedure was investigated. This paper describes the results of the study.

* Paper read at the 8th National Analysis Instrumentation Symposium of the Instrument Society of America, April 30th–May 2nd 1962, in Charleston, W. Va., U.S.A.

SELECTION OF THE GAS–LIQUID COLUMN

The initial experiments with G.L.C. were encouraging but not entirely successful. When Silicone Oil 550 was used as the stationary liquid, the phenols (Table I) were separated in order of their increasing boiling points as shown by the chromatogram in Fig. 1. It was somewhat surprising that one pair of components, *o*- and *p-tert.*-butylphenol, was not resolved satisfactorily.

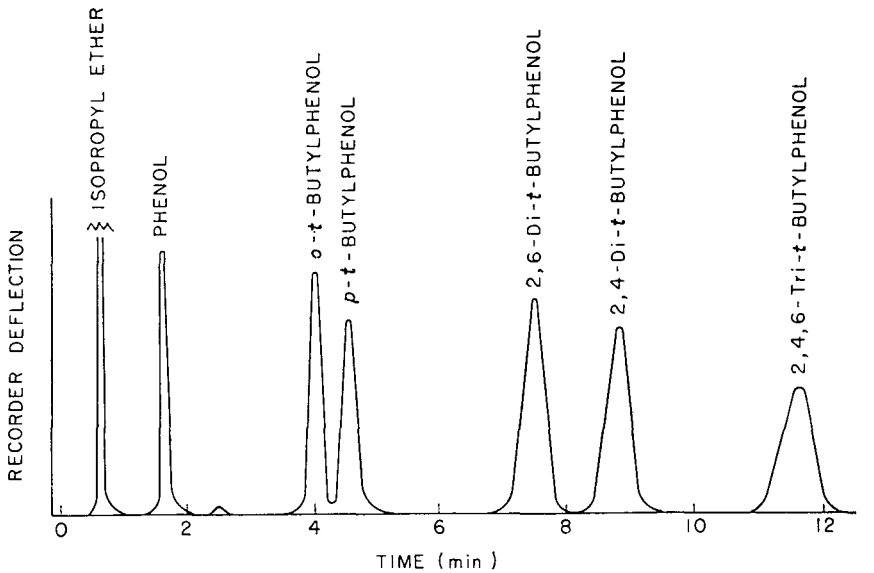

Fig. 1. Chromatogram of a synthetic blend of phenols on a 4 m Silicone Oil 550 column at 220°. Helium flow rate: 80 cm^3/min.

An attempt was made to increase the selectivity of the liquid phase by employing a more polar substance, Carbowax 4000. Again complete separation was not achieved, but unique properties were observed. Both of the completely hindered phenols, 2,6-di-*tert.*-butylphenol and 2,4,6-tri-*tert.*-butylphenol, emerged from the column simultaneously prior to phenol, even though its boiling point is much lower. Moreover, *o-tert.*-butylphenol was widely separated from *p-tert.*-butylphenol, although the latter was not completely resolved from 2,4-di-*tert.*-butylphenol.

These findings indicate the need for a two-stage column. McFADDEN[7] discussed the general case and demonstrated that the properties of a chromatographic column prepared from a mixture of two stationary liquid phases which do not interact chemically are equivalent to those of a two-stage column prepared in the same ratio. Therefore, in order to establish the ratio between Silicone and Carbowax to provide the optimum separation of *tert.*-butylphenols, columns prepared from several different mixtures of the two were examined. The total amount of liquid phase employed in each case was held constant.

Retention times for the *tert.*-butylphenols were measured relative to phenol for Silicone–Carbowax ratios between 5:1 and 1:2, that is between Carbowax concentrations in the liquid phase of 16.5 and 66.7 %, respectively. To facilitate the selection

of the particular stationary phase mixture, the data relative retention times *versus* Carbowax concentration in the stationary phase, were examined graphically as shown in Fig. 2. Unexpectedly, the data revealed not one but four mixtures that give maximum separation, each with a different order of emergence for the individual phenols. These stationary phases were located by determining the widest spacings between the lines representing retention times. The orders of emergence were

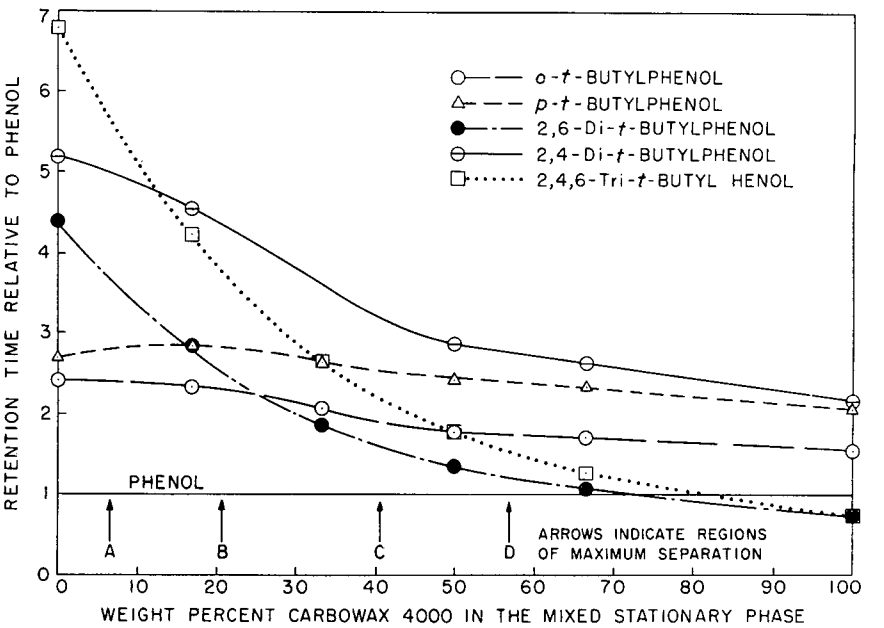

Fig. 2. Chromatography at 220° of phenol and *tert.*-butylphenols on 3 m mixed Silicone Oil 550 and Carbowax 4000 columns.

established by observing the order of the lines relative to that for phenol. The particular mixed liquid phases occur at Carbowax concentrations of 7.0, 21.5, 40.6 and 57.0 % and are indicated in Fig. 2 by the arrows labeled A, B, C, and D, respectively.

Columns were then prepared using these four selected ratios of liquid phases, and then tested in the same manner. The predicted different orders of emergence of the components were achieved as illustrated by the chromatograms shown in Fig. 3. Examination of these chromatograms shows that column C is clearly superior as regards separation ability so it was chosen as the analytical column.

ANALYSIS OF SYNTHETIC SAMPLES

The accuracy of the quantitative determination of the individual phenols by G.L.C. using column C was checked by analyzing two synthetic blends. One of the typical chromatograms appears as Fig. 4. The results, summarized in Table II, show a mean deviation of 0.4 % of the total and a maximum deviation of 1.0 %. It was necessary to employ relative area calibration factors with the blend having the high phenol content.

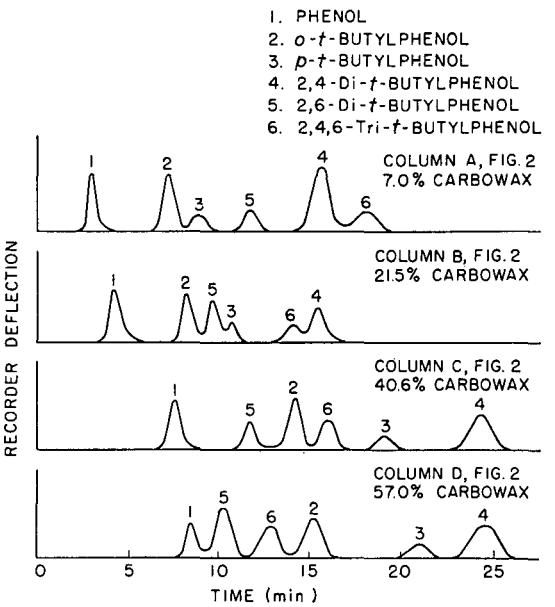

Fig. 3. Chromatography at 220° of *tert.*-butylphenols on selected 3 m columns containing Silicone Oil 550 and Carbowax 4000.

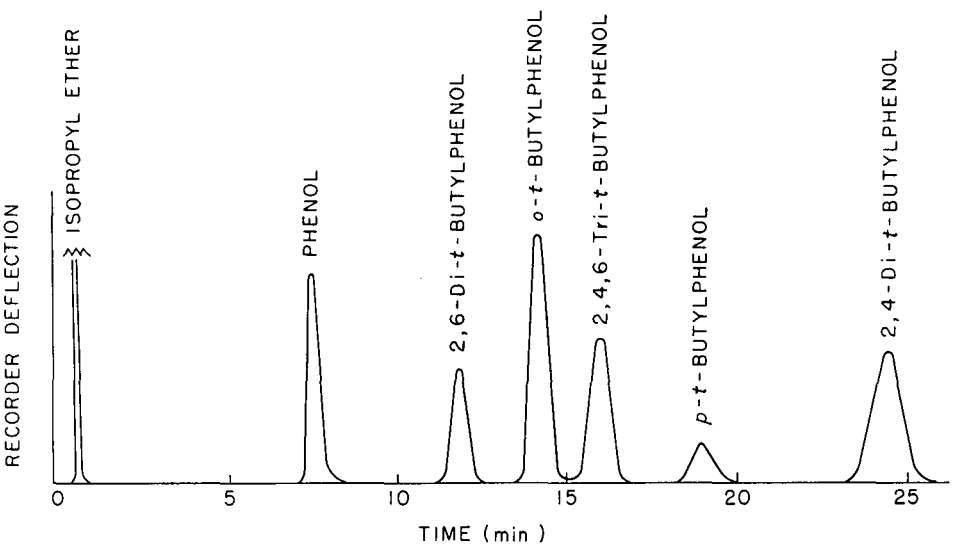

Fig. 4. Chromatogram of a synthetic blend of phenols on a 3 1/2 m column containing a 3:2 mixture of Silicone Oil 550 and Carbowax 4000 at 220°. Helium flow rate: 180 cm³/min.

TABLE II

DEVIATIONS IN ANALYSIS OF SYNTHETIC BLENDS OF PHENOL AND *tert.*-BUTYLPHENOLS

Compound	*Per cent by weight*									
	Weighed	*Found**		*Deviation*		*Weighed*	*Found***		*Deviation*	
		(1)	*(2)*	*(1)*	*(2)*		*(1)*	*(2)*	*(1)*	*(2)*
Phenol	4.8	5.6	5.8	+0.8	+1.0	43.2	43.0	43.4	−0.2	+0.2
o-tert.-Butylphenol	20.0	20.6	20.7	+0.6	+0.7	8.2	8.5	8.5	+0.3	+0.3
p-tert.-Butylphenol	2.4	2.4	2.4	0	0	39.3	39.8	39.5	+0.5	+0.2
2,4-Di-*tert.*-butylphenol	50.1	49.5	49.5	−0.6	−0.6	6.0	5.8	5.6	−0.2	−0.4
2,6-Di-*tert.*-butylphenol	8.6	8.2	8.5	−0.4	−0.1	1.9	1.6	1.7	−0.3	−0.2
2,4,6-Tri-*tert.*-butylphenol	14.1	13.7	13.1	−0.4	−1.0	1.4	1.3	1.3	−0.1	−0.1

* Equivalent to uncorrected area per cent.

** Corrected area %; factors employed: phenol 1.00; mono-*tert.*-butylphenols 1.20, di-*tert.*-butylphenols 1.29, tri-*tert.*-butylphenol 1.32; corrected area = measured peak area × factor.

APPLICATION OF THE PROCEDURE

On the basis of experience gained by application of the method to the analysis of process samples, an alternate procedure was devised. Isopropyl ether employed as a solvent as described in the experimental section was used as an internal standard. The accuracy obtained with this alternate procedure is somewhat poorer, but the amount of an individual phenol can be determined without regard to total area or the presence of uneluted higher boiling components in a sample.

Samples of individual phenols of 0.3 ml and 97–99 % purity were isolated from a synthetic blend by using G.L.C. on a preparative scale. This experiment illustrates the feasibility of collecting chromatographic peaks from process samples. Infrared spectra of such isolated fractions would then reveal the presence of unexpected impurities being eluted along with a particular phenol.

During prolonged constant use of analytical column C, Silicone gradually bleeds away from the solid support. Finally, separation of the phenols is no longer satisfactory, and a fresh column must be prepared. Therefore, mixtures of Carbowax with various other stationary phases, for example, Silicone high vacuum grease or Apiezon wax, should be examined. Perhaps one of these will give a similar separation and at the same time a more stable column.

On the other hand, at temperatures below 200° column C is quite stable and has provided the means of analyzing many other complex mixtures. In addition, by employing matched columns to compensate for substrate bleeding, column C has been employed in temperature programmed operation up to 250°.

BEHAVIOR OF PHENOLS IN MIXED SILICONE–CARBOWAX GAS–LIQUID COLUMNS

It is interesting to speculate regarding the unique orders of emergence of the *tert.*-butylphenols from the mixed Silicone–Carbowax columns. When Carbowax alone is the stationary phase the order is remarkably different from that of the boiling point. This is in contrast to the behavior in Silicone where only simple solubility is involved. The difference can be explained on the basis that the strength of the interaction,

hydrogen bonding, between a phenol and Carbowax is greatly influenced by steric factors. Therefore, the retention time should depend primarily upon the number of substituents *ortho* to the phenolic hydroxyl group, rather than upon volatility. The behavior in mixed columns is more difficult to explain. For Carbowax concentrations greater than 50 % it is apparent that differences in the degree of hydrogen bonding is certainly the dominant factor. Below this concentration volatility gradually becomes of most importance. The completely hindered phenols exhibit the greatest change in retention time. These two compounds have high retention times in Silicone because of their low volatility (high boiling point) but low retention times in Carbowax due to the absence of hydrogen bonding. The mutual effect of these diverse properties in mixed columns causes their retention times to move between those of the other components. It is this phenomenon that results in different orders of emergence from mixed columns and provides the four mixtures giving different maximum separations.

EXPERIMENTAL

Apparatus and procedure

Analytical gas–liquid chromatography was performed using a Beckman Model GC-2 Gas Chromatograph operated at a temperature of 220°. The inlet pressure of the helium carrier gas was 55 p.s.i. and its rate of flow approximately 180 cm^3/min.

The column packings were prepared following the procedure described by JOHNS[8]. Seven parts of Chromosorb-W, 30 to 60 mesh, were coated with three parts of Silicone Oil 550, Carbowax 4000 or mixtures of the two. Stainless steel columns of 6 mm diameter and 3 m length were filled, then purged with carrier gas at 220° until a stable base line was achieved. Higher temperatures should be avoided with mixed packings, since the Silicone would be selectively eluted, changing column characteristics.

A phenol sample was dissolved in an equal quantity of isopropyl ether, then approximately 0.02 ml of the solution was introduced into the column with a hypodermic syringe. Ether was employed since it easily dissolves large quantities of solid phenols and aids in their vaporization. Therefore, it was also employed as an internal standard for determining relative calibration factors.

Preparative scale chromatography was accomplished using a revised Greenbrier Chroma-Lab instrument, Model 940. The injection block was rebuilt to provide for the rapid and complete vaporization of the larger sample. The column vent line between the detector and the fraction collection apparatus was equipped with a heater to prevent condensation of the materials being eluted from the column. Usually 300 g of packing were prepared as described above and filled into copper tubing of 13 mm inner diameter and 6 m length. A phenol sample was dissolved in ether then usually 2 ml of the solution was injected into the column. Fractions were trapped using ice water each time a peak indicated elution of a component from the column.

MATERIALS

The phenols employed in this study were purified by means of fractional distillation and recrystallization. Their purities were certified by comparison of melting points and infrared spectra with those of authentic samples.

Silicone 550 fluid is obtained from Dow Corning Corporation, Midland, Michigan (U.S.A.). Carbowax 4000 is a higher molecular weight polyethylene glycol available from Union Carbide Chemicals Company.

SUMMARY

Complete separation and quantitative analysis of mixtures of phenol and its five *ortho-* and *para-tert.*-butyl derivatives can be accomplished by gas–liquid chromatography. The column, three meters in length, contains a specially selected stationary phase that consists of three parts Silicone Oil 550 and two parts of Carbowax 4000. The particular amounts of Silicone and Carbowax are quite important, since changes in their ratio alter the retention times for the various phenols. This produces different orders of emergence for these phenols from particular mixed columns. Reasons for this occurrence are discussed. Analysis of synthetic samples shows the mean deviation of results to be 0.4 % of the total with a maximum deviation of 1.0 %.

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DIE GAS- UND DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG DER STEREOISOMEREN FARNESOLE UND IHRER DERIVATE

I. MITTEILUNG. *trans-trans* UND *cis-trans*-FARNESOL UND DERIVATE

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(Eingegangen den 21. September 1962)

Anhand der neuesten biogenetischen Untersuchungen sind die Farnesolderivate die Prekursore aller in der Natur vorkommenden sesqui und höheren Terpenoide, Steroide und Carotinoide[1–4].

Stereochemisch kann Farnesol mehrere Isomere haben, dies wurde zuerst durch BATES *et al.*[5,6] bewiesen. Sie bestimmten gaschromatographisch vier stereoisomere Farnesole. Die raumstrukturellen Verhältnisse wurden mit Magnet-Kernresonanz-Spektroskopie untersucht und die Methylabsorptions τ-Werte des *trans-trans*, *cis-trans*, *cis-cis* und *trans-cis*-Farnesole bestimmt. Nach BROOKS UND OVERTON[7] nehmen in der Natur in der Biosynthese des Squalens und Drimenols die *trans-trans*-Farnesolderivate teil. Die Wichtigkeit der Farnesolderivate wird durch die Feststellung von SCHMIALEK[8] unterstrichen. Er isolierte aus dem Exkrement der *Tenebrio* (Mehlwurm) und aus der Hefe eine stark juvenilhormonwirkende Fraktion und stellte durch chemische und chromatographische Methoden fest, dass diese Hormonwirkung hauptsächlich auf die Anwesenheit des Farnesols und dessen Aldehyd (Farnesal) zurückzuführen ist. SCHMIALEK macht keine Erwähnung davon, welche stereoisomere Raumstruktur das von ihm isolierte und verwendete Farnesol besass. In den von WIGGLESWORTH[9] beschriebenen, ähnlichen Untersuchungen werden die raumstrukturellen Verhältnisse des verwendeten Farnesols auch nicht erwähnt.

Das Ziel unserer Arbeit war, die Herstellung einiger *trans-trans* und *cis-trans*-Farnesolderivate und die Trennung der Ausgangsstoffe und ihrer Derivate, mittels Gas- und Dünnschichtchromatographie.

METHODISCHER TEIL*

Herstellung von Farnesolderivaten zu chromatographischen Untersuchungen

Zur Reproduktion der gaschromatographischen Untersuchungen von BATES *et al.*[6], benutzten wir Farnesolmuster von der Firma Fluka A.G. Parallel mit diesen Mustern untersuchten wir die von der Firma Parfümerie und Kosmetik, Budapest, zu unserer Verfügung gestellten Farnesolmuster.

Bei der gaschromatographischen Trennung beider Muster erwies sich, dass sie

* Bei den Untersuchungen waren uns A. GYÖRE, E. SIMONFAI UND E. JÁRAY behilflich.

eine Mischung von *trans-trans*- und *cis-trans*-Farnesol sind, wie dies auch BATES *et al.*[6] von den Mustern der Fluka A.G. feststellten.

Wir stellten die Esterderivate (C_1–C_4) der beiden Farnesolisomere mit Hilfe von Säureanhydriden bzw. Säuren her. Bei Acetylierung war die Ausbeute 90–95 %, während bei der Esterbildung höherer Fettsäuren das Gleichgewicht sich auf Kosten der Esterbildung verschiebt. Wurde Al_2O_3 als Katalysator benutzt, ging die Esterifikation auch bei den letzteren fast vollständig durch (90 %).

Auf die Wirkung der milden Chromsäure- und Braunstein-Oxydation bildeten wir aus Farnesolen Farnesale, welche mit Semicarbazid Farnesalsemicarbazone bildeten.

Wir stelten aus den Farnesolen die aliphatischen Sesquiterpene (Farnesene) mittels Wasserentziehung (konz. H_2SO_4) her.

Die gaschromatographische Trennung des trans-trans- und cis-trans-Farnesols und einiger Derivate

Die gaschromatographische Trennung der von Fluka A.G. und der Firma Parfümerie und Kosmetik, Budapest stammenden handelsmässigen Farnesolmuster wurde mit katherometrischer Detektion mittels Griffin & George Apparat für Gaschromatographie ausgeführt. Die Trennung der beiden stereoisomeren Farnesole erfolgte auf einer SiO_2 Adsorbentsäule, die mit 20 %-iger Apiezon N imprägniert war, während die Trennung der Derivate mit Celite 545/20 %-iger Siliconelastomer Adsorbentsäule, bei 190° durchgeführt wurde. Auf Fig. 1 sieht man das Gaschromatogramm und die Untersuchungsbedingungen der handelsmässigen Farnesolmuster der Fluka A.G. Die sichtbaren und berechenbaren Mengenverhältnisse sind: *trans-trans*-Farnesol: 73.0 % (Retentionszeit 30 Min.) und *cis-trans*-Farnesol: 26.6 % (Retentionszeit 20 Min.).

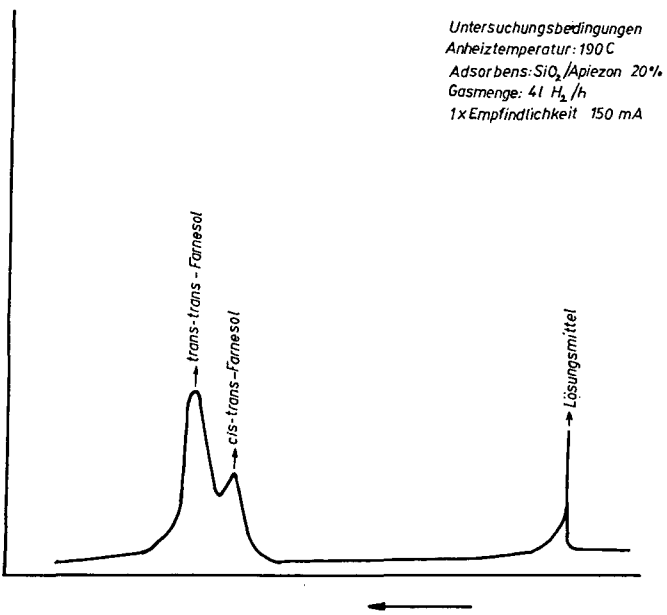

Fig. 1.

Im Falle der gewählten Adsorbentensäule und bei der Trennung des Ausgangsmaterials verwendeten Untersuchungsbedingungen, wurde eine gute Trennung der Farnesolderivate von den Farnesolen erhalten.

Mit unseren gaschromatographischen Untersuchungen ist es uns gelungen die Untersuchungen von BATES *et al.*[6] zu reproduzieren und einen Fortschritt in der Richtung der Trennung der stereoisomeren Farnesole und ihrer Derivate zu machen

Dünnschichtchromatographische Trennung des trans-trans- und cis-trans-Farnesols und einiger Derivate

Die Trennung der zwei stereoisomeren Farnesole und ihrer Derivate ist mit dem TYIHÁK–VÁGUJFALVI-System[10], das mit Erfolg für den dünnschichtchromatographischen Nachweis der ätherischen Ölkomponenten benutzt wurde (Laufstrecke: 16 cm; Lösungsmittel: Benzol–Äthylacetat; Reagens: Vanillin oder Anisaldehyd), gelungen.

Methode: Auf dem Adsorbent "Szialgel 47"* bzw. "Szialgel V"* wurde 150 γ Farnesoltest in Äther aufgebracht und in Benzol–5 % Äthylacetat chromatographiert. Entwickler: 1 % Vanillin in 96 %-iger H_2SO_4, oder 0.5 g Anisaldehyd in 96 %-iger H_2SO_4. Entwicklungstemperatur 105°; Entwicklungszeit: 5 Min. Die Resultate der Untersuchungen sind in Tabelle I, angegeben. Die Flecken der getrennten Farnesole und Derivate sind in der Fig. 2 zu sehen.

TABELLE I

No.	*Substanz*	R_F*-Werte**	*Vanillin-Reagens*	
			U.V.	*Sonnenlicht*
1	Farnesal	0.08	—	rötlich
2	Farnesal	0.14	—	rötlich
3	*trans-trans*-Farnesol	0.27	rötlich	dunkelviolett
4	*cis-trans*-Farnesol	0.36	rötlich	dunkelviolett
5	Farnesalsemicarbazon	0.45	—	gelblich braun
6	Farnesalsemicarbazon	0.53	—	gelblich braun
7	Farnesen	0.90	rötlich	dunkelviolett
8	Farnesen	0.95	rötlich	dunkelviolett
9	"*trans-trans*"-Farnesylacetat	0.66	rötlich	dunkelviolett
10	"*cis-trans*"-Farnesylacetat	0.76	rötlich	dunkelviolett
11	"*trans-trans*"-Farnesylpropionat	0.79	rötlich	dunkelviolett
12	"*cis-trans*"-Farnesylpropionat	0.84	rötlich	dunkelviolett
13	"*trans-trans*"-Farnesylbutyrat	0.82	rötlich	dunkelviolett
14	"*cis-trans*"-Farnesylbutyrat	0.87	rötlich	dunkelviolett
15	"*trans-trans*"-Farnesylvalerianat	0.90	rötlich	dunkelviolett
16	"*cis-trans*"-Farnesylvalerianat	0.90	rötlich	dunkelviolett

* Die R_F-Werte beziehen sich auf "Szialgel V"-Platten.

Aus den Angaben der Tabelle sieht man, dass die beiden stereoisomeren Farnesole bei den angegebenen Bedingungen gut trennbar sind und dass das Verhältnis der Isomere ungefähr mit den gaschromatographischen Werten identisch ist. Während die Farbe der Ausgangsalkohole und ihrer Ester keine Abweichung zeigt, ist die Farbe der übrigen Derivate mit der Vanillinreaktion charakteristisch verschieden. Dies ermöglicht eine Differenzierung auch bei kleinen R_F-Differenzen.

* Unter Herstellung bei der Firma Reanal, Budapest.

Die Bezeichnung " " in der Tabelle, bei den Farnesolestern, weist auf die raumstrukturellen Verhältnisse der Ausgangsalkohole.

Mit dieser dünnschichtchromatographischen Methode ist es uns gelungen den Farnesol und einige Derivate etlicher Pflanzen (*Matricaria chamomilla* L., *Solanum laciniatum* Ait., *Beta vulgaris* var. *conditiva*) nebeneinander nachzuweisen[11–13]. Sie

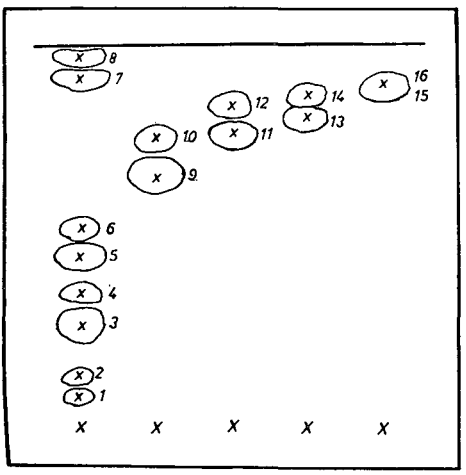

Fig. 2.

ist im allgemeinen gut für die Identifizierung dieser Verbindungen bei physiologischen, genetischen, pflanzenchemischen und botanischen Untersuchungen verwendbar.

ZUSAMMENFASSUNG

Wir stellten für gas- und dünnschichtchromatographische Untersuchungen einige Derivate des *trans-trans* und *cis-trans*-Farnesols her. Die zwei Farnesole wurden auf einer SiO_2/Apiezon N 20 % Adsorbentensäule, ihre Derivate auf Celite 545/Siliconelastomer 20 % Adsorbentensäule gaschromatographisch getrennt. Der verwendete Apparat war vom Typ Griffin & George. Es ist uns auch gelungen die Ausgangsmaterialien und Derivate auf Adsorbenten "Szialgel V" und "Szialgel 47" in Benzol mit 5 %-iger Äthylacetat mit Dünnschichtchromatographie zu trennen. Für den Nachweis erwies sich 1 %-iger Vanillin in H_2SO_4 geeignet.

SUMMARY

Some derivatives of *trans-trans* and *cis-trans*-farnesol were prepared for investigation by gas chromatography and thin-layer chromatography. The two farnesols were separated by gas chromatography on a SiO_2/Apiezon N 20 % column, while a Celite 545/Silicone elastomer 20 % column was used for the derivatives. The apparatus used was Griffin & George type. It was also possible to separate the farnesols and their derivatives by thin-layer chromatography, using "Szialgel V" and "Szialgel 47" as adsorbents and benzene–5 % ethyl acetate as solvent system. For the detection of these substances 1 % vanillin in H_2SO_4 proved suitable.

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EINFLUSS DER SCHICHTBEREITUNGSWEISE AUF DIE REPRODUZIERBARKEIT DER R_F IN DER DÜNNSCHICHTCHROMATOGRAPHIE

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(Eingegangen den 16. Oktober 1962)

Obwohl die Dünnschichtchromatographie (D.C.) seit längerer Zeit bekannt ist[1], wurde sie erst nach der Einführung eines brauchbaren Streichgerätes[2] zu einer allgemeinen analytischen Methode. Zahlreiche Veröffentlichungen auf nahezu allen Arbeitsgebieten[3] haben ergeben, dass die D.C. der Papierchromatographie (P.C.) in vielen Fällen überlegen ist.

Zur Bezeichnung der Lage einer Substanz werden in der D.C., genau wie in der P.C. die R_F angegeben. Sie sind von vielen Faktoren[4], u.a. von der Schichtdicke[5] abhängig. Die Trägerschicht soll, einerseits von Platte zu Platte reproduzierbar, anderseits innerhalb ein und derselben Platte einheitlich sein. Beide Bedingungen werden weitgehend erfüllt, wenn man 200 × 200 mm Glasplatten* mit Hilfe des STAHL'schen Streichgerätes[2] bestreicht.

Es wird auch in der neuesten Zeit, in Anlehnung an frühere Arbeiten[1], gelegentlich vorgeschlagen, die Chromatoplatten manuell zu bereiten[7–9]. Solche Schichten sind unseres Erachtens nicht einheitlich und auch nicht von Platte zu Platte reproduzierbar.

Um diese Annahme zu prüfen, führten wir folgende Experimente durch:

(a) 25 g Kieselgel G wurde mit 50 ml Wasser in einem verschlossenen Erlenmeyer-Kolben (200 ml) 30 sec lang sehr kräftig geschüttelt[10] und mit Hilfe des STAHL'schen Streichgerätes[2] auf 200 × 200 mm Glasplatten gebracht (Streichdauer: 4 sec, Schichtdicke: ca. 0.25 mm). Die Platten wurden über Nacht an der Luft getrocknet[4,10].

(b) 5 g Kieselgel G und 15 ml Wasser wurden in einem verschlossenen Erlenmeyer-Kolben (100 ml) 30 sec lang sehr kräftig geschüttelt und auf eine Glasplatte (200 × 200 mm) gegossen. Die Suspension wurde durch Rütteln möglichst gleichmässig über die ganze Platte verteilt. Die bestrichenen Platten liess man über Nacht an der Luft trocknen.

Die Startpunkte lagen sowohl bei (a) als auch bei (b) 1.5 cm vom unteren Plattenrand entfernt, die Entfernung zwischen zei Auftragstellen betrug mindestens 0.8 cm. Als Laufstrecke verwendeten wir bei allen Versuchen 10 cm. Zur Kammersättigung[11] wurden die Kammern mit Filterpapier vollständig ausgekleidet und vor der Einstellung der Platten mehrmals kräftig geschüttelt. Die Entwicklung erfolgte aufsteigend.

* Die Schichtdicke ist innerhalb ein und derselben Platte nicht konstant, wenn man zur Chromatographie 200 × 50 mm oder sogar kleinere Platten bestreicht[6].

ERGEBNISSE

(1) Wir haben zunächst die Reproduzierbarkeit der R_F an *ein und derselben Platte*, welche nach (a) bzw. nach (b) bereitet wurde, untersucht. Aus diesem Grund trugen wir 1 mm³ des Testgemisches[2] vierzehnmal auf Platten nach (a) und (b) auf. Fig. 1 und 2 zeigen, dass die R_F an Platten, welche nach (b) bereitet wurden viel stärker

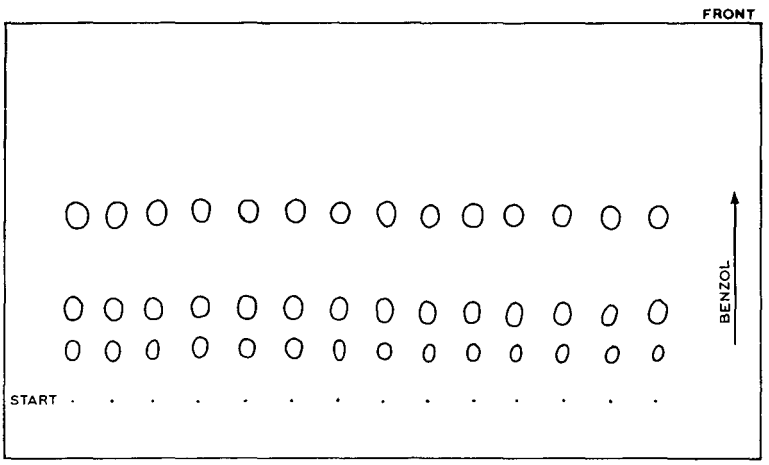

Fig. 1. Dünnschichtchromatogramm von Indophenol, Sudanrot G und Buttergelb (Testgemisch[2]) auf Kieselgel G nach (a) (vgl. Text).

streuen. Es sei bemerkt, dass wir bei Platten, welche nach (b) bereitet wurden, nur diejenige verwendet haben, welche bei Durchsicht einigermassen einheitlich waren. Demgegenüber haben wir bei Platten, welche nach (a) bereitet wurden, keine Auswahl getroffen.

Tabelle I zeigt den statistischen Vergleich zwischen (a) und (b). Wir haben dort

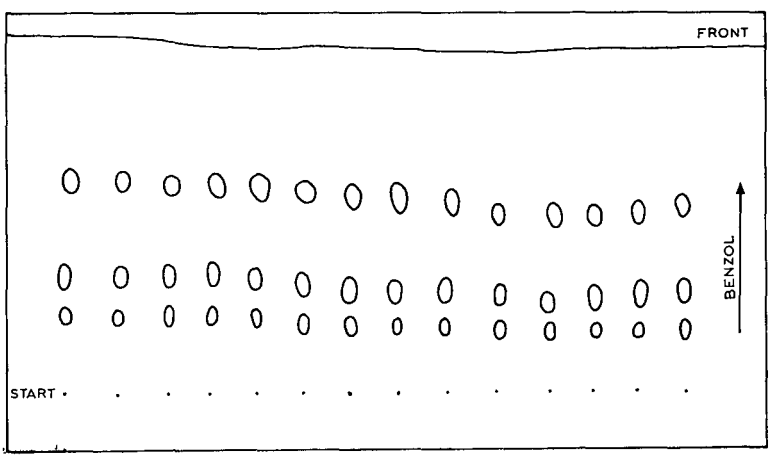

Fig. 2. Dünnschichtchromatogramm von Indophenol, Sudanrot G und Buttergelb (Testgemisch[2]) auf Kieselgel G nach (b) (vgl. Text).

TABELLE I

VERGLEICH DER R_F-STREUUNGEN AUF SCHICHTEN, DIE NACH (a) BZW. (b) BEREITET WURDEN AN EIN UND DERSELBEN PLATTE

(Fliessmittel: Benzol[2])

Verbindung	*(a)*				*(b)*			
	$\bar{R}_F$	n	*SB*	s_{R_F}	$\bar{R}_F$	n	*SB*	s_{R_F}
Buttergelb*	0.494	14	0.49–0.50	0.005	0.610	14	0.58–0.63	0.014
	0.504	14	0.49–0.52	0.008	0.504	14	0.46–0.56	0.033
Sudanrot G*	0.232	14	0.22–0.24	0.006	0.366	14	0.34–0.39	0.014
	0.228	14	0.21–0.25	0.010	0.265	14	0.23–0.30	0.023
Indophenol*	0.143	14	0.14–0.15	0.005	0.258	14	0.22–0.28	0.016
	0.133	14	0.12–0.16	0.012	0.178	14	0.15–0.21	0.018

* Testgemisch[2]; n = Anzahl Bestimmungen; $\bar{R}_F$ = R_F-Mittelwert; SB = Schwankungsbreite der R_F; s_{R_F} = Standardabweichung.

sowohl die Schwankungsbreiten[12] [SB] als auch die Standardabweichungen[4] [s_{R_F}] angegeben.

Bei (a) ist: $0.01 \leqq SB \leqq 0.04$ und $0.005 \leqq s_{R_F} \leqq 0.012$,

bei (b) ist: $0.05 \leqq SB \leqq 0.10$ und $0.014 \leqq s_{R_F} \leqq 0.033$.

(2) Die Reproduzierbarkeit der R_F an *mehreren Platten* (nach (a) bzw. (b)) zeigt die Tabelle II.

Bei (a) ist: $0.02 \leqq SB \leqq 0.07$ und $0.009 \leqq s_{R_F} \leqq 0.020$,

bei (b) ist: $0.07 \leqq SB \leqq 0.11$ und $0.021 \leqq s_{R_F} \leqq 0.041$.

SCHLUSSFOLGERUNGEN

Es gelang uns zu beweisen dass die R_F an manuell bereiteten Schichten grösseren Schwankungen unterworfen sind als jene an mechanisch bereiteten Schichten.

Wir folgern daraus:

(1) Manuell bereitete Chromatoplatten eignen sich nicht für Studien zwischen Struktur und chromatographischem Verhalten[17].

(2) Sie eignen sich wenig für genaue Untersuchungen, z.B. zur Bestimmung von R_F-Werten.

Wir sind auch der Ansicht, dass der Einsatz manueller Chromatoplatten zur Reinheitsprüfungen oder zu orientierenden Versuchen gewisse Gefahren in sich birgt. Dies wird besonders deutlich durch die Fig. 1 und 2 veranschaulicht.

DANK

Den Herren Proff. Dr. R. WIZINGER und Dr. M. BRENNER danken wir für die Förderung dieser Arbeit. Der eine von uns (J.K.) dankt für die Mittel zur Durchführung dieser Arbeit dem Schweizerischen Nationalfond.

TABELLE II

VERGLEICH DER R_F-STREUUNGEN AUF SCHICHTEN DIE NACH (a) BZW. (b) BEREITET WURDEN AN MEHREREN PLATTEN

Verbindung	*(a)*				*(b)*			
	$\bar{R}_F$	*n*	*SB*	s_{R_F}	$\bar{R}_F$	*n*	*SB*	s_{R_F}
Bis-[4-(2,6-diisopropylpyrylo)]-trimethincyanin-perchlorat[13]	0.519[z]	12	0.49–0.55	0.015	0.560[z]	12	0.50–0.61	0.041
Bis-[4-(2,6-di-*tert.*-butyl-pyrylo)]-trimethincyanin-perchlorat[14]	0.598[z]	12	0.58–0.63	0.019	0.572[z]	12	0.52–0.61	0.028
Bis-[4-(2,6-diphenylpyrylo)]-trimethincyanin-perchlorat[15]	0.487[z]	12	0.46–0.53	0.020	0.548[z]	12	0.51–0.61	0.031
Bis-[2-(1,3,3-trimethylindol)]-trimethincyanin-perchlorat[16]	0.492[z]	12	0.48–0.52	0.012	0.562[z]	12	0.51–0.60	0.029
Carbobenzoxy-glycin	0.652[y]	8	0.63–0.67	0.015	0.684[y]	8	0.64–0.72	0.034
Carbobenzoxy-alanin	0.669[y]	8	0.65–0.68	0.011	0.702[y]	8	0.67–0.76	0.032
Carbobenzoxy-norleucin	0.704[y]	8	0.69–0.72	0.010	0.740[y]	8	0.70–0.77	0.026
Carbobenzoxy-norvalin	0.693[y]	8	0.68–0.70	0.009	0.727[y]	8	0.69–0.76	0.021

Fliessmittel: z = Nitromethan[14]; y = Äthanol–Wasser (7:3, v/v)[18].
Bezeichnungen vgl. Tabelle I.

ZUSAMMENFASSUNG

Manuell bereitete Chromatoplatten eignen sich wenig für genaue Untersuchungen. Dies wurde durch statistischen Vergleich der R_F-Schwankungen bei der Standardmethode und bei der manuellen Methode bewiesen. Die Anwendung manuell bereiteter Chromatographierschichten empfiehlt sich auch nicht zu Reinheitsprüfungen oder zu orientierenden Untersuchungen.

SUMMARY

Chromatoplates prepared manually are not very suitable for exact determinations. This was proved by a statistical comparison of the deviations in the R_F values obtained in the standard method and in the manual method. It is also not advisable to use such chromatoplates for purposes of purity control or for informative investigations.

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DÜNNSCHICHTCHROMATOGRAPHIE VON AROMATISCHEN KOHLENWASSERSTOFFEN UND EINIGEN HETEROCYCLISCHEN VERBINDUNGEN

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Die Chromatographie von aromatischen Kohlenwasserstoffen auf imprägnierten und acetylierten Papieren wurde schon vor einigen Jahren beschrieben. So trennten WIELAND UND KRACHT[1] erfolgreich einige höhere Kohlenwasserstoffe auf acetyliertem Papier, das Durchlaufen dauerte jedoch 18–20 Stunden. Im System *n*-Hexan, das mit Dimethylformamid gesättigt war, trennten TARBELL UND HUANG[2] Kohlenwasserstoffe und ihre Derivate. Die Differenzen in R_F-Werten einzelner Kohlenwasserstoffe waren aber sehr gering. Die meisten anderen Arbeiten über die Chromatographie von aromatischen Kohlenwasserstoffen befassten sich nur mit Spezialfällen der einzelnen Kohlenwasserstoffe[3–9].

Bei Isolierung einzelner Stoffe aus höhersiedenden Steinkohlenteerfraktionen wurde das Problem einer schnellen und zugleich einfachen Reinheitskontrolle sehr aktuell. Auf Grund der Erkenntnisse der Säulenchromatographie der aromatischen Kohlenwasserstoffe und der Erfolge der Dünnschichtchromatographie, versuchten wir eine schnelle dünnschichtchromatographische Methode für die Trennung von aromatischen Kohlenwasserstoffen und einigen heterocyclischen Verbindungen auszuarbeiten.

Als Fliessmittel wurden *n*-Hexan, Tetrachlormethan und andere Chlorkohlenwasserstoffe benützt.

Die Detektion wurde mit Tetracyanäthylen-lösung nach TARBELL UND HUANG[2] und PEURIFOY, SLAYMAKER UND NAGER[10] durchgeführt. Erfolgreich wurden die Platten auch mit Formaldehyd–Schwefelsäure nach LE ROSEN, MORAVEK UND CARLTON[11] besprüht oder im U.V.-Licht betrachtet.

EXPERIMENTELLER TEIL

Testsubstanzen

Die Testsubstanzen der Kohlenwasserstoffe wurden in unserem Institut hergestellt und an Hand physikalischer Konstanten, die den Literaturangaben entsprachen, als rein betrachtet.

Adsorbentien

Es wurden benützt:

(1) Aluminiumoxyd für Chromatographie, Erzeugniss der Lachema Brno, ČSN 6851 31, neutral, Aktivität I-II nach Brockmann.

(2) Kieselgel PHH, Erzeugniss der Spolana n.p. Neratovice, durchschnittliches Schüttgewicht 0.5 kg/l, Körnung 0.05–0.15 mm, Maximalsorption von Wasser 97 % (bestimmt nach PITRA[12]), aktiviert durch 4-stündiges Erwärmen auf 120° im Vakuum 10 mm Hg.

Glasplatten

Wir verwendeten Glasplatten 200 × 120 mm, Schichtdicke 0.5 mm. Die Laufzeit war 20–45 min.

Fliessmittel

Die benutzten Fliessmittel waren:

n-Hexan p.a.,
Tetrachlormethan p.a.,
Trichloräthylen, rein,
1,2-Dichloräthan.
Alle Erzeugnisse der Lachema, Brno.

Sprühreagenzien

(1) 10 % Lösung von Tetracyanäthylen in Benzol.

(2) Lösung von 0.2 ml 37 % Formaldehyd in 10 ml konzentrierter Schwefelsäure.

Die Besprühung wurde sofort nach dem Herausnehmen der Platten aus den chromatographischen Kammern durchgeführt.

R_F-Werte

Die R_F-Werte der einzelnen Kohlenwasserstoffe sind durchschnittliche Werte von mindestens drei Einzelmessungen unter Einhaltung der gleichen Bedingungen bei 20° (Tabelle I und II).

Sichtbarmachung

Das Sichtbarmachen von aromatischen Kohlenwasserstoffen und heterocyclischen Verbindungen mit Tetracyanäthylen ist uns nur auf den Kieselgelschichten gelungen, auf den Aluminiumoxydschichten erhielten wir nur Gelbfärbung des Untergrundes. Mit Tetracyanäthylen färben sich beinahe alle Kohlenwasserstoffe und heterocyclische Verbindungen, die wir untersuchten. Die Färbung ist nicht mit Anthracen, 9,9'-Dianthryl zu erzielen und weiter wahrscheinlich mit den Verbindungen, die sehr schnell nach Diels-Alder zu farblosen Produkten reagieren. Die Färbung ist je nach dem Stoff intensiv bis sehr schwach, der Farbton ändert sich nach kurzer Zeit und verschwindet sehr oft.

Nach unseren Erfahrungen ist das Sichtbarmachen mit Formaldehyd–Schwefelsäure viel empfindlicher als mit Tetracyanäthylen. So ist es z.B. möglich durch Besprühen mit diesem Reagent Fluoren noch in einer Menge von 1 γ, Naphthalin in einer Menge von 4 γ, nachzuweisen. Die Farbtöne sind hier auch nicht von Dauer. Als Nachteil der Formaldehyd–Schwefelsäure muss die Ätzwirkung und Reizung der Atmungsorgane betrachtet werden. Es zeigte sich, dass auch *n*-Hexan mit diesem Reagent eine rote Färbung gibt. Mit Ausnahme von Chinolin färben sich alle untersuchten Stoffe.

Eine befriedigende Sichtbarmachung mittels U.V.-Licht kann erreicht werden, wenn die Stoffe genügend fluoreszieren. Die Verbindungen, die stark im benützten

TABELLE I

DIE DETEKTION DER VERBINDUNGEN AUF DÜNNSCHICHTEN

Stoff	*Die Färbung der Flecke bei der Detektion*		
	Tetracyanathylen	*Formaldehyd–H_2SO_4*	*U.V.-Licht*
Durol	ziegelrot	rotviolett	—
Naphthalin	violett	blau	schwach violett
1-Methylnaphthalin	gräulich-blau	gräulich-blau	violett
2-Methylnaphthalin	gräulich-blau	gräulich-blau	violett
1,5-Dimethylnaphthalin	grün	gräulich-blau	violett
1,6-Dimethylnaphthalin	gräulich-blau	blau	violett
2,6-Dimethylnaphthalin	hellgrau	blaugrün	violett
2,3-Dimethylnaphthalin	grau	blau	violett
2,7-Dimethylnaphthalin	grau	gräulich-blau	—
Inden	violett	rotviolett	—
Diphenyl	orange	blau	—
Acenaphthen	grün	gräulich-grün	—
Fluoren	hellbraun	blaugrün	—
Anthracen	färbt sich nicht	gelbgrün	blauviolett
Phenanthren	braun	grün	—
Fluoranthen	violett	dunkelgrün	grün
Pyren	braun	braun	hellgrün
Chrysen	blau	dunkelblau	violett
3:4-Benzpyren	hellbraun	hellgrau	blau
9,9'-Dianthryl	färbt sich nicht	rotbraun	grün
Benzo[*b*]thiophen	violett	hellblau	—
Dibenzothiophen	violett	blau	—
Indol	rotbraun	hellviolett	—
Carbazol	blau	dunkelgrün	hellgrün
Chinolin	gelb	färbt sich nicht	—
Diphenylenoxyd	braun	blauviolett	—
Diphenyloxyd	grün	gräulich-grün	blau
p-Cresol	braun	—	—
3,5-Dimethylphenol	rotbraun	—	—
Tetrahydronaphthalin	—	braun	—

U.V.-Bereich absorbieren, kann mann als dunkle Flecke auf schwach fluoreszierendem Untergrund auffinden, wenn auch diese Weise weniger empfindlich ist.

Anwendungsbeispiele

(1) Es wurde eine Reinheitskontrolle bei "reinem" 2,3-Dimethylnaphthalin durchgeführt. Dabei stellte sich heraus, dass diese Substanz noch zwei andere Stoffe in kleiner Menge enthält (Fig. 1).

(2) Bei der chromatographischen Analyse des flüssigen Anteiles einer schmalen Fraktion des Anthracenöls, wurden sechs Stoffe aufgefunden (Fig. 2), einer von ihnen wurde als Fluoren identifiziert (Fluoren kann in der Form des Fluorenonoxims beseitigt werden).

TABELLE II

R_F-WERTE DER AROMATISCHEN KOHLENWASSERSTOFFE UND HETEROCYCLEN

Stoff	*Kieselgel*		*Al_2O_3*	
	n-Hexan	*CCl_4*	*n-Hexan*	*CCl_4*
Durol	0.62	0.76	—	0.85
Naphthalin	0.59	0.77	0.63	0.85
1-Methylnaphthalin	0.54	0.74	0.55	0.78
2-Methylnaphthalin	0.50	0.87	0.52	0.85
1,5-Dimethylnaphthalin	0.50	0.72	0.46	0.85
1,6-Dimethylnaphthalin	0.54	0.75	0.46	0.83
2,6-Dimethylnaphthalin	0.50	0.72	0.52	0.85
2,3-Dimethylnaphthalin	0.48	0.75	0.39	0.81
2,7-Dimethylnaphthalin	0.53	—	0.42	0.80
Inden	0.55	0.84	0.67	0.81
Diphenyl	0.45	0.74	0.58	0.79
Acenaphthen	0.44	0.83	0.44	0.70
Fluoren	0.32	0.66	0.35	0.73
Anthracen	0.37	0.65	0.35	0.59
Phenanthren	0.33	0.65	0.20	0.66
Fluoranthen	0.29	0.66	0.10	0.55
Pyren	0.32	0.64	0.10	0.65
Chrysen	auf dem Start	0.11	auf dem Start	auf dem Start
3:4-Benzpyren	0.17	0.65	auf dem Start	0.10
9,9'-Dianthryl	0.10	0.58	auf dem Start	0.06
Benzo[*b*]thiophen	0.53	0.73	0.50	0.83
Dibenzothiophen	0.42	0.70	0.18	0.67
Indol	auf dem Start	0.12	auf dem Start	0.05
Carbazol	auf dem Start	0.15	auf dem Start	0.05
Chinolin	auf dem Start	auf dem Start	auf dem Start	auf dem Start
Diphenylenoxyd	0.39	0.73	0.11	0.73
Diphenyloxyd	0.47	0.75	0.35	0.62
p-Cresol	auf dem Start	0.12	auf dem Start	—
3,5-Dimethylphenol	auf dem Start	0.07	auf dem Start	—
Tetrahydronaphthalin	—	—	—	0.75

(3) Sehr interessant erwies sich die Dünnschichtchromatographie des Acetonextraktes vom Russ Typ HAF (Fig. 3). Es wurden sieben Stoffe nachgewiesen, mittels erreichbarer Testsubstanzen wurden drei von ihnen als Pyren, Fluoranthen und 3:4-Benzpyren identifiziert.

In allen drei Beispielen ging es um schnell durchführbare informative Analysen,

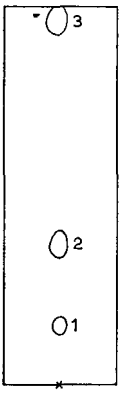

Fig. 1. Reinheitskontrolle des 2,3-Dimethylnaphthalins im System Al_2O_3/Trichloräthylen. Die Detektion unter U.V.-Licht: (1) blau, $R_F = 0.16$; (2) dunkel, $R_F = 0.37$ (Absorption); (3) violett, $R_F = 0.96$ (2,3-Dimethylnaphthalin).

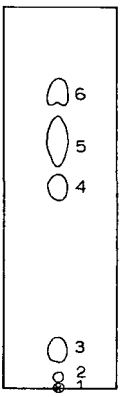

Fig. 2. Analyse einer Fraktion (306–310°) des Anthracenöles nach Beseitigung des kristallinischen Anteils im System Kieselgel/CCl_4. Detektion mit Formaldehyd–Schwefelsäure: (1) dunkelblau (auf dem Start); (2) braun, $R_F = 0.03$; (3) gelb, $R_F = 0.10$; (4) gelbgrün, $R_F = 0.53$; (5) blaugrün, $R_F = 0.65$ (Fluoren); (6) gräulich-grün, $R_F = 0.78$.

welche als Ergänzung zu den Resultaten anderer analytischer Methoden durchgeführt wurden.

Für präparative Zwecke bewährte es sich, grössere Platten mit Schichtdicke von 1 mm zu benützen, so dass die Stoffgemische in einer Menge von 50–100 mg ohne Schwierigkeiten getrennt werden konnten. Die getrennten Zonen wurden durch Abkratzen von der Platte in ein Glasröhrchen eingetragen und mit Chloroform oder Benzol eluiert. In einigen Fällen wurde gute Trennung durch zweidimensionale Chromatogramme auf Viereckplatten erzielt.

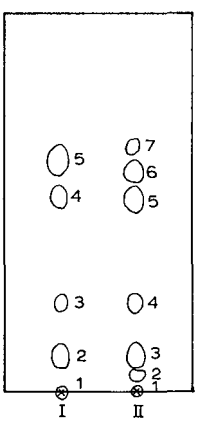

Fig. 3. Analyse des Acetonextraktes vom Russ im System Al_2O_3/CCl_4 (es handelt sich um einen Ofenruss aus Anthracenöl—Typ HAF nach der amerikanischen Klassifizierung). I. Detektion unter U.V.-Licht: (1) dunkel (Absorption); (2) blau; (3) hellblau; (4) grün; (5) dunkelviolett. II. Detektion mit Formaldehyd–Schwefelsäure: (1) braun, auf dem Start; (2) blau, $R_F = 0.05$; (3) hellblau, $R_F = 0.10$ (3:4-Benzpyren); (4) grau, $R_F = 0.24$; (5) grün, $R_F = 0.52$ (Fluoranthen); (6) braun, $R_F = 0.58$ (Pyren); (7) blau, $R_F = 0.65$.

DISKUSSION

Nach Angaben von KISELEV[13] und PITRA[14] sowie von STAHL[15] beeinflussen die Adsorptionsaffinität von Stoffen zu Kieselgel oder Aluminiumoxyd:

(1) Wasserstoffsbrücken zwischen Adsorbent und dem Stoff,

(2) Dispersionskräfte (Entstehung von Komplexen),

(3) Geometrische Eigenschaften des Stoffes sowie des Adsorbenten.

Die R_F-Werte der untersuchten Stoffe stimmen damit gut überein. Die grösste Adsorptionsaffinität haben Stoffe, die Wasserstoffbrücken bilden können (Indol, Carbazol, Chinolin, Phenole), was ja allgemein schon bekannt ist. Sehr interessant ist, dass Diphenylenoxyd und Diphenyloxyd schon schwächer adsorbiert werden und schwefelhaltige heterocyclische Verbindungen eine relativ kleine Adsorptionsaffinität aufweisen. Bei Kohlenwasserstoffen wächst die Adsorptionsaffinität mit dem Anwachsen von Ringen. Beim Eintreten von Methylgruppen in die Molekel wird die Adsorption stärker, was nach STAHL[15] zu erwarten ist, in unserem Fall beweisen es die R_F-Werte des Naphthalins und seiner Mono- und Dimethylhomologe.

Nach den erhaltenen R_F-Werten der geprüften Stoffe in verschiedenen Laufmitteln ist es möglich die folgende Lösungsmittelreihe aufzustellen: *n*-Hexan–Tetrachlormethan–Trichloräthylen–Dichloräthan. Zur Trennung von Stoffen bei welchen niedrige R_F-Werte erwartet werden, können die letzten zwei Fliessmittel gebraucht werden, für hohe R_F-Werte die ersten.

Ein Nachteil dieser schnellen Methode ist, dass die R_F-Werte sehr abhängig von Änderungen der Arbeitsbedingungen und Adsorbentengüte sind. Der Vergleich mit Testsubstanzen ist deshalb zu empfehlen.

ZUSAMMENFASSUNG

Es ist möglich aromatische Kohlenwasserstoffe und heterocyclische Verbindungen in Gemischen mittels Dünnschichtchromatographie auf Kieselgel oder Aluminiumoxyd zu trennen. Als Laufmittel dienen *n*-Hexan, Tetrachlormethan, Trichloräthylen und Dichloräthan. Die Sichtbarmachung erfolgt durch Besprühen der Platten mit Formaldehyd–Schwefelsäure, mit 10%iger Tetracyanäthylenlösung oder durch Betrachten unter U.V.-Licht. Die R_F-Werte von 30 Verbindungen sind in Tabellen zusammengefasst und drei Beispiele von Analysen komplizierterer Gemische aus der Praxis der Steinkohlenteerverarbeitung beschrieben.

SUMMARY

The separation of mixtures of aromatic hydrocarbons and heterocyclic compounds was carried out on thin layers of silica gel or alumina. The following solvents were used: *n*-hexane, carbon tetrachloride, trichloroethylene and dichloroethane. Detection was carried out by spraying with formaldehyde–sulphuric acid, with tetracyanoethylene solutions and by examination in U.V. light. The R_F values of 30 compounds are listed in tables, and three chromatograms of more complicated mixtures met with in coal-tar analysis are given as illustration of the method.

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PAPIERCHROMATOGRAPHIE DER TETRACYCLINSTOFFE

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Analytische Arbeiten, die sich mit der Papierchromatographie der Tetracycline beschäftigen, beschränken sich auf die Trennung der Tetracycline, die in der allgemeinen klinischen Praxis verwendet werden (Chlortetracyclin, Tetracyclin, Oxytetracyclin). Die Autoren wenden entweder das System: Butanol–Essigsäure–Wasser in verschiedenen Verhältnissen[1–3] oder andere, manchmal ziemlich komplizierte Gemische als Entwicklungssysteme an[4–6]. Arbeiten, die sich mit dem Studium der Struktur der Tetracyclinstoffe beschäftigen, beschreiben dagegen eine Reihe von Systemen, in welchen sich die Grundstoffe von ihren Epimeren trennen[7–10] und Demethyl- und Dehydroderivate unterschieden werden können.

Von diesen ist für rasche Orientierungsarbeiten die Methode von SELZER[8] besonders geeignet, die mit dem System Chloroform–Nitromethan–Pyridin (10:20:3) arbeitet und das Rundpapier mit McIlvaine's Puffer befeuchtet.

Wir haben ein anderes System gesucht, das dieselbe Trennfähigkeit aufweisen würde und aus solchen Lösungsmitteln besteht, die allgemein zur Verfügung stehen. Wir hatten eine Schnellmethode auszuarbeiten, welche für die Analyse der kristallinischen Stoffe ebenso wie für Filtrate der Gärlösungen benutzt werden könnte.

Unseren Ansprüchen entsprach das zweiphasige System: McIlvaine's Pufferlösung pH 4.5/Chloroform–Butanol (4:1) am besten. Das System haben wir bei dem von MISTRETTA[11] beschriebenen Verteilungsverfahren der Tetracycline in Anwendung gebracht.

Wie nach den bei der Verteilung der Tetracycline gewonnenen Erfahrungen vorauszusetzen war, hat sich das angeführte System besonders bei der Chromatographie des Gemisches von Chlortetracyclin, Tetracyclin und ihrer Demethylderivate bewährt. Für diese Stoffe geben wir auch die R_F-Werte an.

EXPERIMENTELLES

Chemikalien und Geräte

Frisch destilliertes Chloroform und *n*-Butanol
0.1 *M* Citronensäurelösung p.a.
0.2 *M* sekundäres Natriumphosphat p.a.
Wässerige Ammoniaklösung konz.
Rundpapier Whatman No. 1, Durchmesser 28 cm, oder 45 cm lange Streifen
Chromatographiekammern
U.V.-Strahlungsquelle: Phillora HPW 125 W, Typ 57202 E/70.

Arbeitsweise

Chloroform und *n*-Butanol mischen wir im Verhältniss 4:1 (Entwicklungsphase). Citronensäurelösung und sekundäres Natriumphosphat werden im Verhältniss 10.92: 9.08 gemischt und auf pH-Wert 4.5 eingestellt (wässerige Phase). Beide Phasen sättigt man gegenseitig durch 12-stündiges Rühren. Dann füllt man beide Phasen getrennt in die Chromatographiekammern und lässt diese vollkommen mit den Dämpfen sättigen. Auf dem Rundpapier Whatman No. 1 bezeichnet man, 1 cm von der Mitte entfernt, den Start und trägt 6 Proben von je 10 μl der Lösung in einer Konzentration von 500–1000 γ des Antibioticums per ml auf. Unmittelbar vor der Entwicklung bespritzt man das Chromatographiepapier mit McIlvaine's Pufferlösung pH 4.5 (die mit der Entwicklungsphase gesättigt wurde) so, dass es gleichmässig befeuchtet ist und stellt es gleich in die Chromatographiekammer ein. Die Entwicklung dauert ca. 90 Minuten. Die Front wandert 12 cm vom Start (Fig. 1). Das entwickelte getrocknete Chromatogramm sättigt man mit Ammoniakdämpfen und führt die Detektion der gelb fluores-

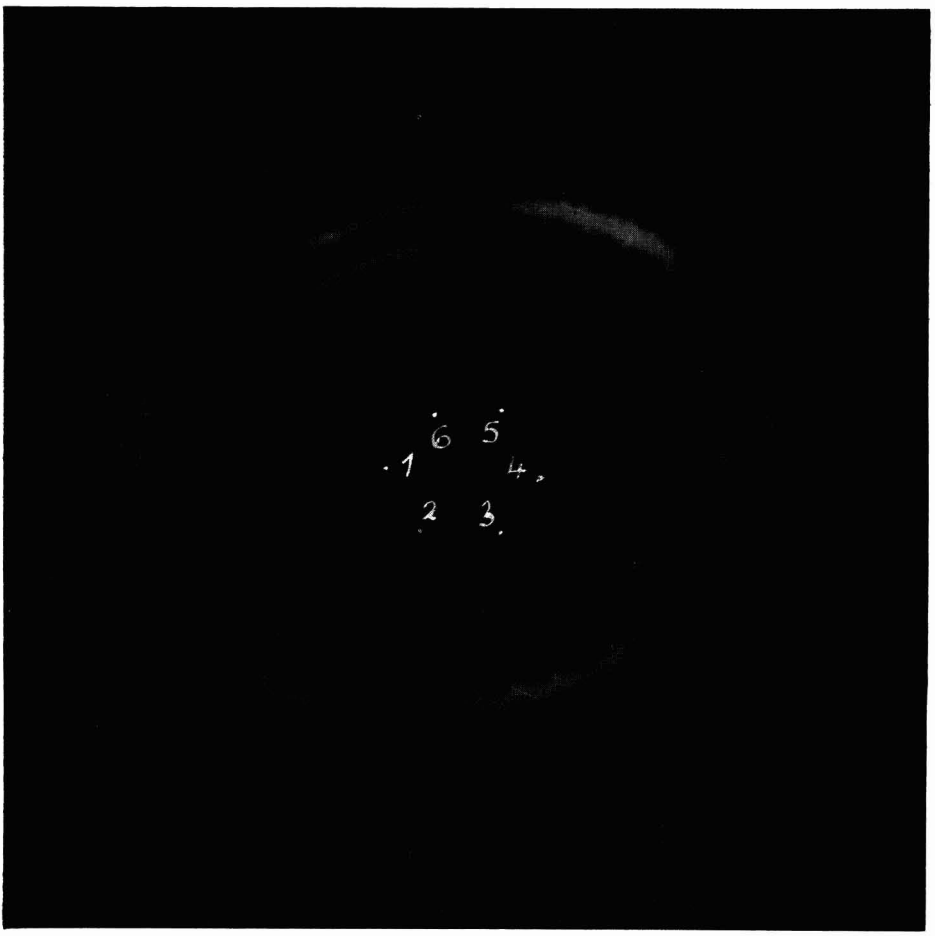

Fig. 1. Chromatogramm am Rundpapier. 1 = Chlortetracyclin; 3 = Tetracyclin; 5 = Demethylchlortetracyclin; 2,4,6 = Gemisch aller Tetracycline.

zierenden Flecken der Tetracyclinstoffe mittels U.V.-Licht durch. Bei aufsteigender Entwicklung folgt man dieselbe Arbeitsweise. Die Entwicklung dauert aber 24 Stunden. Die Front wandert hier 34 cm vom Start (Fig 2).

ERGEBNISSE

Die Rundpapiermethode ist besonders deswegen vorteilhaft, weil sie die Möglichkeit rascher Orientation über die Zusammensetzung des Tetracyclingemisches bietet. Abgesehen von der für die aufsteigende Entwicklung des Chromatogramms benötigten längeren Zeit ergibt dieses Verfahren gleichwertige Resultate. Bei der absteigenden Entwicklung bilden sich regelmässig "Schwänze".

Die R_F-Werte einzelner Stoffe dieser Gruppe sind soweit verschieden (siehe Tabelle I), dass sich die Stoffe voneinander vollkommen trennen mit Ausnahme der Epimere, die sich zwar von ihren Grundstoffen, nicht aber voneinander trennen.

Wenn man mit reinen Substanzen arbeitet, stellt man in der Regel Flecke fest, die einer Menge von 5–10 γ entsprechen. Die Grenze der Empfindlichkeit dieser

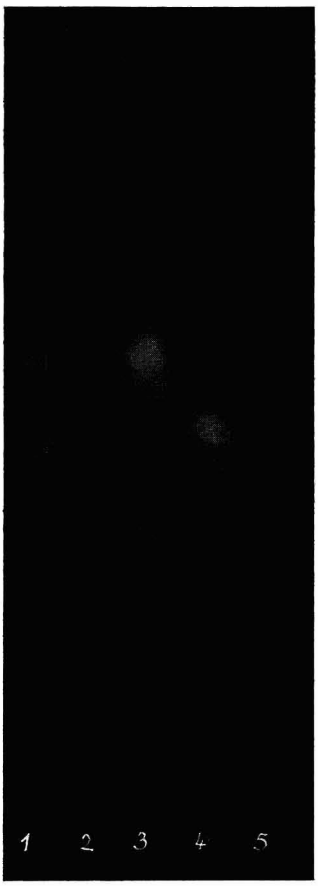

Fig. 2. Aufsteigende Entwicklung des Chromatogramms. 2 = Chlortetracyclin; 3 = Demethylchlortetracyclin; 4 = Tetracyclin; 1,5 = Gemisch aller Tetracycline.

Methode ist jedoch weit niedriger. Man kann Zonen, die einer Menge von 0.5 γ der Substanz entsprechen, noch verlässlich nachweisen, mit einiger praktischen Erfahrung kann man 0.2 γ der Substanz noch wahrnehmen. Die Empfindlichkeit

TABELLE I

	R_F-Werte
Chlortetracyclin	0.695–0.727
Demethylchlortetracyclin	0.561–0.597
Tetracyclin	0.47 –0.50
Demethyltetracyclin	0.38 –0.414
Epimere	0.27 –0.30
X*	0.24 –0.27

* Der Stoff X hat im U.V.-Licht die Eigenschaften der Tetracycline und kann durch Einwirkung von Chlorwasserstoffsäure in das Anhydroderivat überführt werden. Seine genaue chemische Zusammensetzung ist uns jedoch unbekannt.

wird selbst durch Begleitstoffe, die in der Gärlösung enthalten sind, nicht beeinflusst. In einem Tetracyclingemisch wird eine Komponente, die 5 % der Tetracycline ausmacht auch bei einer Gesamtmenge aller Tetracycline von 10 γ noch verlässlich nachgewiesen.

ZUSAMMENFASSUNG

Wir haben eine Methode der Papierchromatographie der Tetracyclinstoffe im zweiphasigen System: McIlvaine's Pufferlösung pH 4.5/Chloroform–*n*-Butanol (4:1) beschrieben. Am Rundpapier findet im Laufe von 90 Minuten die vollkommene Trennung des Chlortetracyclins und Tetracyclins von den Demethylanalogen und Epimeren statt. Mittels Fluoreszenzdetektion können noch 0.5–0.2 γ der Stoffe wahrgenommen werden.

SUMMARY

A description is given of a method for the paper chromatography of tetracycline antibiotics, using the two-phase system McIlvaine's pH 4.5 buffer/chloroform–*n*-butanol (4:1). With circular development, it is possible to obtain a complete separation of chlorotetracycline and tetracycline from their demethyl-analogues and epimers within 90 minutes. By observing the fluorescence, it is possible to detect as little as 0.5–0.2 γ of the substances.

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"MOLECULAR-SIEVE" ELECTROPHORESIS IN CROSS-LINKED POLYACRYLAMIDE GELS

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INTRODUCTION

Electrophoresis of proteins in starch gels according to the method developed by SMITHIES[1] shows in general a much higher resolution than electrophoresis in media like agar gel and paper. Probably, this is in part due to the fact that the dimensions of proteins are of the same order of magnitude as the pores in the starch gel. The proteins will therefore be more or less restricted in their migration through such a gel. The possibility of utilizing this molecular sieving action of the stabilizing medium was first pointed out by SYNGE AND TISELIUS[2], when separating uncharged molecules by forcing them through an agar gel with the aid of electroendosmosis.

As a complement to starch RAYMOND AND WEINTRAUB[3] and DAVIS AND ORNSTEIN[4] introduced a synthetic polymer, cross-linked polyacrylamide, as a stabilizing agent. Unaware of these papers and before they were published the author noticed the high resolving power of gels of polyacrylamide when he was searching for a chemically better defined gel than starch and one with a pore size that could be easily adapted to the sizes of the substances to be separated*.

It is now three years since the first papers[3,4] on electrophoresis in polyacrylamide gels appeared. It is therefore surprising that the potentialities of molecular sieving observed by the author, which the flexibility of the polyacrylamide gel offers, have not been more extensively pointed out and utilized. We, therefore, now wish to publish some earlier performed experiments which clearly indicate these potentialities. Besides showing that the migration velocities decrease with increasing concentration of the gel, these experiments indicate that the relative decrease in velocity is not the same for all proteins, but, as expected, is most pronounced for those of higher molecular weight. Similar experiments have recently been published by RAYMOND AND WANG[5], but as they used hemoglobins of the same molecular weight, no effect of the gel concentration was observed.

EXPERIMENTAL

Materials

R-Phycoerythrin and R-phycocyanin were extracted as described by KYLIN[6], only small modifications of the method being introduced[7]. The separation of phycoerythrin and phycocyanin was performed by electrophoresis in agarose suspensions[8,9].

* Dr. R. MOSBACH of this Institute proposed the use of polyacrylamide when consulted on gels suitable for "molecular-sieve" electrophoresis.

Human carboxyhemoglobin was prepared according to the method of ADAIR AND ADAIR[10].

Bovine serum albumin was an Armour product, containing both monomer and dimer molecules.

Preparation of the gel

Polymerization was performed in the buffer to be used for the electrophoresis, exactly as described in ref. 11. The quantities total concentration T and cross-linking concentration C, which are used in the following, are also defined in ref. 11.

Investigation of the relationship between gel concentration, migration velocity and molecular weight

The column tubes were ordinary glass tubes with an inner diameter of 0.6 cm and a length of 25 cm. They were closed with the aid of thin dialysis membranes moistened with buffer and fixed to the glass tube by means of rubber bands. The deaerated solution of acrylamide and N,N'-methylene-bisacrylamide containing the catalyst system was poured into the vertical column tube. Three ml of petroleum ether was layered above the monomer solution. After some minutes the polymerization was completed; the petroleum ether was then removed and replaced by buffer. The petroleum ether prevents air — which inhibits the polymerization—from entering the monomer solution. Furthermore, a sharp boundary is obtained between the gel and the buffer above, which is of importance in obtaining a narrow starting zone. The sample was applied by layering under buffer[11]. The lower end of the column tube was dipped into the anode vessel, and the upper end was connected to the cathode vessel by means of a piece of polyvinyl chloride tubing filled with buffer. To avoid deformation of the applied sample zone by heat convection, the current was kept at a comparatively low value (about 2 mA) during the migration of the sample into the gel. The current was then increased to about 5 mA.

Each sample applied contained two colored proteins, A and B. B consisted of R-phycoerythrin in all experiments. The distances these two proteins had migrated

TABLE I

THE INFLUENCE OF THE TOTAL CONCENTRATION T OF THE GEL UPON THE RELATIVE MIGRATION VELOCITIES OF PROTEINS OF DIFFERENT MOLECULAR WEIGHTS

The migration velocities are measured against R-phycoerythrin (mol. wt. 290,000).

Buffer	*Protein*	*Molecular weight*	*Total conc. of the gel*	*Relative migration velocity* (v_A/v_B)
0.05 *M* sodium acetate buffer, pH 5.4	R-Phycocyanin	270,000	2.5	0.48
			6.0	0.50
			10	0.48
0.02 *M* sodium phosphate buffer, pH 7.3	R-Phycocyanin	135,000	2.5	0.53
			6.0	0.67
			10.0	1.1
Sodium veronal buffer, pH 8.6, $\Gamma/2 = 0.05$	Human carboxy-hemoglobin	68,000	2.5	0.48
			6.0	0.77
			10.0	1.4

were measured at different times and the ratio between them calculated. This ratio represents the migration velocity of the protein A, relative to that of R-phycoerythrin (v_A/v_B). These measurements were carried out at three different total concentrations, T, of the gel. The cross-linking concentration was the same in all experiments and equal to 5 %. The proteins used, their molecular weights, the buffer and the total concentration of the gel are listed in Table I, in which the calculated relative migration velocities are also given. In Fig. 1 these velocities are plotted against the total concentration T of the gel. No correction was made for electroendosmosis.

It should be pointed out that during electrophoresis in the 10 % gels, phycoerythrin was split into two zones. Judging from the color intensity, the concentration of the fastest moving component was 10–15 % of that of the slower component. The migration velocities given in Table I are related to the latter component. Examination of the phycoerythrin solution in the ultracentrifuge revealed the presence of two components, the smallest of them being present in a concentration approximately equal to that of the fastest electrophoresis component. The heterogeneity of R-phycoerythrin has earlier been observed during chromatography on calcium phosphate columns[12,7].

Separation of monomer and dimer of bovine serum albumin

The column was made of plexiglass and was of a type very similar to that used by PORATH, LINDNER AND JERSTEDT[9,13] and VARGAS, TAYLOR AND RANDLE[14] for migration-elution electrophoresis. The electrophoresis tube had an inner diameter of 1 cm and the water-cooled part a length of 30 cm. A plexiglass tube with an inner diameter of 1 mm was inserted into the electrophoresis column, 1.6 cm from the lower end of the column and glued into place. To this tube a piece of polyethylene tubing was attached, the outlet of which was placed above a fraction collector. The lower end of the electrophoresis tube was supplied with a disc of vyon*. The column was packed with Pevikon[15], a copolymer of vinyl chloride and vinyl acetate, to a height of 2 cm. This packing was performed in buffer to which some sucrose had been added in order to increase the density of the buffer. The electrophoresis tube was then closed with a dialysis membrane fastened by means of rubber bands. The solution of acrylamide and N,N'-methylene-bisacrylamide together with the catalyst system was carefully layered above the Pevikon column. Application of the petroleum ether** and layering of the sample was performed as in the preceding experiment. Owing to the higher density of the buffer in the Pevikon column, due to the presence of sucrose, a well-defined boundary was formed between this column and the gel; furthermore, the risk that polymerization would take place inside the Pevikon column was eliminated. The polyacrylamide gel had the composition $T = 6\,\%$, $C = 5\,\%$; the polymerization was performed in sodium borate buffer, pH 8.9, $I/2 = 0.03$. The length of the gel column was 22 cm. About 0.25 ml of a 2 % protein solution, containing monomers and dimers of bovine serum albumin, was layered under the buffer. The dialysis membrane was removed before the voltage was applied. After the sample had entered the gel, the current was increased to 22 mA (from 8 mA). The elution rate was adjusted to 1 ml/h.

* A porous plastic material[9], available from Porous Plastics Limited, Dagenham Dock, Essex, England.

** In many cases the petroleum ether can with advantage be replaced by deacerated distilled water[16].

After about 50 hours the experiment was stopped. The protein contents of the collected 0.5 ml fractions were determined by absorption measurements in a 1-cm cell. Owing to the elution procedure, the protein concentrations in the collected fractions are very low, and we chose, therefore, to carry out these measurements at 230 mμ, where the absorption coefficient for proteins is much greater than at 280 mμ. The electropherogram is given in Fig. 2. The ultraviolet-absorbing fractions in front of the albumin peaks are due to impurities and non-polymerized material etc. Analysis by ultracentrifugation showed that peak I contained the monomers and peak II the dimers.

DISCUSSION

Curve I in Fig. 1 shows that at pH 5.4, where R-phycoerythrin and R-phycocyanin have about the same molecular weight (290,000 and 270,000 respectively), changes in gel concentration, *i.e.* the pore size of the gel, have no observable influence upon the

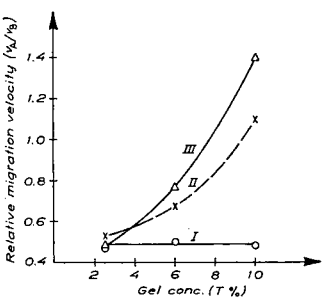

Fig. 1. The influence of the total concentration T of the gel upon the relative migration velocities of proteins of different molecular size. Curve I represents whole molecules of R-phycocyanin (mol. wt. 270,000), curve II dissociated molecules of R-phycocyanin (mol. wt. 135,000), and curve III human carboxyhemoglobin (mol. wt. 68,000). The migration velocities are measured against R-phycoerythrin (mol. wt. 290,000).

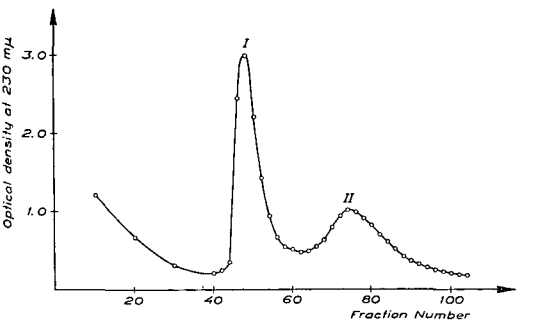

Fig. 2. Separation of monomers (I) and dimers(II) of bovine serum albumin on a gel of cross-linked polyacrylamide. The zones were continuously eluted during the electrophoresis.

relative migration velocities of these proteins. However, at pH 7.4, R-phycocyanin is dissociated into half molecules and the slope of curve II (Fig. 1) indicates that these have a lower value for the "friction coefficient" than the undissociated R-phycoerythrin molecules and even migrate faster than the latter at high gel concentrations ($v_A/v_B > 1$). For carboxyhemoglobin, which has a lower molecular weight (68,000) than dissociated R-phycocyanin, this effect is still more pronounced as curve III is steeper than curve II.

From the above one can expect a high resolution if the starting material is first purified by electrophoresis in a bed completely devoid of "molecular sieving" properties, such as cellulose powder or agarose suspensions. The fractions containing the material to be isolated, are then rerun on a polyacrylamide gel of suitable composition;

if these fractions consist of substances of different molecular size, they can in many cases be resolved. With "molecular-sieve" electrophoresis performed in this way, one can thus achieve separations similar to those obtainable with "molecular-sieve" chromatography[11]. The experiment corresponding to Fig. 2 may serve as an example of this.

Too high a gel concentration should be avoided, since the migration velocities of all proteins decrease as the concentration of the gel is increased.

ACKNOWLEDGEMENTS

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SUMMARY

The migration velocity of a protein in cross-linked polyacrylamide gels is dependent not only on the charge which the protein carries, but also to a large extent on the molecular size of the protein. The latter factor is so pronounced that reversal of the migration velocities of two proteins of different molecular weights can often be accomplished by mere changes in the gel concentration, *i.e.* the pore size. If this "molecular-sieve" electrophoresis is combined with electrophoresis in a medium without molecular sieving properties a high resolution can be expected. "Molecular-sieve" electrophoresis, which can be used both for analytical and small scale preparative purposes, can in some cases give separations similar to those obtained in "molecular-sieve" chromatography.

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RAPID SEPARATION OF SERUM MUCOPROTEINS FROM OTHER FOLIN–CIOCALTEU-POSITIVE SUBSTANCES BY MEANS OF GEL FILTRATION

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The existing methods for isolating mucoproteins from human blood are rather time-consuming and too drastic. These mucoproteins are usually prepared by fractional salting out with ammonium sulphate according to WEIMER *et al.*[1]. For analytical purposes mucoproteins can be isolated from an acid serum filtrate by means of an acid solution of phosphotungstic acid[2]; the denatured mucoprotein must then be dissolved in sodium hydroxide. For the electrophoretic separation of blood mucoproteins into fractions MP-1, MP-2 and MP-3, the filtrate must be dialysed for a long time (especially sulphosalicylic acid is very difficult to remove), and then concentrated from a large volume by freeze-drying[3,4]. During the dialysis considerable losses of mucoproteins occur[5].

The use of gel filtration with Sephadex permits a simple and less drastic isolation of blood mucoproteins. So far Sephadex, has been used for this purpose only by KOČENT[6], who separated sulphosalicylic acid serum filtrates after lyophilisation, and observed a perfect separation from the acid.

METHODS

Preparation of serum filtrates

Human blood sera were deproteinized with perchloric acid as follows: 4 ml of serum was dissolved in 15.2 ml distilled water and 12.8 ml 0.75 *M* perchloric acid was added slowly, under continual mixing. After 10 min the mixture was filtered through Whatman No. 1 filter paper.

In a number of experiments the filtrate was dialysed in a cellophane bag in running water (electrodialysis is not recommended in this case—see ref.[5]). The dialysate was then concentrated by vacuum distillation below 45°.

In the other experiments the filtrates were concentrated by dialysing against a cellophane bag containing a 30 % aqueous dextran solution, which was exchanged at 8 h intervals.

Packing of the columns

8.5 g of dry Sephadex G-25 medium, 100–250 mesh (Pharmacia, Sweden) was stirred in a dilute salt solution, the suspension was packed into a tube and the column (total volume 36.9 ml) was then washed with water. 3–15 ml of the blood filtrate (concen-

trated or non-concentrated) were then slowly pipetted onto the top of the bed. As soon as the sample had entered the column, the latter was eluted with a large quantity of distilled water. It was not necessary to regulate the flow rate, which varied in the range from 28.8 to 49.2 ml/h. Distilled water was also used for the regeneration of the column. All the curves relating to Sephadex G-25 mentioned in this paper were obtained with the same column.

A column of Sephadex G-200 (140–400 mesh) was prepared in an analogous manner. For the preparation of a column with a total volume of 47.7 ml, 1.8 g of dry Sephadex was necessary. The flow rate was very low: 3.9 ml/h.

Analysis of the eluate

The effluent was collected in 3 ml amounts, 2 ml of which was used for the determination of Folin–Ciocalteu-positive substances as follows: 2 ml of a 20 % sodium carbonate solution and 0.2 ml of Folin–Ciocalteu reagent (diluted 1:3) were added, and after 30 min the solution was measured in a 1 cm cell at 610 mμ against a blank. In the remaining 1 ml of the effluent the acidity was determined by titration with 0.1 *N* sodium hydroxide (using phenolphthalein).

Determination of the amount of protein-bound hexoses in the filtrate

For the determination an orcinol reaction was used. The orcinol reagent was prepared according to STARY *et al.*[7] and standardized against a glucose solution; the reaction was performed according to SÖRENSEN AND HAUGAARD[8]. In the first place, the total hexose concentration of the filtrate was determined. Then the mucoproteins (or mucopeptides) were precipitated with an acid solution of phosphotungstic acid, the precipitate redissolved, and the protein-bound hexoses determined. The difference in the results of the two determinations is due to the presence of free blood sugar. As in other investigations we found that under the given conditions free glucose leaves the column almost simultaneously with fraction II (see below). Determination of bound hexoses in fraction III was therefore not necessary.

Polarographic activity of the fractions

This was measured in a cobalt (III) solution (composition: 0.001 *M* $Co(NH_3)_6Cl_3$, 0.1 *N* NH_4Cl and 1 *N* NH_3). 5 ml of this solution was mixed with 0.5 ml of the sample, and the height of the polarographic double-wave, beginning at —800 mV was registered (at galvanometer sensitivity 1:150).

RESULTS

Fig. 1 shows the results of gel filtration on Sephadex G-25 of deproteinized perchloric acid filtrates. The upper curve shows clearly that gel filtration separates the Folin–Ciocalteu-positive substances into three fractions, designated I, II and III; the same results are obtained with the original untreated filtrate (without concentration or dialysis). The peak of the eluted perchloric acid coincides for the most part with that of fraction II. The quality of separation of the first mucoprotein fraction from the perchloric acid wave is improved considerably by previous concentration of the filtrate, although this process takes more time. In some of the sera investigated it was found that the peak of fraction II was split into two fractions.

The three lower curves of Fig. 1 show the result of an experiment in which three separate portions of the effluent were collected, corresponding to the peaks of the above-mentioned three fractions. These portions were poured once more onto the same column of Sephadex G-25, and their identity ascertained. Fig. 1 (B–D) proves that gel filtration gives rise to three separate fractions with standard properties, their peaks always being localised in the same place on the elution curve.

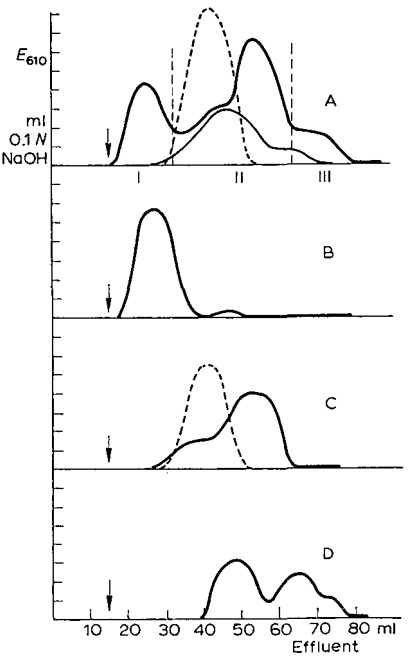

Fig. 1. Separation of the Folin–Ciocalteu-positive substances of the perchloric acid serum filtrates on Sephadex G-25 (A) and repeated gel filtration of the portions I–III (B–D). Solid line: Folin phenol reagent; dotted line: acidity; thin line: Folin colour obtained with a mixture of tyrosine, tryptophan and uric acid (see text).

Chemical and physico-chemical analysis yielded more detailed data on the composition of these fractions (Table I).

Further data on the properties of the above-mentioned three fractions were obtained from experiments in which the filtrates were concentrated and dialysed. Fig. 2 shows clearly that the values obtained with fractions II and III decrease after 24 h, and more so after 48 h, of concentration and partial dialysis. Especially curve D proves that only the macromolecular fraction I remains after total dialysis.

The conclusion can be drawn that fraction I is identical with blood mucoprotein (seromucoid, orosomucoid), whereas fraction II has the character of a mixture of glycopolypeptides and low-molecular nitrogen substances. In Fig. 1A the thin line demonstrates the result of the reaction of the Folin–Ciocalteu reagent with an artificial mixture of L-tyrosine, D,L-tryptophan and uric acid in concentrations (1.0, 1.0 and 3.5 mg/100 ml, respectively) corresponding to their ratio in normal serum; the procedure for the mixture was identical to that for serum. The results show that a considerable part of fraction II and an appreciable amount of fraction III consist

TABLE I

CHEMICAL AND PHYSICO-CHEMICAL ANALYSIS OF THE FRACTIONS

	*Hexoses**	*Coagulability with phosphotungstic acid*	*Specific polarographic activity***
Fraction I	8.23	+	0.956
Fraction II	1.49	+***	0.177
Fraction III		—	0.195

* In per cent of the total amount of the Folin–Ciocalteu-positive components.
** Ratio of the wave-height in mm and the concentration of Folin–Ciocalteu-positive substances in mg/100 ml. The activity of the total filtrate was 0.517.
*** Only after reduction of the perchloric acid concentration by filtration through a column of the anion-exchanger Anionit EGE (USSR) in OH^- form.

of these low-molecular Folin–Ciocalteu-positive components of blood serum. This fact explains the low polarographic activity of fractions II and III (see Table I). Wave III has no mucoid character; it is a mixture of peptides. These observations were supported by some further experiments. Paper electrophoresis of the isolated fraction I in barbital buffer of pH 8.6 yielded two zones (probably corresponding to mucoproteins MP-1 and MP-2 according to MEHL AND GOLDEN[3]); after dialysis, however, only one strongly marked zone remained on the electrophoregram. An

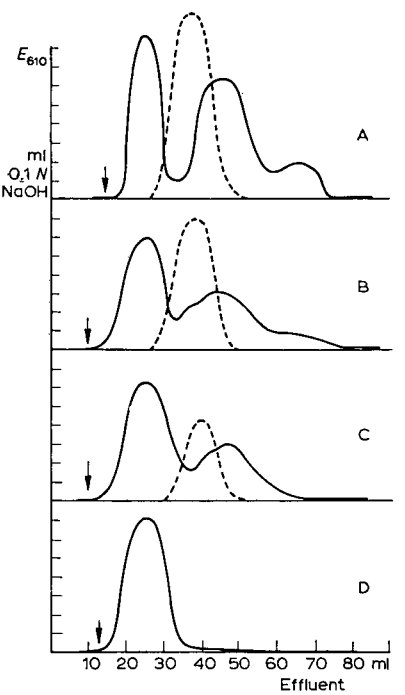

Fig. 2. Influence of concentration and dialysis on the separation of the Folin–Ciocalteu-positive substances from the perchloric acid serum filtrates on Sephadex G-25. A = result without concentration of the filtrate; B = after 24 h concentration against 30 % dextran; C = the same after 48 h; D = result after 24 h dialysis against water.

attempt was then made to find out whether fraction I could be separated in a similar fashion by means of repeated gel filtration on Sephadex G-200 (see Fig. 3). Also in this case the single mucoprotein fraction divided to give at least two distinct peaks.

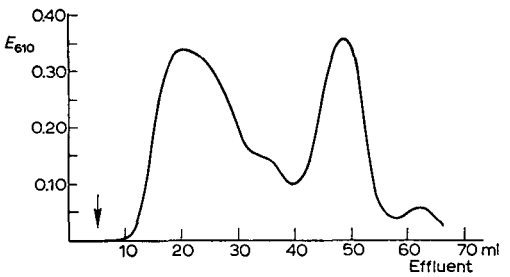

Fig. 3. Separation of the mucoprotein fraction I on a column of Sephadex G-200. 5 ml of fraction I solution was poured on to the column.

Because the second main peak obtained in this separation has the same position as in the case of the separation of the whole filtrate on Sephadex G-25, it may be assumed that the second peak could be formed from fraction I by hydrolysis or depolymerisation.

Seromucoid present in crude state in fraction I can be purified by means of some of the known preparative methods. Salting out with ammonium sulphate according to WEIMER *et al.*[1] proved to be successful.

Fractions II and III were further investigated after preliminary preparation and concentration by vacuum distillation below 45°. Desalting of the fraction II, which could not be done by dialysis, caused some difficulty. We therefore chose another procedure: the solution of fraction II was filtered through a cation-exchange column of Dowex 50 W (X2, 200–400 mesh) in H-form; perchloric acid and uncharged substances passed through, whereas amino acids and peptides were retained. These were later eluted with 6 % ammonia, which was then removed from the solution by evaporation. A small residue was finally investigated by paper chromatography. Also adsorption from the acid solution on cellulose powder (Schleicher & Schüll No. 449a) or on aluminium oxide followed by elution with ammonia was successful.

DISCUSSION

The results obtained show that deproteinized filtrates of blood serum contain not only mucoprotein, but also two further fractions of peptide nature, which are of considerable importance. This may explain the fact that results of mucoprotein estimation by the polarographic filtrate reaction (according to BRDIČKA), and results of the estimation of mucoprotein tyrosine or peptide linkage are not strictly comparable, although according to other authors[9,10], they are, as a rule, correlated.

The above-mentioned results are not identical with those obtained in the separation of sulphosalicylic acid serum filtrates on diethylaminoethylcellulose[5].

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The author wishes to express his thanks to Dr. Z. BRADA and Dr. A. KOČENT, Cancer Research Institute, Brno, for their advice during the course of this work.

SUMMARY

Gel filtration on Sephadex G-25 permits a rapid and relatively mild isolation of serum mucoproteins, directly from deproteinized perchloric acid filtrates of blood sera, even without previous concentration and neutralisation. During the elution mucoproteins (fraction I) separate from a fraction of glycopolypeptide character (fraction II) and from a third, easily dialysable fraction of peptides (fraction III). The peak of the deproteinizing agents and of low-molecular Folin–Ciocalteu-positive substances coincides with the peak of polypeptides (fraction II). The isolated mucoproteins of fraction I can be separated by paper electrophoresis at pH 8.6 to give two subfractions; on Sephadex G-200 at least two subfractions are also detectable. By the method described it is possible to perform a fractional separation of mucoproteins with a small amount of serum.

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THE PAPER CHROMATOGRAPHY OF ESTERS OF PHOSPHORIC AND PHOSPHOROUS ACIDS

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INTRODUCTION

Few methods have been published for the analysis of trialkyl or triaryl phosphites. BERNHART AND RATTENBURY[1] described an acid–alkali titration procedure for determining mono-, di- and trialkyl phosphites in mixtures of these compounds, while a method for titrating trialkyl phosphites with a methanolic solution of iodine in the presence of pyridine has been described by THOMPSON[2]. With either method the reaction proceeds slowly with triaryl phosphites, while neither will differentiate between several trialkyl phosphites together in a mixture. In addition certain phosphites, such as tris-2-chloroethyl phosphite, lose chloride with alkali, leading to erroneous results if the acid–alkali titration procedure is used.

Acid esters of phosphoric acid can be analysed by acid–alkali titration methods, which make use of the several end points of phosphoric acid and its esters, but if trialkyl phosphate is present this does not react, being neutral. Further, if esters of pyrophosphoric acid are present, as may often be the case, the results obtained by titration are difficult to interpret. Similarly, the 2-chloroethyl esters lose chloride with alkali.

Separation on paper of phosphoric acid esters, such as hexose monophosphates and adenosine triphosphate was described by HANES AND ISHERWOOD[3], who used various mobile phases including alcohol–water–ammonia mixtures. Their method of revealing the spots was to spray the papers with an acid molybdate solution, and then to expose them to hydrogen sulphide gas. Since this paper was published there have been many papers dealing with the separation on paper of mixtures of phosphoric acid esters of biological importance.

The separation of phosphoric acid, monobutyl phosphate and dibutyl phosphate has been reported by several workers. CERRAI, CESARANO AND GADDA[4] used a *n*-butanol–formic acid–water solvent, and revealed the spots by the HANES–ISHERWOOD[3] method, or by spraying with bromophenol blue solution. HARDY AND SCARGILL[5] employed a *n*-butanol–acetone–water–ammonia solvent mixture, and by spraying with a ferric iron solution followed by a sulphosalicylic acid solution, as recommended by WADE AND MORGAN[6], obtained colourless spots on a mauve background. SHVEDOV AND ROSYANOV[7] used several mobile phases, including alcohol–water–ammonia mixtures, and showed up the spots by the HANES–ISHERWOOD[3] technique.

PLAPP AND CASIDA[8] have described the separation of certain mono- and dialkyl

phosphates derived from the breakdown of insecticides. They used isopropanol–water–ammonia mobile phases, and to reveal the spots they sprayed the papers with an acid molybdate solution and then exposed them to ultraviolet light, as recommended by CROWTHER[9].

WEIL[10] employed a mobile phase containing *n*-butanol, water and ammonia to separate mixtures of phosphonous, phosphonic and phosphinic acids and certain of their esters, showing up the spots by spraying with a saturated alcoholic solution of silver nitrate, or with a slightly alkaline phenol red solution.

We have utilised paper chromatographic techniques to separate technical samples of a number of esters of phosphorous acid. These include tris-2-chloroethyl phosphite, octyl diphenyl phosphite, triallyl phosphite and trioctyl phosphite. Methods have also been developed for the separation of alkyl phosphate samples, which may contain mono-, di- or trialkyl-phosphates, as well as free phosphoric and pyrophosphoric acids and esters of pyrophosphoric acid. The separated components may be determined quantitatively by wet ashing the spots and determining the phosphorus, as suggested by HANES AND ISHERWOOD[3].

EXPERIMENTAL

Reagents

All reagents were AnalaR or AR quality wherever possible.

1. Stationary phases

20 % v/v liquid paraffin in ether: Mix 10 ml of liquid paraffin (sp. gr. 0.865–0.890) with 40 ml of ether as required.

Alkaline 20 % v/v polyethylene glycol 400 in methanol: Mix 10 ml of polyethylene glycol 400 with 35 ml of methanol and 5 ml of 0.5 *N* ethanolic potassium hydroxide solution as required.

Sodium carbonate solution: Mix 15 ml of 1 % w/v aqueous sodium carbonate solution with 35 ml of methanol as required.

2. Mobile phases

90/10 petrol–*n*-butanol mixture: Mix 90 ml of 100°–120° petroleum spirit with 10 ml of *n*-butanol.

80/20 petrol–chloroform mixture: Mix 80 ml of 100°–120° petroleum spirit with 20 ml of chloroform.

Nitromethane.

2-Methoxyethanol: Redistilled. Boiling range 122°–124°.

Methanol.

n-Propanol–water–ammonia mixture: Mix 70 ml of *n*-propanol with 30 ml of 2 *N* aqueous ammonia.

n-Propanol–*n*-butanol–water–ammonia mixture: Mix 50 ml of *n*-propanol, 20 ml of *n*-butanol and 30 ml of 2 *N* aqueous ammonia.

3. Spray reagents

Alkaline potassium permanganate solution: Dissolve 0.15 g potassium permanganate and 0.1 g anhydrous sodium carbonate in 100 ml of water.

Alkaline universal indicator solution: Dissolve 0.1 g anhydrous sodium carbonate

in 100 ml of Universal Indicator solution, as supplied by British Drug Houses, Ltd., England.

Ferric thiocyanate solution: Mix 10 ml of 2.5 % w/v ferric ammonium sulphate solution with 40 ml of 5 % w/v ammonium thiocyanate solution, add 1 ml of *N* hydrochloric acid solution and dilute to 100 ml with water.

Preparation of papers

Whatman No. 54 papers are usually employed, since they have good wet strength and rapid flow characteristics; a 2 in. wide strip has been found convenient. Where an added stationary phase is necessary the paper is dipped in the appropriate stationary phase solution, the solvent is dried off with an air current and the paper is rolled and stored in an air tight vessel. In the case of the polyethylene glycol 400 and sodium carbonate stationary phases a small dish of water is placed in the vessel, to maintain a water saturated atmosphere. Where no added stationary phase is used the papers are allowed to equilibrate in the chromatographic tank for 2–3 h before use.

General chromatographic procedure

Papers are cut from the prepared roll in lengths of about 18 in. to which is applied a droplet of sample (about 0.25–1.0 μl); the descending technique is used. The time taken for the solvent to flow varies considerably: with sodium carbonate papers it may be 1–1½ h, while with paraffin papers it may be 7–8 h. When the solvent front has travelled to within 2 in. of the lower edge the paper is removed from the tank and the solvent is evaporated by a current of air. The spots are revealed by spraying the papers.

Phosphites are revealed as yellow spots on a pink background by the use of alkaline permanganate: the background colour fades rapidly, and the spots must be outlined as soon as possible. For acid phosphates alkaline Universal Indicator shows the spots distinctly and is quite sensitive. The traces are yellow on a blue-green background, fading slowly as the paper dries to red on a yellow background.

If trialkyl phosphates are suspected the paper should be sprayed with the ferric thiocyanate solution. Trialkyl phosphates from C_3 upwards show as bright red spots, while acid phosphate esters give colourless spots on a brown-pink background. This reagent is not as sensitive as Universal Indicator to the acidic components.

Individual procedures

I. Esters of phosphorous acid. Samples of tris-2-chloroethyl phosphite or tris-2-chloropropyl phosphite may be separated on sodium carbonate papers with 90/10 petrol–*n*-butanol mixture as mobile phase. The sodium carbonate serves only to maintain a mildly alkaline environment for the sample, which is sensitive to acids. As might be expected the moisture content of the paper affects the R_F' values, and Table I gives typical values obtained with papers treated as described above.

Technical octyl diphenyl phosphite may contain a variety of chemical entities, including trioctyl phosphite, dioctyl phenyl phosphite, octyl diphenyl phosphite, diphenyl phosphite, triphenyl phosphite and phenol. A complete separation of these compounds can be achieved by the use of methanol as mobile phase and liquid paraffin as stationary phase, while nitromethane will separate all but di- and triphenyl phosphite. The appropriate R_F' values are given in Table II.

TABLE I

R_F' VALUES FOR ESTERS OF PHOSPHOROUS ACID

(Sodium carbonate stationary phase, 90/10 petrol–*n*-butanol mobile phase)

Tris-2-chloroethyl phosphite	0.95
Bis-2-chloroethyl phosphite	0.35
Tris-2-chloropropyl phosphite	1.00
Bis-2-chloropropyl phosphite	0.73

Some trialkyl phosphites, such as trioctyl and trilauryl phosphites, can be separated on liquid paraffin papers with 2-methoxyethanol as mobile phase. The usual impurity in these materials is dialkyl phosphite, sometimes with traces of alkyl diphenyl phosphite and phenyl dialkyl phosphite. The R_F' values for these compounds will also be found in Table II.

Papers with alkaline polyethylene glycol 400 as stationary phase will effect a separation of technical triallyl phosphite, as well as tris-2-chloroethyl phosphite and tris-2-chloropropyl phosphite. The alkali, which rapidly becomes carbonate, maintains mildly alkaline conditions. In this case the mobile phase is 80/20 petrol–chloroform solution, and the R_F' values from typical samples are listed in Table III.

TABLE II

R_F' VALUES FOR ESTERS OF PHOSPHOROUS ACID

(Liquid paraffin stationary phase)

		Mobile phase		
		Methanol	*Nitromethane*	*2-Methoxy-ethanol*
Trioctyl phosphite	$(C_8H_{17}O)_3P$	0.07	0.03	0.12
Phenyl dioctyl phosphite	$C_6H_5OP\ (OC_8H_{17})_2$	0.20	0.12	0.40
Octyl diphenyl phosphite	$C_8H_{17}OP\ (OC_6H_5)_2$	0.36	0.51	0.74
Triphenyl phosphite	$(C_6H_5O)_3P$	0.65	0.92	0.96
Dioctyl phosphite	$(C_8H_{17}O)_2\ POH$	DNS*	DNS	0.88
Diphenyl phosphite	$(C_6H_5O)_2POH$	0.80	1.00	0.96
Trilauryl phosphite	$(C_{12}H_{25}O)_3P$	0.03	0.00	0.03
Dilauryl phosphite	$(C_{12}H_{25}O)_2POH$	DNS	DNS	0.55
Phenol	C_6H_5OH	1.00	1.00	0.96

* DNS = Did not show.

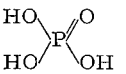
TABLE III

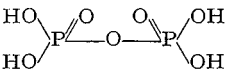
R_F' VALUES FOR ESTERS OF PHOSPHOROUS ACID AND ASSOCIATED IMPURITIES

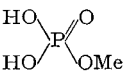
(Polyethylene glycol 400 stationary phase, 80/20 petrol–chloroform mobile phase)

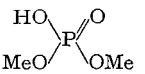
	Ester	R_F' *value*
Triallyl phosphite	$(CH_2=CH\cdot CH_2O)_3P$	0.97
Diallyl phosphite	$(CH_2=CH\cdot CH_2O)_2POH$	0.43
Triallyl phosphate	$(CH_2=CH\cdot CH_2O)_3PO$	0.67
Diallyl phosphate	$(CH_2=CH\cdot CH_2O)_2POOH$	0.31
Tris-2-chloroethyl phosphite	$(ClCH_2\cdot CH_2O)_3P$	0.56
Bis-2-chloroethyl phosphite	$(ClCH_2\cdot CH_2O)_2POH$	0.09
Tris-2-chloropropyl phosphite	$[CH_2\cdot CH(Cl)\cdot CH_2O]_3P$	0.93
Bis-2-chloropropyl phosphite	$[CH_2\cdot CH(Cl)\cdot CH_2O]_2POH$	0.25

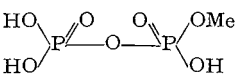
II. Esters of phosphoric acid. Technical samples of acid alkyl phosphate esters may contain monoalkyl orthophosphate and dialkyl orthophosphate with some free orthophosphoric acid. In addition there may be free pyrophosphoric acid and esters of pyrophosphoric acid. The possible components of, for example, acid methyl phosphates are:

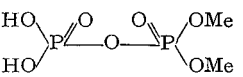

orthophosphoric acid (1)

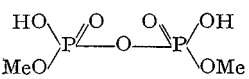

pyrophosphoric acid (2)

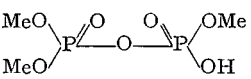

monomethyl orthophosphate (3)


dimethyl orthophosphate (4)


monomethyl pyrophosphate (5)


asym. dimethyl pyrophosphate (6)


sym. dimethyl pyrophosphate (7)


trimethyl pyrophosphate (8)

Further, trialkyl phosphate may contain dialkyl phosphate as impurity. The components of such samples can be separated very efficiently using Whatman 54 paper with no prior treatment, apart from equilibration in the chromatographic tank, and 70/30 *n*-propanol–2 *N* aqueous ammonia mixture as mobile phase. This solvent was recommended by HOWE[11] for the separation of organic acids.

The R_F' values of the components of various acid alkyl phosphates are given in Table IV. Those components which have been identified by the use of reference com-

TABLE IV

R_F' VALUES FOR ESTERS OF PHOSPHORIC ACID

(70/30 *n*-propanol–2 *N* aqueous ammonia as mobile phase)

Alkyl radicle	*Mono-alkyl*	*Di-alkyl*	*Tri-alkyl*	*Pyro acid*	*Ortho acid*	*Other traces (probably esters of pyro acid)*
Methyl	0.22	0.65	1.00	0.04	0.12	0.08, 0.31, 0.42, 0.72
Ethyl	0.27	0.77	1.00			0.18, 0.47
Isopropyl	0.33	0.86	1.00			0.20, 0.61
n-Butyl	0.48	0.94	1.00			
n-Amyl	0.55	0.95	1.00			0.14, 0.28, 0.39
n-Hexyl	0.62	0.95	1.00			
Octyl	0.68	0.95	1.00			
Nonyl	0.75	0.98	—			
Lauryl	0.79	0.98	—			
2-Chloroethyl	0.33	0.84	—			0.19, 0.59
2-Hydroxyethyl	0.20	0.49	—			0.29

pounds are indicated. The identity of the other traces is conjectural, but they are probably esters of pyrophosphoric acid. Thus a sample of acid methyl pyrophosphate gave eight spots, which were considered to be as follows:

0.04	pyrophosphoric acid	(2) above
0.08	monomethyl pyrophosphate	(5) above
0.13	orthophosphoric acid	(1) above
0.21	monomethyl orthophosphate	(3) above
0.31	dimethyl pyrophosphate (*sym.*)	(7) above
0.42	dimethyl pyrophosphate (*asym.*)	(6) above
0.62	dimethyl orthophosphate	(4) above
0.72	trimethyl pyrophosphate	(8) above

As can be seen from Table IV the trialkyl phosphates travel with the solvent front in all cases. A slightly better separation of trialkyl phosphate from dialkyl phosphate can be achieved by substituting 50:20:30 *n*-propanol–*n*-butanol–2 *N* aqueous ammonia solution as mobile phase.

Quantitative analyses

The spots which have been revealed by the spray reagent can be cut out and wet ashed, using nitric, sulphuric and perchloric acids, and the phosphorus content determined. This technique has been used on a routine basis for the analysis of phosphite and phosphate esters.

The experimental procedure is the same as has been described for the qualitative separations, except that in order to obtain enough phosphorus to permit reasonably accurate determinations the sample weight must be about 3–4 mg. Such an amount of sample cannot be applied as a single spot, and it is our practice to streak the sample on to the starting line, as evenly as possible, from a weighed glass capillary tube. The sample weight is then obtained simply by reweighing the capillary. A micro balance should be used for this purpose.

Duplicate determinations normally agree to within $\pm$ 3%, but for complete recovery the sample must be substantially involatile. This is not the case with, for example, triallyl phosphite or the lower trialkyl phosphites. Most of the phosphites and acid alkyl phosphates mentioned in the paper can be determined in this manner.

SUMMARY

Procedures are described for the separation on paper of the components of technical samples of alkyl and alkyl–aryl phosphites and of esters of phosphoric acid. Suitable stationary and mobile phases, as well as spray reagents, are noted, and typical R_F' values are presented. The quantitative analysis of these esters, by wet ashing the spots and determining the phosphorus content, is briefly indicated.

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CHELATBILDENDE AUSTAUSCHERHARZE

III. SELEKTIVE UND SPEZIFISCHE IONENAUSTAUSCHER AUF HYDRAZIDBASIS

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Ionenaustauscher erhalten im allgemeinen eine ausgeprägte Selektivität oder Spezifität für bestimmte Ionen, wenn die aktive Gruppe des Austauschers die Konfiguration eines selektiv oder spezifisch wirkenden Reagenzes aufweist. Eine vollständige Übereinstimmung der Spezifität oder Selektivität von Monomerem und Polymerem ist allerdings nicht zu erwarten; denn die Bindung der funktionellen Gruppe an das Harz verändert in mehr oder weniger starkem Masse das Molekül[1]. Auch muss eine sterische Hinderung bei der Ausbildung von Komplexen in Betracht gezogen werden.

Die bisher von uns untersuchten Austauscher, die Derivate[2] und Strukturanaloga[3] der Iminodiessigsäure enthalten, entsprechen in bezug auf die Chelatbildung im wesentlichen den Eigenschaften des Monomeren. Über das Verhalten hydrazinhaltiger Harze wurde dagegen bisher auch von anderer Seite nicht berichtet.

Die Untersuchungen über die Komplexbildung von Hydraziden und die Herstellung von Ionenaustauscherharzen auf Hydrazidbasis wurden angeregt durch die bekannte Komplexbildungsfähigkeit des Hydrazins sowie durch einige Hinweise auf den sauren Charakter einiger Carbonsäurehydrazide[4] und deren Komplexbildung in alkalischer Lösung[5–7]. Die näher untersuchten Hydrazide können im wesentlichen drei Gruppen zugeordnet werden, die zum Teil völlig verschiedene Eigenschaften in bezug auf die Acidität bzw. Basizität und Komplexbildung besitzen:

a. Hydrazide gesättigter Carbonsäuren $R–CH_2–CO–NH–NH_2$

b. Ringförmige Hydrazide, z.B. das Maleinylhydrazid

c. Lineare Hydrazide, deren an Stickstoff gebundenen Wasserstoffatome sauren Charakter tragen, z.B. das Dimaleinyl-bernsteinsäuredihydrazid.

Von den genannten Verbindungsgruppen sind die Hydrazide gesättigter Carbonsäuren nur von geringem Interesse, da infolge ihres sehr schwach basischen Charakters das freie Elektronenpaar des Stickstoffs für eine Komplexbildung kaum zur Verfügung steht.

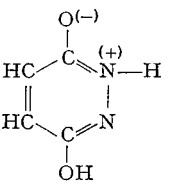
Die Bestimmung der Acidität und der Komplexbildung der monomeren Verbindungen erfolgte durch Auswertung potentiometrischer Titrationskurven. Über Einzelheiten wurde bereits früher[3] berichtet. Abweichend von den dort angegebenen Bedingungen wurden die Me^{2+}-Ionen in äquivalenter Menge zugesetzt. Die Messergebnisse wurden in Diagrammen dargestellt, auf deren Ordinate die pH-Einheiten

und auf deren Abszisse die Anzahl der pro Mol Komplexbildner verbrauchten Mole NaOH aufgetragen sind. Die Titrationskurven der Chelatbildner sind innerhalb von 0.1 pH-Einheiten gut reproduzierbar. Bei der Bildung löslicher Chelate ist die Bildungs- oder Umwandlungsgeschwindigkeit der Komplexe nur in Ausnahmefälle so gering, dass grössere Abweichungen zwischen den einzelnen Messreihen auftreten.

Die verwendeten Chemikalien wurden, soweit nicht anders angegeben, von der Fa. E. Merck, Darmstadt im höchsten Reinheitsgrad bezogen. Das Maleinylhydrazid und die Rohstoffe zur Herstellung der Harze stammten von den Firmen Th. Schuchardt, München und Riedel-de-Haen, Hannover.

AUSTAUSCHER AUF MALEINYLHYDRAZID-BASIS

Das Maleinylhydrazid $C_4H_4O_2N_2$



(I)

ist eine schwache einbasige Säure[8] mit dem pK-Wert 5.58. Ein zweites Proton kann in wässriger Lösung mit Natronlauge nicht titriert werden (Fig. 1).

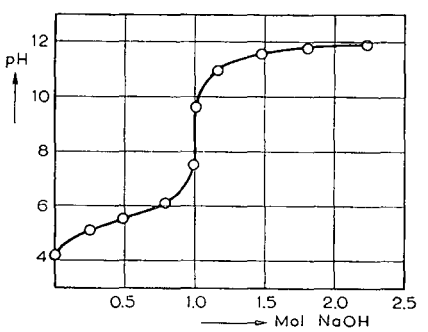


Fig. 1. Neutralisationskurve des Maleinylhydrazids.

Mit einer Reihe von Schwermetallkationen bildet Maleinylhydrazid in saurer bis neutraler Lösung schwer lösliche Komplexe. Am schwersten löslich sind die Hg(I)-, Hg(II)-, Ag(I)- und Tl(I)-Verbindungen. Sie fallen bereits beim Zusammengiessen von wässrigen Lösungen des Hydrazids und des entsprechenden Nitrats aus, wobei die Lösungen durch die Bildung freier Salpetersäure stark sauer werden. Das Maleinylhydrazid verhält sich also wie ein Pseudohalogenid. Bei Zusatz weiterer Salpetersäure lösen sich die Niederschläge wieder auf. In neutraler bis alkalischer Lösung reagiert Maleinylhydrazid in Gegenwart von Ag^+-, Cu^{2+}-, Co^{2+}- und Ni^{2+}-Ionen wie eine zweibasige Säure. Näher untersucht wurde das Verhalten des Hydrazids gegenüber von Ag^+-Ionen.

Bei Zusatz einer neutralen $AgNO_3$-Lösung zu einer wässrigen schwach sauer reagierenden Maleinylhydrazidlösung fällt die Monosilberverbindung als weisser silberchloridähnlicher Niederschlag aus. Bei Zusatz von NaOH wandelt sie sich in die gelbe Disilberverbindung um. Diese Reaktionen können durch folgende Gleichungen beschrieben werden (A = Anion der Säure H_2A):

$$H_2A + AgNO_3 + NaOH \longrightarrow AgHA + NaNO_3 + H_2O$$

$$2\ AgHA + NaOH \longrightarrow Ag_2A + NaHA + H_2O$$

Setzt man dagegen einer alkalischen Hydrazidlösung 2 Mol $AgNO_3$ pro Mol Hydrazid zu, so bildet sich sofort die Disilberverbindung:

$$H_2A + 2\ AgNO_3 + 2\ NaOH \longrightarrow Ag_2A + 2\ NaNO_3 + H_2O$$

In Gegenwart von Cu^{2+}-, Co^{2+}- oder Ni^{2+}-Ionen fallen beim Neutralisieren mit NaOH hydroxidartige Niederschläge mit nicht ganz stöchiometrischer Zusammensetzung aus. Sie enthalten ca. 2 Mole Metallionen pro Mol Hydrazid. Aus einer Zn^{2+}-haltigen Maleinylhydrazid-Lösung fällt dagegen $Zn(OH)_2$.

Die Verbindungsbildung kann an Hand von Titrationskurven verfolgt werden. Wegen der Niederschlagsbildung treten jedoch geringe Unterschiede zwischen Paralleluntersuchungen auf.

In stark saurer Lösung wirkt Maleinylhydrazid als schwache Base. Durch Anlagerung eines Protons entstehen Maleinylhydrazidiumsalze.

Aus einer mit Maleinylhydrazid in der Hitze gesättigten 50%igen H_2SO_4 kristallisiert z.B. beim Abkühlen das Maleinylhydrazidiumsulfat aus. Die Kristalle werden mit wasserfreiem Alkohol gewaschen und im Vakuum bei 60° getrocknet. An der Luft ziehen sie schnell Feuchtigkeit an und hydrolysieren. Auf Grund der Titrationskurven muss der Verbindung die Formel $(C_4H_5O_2N_2)_2SO_4$ zugeschrieben werden.

Bei der Kondensation von Resorcin mit Formaldehyd in Gegenwart von Maleinylhydrazid entsteht ein Austauscher, in dem das Hydrazid fest am Grundgerüst verankert ist. Der Formaldehyd greift dabei vermutlich ebenso wie bei der Mannich-Reaktion[9] an einem Stickstoffatom des Maleinylhydrazids an (Fig. 2).

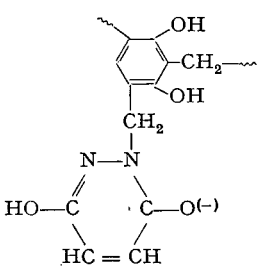


Fig. 2. Struktur des Maleinylhydrazid-Austauschers.

Zur Darstellung eines geeigneten Produktes werden Lösungen von 0.2 Mol Resorcin (22 g) in 40 ml Wasser und von 0.1 Mol (11 g) Maleinylhydrazid und 0.1 Mol

(4 g) NaOH in 60 ml Wasser gemischt. Reagiert diese Lösung noch nicht alkalisch, setzt man noch etwas NaOH zu. Nach Zusatz von 70 ml 35 %iger Formalinlösung wird die Mischung in einer abgedeckten Porzellanschale auf dem Wasserbad erwärmt. Schon nach kurzer Zeit bildet sich ein klares Gel, das im Laufe von ca. 20–30 Std. zu einem spröden, rotbraun gefärbten Harz erstarrt und sich dann leicht mahlen lässt. Die Siebfraktion mit einer Korngrösse von 0.2–0.4 mm wird dann in eine Austauschersäule gefüllt und sorgfältig durch wiederholtes Behandeln mit HCl und NaOH von nicht kondensierten Bestandteilen und Polyelektrolyten befreit. Der Austauscher ist gegen die meisten verdünnten Säuren und Laugen beständig. Bei Behandlung mit verdünnter HNO_3 tritt jedoch bereits nach 24 Stunden stärkere Zersetzung auf.

Der Maleinylhydrazid-Austauscher ist in saurer Lösung (pH 1) quecksilberspezifisch. Die Säureform bindet Hg_2^{2+}- und Hg^{2+}-Ionen aus den mit Salpetersäure schwach angesäuerten Lösungen. Beim Nachwaschen mit 2 %iger Essigsäure wird kein Quecksilber eluiert. Ag^+-Ionen werden dagegen selbst aus neutraler Lösung nicht quantitativ gehalten. 2 %ige Essigsäure eluiert den gebundenen Anteil schnell und vollständig. Ebenso verhalten sich auch Tl^+-, Cu^{2+}-, Ni^{2+}-, Co^{2+}-, Zn^{2+}-, Mg^{2+}- und andere Ionen.

Nach den beschriebenen Fällungsreaktionen war ausser für Quecksilber auch für Ag^+- und Tl^+-Ionen eine erhöhte Bindungsfestigkeit an das Harz zu erwarten. Für das abweichende Verhalten ist vermutlich die Brücke verantwortlich, die das Maleinylhydrazid an das Harzgerüst bindet.

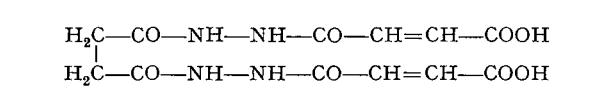
Auf Grund der schwachen Basizität des Maleinylhydrazids reagiert das Harz auch als schwach basischer Anionenaustauscher. So ist z.B. eine Trennung von As(III) und Sb(III) über die Chlorokomplexe in 1.5–2 *N* HCl möglich.

Die Spezifität für Quecksilberionen in saurer Lösung ermöglicht die Abtrennung von anderen Kationen und eine störungsfreie potentiometrische Titration nach der Elution mit 1 *N* $HClO_4$. Als Beispiel sei die Trennung von Hg_2^{2+}- und Ag^+-Ionen angeführt.

Die je ca. 0.5–2 mMol $AgNO_3$ und $Hg_2(NO_3)_2$ enthaltende Lösung wird am Kopf der 40 ml Maleinylhydrazid-Austauscher (H^+-Form) enthaltenden Säule eingefüllt. Nach dem Ablauf wird mit 150 ml 2 %iger Essigsäure nachgewaschen. Im Eluat befindet sich quantitativ das Silber. Nach dem Wechsel der Vorlage wird das Quecksilber mit 150 ml 1 *N* $HClO_4$ eluiert. Die Durchlaufgeschwindigkeit soll ca. 5 ml/Minute betragen. Beide Ionen lassen sich allein leicht potentiometrisch mit HCl titrieren. Für die Silberbestimmung benutzt man dabei eine Silberelektrode, für die Quecksilberbestimmung ein amalgamiertes Silberblech als Indikatorelektrode. In einem Gemisch beider Ionen titriert man jedoch nur ihre Summe. Die Trennung ist quantitativ. Die hier erreichte mittlere Varianz der Einzelwerte von 0.5 Rel.-% hängt von der Genauigkeit der Bestimmungsmethode ab.

AUSTAUSCHER MIT DIMALEINYL-BERNSTEINSÄUREDIHYDRAZID-STRUKTUR

Das Dimaleinyl-bernsteinsäuredihydrazid $C_{12}H_{14}O_8N_4$ hat die Struktur:



Die genannte Verbindung fällt in über 90%iger Ausbeute beim Zusammengiessen einer Lösung von 5.7 g Maleinsäureanhydrid in 30 ml Eisessig und einer Lösung von 5 g Bernsteinsäuredihydrazid in 60 ml Eisessig als weisser körniger Niederschlag aus. Nach dem Waschen mit Alkohol und Trocknen im Vakuum hat das Produkt einen Schmelzpunkt von 169°. Es konnte nicht ohne erhebliche Zersetzung umkristallisiert werden. Die Elementaranalyse ergab befriedigende Übereinstimmung mit den theoretischen Werten. In wässriger Lösung zerfällt die Verbindung bei Zimmertemperatur allmählich, beim Kochen jedoch schon in wenigen Minuten. Als Zersetzungsprodukt konnte cyclisches Maleinylhydrazid isoliert werden.

Das Dimaleinyl-bernsteinsäuredihydrazid ist eine schwache bis mittelstarke Säure, die in saurer bis neutraler Lösung zwei Protonen abspaltet. Die Lage der Titrationskurve im alkalischen Bereich zeigt aber, dass weitere H^+-Ionen abdissozieren.

In Fig. 3 ist zum Vergleich die Neutralisationskurve von HCl eingezeichnet, so dass die pH-Erniedrigung deutlich wird. Auch die Neutralisationskurven in Gegenwart von Schwermetallkationen weisen darauf hin, dass in diesem Gebiet 4 Protonen abgespalten werden. Die Verbindung, die in saurem Medium farblos ist, erteilt der Lösung bei hohen pH-Werten eine schwach gelbliche Färbung.

Die Neutralisation kann formal durch die Gleichungen

$$\text{(a)} \quad H_6A + 2\ NaOH \longrightarrow Na_2H_4A + 2\ H_2O$$

und

$$\text{(b)} \quad Na_2H_4A + 4\ NaOH \longrightarrow Na_6A + 4\ H_2O$$

beschrieben werden.

In Gegenwart von ein oder zwei Molen eines Me^{2+}-Kations pro Mol Dimaleinyl-bernsteinsäuredihydrazid (Fig. 3) bilden sich lösliche Chelatkomplexe. Die Komplexstabilität nimmt in der Reihenfolge Cu > Ni > Zn > Co > Fe(II) > Mg ab.

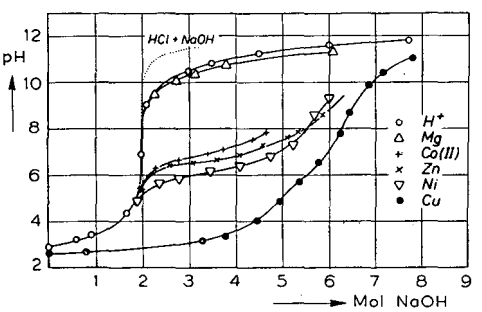


Fig. 3. Titration von Dimaleinyl-bernsteinsäuredihydrazid in Gegenwart von Schwermetallkationen. Hydrazid:Me^{2+} = 1:2.

Für die Mesomerie im Anion des Dimaleinyl-bernsteinsäuredihydrazids sind im wesentlichen vier Grenzstrukturen zu diskutieren (Fig. 4 a–d).

Das Auftreten einer leicht gelb gefärbten Lösung bei höheren pH-Werten deutet auf ein Gleichgewicht zwischen Struktur (d) und wahrscheinlich (b). (b) ist sicher gegenüber (c) infolge der Konjugation der Doppelbindungen im Maleinylrest bevor-

zugt. Der Metallchelatverbindung wäre etwa die folgende Struktur zuzuordnen (Fig. 5).

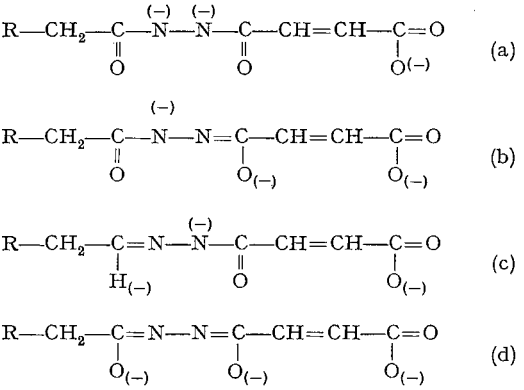


Fig. 4. Mesomere Grenzstrukturen.

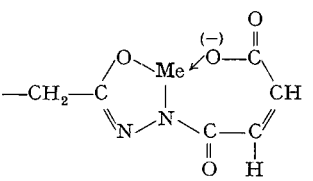


Fig. 5. Struktur des Dimaleinyl-bernsteinsäuredihydrazid–Metallchelatkomplexes.

Die entstehenden Ringe sind spannungsfrei, wie sich mit Hilfe von Atomkalotten zeigen lässt. Die Struktur des fünfgliedrigen Ringes entspricht im Prinzip dem Aufbau des Fünfrings in den Dithizonaten[10].

Ein Austauscherharz mit der Struktur des Dimaleinyl-bernsteinsäuredihydrazids entsteht durch Mischpolymerisation von Styrol, Divinylbenzol und Maleinsäureanhydrid, Umsetzung mit Hydrazinhydrat und anschliessende Maleinylierung.

Beim Erwärmen einer Lösung von 50 g Maleinsäureanhydrid, 50 g Styrol und 8 ml 50%igem Divinylbenzol (DVB) in 100 ml Aceton auf dem Wasserbad bildet sich in wenigen Stunden ein Gel, das über Nacht bei 100° im Trockenschrank nachgehärtet und dann in einer Schlagmühle gemahlen wird. Der DVB-Gehalt entspricht einem Vernetzungsgrad von etwa 4%. Das gemahlene Harz reagiert mit Hydrazinhydrat unter Wärmeentwicklung und Quellung, wobei 100 g Harz 250 ml Hydrazinhydrat fast vollständig aufnehmen. Das Reaktionsprodukt wird noch einige Stunden auf dem Wasserbad erhitzt und dann nacheinander mit Wasser, 2 *N* HCl, Wasser, verdünnter NH_3-Lösung und Wasser gewaschen und bei 50° im Vakuum getrocknet.

Die Maleinylierung verläuft glatt, wenn man langsam über 40 ml in Eisessig eingequollenes und in eine Säule gefülltes Harz eine Lösung von ca. 15 g Maleinsäureanhydrid in 100 ml Eisessig laufen lässt. Um eine möglichst quantitative Umsetzung zu gewährleisten, lässt man das Harz noch über Nacht unter der Lösung stehen. Am nächsten Tag wird es dann mit Wasser säurefrei gewaschen und mit

einem NH_4^+-Salz–NH_3-Puffer (pH 9–10) behandelt. Das Harz ist jetzt gebrauchsfertig.

Der Austauscher quillt bei der Umladung aus der Säureform in die NH_4^+-Form um ca. 50 %. Die geringe Stabilität des Dimaleinyl-bernsteinsäuredihydrazids macht sich am Austauscher wesentlich weniger bemerkbar. Das Harz kann monatelang benutzt werden, ohne dass eine merkliche Verschlechterung der Eigenschaften eintritt. Die Kapazität für chelatartig gebundenes Cu^{2+} beträgt 3.2 mval/g trockenes Harz.

Der chelatbildende Austauscher wirkt in der NH_4^+-Form selektiv für Schwermetallionen. Als Elutionsmittel dienen Ammoniumacetat-Lösungen, die durch Ammoniak-Zusätze auf den gewünschten pH-Wert eingestellt werden. Unter diesen Bedingungen ist eine Trennung der Schwermetallionen von Erdalkali- und Alkaliionen möglich. Darüber hinaus können auch Schwermetallionen untereinander durch Elutionschromatographie getrennt werden. Als Modellanalyse sei eine Ni^{2+}–Zn^{2+}-Trennung beschrieben. Sie ist besonders geeignet, die Trennfähigkeit des Austauschers zu zeigen, weil die Komplexbildungskonstanten beider Ionen sehr ähnlich sind (Fig. 3).

Die Ni^{2+} und Zn^{2+} enthaltende Lösung wird mit 4–5 g NH_4Cl und etwas konz. NH_3 versetzt und auf 100 ml aufgefüllt. Pro Analyse werden je 0.5–2 mMol Zn und Ni eingesetzt. Die Lösung wird am Kopf der 40 ml Austauscher (NH_4^+-Form) enthaltenden Säule einpipettiert. Das Zn^{2+} wird mit 1200 ml 0.75 *N* NH_4CH_3COO-Lösung (pH 10.75) eluiert. Das Eluat wird auf dem Sandbad eingeengt. Dabei verflüchtigt sich das Acetat vollständig, so dass anschliessend das Zn^{2+} komplexometrisch bestimmt werden kann. Ni^{2+} kann leicht mit 100 ml ammoniakalischer KCN-Lösung abgelöst und nach Ansäuern und Vertreiben des HCN titriert werden. Die mittlere Varianz der Einzelwerte beträgt 1 Rel.-%.

AUSTAUSCHER MIT DIOXALYL-BERNSTEINSÄUREDIHYDRAZID-STRUKTUR

Ein chelatbildender Austauscher, der wahrscheinlich die Struktur des Dioxalylbernsteinsäuredihydrazids besitzt, entsteht aus vorstehend beschriebenem Mischpolymerisat durch Umsetzung mit Hydrazinhydrat und anschliessende Oxalylierung.

Das mit Hydrazinhydrat umgesetzte Mischpolymerisat wird mit etwa der gleichen Gewichtsmenge Oxalsäureäthylesterchlorid übergossen und eine Stunde stehen gelassen. Während dieser Zeit beginnt bereits eine Reaktion unter Erwärmung und HCl-Entwicklung. Beim anschliessenden vorsichtigen Erhitzen wird die Reaktion so lebhaft, dass eine Kühlung mit kaltem Wasser notwendig ist. Dabei quillt das Harz und nimmt die gesamte Flüssigkeit auf. Das überschüssige Esterchlorid wird mit Tetrahydrofuran ausgewaschen. Danach braucht man das Harz nur mit Wasser neutral und eventuell mit einer verdünnten NH_3-Lösung zu waschen, damit es voll reaktionsfähig wird. Bei dieser Behandlung werden die Estergruppen des Substituenten bereits verseift.

Die Quellung der NH_4^+-Form gegenüber der H^+-Form des Austauschers beträgt ca. 60 %. Das Harz ist über 2 Monate stabil. Die Stabilität kann durch vollständige Beladung mit Cu^{2+}-Ionen erhöht werden. Die Kapazität für chelatartig gebundenes Cu^{2+} beträgt 3.2 mval/g trockenes Harz.

Die Säureform des oxalylierten Austauschers bindet Cu^{2+}-Ionen aus 0.1 *N* HCl

vollständig. Allerdings darf die Durchlaufgeschwindigkeit nicht zu hoch sein, weil sich sonst die geringe Reaktionsgeschwindigkeit des Harzes nachteilig bemerkbar macht.

Bei der Elution mit HCl verschiedener Konzentration bricht das Cu bei 0.1 *N* HCl nach 2, bei 0.05 *N* HCl nach ca. 9 Säulenvolumina Elutionsmittel durch. Andere Schwermetallionen wie Ni^{2+} werden nur sehr schwach gebunden und können schnell und vollständig eluiert werden. Dadurch wird eine schnelle Abtrennung von Cu^{2+}-Ionen ermöglicht.

Die im Vergleich zum Dimaleinyl-bernsteinsäuredihydrazid-Austauscher wesentlich höhere Bindungsfestigkeit des Dioxalyl-bernsteinsäuredihydrazid-Austauschers gegenüber Cu^{2+}-Ionen beruht wahrscheinlich auf dem Ersatz des siebengliedrigen Maleinylringes im Metallchelatkomplex durch den fünfgliedrigen Oxalylring (Fig. 6).

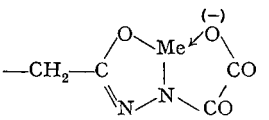


Fig. 6. Struktur des oxalylierten Bernsteinsäuredihydrazid–Metallchelatkomplexes.

Neben dieser Struktur muss aber auch die Existenz eines sechsgliedrigen Ringes in Erwägung gezogen werden (Fig. 7).

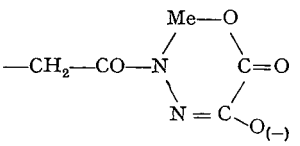


Fig. 7. Struktur des oxalylierten Bernsteinsäuredihydrazid–Metallkomplexes.

Während das Kalottenmodell des Komplexes (Fig. 7) geringe Spannung hat, ist der Sechsring völlig spannungsfrei.

Zur Kennzeichnung der Wirkungsweise des Austauschers ist im folgenden eine Cu^{2+}–Ni^{2+}-Trennung als Modellanalyse beschrieben.

Eine mit 40 ml Austauscher (H^+-Form) gefüllte Säule wird mit 20 ml einer Lösung, die je ca. 0.5–1 mMol Cu^{2+}- und Ni^{2+}-Ionen enthält, beschickt. Die Tropfgeschwindigkeit beträgt während des Austausches 20 ml/Std. Als Elutionsmittel dient eine 2 *N* KCl-Lösung mit dem pH-Wert 2 (0.01 *N* HCl). Die Tropfgeschwindigkeit wird jetzt nach Durchlauf der ersten 20 ml auf 3 ml/Min. erhöht. Nach etwa 300 ml Elutionsmittel ist das Ni quantitativ vom Harz entfernt. Anschliessend kann das Cu mit 100 ml 2 *N* HCl und 100 ml Wasser zum Nachwaschen vollständig eluiert werden. Die Bestimmung der Schwermetallionen im Eluat erfolgt komplexometrisch. Die mittlere Varianz der Einzelwerte beträgt 1 Rel.-%.

DANK

Herrn Prof. Dr. G. SCHWARZENBACH danken wir für wertvolle Diskussionsbeiträge sowie der Verwaltungsstelle für ERP-Vermögen und der Deutschen Forschungsgemeinschaft für die Bereitstellung von Mitteln und Apparaten.

ZUSAMMENFASSUNG

Cyclisches Maleinylhydrazid ist eine schwache Säure, die mit Ag^{+}-, Hg_2^{2+}-, Hg^{2+}- und Tl^{+}-Ionen schwer lösliche Salze bildet. Es verhält sich also wie ein Pseudohalogenid. In stark saurer Lösung addiert Maleinylhydrazid ein Proton unter Bildung von Hydrazidiumsalzen. Bei der Kondensation mit Formaldehyd in Gegenwart von Resorcin entsteht ein Polykondensations-Ionenaustauscher, der in schwach saurer Lösung spezifisch für Hg^{2+}- und Hg_2^{2+}-Ionen ist. In stark saurer Lösung verhält er sich wie ein schwach basischer Anionaustauscher.

Dimaleinyl-bernsteinsäuredihydrazid bildet mit Schwermetallionen lösliche Chelatkomplexe. Ein chelatbildender Ionenaustauscher mit analoger Struktur entsteht durch Umsetzung eines Styrol–Divinylbenzol–Maleinsäureanhydrid-Mischpolymerisats mit Hydrazin und anschliessende Maleinylierung. Der Austauscher ist selektiv für Schwermetallionen und erlaubt die Trennung von Ionen mit sehr ähnlicher Komplexstabilität durch Elutionschromatographie. Durch Oxalylierung des mit Hydrazin umgesetzten Mischpolymerisats erhält man einen Austauscher, der in 0.01–0.1 *N* HCl spezifisch für Cu^{2+}-Ionen ist.

SUMMARY

Cyclic maleic hydrazide is a weak acid, which forms difficultly soluble salts with Ag^{+}, Hg_2^{2+}, Hg^{2+} and Tl^{+}-ions, and thus behaves as a pseudohalide. In strong acid solution maleic hydrazide adds one proton and forms hydrazidium salts. Condensation with formaldehyde in the presence of resorcinol leads to the formation of a polycondensed ion exchanger, which in weak acid solution is specific for Hg^{2+} and Hg_2^{2+}-ions. In strong acid solution it behaves as a weakly basic anion exchanger.

Dimaleyl succinic dihydrazide forms soluble chelate complexes with heavy metal ions. An ion exchanger with similar structure, which forms chelate complexes, is obtained when a styrene–divinylbenzene–maleic anhydride copolymer is treated with hydrazine and subsequently converted to the maleyl derivative. The exchanger is selective for heavy metal ions and can be used to separate ions that form complexes of similar stability by means of elution chromatography. Conversion of the hydrazine-treated copolymer to the oxalyl derivative produces an exchanger, which in 0.01–0.1 *N* HCl is specific for Cu^{2+}-ions.

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CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY OF INORGANIC IONS

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Since the original work of CONSDEN, GORDON AND MARTIN[1] paper chromatography has been applied to the problems of separation of both organic and inorganic mixtures that were difficult or time-consuming to separate by more conventional methods of analysis. The period of the order of hours usually necessary for the development of chromatograms in order to obtain satisfactory separation has been, however, the major drawback to applying this technique to the analysis of labile compounds, unstable valency states, and radioelements of short half-life. Attempts have recently been made to speed up the flow of solvent through the paper by the use of volatile solvents[2], or by developing the chromatograms at higher temperatures[3–10]. Although these modifications when applied to some particular problems gave more satisfactory results than conventional paper chromatography, they suffer, however, from the drawback that it is difficult to obtain equilibrium conditions with volatile solvents, and that changes in composition of the species or valency states may occur at higher temperatures.

An interesting innovation recently introduced by CARONNA[11] and developed in particular by MCDONALD and coworkers[12–17] has permitted the development of chromatograms in very short times by combining chromatography with centrifugal acceleration. This procedure has been successfully applied to the separation of dyes[15], amino acids[15,16], chloroplast pigments[18], ^{82}Br- and ^{131}I-labelled organic substances[19], human serum lipoproteins[17], 2,4-dinitrophenylhydrazones of carbonyl compounds[20], and some substances of the vitamin B_6 group[21]. The only separation of inorganic ions by this technique reported in the literature is that of Cu, Cd, Bi, and Hg, by INDOVINA AND RICOTTA[22]. The success achieved in the rapid separation of organic compounds and the improved results over those given by ordinary chromatographic techniques prompted us to extend the application of centrifugally accelerated paper chromatography to inorganic and radiochemical problems. The present communication reports the initial results of a study of fundamental factors that govern good and reproducible separation of inorganic ions by this technique. The study was made with Fe(III), Co(II), Ni(II), Mn(II), Cu(II), and Zn(II) ions with R_F values that extend over a wide range.

APPARATUS

In the literature descriptions have been given of several apparatus for centrifugal

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paper chromatography[11–17, 19, 23–28] as well as various modifications in the procedure. When we undertook the present work two models of the apparatus (Chromatofuge Modèle T.65, and Centrifugeur pour chromatographie par Ets. Jouan) were commercially available. Because of the lower price and its simplicity, the apparatus made by Etablissements Jouan was chosen for these studies. The apparatus is shown in Fig. 1. Its chief characteristics are: rate of rotation: variable between 500 to 1200 rev./min; size of paper: up to 45 cm diameter. Dimensions of the apparatus: diameter 50 cm; height 26 cm; weight 4 kg.

The apparatus consists essentially of a detachable developing tank (of Perspex or stainless steel, that of Perspex being used in the present work) provided with a removable cover fastened by three pins. A Petri dish with the solvent placed in the tank permits an easy saturation of the atmosphere within the tank with the vapour of the solvent. The circular sheet of paper is held in position at the centre of the tank on a Teflon disk coupled magnetically to the motor. During rotation the paper disk assumes a taut horizontal position. Each tank is equipped with a magnetic disk covered with Teflon to which the rotation is imparted by a magnetic drum attached to the motor. Thus every tank is air-tight.

The circular paper is spotted, by means of a micro pipette, with the solution to be analysed on previously marked points on a circle of 4.5 cm radius. To prevent inward movement of the spots, the developing solvent was fed at 3 cm from the centre of the paper with the aid of a glass atomiser (Fig. 2). The desired delivery of the solvent in the form of a fine continuous stream could be adjusted by regulating the pressure of air and the diameter of the capillary connecting the atomiser with the solvent vessel.

EXPERIMENTAL AND RESULTS

Fe(III), Co(II), Ni(II), Mn(II), Cu(II), and Zn(II) chloride solutions in *N* HCl were spotted on the paper (diameter 38 cm), either separately or as a mixture. Butanol–HCl (1:1, v/v) mixtures, in which the concentration of the acid was varied, were used as solvents. Acetone–HCl–H_2O mixtures, well known for giving good resolution of many of the above ions in conventional chromatography[29], were also tried. The spots on the chromatograms were detected by means of an ammoniacal solution of 8-hydroxyquinoline in ethanol.

The following types of paper were used in the present studies: Whatman No. 1, Whatman No. 3 MM, Arches No. 302, Arches No. 304 and Ederol No. 202.

Effect of the type of paper on the nature of the chromatogram

As already observed by McDONALD *et al.*[15] in the case of Whatman No. 1 paper, our solvents gave with Whatman No. 1, Whatman No. 3 MM, Arches No. 302 and Arches No. 304, elliptical chromatograms, the long axis coinciding with the machine direction of the paper. Almost circular patterns were obtained with Ederol No. 202. Measurement of the R_F values of Fe(III) in different directions in the case of elliptical chromatograms showed variations of about 12 %, while in the case of Ederol No. 202 the R_F was found to be constant, *viz.* 0.17 for butanol–*N* HCl, in all directions of the paper. The detailed study reported in the present article was therefore carried out with Ederol No. 202. In order to compare the R_F values on centrifugal chromatograms with those obtained with conventional chromatographic procedures, separa-

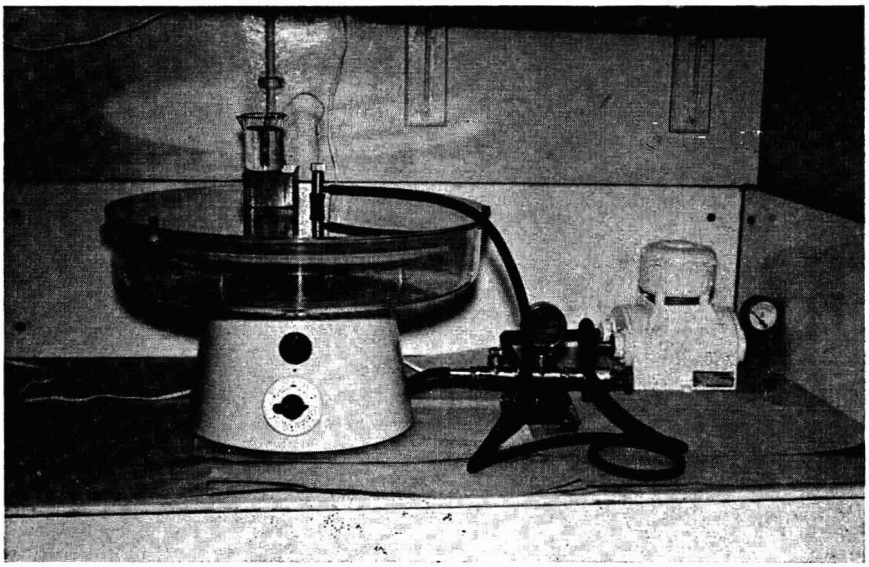

Fig. 1. Apparatus used for centrifugal chromatography.

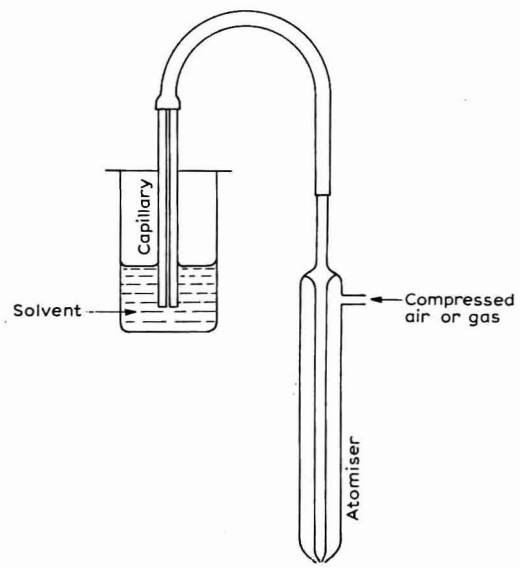

Fig. 2. Device for feeding the developing solvent to the paper.

tions were also realised side by side by the ascending technique on 3.5 cm wide Ederol No. 202 paper strips, since the R_F values of these ions on this paper have not been reported in the literature.

Effect of the solvent and its flow rate on the chromatograms

The pattern of the chromatograms and the quality of separation were greatly influenced both by the nature of the solvent and by its rate of flow. Volatile solvents, *e.g.* acetone–acid mixtures, are not favourable for centrifugal chromatography owing to evaporation of the solvent from the paper. A higher delivery rate of the solvent in order to compensate for the evaporation often leads to flooding of the paper, which gives rise to elongated and diffused bands on the chromatogram. Thus volatile solvents demand a careful adjustment of the flow rate for a given speed of rotation of the paper. Less volatile solvents, *e.g.* butanol–HCl mixtures, are most suitable for this technique and give a wider operation range. In the present work the flow rate of the solvent for "minimal wetting" of the paper was always determined in each case by preliminary experiments guided by the conditions described by McDONALD and coworkers[15], *viz.*, "the ideal flow rate ought to be such that on stopping both the flow of solvent and the rotor, the perimeter of the wetted area does not increase appreciably on standing". For Ederol No. 202 paper this flow rate varied within the range 1.5–2.25 ml/min, depending on the rate of rotation of the paper and composition of the solvent.

Effect of the rate of rotation of the paper on the chromatograms

Thick papers, namely Whatman No. 3 MM, Arches No. 304, and Ederol No. 202, could be utilised for the whole range of the rotation speeds of the apparatus; Arches No. 302 was less suitable, while the disks of Whatman No. 1 could not stand a speed higher than 500 rev./min with the solvents used.

Since the front portion of the spots moves slower than the rear portion, the spots in centrifugal chromatography are more compact than those observed with other chromatographic procedures. Thus a greater resolution for a relatively shorter run of the solvent is obtained. At higher speeds the spots are more compact and the separation therefore more complete.

Separation of Fe(III), Co(II), Ni(II), Mn(II), Cu(II) and Zn(II)

Mixtures of acetone, water and HCl, which gave satisfactory results with Ederol No. 202 paper strips in ascending paper chromatography, were not convenient solvents for centrifugal chromatography for the reasons already pointed out. Table I lists the variation of the R_F values of these ions when the concentration of HCl is increased in the butanol–HCl (1:1, v/v) mixture. The R_F values obtained with the ascending technique are also given for comparison. For the six ions examined and over the entire range of solvents used the R_F values on the centrifugal chromatograms are higher than those obtained by the ascending technique. INDOVINA AND RICOTTA[22] have also observed higher R_F values for Cu, Cd, Bi and Hg ions, in the solvent butanol–acetic acid–conc. HCl–water (45:10:1:44) in centrifugal chromatography than in the stationary technique. The R_F values have been shown to depend on the partition coefficient and the relative amounts of the two phases in contact[29]. Since the experiments with the centrifugal and ascending techniques were carried out

with the same solvent and paper system, the partition coefficient can be assumed to be practically constant. The increase in the R_F values in the case of centrifugally accelerated chromatography can therefore be attributed to the higher rate of flow of the organic phase in relation to the aqueous phase.

TABLE I

R_F VALUES OF Fe(III), Co(II), Ni(II), Mn(II), Cu(II) AND Zn(II) OBTAINED IN CENTRIFUGAL CHROMATOGRAPHY AND BY ASCENDING DEVELOPMENT

Solvent: butanol–HCl (1:1, v/v) mixtures. Paper: Ederol No. 202. Speed of rotation: 1200 rev. min. The values in parentheses are the R_F values obtained by the ascending technique.

Normality of acid	*Fe(III)*	*Co(II)*	*Ni(II)*	*Mn(II)*	*Cu(II)*	*Zn(II)*
1	0.17	0.091	0.08	0.10	0.15	0.76
	(0.092)	(0.04)	(0.043)	(0.059)	(0.086)	(0.62)
2	0.37	0.17	0.16	0.20	0.28	0.90
	(0.17)	(0.088)	(0.077)	(0.092)	(0.18)	(0.73)
3	0.49	0.26	0.26	0.31	0.37	0.94
	(0.28)	(0.17)	(0.15)	(0.15)	(0.23)	(0.78)
4	0.72	0.51	0.48	0.51	0.58	0.97
	(0.52)	(0.34)	(0.29)	(0.25)	(0.42)	(0.96)
4.25	0.76	0.66	0.65	0.67	0.70	0.97
	(0.61)	(0.46)	(0.46)	(0.46)	(0.52)	(0.96)
5	0.81	0.66	0.65	0.68	0.71	0.93
	(0.57)	(0.48)	(0.46)	(0.45)	(0.56)	(0.95)
6	0.90	0.68	0.66	0.70	0.72	0.92
	(0.98)	(0.48)	(0.43)	(0.49)	(0.59)	(0.95)
7	0.90	0.62	0.62	0.66	0.70	0.91
	(0.97)	(0.48)	(0.44)	(0.46)	(0.57)	(0.91)
8	0.98	0.63	0.60	0.67	0.73	0.89
	(0.96)	(0.45)	(0.39)	(0.43)	(0.56)	(0.82)
9	0.98	0.65	0.62	0.64	0.71	0.88
	(0.98)	(0.47)	(0.34)	(0.38)	(0.56)	(0.72)
10	0.98	0.65	0.60	0.63	0.71	0.86
	(0.98)	(0.57)	(0.31)	(0.38)	(0.56)	(0.67)
11	0.98	0.64	0.57	0.61	0.70	0.85
	(0.97)	(0.63)	(0.29)	(0.36)	(0.54)	(0.67)
12	0.98	0.67	0.51	0.55	0.72	0.83
	(0.96)	(0.65)	(0.26)	(0.37)	(0.56)	(0.67)

Except that the values were higher, the manner in which the R_F's of the ions examined varied with the concentration of hydrochloric acid in the mixture was found to be similar to that observed in the ascending technique. This indicates that the mechanism of separation of these inorganic ions in centrifugal chromatography is essentially the same as that in conventional chromatography.

The best separation of the six ions examined is obtained (Fig. 3) with butanol–12 *N* HCl (1:1, v/v) in 45 minutes by centrifugal chromatography. Although the separation is better in this solvent than in the other solvents used, the ascending technique does not permit (Fig. 4) the separation of Co(II) and Zn(II).

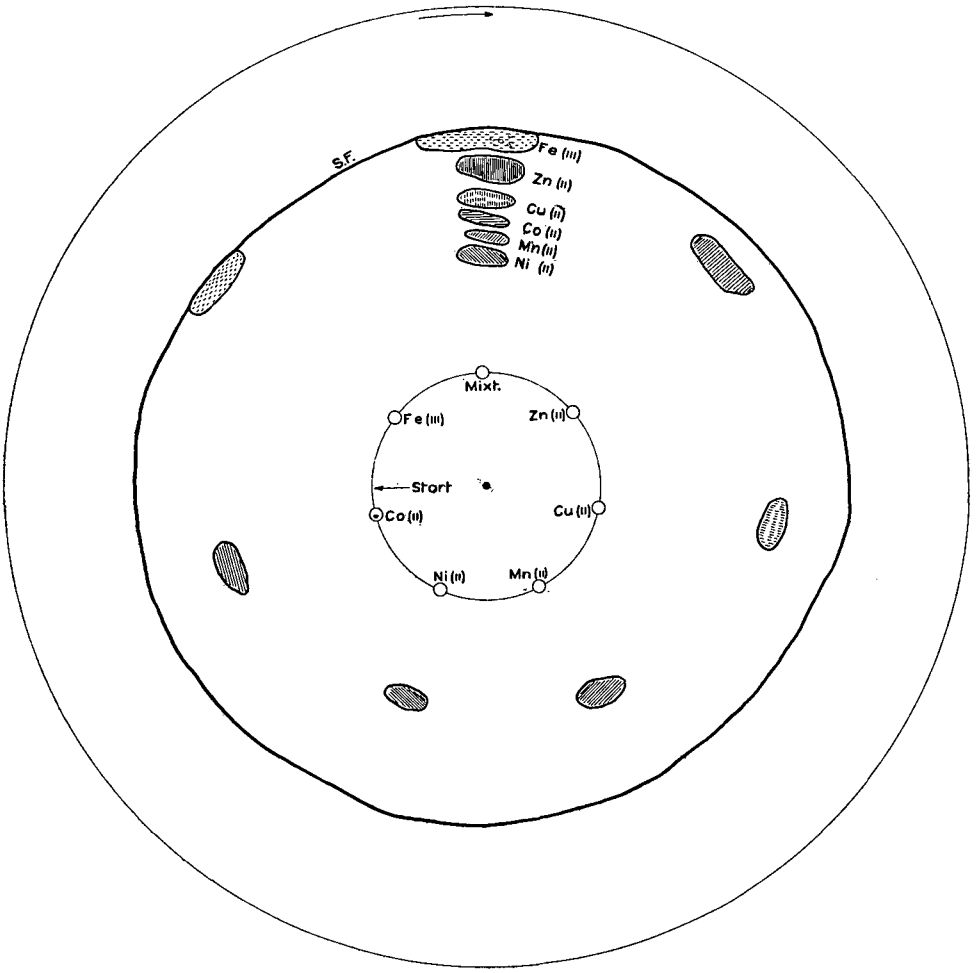

Fig. 3. Centrifugal chromatogram of Fe(III), Co(II), Ni(II), Mn(II), Cu(II) and Zn(II), and of a mixture of these ions. Paper: Ederol No. 202 (radius = 19 cm). Solvent: butanol–12 *N* HCl (1:1, v/v). Speed of rotation: 1200 rev./min. Time of development: 45 min.

The advantages of centrifugal chromatography are thus: (1) better resolution in a fraction of the time (10–15 times faster) required by other chromatographic methods; (2) possibility of studying the separation of many substances at the same time and under the same experimental conditions.

SUMMARY

Centrifugally accelerated paper chromatography has been applied to the separations of inorganic ions. A study has been made of the various factors which favour good and reproducible fractionation. A comparative study of the separation of Fe(III), Co(II), Ni(II), Mn(II), Cu(II) and Zn(II) by this procedure and by ascending chromatography is reported. The developing solvent consisted of butanol–HCl

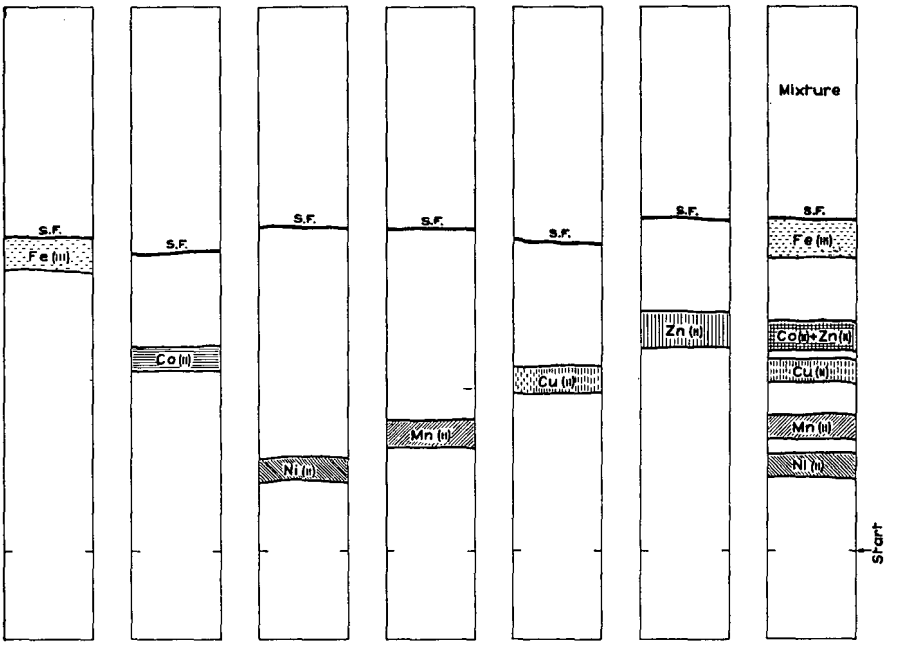

Fig. 4. Chromatograms of Fe(III), Co(II), Ni(II), Mn(II), Cu(II) and Zn(II), and of a mixture of these ions, obtained by ascending development. Paper: Ederol No. 202 (3.5 cm wide strips). Solvent: butanol – 12 *N* HCl (1:1, v/v). Time of development: 7 h.

(1:1, v/v) mixtures in which the concentration of the HCl was varied in the concentration range from 1 to 12 *N*. The best separation of all the six ions was obtained with the solvent butanol–12 *N* HCl using centrifugal chromatography only.

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INVESTIGATION OF COMPLEX EQUILIBRIA BY MEANS OF CHROMATOGRAPHY WITH ION-EXCHANGE PAPERS

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It has been shown[1-3] that chromatography on resin papers makes it possible to obtain information about the nature of ions existing in solution, to determine their charge and consequently to establish whether complexes are formed; the conclusions obtained with these procedures are, however, essentially qualitative.

Since chromatography with ion-exchange papers has a number of interesting features, such as rapidity and simplicity of operation, the present work was carried out with the aim of developing a suitable treatment of the chromatographic data in order to obtain quantitative information about the equilibria in solutions and to determine the stability constants.

To achieve this aim the method of calculation suggested by FRONAEUS for the quantitative determination of stepwise constants[4-7] has been extended to resin paper chromatography. The procedure developed has been applied to the study of the system copper–acetate, which had previously been investigated by FRONAEUS by means of cation-exchange resins[4].

THEORETICAL

Complex formation between a bivalent central ion M^{2+} and a monovalent ligand A^- according to the reaction:

$$M^{2+} + jA^- \rightleftarrows MA_j^{(2-j)+} \qquad (1)$$

the constants of which are $\beta_1, \beta_2 \ldots \beta_N$, can be determined by means of the exchange equilibria of the ionic species existing in the solution on a cationic exchanger (Na_R^+):

$$MA_j^{(2-j)+} + (2 - j)\ Na_R^+ \rightleftarrows (2 - j)\ Na^+ + MA_{jR}\ (j = 0,1) \qquad (2)$$

The relationship between exchange equilibria and equilibria of complex formation can be obtained according to FRONAEUS by introducing a distribution function φ; this function, which can be experimentally determined, is given by the following relationship:

$$\varphi = \frac{C_{MR}}{C_M}$$

where C_{MR} and C_M are the total concentrations of the metal species at the equilibrium on the resin and in the solution respectively; φ can also be expressed as a function

of the formation constants and of the concentration of ligand

$$\varphi = \frac{[M^{2+}]_R + [MA^+]_R}{[M^{2+}]\,(1 + \sum_{j=1}^{N} \beta_j\,[A^-]^j)} \tag{3}$$

It has been shown by MARTIN AND SYNGE[8] that the partition coefficent, α, of a certain species which distributes between a stationary and a mobile phase is related to the chromatographic R_F value by the equation:

$$\alpha = \left(\frac{1}{R_F} - 1\right)\frac{A_L}{A_S} \tag{4}$$

where A_L/A_S is the ratio of the amount of solvent to the amount of resin in a cross section of the paper; the validity of the above relationship has been verified in the case of equilibria on ion-exchange papers[1,9].

In the absence of a ligand the equilibrium constant K_e, of an exchange reaction

$$M^{2+} + 2\ Na_R^+ \rightleftarrows M_R^{2+} + 2\ Na^+$$

can be expressed as

$$\log K_e = \log \frac{[M^{2+}]_R}{[M^{2+}]} + \log [H^+] - 2 \log [H^+]_R$$

where:

$$\log \frac{[M^{2+}]_R}{[M^{2+}]} = \log \alpha\,.$$

In the presence of a ligand the numerator represents the total concentration of the metal species on the resin and the denominator the total concentration of the metal ions in solution; as a consequence α has the same meaning as φ (eqn. (3)), and the same theoretical treatment applied to the φ function can be extended to α.

The concentration of the metal species in the resin phase (eqn. (3)) can be expressed by means of the exchange constant K_j (eqn. (2)).

$$[MA_j^{(2-j)+}]_R = [MA_j^{(2-j)+}]\,\frac{[Na^+]_R{}^{2-j}}{[Na^+]^{2-j}}\,K_j = [MA_j^{(2-j)+}]\,l_j$$

and the function α by means of the following equation:

$$\alpha = l_0\,\frac{1 + l\,[A^-]}{1 + \sum_{j=1}^{N} \beta_j\,[A^-]^j} \tag{5}$$

where:

$$l = \frac{\beta_1 l_1}{l_0} \text{ and } l_0 = \frac{[M^{2+}]_R}{[M^{2+}]} = \frac{\lim \alpha}{[A^-] \to 0}. \tag{6}$$

The terms K_j and l_j are functions of the activity coefficients in the resin paper and in the solution; in the case of ion-exchange papers, if a medium of constant ionic strength, is used, the activity coefficients in solutions may be considered to be constant. To

relate the α values to a constant C_{MR}, according to the FRONAEUS method, experiments were carried out in which the metal ion concentration was changed over a fairly wide range; it was found that the effect upon the α value is within the range of the experimental errors. We conclude therefore that changes of activity coefficients in the resin paper have only a minor influence and may be neglected in the present treatment.

The formation constants have been calculated by means of the following procedure. From the experimental data, α and A^-, the following function is calculated

$$\alpha_1 = \left(\frac{l_0}{\alpha} - 1\right) \frac{1}{[A^-]} \tag{7}$$

which by extrapolation for $[A^-] \to 0$ gives $\alpha_1^\circ = \beta_1 - l$. From this value a new function containing the two quantities l_0 and $\beta_1 - l$ is introduced

$$f = \frac{l_0}{\varphi} \{[(\beta_1 - l)\,[A^-] - 1] + 1\} \frac{1}{[A^-]^2} \tag{8}$$

and by extrapolation, for $[A^-] \to 0$, the limiting value f° is obtained:

$$f^\circ = \beta_1 (\beta_1 - l) - \beta_2 \tag{9}$$

To obtain the formation constants the following function can be determined

$$\frac{\Delta f}{[A^-]} = \beta_1 \frac{\Delta \alpha_1}{[A^-]} - \sum_{j=3}^{N} \beta_j [A^-]^{j-3} \tag{10}$$

where $\Delta f = f - f^\circ$ and $\Delta \alpha_1 = \alpha_1 - \alpha_1^\circ$.

By plotting $\Delta f/[A^-]$ against $\Delta \alpha_1/[A^-]$, β_1 can be obtained. When $\Delta f/[A^-]$ and $\Delta \alpha_1/[A^-]$ are almost constant over the entire $[A^-]$ range investigated, eqn. (10) cannot be applied, and β_1 is approximatively given by the quotient $\Delta f/\Delta \alpha_1$. In order to obtain higher complexity constants, the function

$$g = \{f - \beta_1 \alpha_1 + \frac{l_0}{\alpha} \beta_2\} \frac{1}{[A^-]} \tag{11}$$

is calculated. By introducing the functions α_1, f and g, eqn. (4), at small ligand concentrations, can be written in the form:

$$g = \beta_1 \alpha_1 - \beta_3 \tag{12}$$

Thus g is a linear function of α_1, the slope gives β_2 and the intercept β_3.

EXPERIMENTAL

Amberlite ion-exchange paper SA-2 (Rohm & Haas) containing *ca.* 45 % of a strong cation exchanger (Amberlite IR-120 sulphonic resin in the sodium form) was used.

The paper was cut in pieces of about 22 × 3 cm size. These strips were kept for about 30 min in 1 *M* NaCl and then washed with distilled water. The treatment was repeated several times; the papers were air-dried before use.

Chromatograms were run according to the ascending technique, keeping the temperature constant at 30°. The eluting solution was allowed to reach the same height in every strip; 25 min were needed for this. The copper solution was about 0.1 M in copper perchlorate; a few microliters were used for each chromatogram.

In order to realize the conditions required for this investigation (to keep the ionic strength and the hydrogen ion concentration in the solution constant and to carry out chromatograms on a wide range of ligands) the eluants were prepared by mixing sodium acetate and acetic acid, of various concentrations, in the molar ratio 2:1 and adding sodium perchlorate to keep the ionic strength constant. The composition of every eluant was: $C_A' = $ mM AcONa, 0.5 $C_A' = $ mM AcOH and 1000 — $C_A' = $ mM $NaClO_4$. The ligand concentration was varied in the range 10–400 mM.

The spots were detected with $K_4Fe(CN)_6$; A_L was obtained from the difference in weight of a strip of pure air-dried exchange paper in the sodium form before and after impregnation with the eluting solution; A_S is known from the concentration of the resin in the paper (45 %).

The ratio A_L/A_S was calculated for several concentrations of the eluting solutions and the results were plotted *versus* the acetate concentration; by graphic interpolation this ratio could therefore be obtained for any concentration of ligand.

The experimental results are collected in Table I. In the treatment of these data according to the procedure described it was assumed that the equilibrium concentration of the ligand [A^-] is equal to the analytical concentration of the eluting solution; the factor due to the swelling of the resin and the correction due to the change of concentration of the ligand because of the formation of complexes in solution have been neglected. These approximations are justified considering the experimental conditions of the chromatographic technique and the degree of accuracy obtainable in the measurement of the R_F value.

TABLE I

CORRESPONDING VALUES OF [A^-], R_F, A_L/A_S AND α FOR THE COPPER–ACETATE SYSTEM

[A^-] mM	R_F	A_L/A_S	α
0			23.7
10	0.16	3.27	17.2
20	0.21	3.26	12.3
30	0.23	3.26	10.9
40	0.24	3.25	10.3
60	0.32	3.25	6.59
80	0.39	3.24	5.06
100	0.45	3.23	3.94
120	0.48	3.23	3.48
140	0.54	3.22	2.74
160	0.56	3.21	2.54
180	0.58	3.21	2.31
200	0.65	3.20	1.73
225	0.63	3.19	1.88
250	0.66	3.18	1.62
275	0.76	3.16	0.98
300	0.71	3.15	1.29
360	0.74	3.14	1.10
400	0.80	3.13	0.78

In Fig. 1 α is plotted against the ligand concentration [A$^-$]. To calculate l_0 according to eqn. (6), $1/\alpha$ is plotted against [A$^-$] (Fig. 2); by extrapolating to zero free ligand concentration the l_0 value (23.7) is obtained.

In Table II the calculated values for α_1, f, g, $\Delta f/[A^-]$ and $\Delta\alpha_1/[A^-]$ are reported; $\alpha_1{}^\circ$ and f° were determined by graphical extrapolation as shown in Fig. 3.

Since the values of $\Delta f/[A^-]$ and $\Delta\alpha/[A^-]$ in Table II are, within the limits of experimental error, almost constant, β_1 was calculated as the ratio $\Delta f/\Delta\alpha_1$:

$$\beta_1 = (43 \pm 10)\ M^{-1}$$

From the limiting values of l_0 and α°, it is evident that $l_0 > l$, as Cu^{2+} is more strongly sorbed than $CuAc^+$.

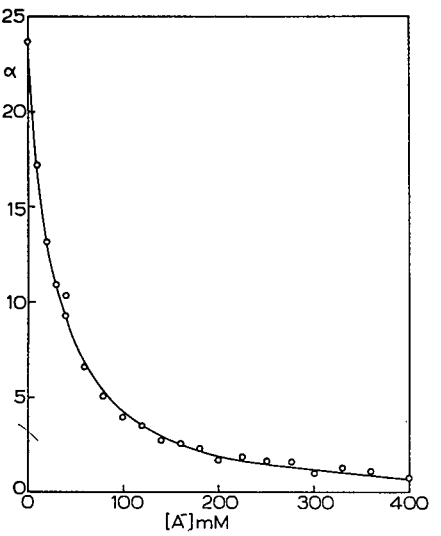

Fig. 1: Plot of the partition coefficient α *versus* ligand concentration.

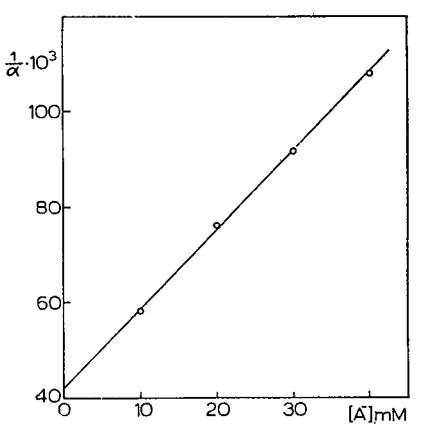

Fig. 2. Determination of the limiting value of α. Plot of $1/\alpha$ as a function of [A$^-$].

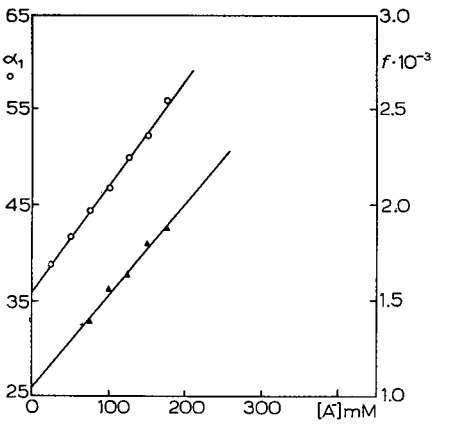

Fig. 3. Determination of the limiting values of α_1 and f. Plot of α_1(–O–O–) and f(–▲–▲–) as functions of [A$^-$].

TABLE II

CORRESPONDING VALUES OF $[A^-]$, α_1, f, g, $\Delta f/[A^-]$ AND $\Delta\alpha_1/[A^-]$

$[A^-]$ mM	α_1 M^{-1}	$f \times 10^{-2}$ M^{-2}	$g \times 10^{-3}$ M^{-3}	$\Delta f/[A^-] \times 10^{-3}$ M^{-3}	$\Delta\alpha_1/[A^-]$ M^{-2}
0	36	1.06			
25	38.8	1.33			
50	41.6	1.40	22.2	6.80	112
75	44.3	1.39	21.4	4.40	110
100	46.6	1.57	23.3	5.10	106
125	49.8	1.64	24.2	4.67	111
150	52.3	1.81	25.8	5.03	108
175	55.8	1.87	27.0	4.65	113
200	56.5	1.93	27.6	4.37	102
225	57.3	1.95	27.9	3.89	94.8
250	58.4	2.03	28.5	3.89	89.6
275	60.4	2.09	29.3	3.76	88.6
300	60.9	2.11	29.6	3.50	83.0
325	63.2	2.19	30.8	3.47	83.8
350	68.3	2.38	33.1	3.76	92.6
375	76.3	2.61	36.8	4.14	107
400	96.3	3.31	46.1	5.63	151

Using this value of β_1, and that of β_2 obtained from eqn. (9), the function g can be calculated from eqn. (11) (Table II). According to eqn. (12) g is plotted against α_1; β_2 and β_3 are respectively obtained from the slope and the intercept with the g-axis (Fig. 4):

$$\beta_2 = (500 \pm 100)\ M^{-2} \qquad \beta_3 = (800 \pm 600)\ M^{-3}$$

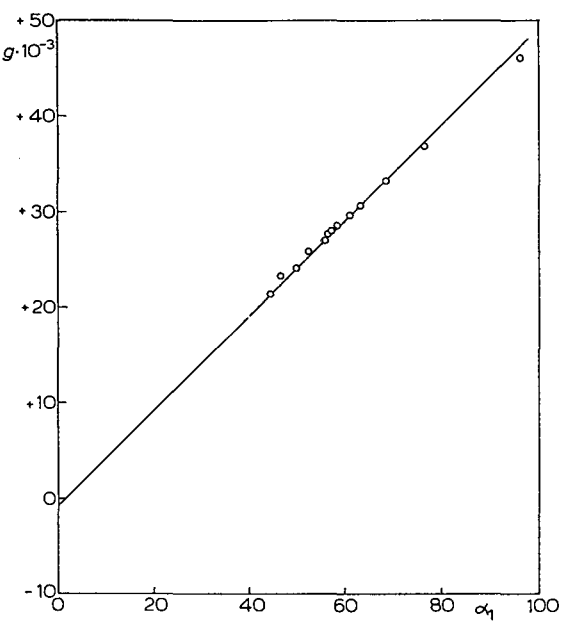

Fig. 4. Determination of the formation constants. Plot of g as a function of α_1.

CONCLUSION

A comparison between the complexity constants obtained in this work and those obtained by ion exchange, is presented:

Fronaeus investigation (t = 20°)	*Ion-exchange paper investigation (t = 30°)*
$\beta_1 = 45 \pm 2\ M^{-1}$	$\beta_1 = 43 \pm 10\ M^{-1}$
$\beta_2 = 440 \pm 60\ M^{-2}$	$\beta_2 = 500 \pm 100\ M^{-2}$
$\beta_3 = 1000 \pm 300\ M^{-3}$	$\beta_3 = 800 \pm 600\ M^{-3}$

The agreement is quite good; the use of ion-exchange paper yielding, as it was expected, a lower degree of accuracy. This result shows that the assumptions and approximations made in applying the FRONAEUS method are justified and that the method may be safely extended to the study of complex equilibria by resin paper chromatography. Though the use of chromatography with ion-exchange papers in the study of complex equilibria yields less accurate results because of the limited degree of precision afforded by paper chromatography in the determination of the R_F value, its application can be very useful because of the simplicity of operation and the speed with which experimental results can be obtained. The method should be very valuable for studying equilibria which occur on paper impregnated with ion exchangers of various nature.

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SUMMARY

Chromatography with ion-exchange papers has been used for the study of complex equilibria. The FRONAEUS method of calculation has been applied to the quantitative determination of equilibria in solution and the procedure has been checked with the system copper–acetate.

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Short Communications

The behavior of isobutane on a molecular sieve gas chromatography column

People who routinely use a Linde 5 A molecular sieve gas chromatography column for the analysis of mixtures of permanent gases and light hydrocarbons occasionally find a shifting peak in their gas chromatograms where identification is not obvious. Such a peak may be due to the presence of isobutane. Using vapor pressure considerations only, one would expect the retention time of isobutane to be several times that of methane. Actually, there can be a certain column operating temperature where the retention volume of isobutane is the same as nitrogen. At another operating temperature, isobutane in the gas chromatogram comes out just barely beyond methane.

In an experiment to study the retention of isobutane on molecular sieve, we

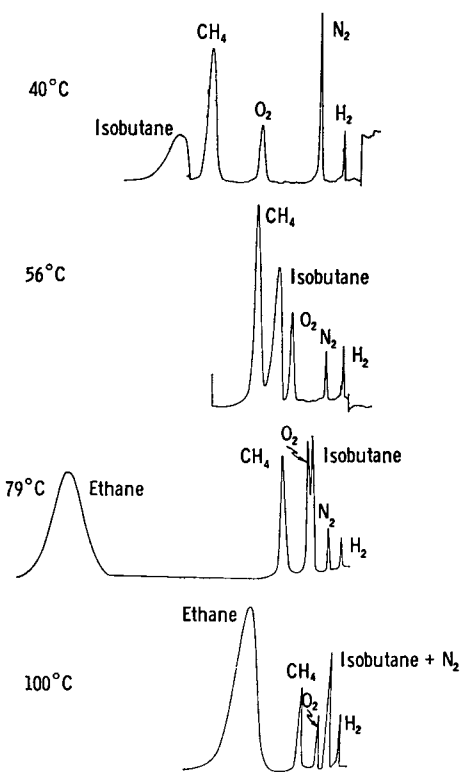

Fig. 1. Gas chromatograms at 4 different temperatures of a mixture of permanent gases and light hydrocarbons on a Linde 5A molecular sieve column.

separated a mixture of permanent gases and light hydrocarbons using a 10 ft. Linde 5A molecular sieve gas chromatography column. The gaseous mixture contained hydrogen, nitrogen, oxygen, methane, ethane, propane, isobutane, and *n*-butane. Separations were performed isothermally at 40°, 56°, 79°, and 100°. A mass spectrometer connected to the exit of the gas chromatograph[1] was used to identify each compound as it emerged from the column.

We see in Fig. 1 that isobutane is in a different spot in each gas chromatogram. It has a much smaller retention volume than either ethane, propane, or *n*-butane. Propane and *n*-butane were not observed in any of the chromatograms. Ethane was eventually eluted at 56° but was not observed at 40°. The relative retention volume of isobutane, relative to methane or any of the permanent gases, is observed to decrease as the operating temperature of the molecular sieve column increases.

Separations are made in a molecular sieve column by gas–solid chromatography coupled with a selective screening by the pores of the molecular sieve. An isobutane molecule has too large a critical diameter to pass through the screening pores; thus adsorption and desorption of isobutane can only occur on the external surface of the molecular sieve. This difference in surface areas, 1–3 m^2/g for the external area versus 700–800 m^2/g for the internal surface area[2], can account for the small retention volume of isobutane relative to the other compounds.

Because isobutane is seemingly adsorbed only on the external surface of the molecular sieve, its position in chromatograms is far more sensitive to flow rate, temperature, and previous column history than the permanent gases or small hydrocarbons which are adsorbed on the internal surface. If isobutane is present, care must be taken to see that it is not reported as one of the permanent gases.

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Notes

Centrifugal chromatography

IX. Centrifugal paper chromatography of soluble collagens*

The existence of two soluble degradation products of collagen has been repeatedly reported[2,3]. Recent work by OREKHOVICH[4] indicated that these fractions could be separated by means of precipitation. PIEZ *et al.*[5] described the chromatographic behaviour of these substances: for chromatographic separation they applied ion-exchange chromatography on a column of carboxymethyl-cellulose, the results being verified by ultracentrifugal analysis. The separation technique, however, was unsuitable for routine analysis because of its complexity.

We recently described simple ion-exchange chromatography of soluble collagens[6]. The time-wasting character of this separation led us to develop a quick type of paper

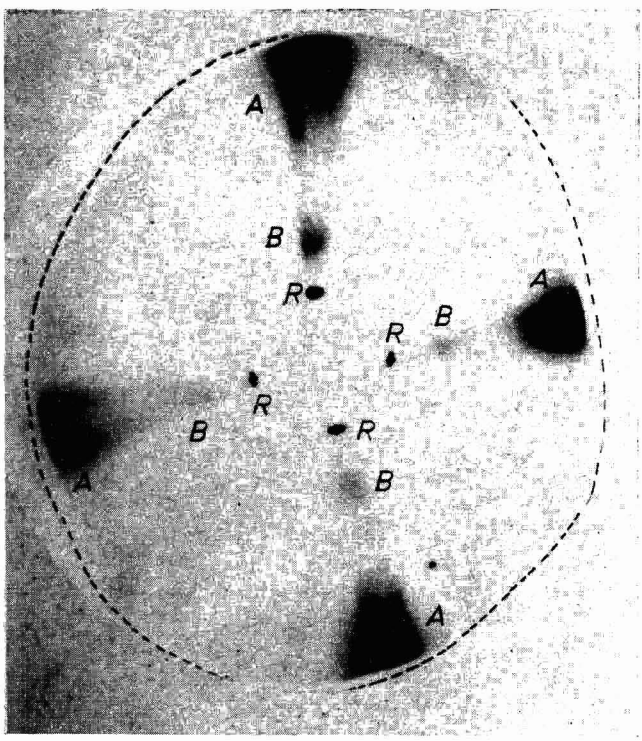

Fig. 1. Centrifugal chromatographic separation of soluble collagens (A = α fraction; B = β fraction). Paper W-3; gradient elution. Run 17 min. Detection: bromphenol blue[8].

* For Part VIII see ref.[1].

chromatography. Using the pressureless apparatus with radial development for centrifugal chromatography, which has been described elsewhere[7], it is possible to obtain perfect results within 20 min.

The bovine collagen (2.5 g) destroyed partially by alkali was extracted with 20 ml of acetate buffer pH 4.8 (μ = 0.1) at 38° for 24 h. The extract was centrifuged and the supernatant liquid applied directly on the chromatogram. Whatman paper No. 3 was found to be the most suitable. Samples of the collagenous mixture were applied in quantities of 10 μl to the start, which was 3 cm from the center of the chromatographic disc. Up to six samples could be developed at a time.

The mobile-phase gradient was made by running glacial acetic acid into 5 ml of amyl alcohol–acetic acid mixture (2:1). The necessary mobile-phase inlet was 1.2 ml per min.

Fig. 1 is a chromatogram on which the separation of two collagenous fractions is shown. In order to identify the separated fractions with those isolated by PIEZ[5] on carboxymethyl-cellulose we made parallel separations on the carboxymethyl-cellulose column. The results obtained by means of ion-exchange chromatography agreed with our results and therefore we designated the first fraction (R_F = 0.9) as the α fraction of collagen and the second (R_F = 0.45) as the β fraction. Further investigation being made in our laboratory is necessary for the identification of soluble collagens from various sources.

Thanks are due to Dr. MILOŠ CHVAPIL of the Institute of Industrial Hygiene and Occupational Diseases, Prague, for his help with the classification of soluble collagens.

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A method for diazotising (2,4-dinitro-5-aminophenyl)-amino acids on thin layer chromatograms

Recently, BERGMANN AND BENTOV[1] introduced 2,4-dinitro-5-fluoroaniline as a reagent for the formation of amino acid derivatives. Its behavior is similar to that of 2,4-dinitrofluorobenzene and it can be used in similar circumstances. The dinitroaminophenyl (DNAP) derivatives have a free amino group which can be diazotised and coupled with phenols to form intensely colored azo compounds.

BERGMAN AND BENTOV suggested spray reagents for conducting the diazotisation and coupling directly on paper chromatograms. When their aqueous spray reagents were tried on silica gel G thin layer chromatograms, the water soluble diazonium salts diffused to produce large irregular spots. For this reason, the non-aqueous system reported here was developed.

The thin layer chromatograms of the DNAP amino acids were first sprayed with a toluene solution of nitrogen trioxide (N_2O_3) and then with a toluene solution of phenol and diethylamine. The DNAP amino acid spots, which were originally light yellow turned rusty brown following this treatment. Since neither the DNAP amino acids, nor their diazonium salts nor the azo dyes were eluted from silica gel by toluene, no diffusion of the spots was noticed. This reagent system permitted the detection of 10^{-9} moles of DNAP amino acid.

Toluene solution of nitrogen trioxide: 10 ml of toluene was layered on 10 ml of 6 *N* HCl and 10 ml of 3 *M* $NaNO_2$ was added. The aqueous solution turned green and gas was evolved vigorously. Nitrogen trioxide and other materials were extracted into the toluene by swirling. The upper layer was used immediately as the spray reagent and discarded after use. Toluene solution of phenol and diethylamine: 9.4 g (0.1 mole) of solid phenol and 14.6 g (0.2 mole (20.6 ml)) of diethylamine were mixed with toluene to make 100 ml of solution. The mixture was initially a pale yellow but darkened on standing for a few weeks.

This method is similar to that used by GRIESS[2] in the original discovery of diazonium salts. In his synthesis, "nitrous acid gas" was prepared by the reaction of nitric acid with arsenious oxide and bubbled into the amine salt in ethanol; the diazonium salt crystallized out as it formed.

When hydrochloric acid and sodium nitrite solutions are mixed, nitrous acid is produced but it decomposes almost immediately to nitrogen trioxide which can be extracted into toluene. The solution so obtained will diazotise aromatic amines. Diazonium salts couple with salts of phenols. Here, the diethylamine salt of phenol was used although other phenols and other organic bases are suitable. Neither phenol nor diethylamine are strong electrolytes and toluene is not a good ionizing solvent but nevertheless there is a small equilibrium concentration of phenoxide ion to couple with the diazonium salt.

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Recording chromatograms of phosphatides on silicic acid-impregnated filter paper

Chromatography of phosphatides on silicic acid-impregnated filter paper is now a commonly used procedure. In this laboratory, while investigating the phosphatides of biological membranes, the need arose for a simple routine method to record chromatograms of different patterns. GROSSMAN[1], and CONDREA *et al.*[2] recently published photographs of rhodamine 6G stained chromatograms obtained by a camera, a method unsuitable for routine work. Photostating techniques, though simpler, need special light filters to sensitively record chromatograms of substances that either absorb[3] or fluoresce[4] in ultraviolet light. Moreover, phosphatides in the concentrations usually applied to paper chromatograms do not absorb sufficiently in the widely used range of 2537 Å to enable their easy recording by these techniques.

It is found that the phosphatide spots on silicic acid paper chromatograms can be photostatically recorded when their absorption in ultraviolet light is increased by either of the following treatments.

(1). Treating the chromatograms with nitric acid to hasten the oxidation of the phosphatides by heating. This is carried out by immersing the chromatograms in 7 *N* nitric acid for 5 min. They are then hung in an oven at 100° for 45–60 sec, washed for 1 min in running tap water, and returned to the oven for 8 min. This treatment

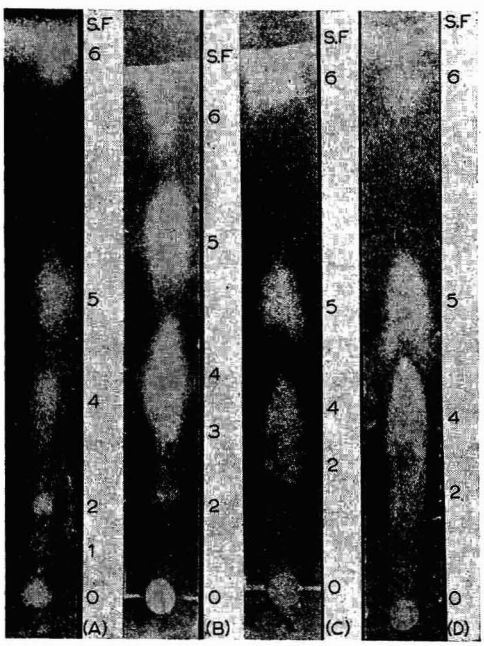

Fig. 1. Chromatograms A–D are treated with dinitrophenylhydrazine, *p*-nitrophenylhydrazine, phenylhydrazine, and by oxidation respectively. Total lipid phosphorus load is 7 μg, 14 μg, 18 μg, 11 μg in the above order. Abbreviations are: SF = solvent front; O = origin. Numbers indicate: 1 = diphosphoinositide; 2 = monophosphoinositide; 3 = sphingomyelin, 4 = phosphatidylcholine; 5 = phosphatidylethanolamine; 6 = non-phospholipids.

causes some loss of the phosphatide phosphorus, and chromatograms treated accordingly are therefore unsuitable for quantitative analysis.

(2). Immersing the chromatograms in an aqueous solution of an ultraviolet absorbing organic compound, such as 2,4-dinitrophenylhydrazine, *p*-nitrophenylhydrazine, and phenylhydrazine, (in 0.15 % solutions in 2*N* HCl). These become adsorbed on the lipid spots and increase their ultraviolet absorption, within 1/2 h, 1 h, and overnight respectively. The chromatograms are then successively washed with 2*N* HCl (10 min), running tap water (1 min), and finally dried. The adsorption treatment causes no loss of the phosphatide phosphorus, and the chromatograms are therefore suitable for purposes of quantitative analysis.

The dry chromatograms are imposed on a Kodak document paper (Duostat Rapid Reflex 23) and pressed between two thin plates of glass. Ultraviolet light (from a low pressure mercury resonance tube "Hanovia Chromatolite") is shone through for 5–10 sec, at a distance of 10 cm. It is found necessary to lower the fore-end of this lamp by 2 cm to shine a light of uniform intensity along the chromatograms. The prints shown in Fig. 1 are typical. The total lipid phosphorus load is indicated on each chromatogram after analysis of an identical but untreated chromatogram in the case of the first treatment, and a duplicate in the case of the second.

I thank Dr. R. P. Cook for his interest and advice during this work.

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Relative response of fatty acid methyl esters on the flame ionization detector

Considerable discussion has dealt with the relative response of the flame ionization detector for different organic substances. It was mentioned first by McWilliam and Dewar[1] that for hydrocarbons, the relative molar responses seem to be directly proportional to the carbon number; detailed data on this subject were presented at the 1961 International Gas Chromatography Symposium, in East Lansing, Mich.[2]. However, only very few values are available regarding substituted organic compounds[3].

Farquhar *et al.*[4] reported in 1959 that when a beta-ray (Argon) ionization detector is used, for higher fatty acid methyl esters, the peak area per cent can be taken with good approximation as concentration by weight of the individual components. We could not, however, find any published data on the relative response of a flame ionization detector for fatty acid methyl esters. Therefore, we carried out some inves-

tigations with two standard mixtures of known concentration obtained from the Applied Science Laboratories, Inc., University Park, Pa. The samples were analyzed under isothermal conditions, at 190°, on a 60 m long open tubular (Golay) column with 0.50 mm internal diameter, coated on the inside wall with butanediol succinate liquid phase: 0.1 μl liquid samples were injected directly into the carrier gas using Hamilton syringes of 0.5 μl capacity and the entire gas flow (without any split) was conducted into the column. The peak area values were evaluated with a commercial integrator.

As shown in columns 2–4 of Table I, the agreement between nominal concentration and peak area per cent is very good particularly if one keeps in mind that the samples also contained some impurities originating in the "pure" fatty acid methyl esters used for sample preparation. Our results demonstrate that with the flame

TABLE I

Components methyl esters of:	*Concentration wt-%*	*Peak area %*	*Deviation %*	*Response per equal weight*[a]	*Relative molar response*[a]	*Calculated relative molar response*[b]	*Relative deviation %*
Mixture I							
Caprylic acid	1.56	1.53	−0.03	96.8	56.6	58.0	−2.3
Capric acid	3.00	3.07	+0.07	101.0	69.6	68.5	+1.6
Lauric acid	5.96	6.01	+0.05	99.5	79.0	79.0	—
Myristic acid	11.96	11.66	−0.30	96.2	86.2	89.5	−3.6
Palmitic acid	19.42	19.68	+0.26	100.0	100.0	100.0	—
Stearic acid	24.91	25.21	+0.30	100.0	110.3	110.5	−0.2
Arachidic acid	33.19	32.84	−1.16	97.6	117.9	121.0	−2.5
Mixture II							
Myristic acid	11.83	12.23	−0.40	100.5	89.9	89.5	+0.4
Palmitic acid	23.62	23.59	−0.03	100.0	100.0	100.0	—
Palmitoleic acid	6.84	6.85	+0.01	100.3	101.9	100.0	+1.9
Stearic acid	13.09	13.43	+0.34	102.7	113.4	110.5	+2.6
Oleic acid	44.62	43.90	−0.72	98.5	107.9	110.5	−2.3

[a] Relative to methyl palmitate (= 100).
[b] Calculated from equation (1).

ionization detector, the relative peak area values can be taken as concentration by weight or in other words, that the relative response per equal weight of the detector for these substances is very close to unity (the fifth column of the table). It can also be seen that the difference between saturated and unsaturated fatty acids in this respect is very small.

Since in our previous work[2,3] we demonstrated that the relative *molar* response of various homologous series is linearly proportional to the carbon number of the molecules, we also calculated these values for the fatty acid methyl esters (sixth column of the table). For this calculation, the molar response of methyl palmitate was arbitrary taken as 100. The values obtained are plotted in the Fig. 1. It can be seen that these points form with good approximation a straight line which was obtained by connecting the points of methyl laurate and palmitate from the analysis

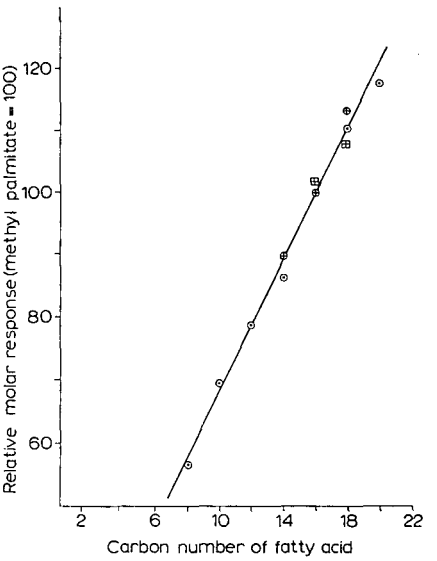

Fig. 1. Relative molar response of fatty acid methyl esters *vs.* carbon number of the molecules. ⊙ = Mixture No. I; ⊕ = Mixture No. II (saturates); ⊞ = Mixture No. II (unsaturates).

of the first mixture; this straight line corresponds—in the given system—to the following equation:

$$R_{\mathrm{Mol}} = 5.25\, C_A + 16 \qquad (1)$$

where R_{Mol} is the relative molar response and C_A the carbon number of the respective fatty acids; R_{Mol} for methyl palmitate is equal to 100. The seventh column of the table gives the relative molar responses calculated from this equation and the eighth column, the relative deviation of the actual values from these data. As a conclusion, one can say that the relative molar response of the higher fatty acid methyl esters follow the same relationship as was demonstrated previously for other homologous series: it is linearly proportional to the carbon number.

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A simple pattern for drawing the start line in paper chromatography

In this communication an improved method of drawing the start line in routine paper chromatographic analysis is described. Usually the places where the sample is to be spotted are indicated by means of a transparent graduated ruler, the place of each spot being dependent on the quality and quantity of the sample to be analysed.

We made a practical pattern of plexiglas, 2 mm thick, with the dimensions 10 × 25 cm. The width was the same as the distance between the start and the end of the paper sheet.

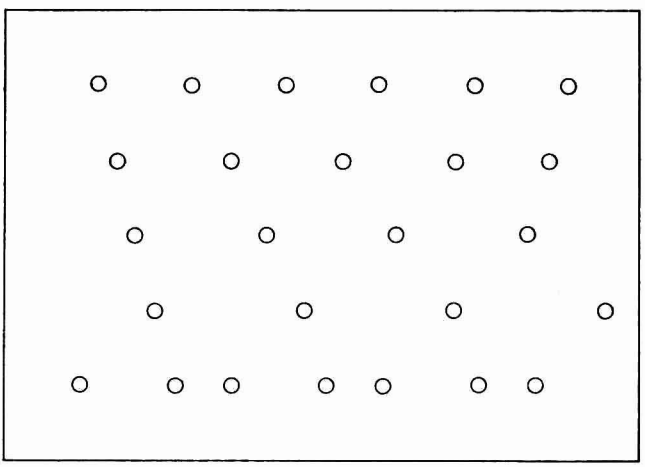

Fig. 1. The pattern of plexiglas with holes at various distances from each other.

The start line is drawn with a pencil parallel to the longest side and the suitable holes are then placed on this line. On the pattern there are several lines of holes placed at various distances from each other. The distances vary from 25 to 50 mm, and the diameters of the holes are about 2 mm.

We found this simple arrangement advantageous, when used daily in routine chromatographic analysis, because it saved much time in preparing the chromatographic sheets.

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Autoradiography of tritium chromatograms

Good autoradiographic pictures of many radio-isotopes can easily be obtained from paper chromatograms by pressing the chromatogram against an X-ray film for a suitable exposure time. With tritium, however, this technique is unsatisfactory because of the very low energy of the emitted beta particle, which has a mean penetration range of less than one micron (*cf.* ref. [1]) when the density of the medium exceeds 1.

ROGERS has described a method to overcome this difficulty by submerging the chromatogram in a liquid nuclear emulsion[2]. In this way an intimate contact between the isotope and the silver bromide is established. However, many substances would be extracted from the chromatogram by this technique. In the present paper a modification of ROGERS' procedure is described which permits autoradiographic analysis also of highly soluble tritiated substances in chromatograms. Instead of submerging the chromatogram in the liquid nuclear emulsion, the emulsion is sprayed on the chromatogram. Also some additional data are given here, which might be of experimental value.

Fig. 1 illustrates the application of the technique to a tritium and ^{14}C chromato-

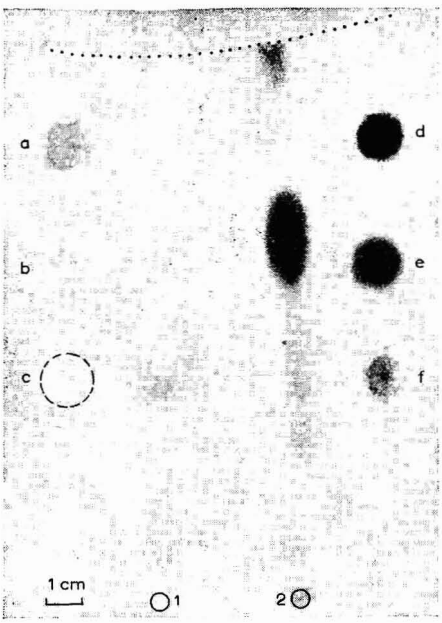

Fig. 1. Paper chromatogram of (1) $8 \cdot 10^{-3}$ μC of ^{14}C-valine and (2) 10 μC of ^{3}H-leucine run for 6 h in a mixture of butanol, acetic acid, and water (780:50:170). It was sprayed with a G5 emulsion, exposed for 4 days, and processed for 2 min in D–19b. The reference spots a–f were applied after chromatography: a–c contained $6 \cdot 10^{-3}$ μC, $2 \cdot 10^{-3}$ μC, and $7 \cdot 10^{-4}$ μC of ^{14}C-leucine (Radiochemical Centre, Amersham); d–f contained 5 μC, 1.7 μC, and 0.6 μC of ^{3}H-leucine (New England Nuclear Corp.).

gram. Further experimental data pertaining to this method are given in Table I. The chromatographic paper used in these experiments was Whatman No. 1. This

TABLE I

AMOUNT OF ISOTOPE AND EXPOSURE TIME IN AUTORADIOGRAPHY OF CHROMATOGRAMS

Isotope	*Amount of isotope to give a spot just visible after 10 days of exposure*		*Grain yield per disintegration in just visible spot**
	μC per cm²	*Number of disintegrations per min and cm²*	
^{3}H**	$3 \cdot 10^{-2}$	$7 \cdot 10^{4}$	0.3
^{14}C***	$1 \cdot 10^{-3}$	$2 \cdot 10^{3}$	10

* The calculations were based on the observation that 50 grains per 100 μ^2 give a just visible spot in ordinary autoradiograms with G5 emulsions.
** ^{3}H-DL-Leucine (New England Nuclear Corp., Boston, Mass.).
*** ^{14}C-L-Leucine (Radiochemical Centre, Amersham, England).

paper was, however, found to desensitize the nuclear emulsion for beta particles, an effect that was overcome by treating the paper before use with diluted (0.3 *M*) hydrochloric acid for a few hours. The paper was then washed for 1 h in running tap water and finally rinsed several times in distilled water.

The nuclear emulsion used was Ilford G5. It was melted by heating to 50° and diluted with 7 volumes of distilled water. The chromatogram was then sprayed with the emulsion using an all-glass spray and nitrogen or carbon dioxide for the gas stream. 10 ml of emulsion was found sufficient for 400 cm² of paper. Drying of the chromatogram was completed in about 1 h in a box with an electric fan and calcium chloride as desiccant. The dry chromatogram was carefully wrapped in aluminium foil to prevent light coming into contact with the emulsion. All these operations were carried out in a dark room illuminated with a 25 Watt bulb, and using a Wratten filter No. 1.

The exposure was carried out in a refrigerator. A suitable exposure time might be calculated from the data in Fig. 1 and Table I. The exposed chromatogram was processed in Kodak D–19b developer for 2 min followed by a stop bath. It was then fixed in an acid hypo for 15 min, and rinsed for at least 1 h in running tap water.

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Herstellung von Eichgemischen im Bereich der inerten Gase und leichten Kohlenwasserstoffe, insbesondere für die Gaschromatographie

Eine Reihe von physikalischen Analysengeräten (Spektrophotometer, Gaschromatographen) ist für die quantitative Analyse auf die Herstellung und Anwendung von Eichgemischen angewiesen. An dieser Stelle soll insbesondere die Gaschromatographie näher in diesem Zusammenhang betrachtet werden. Die meisten kommerziellen Gaschromatographiegeräte sind so ausgerüstet, dass die quantitative Eichung mit Eichgemischen erfolgen muss. Unter Einhaltung der Analysenbedingungen (Art der Säule, Temperatur der Säule, Strömungsgeschwindigkeit des Trägergases etc.) wird zumeist im Bereich des Wasserstoffs, der inerten Gase, der C_1-, C_2-, C_3-, C_4-Kohlenwasserstoffe ein volumetrisch hergestelltes Eichgemisch verwendet. Aus der Literatur[1] und aus eigenen Experimenten war uns bekannt, dass vor allem im Bereich der realen Gase (C_2-, C_3-, C_4-Kohlenwasserstoffe) erhebliche Abweichungen vom Dalton'schen Gesetz eintreten, d.h. ein synthetisches Gemisch, welches C_2-, C_3-, und C_4-Kohlenwasserstoffe enthält, kann infolge der Kompressibilität dieser Gase mehr C_4 und C_3 enthalten als laut volumetrischer Messung angezeigt wird. Wird mit solchen Gemischen die Eichung vorgenommen, so wirkt sich dies bei der Analyse von derartigen Kohlenwasserstoffen so aus, dass zu wenig von der Komponente gefunden wird, die die höchste Kompressibilität aufweist.

Für Gaschromatographiegeräte, die nicht mit einem Eichsystem nach dem Verfahren von VAN DE CRAATS[2] ausgerüstet und daher auf die Eichung mit Eichgemischen angewiesen sind, wurde folgendes Verfahren zur Herstellung von exakten Eichgemischen im Bereich der inerten Gase und niederen Kohlenwasserstoffe für Mengen bis zu *ca.* 40 g mit Erfolg angewendet.

In eine kleine geeignete Stahlflasche (Probeflasche C nach DIN 51 610):

Inhalt:	0.1 l
Leergewicht:	0.6–0.7 kg
Füllgewicht:	40 g
Betriebsdruck:	16.7 atü
Probedruck:	25 atü

werden die benötigten Eichkomponenten (Reinst-Kohlenwasserstoffe) nach sorgfältigem Evakuieren gepresst bzw. mit Hilfe von flüssigem Stickstoff kondensiert. Die Komponenten werden in der Reihenfolge steigender Dampfdrucke kondensiert, um ein Rückdiffundieren aus der Stahlflasche in die zu dosierenden Reinkomponenten zu verhindern. So wird z.B. zuerst *n*-Butan und dann Propan usw. aufgepresst. Von jeder Komponente wird das Gewicht durch Wägung auf einer Analysenwaage ermittelt. Dazu ist der Einsatz einer speziellen Analysenwaage notwendig, deren Tragkraft für das Gewicht der Stahlflasche einschliesslich des Gewichts der Komponentenmischung geeignet ist. Mit gutem Erfolg wurde hierfür die Analysenwaage B 5 C 1000 der Firma Mettler mit einer Tragkraft von 1 kg, einer Empfindlichkeit von 0.1 mg und einer Reproduzierbarkeit von $\pm$ 0.1 mg eingesetzt.

(1) Bei einem Füllgewicht von *ca.* 40 g beträgt der durch Einwaage mögliche relative Fehler bei einem binären flüssigen Gemisch von Propan–Butadien-1,3 von *ca.* 70 Gew.-% Propan und 30 Gew.-% Butadien:

Bei einer Einwaage von 28 g: ± 0.00035 % rel. C_3H_8.

Bei einer Einwaage von 12 g: ± 0.001 % rel. C_4H_6-1,3.

Auch für die Eichung von Spurenkomponenten ist das Verfahren geeignet:

(2) Bei einer Einwaage von 0.04 g Propin in 39.06 g C_3- oder C_4-Kohlenwasserstoffen in Flüssigphase in der Probeflasche C beträgt der rel. Fehler für Propin: ± 0.25 %.

(3) Von einem Gasgemisch von 70 Vol.-% H_2 und 30 Vol.-% CH_4 lassen sich bei 10 atü Druck in 0.1 l Stahlflaschen 63 mg H_2 und 215 mg CH_4 einbringen mit einem rel. Fehler von ± 0.16 % für H_2 und ± 0.05 % für CH_4.

Gemische aus inerten Gasen und unter Druck bei Zimmertemperatur leicht verflüssigbaren Komponenten (C_3-, C_4-Kohlenwasserstoffe) werden zweckmässig gasförmig in Gaspipetten eingewogen und zur Eichung eingesetzt, da derartige Gemische in Stahlflaschen unter Druck leicht zweiphasig vorliegen und daher zu Eichzwecken ungeeignet sind.

Auch bei Anwendung einer kleineren Gemischmenge, z.B. in einer Gaspipette in Gasphase erwies sich die Methode als sehr gut brauchbar, wie aus dem folgenden Beispiel hervorgeht:

(4) Bei einer Einwaage von C_4H_8-1 und *i*-C_4H_8 in einer *ca.* 1 l Gaspipette (Leergew.: 0.3–0.5 kg) im Verhältnis von z.B. 40:60 wird dieses Gemisch mit einem rel. Fehler erhalten von:

(a) bei einer Einwaage von 1,000 g: ± 0.1 mg = ± 0.01 % C_4H_8-1
bei einer Einwaage von 1,500 g: ± 0.1 mg = ± 0.007 % *i*-C_4H_8
für eine Reproduzierbarkeit der Waage von ± 0.1 mg,

(b) bei einer Einwaage von 1,000 g: ± 1 mg = ± 0.1 % C_4H_8-1
bei einer Einwaage von 1,500 g: ± 1 mg = ± 0.07 % *i*-C_4H_8
für eine Reproduzierbarkeit der Waage von ± 1 mg.

Aufgrund dieser Überlegungen und der gewonnenen Erfahrungen lässt sich sagen, dass auch die preisgünstigere Waage B 4 C 1000 der Firma Mettler mit ebenfalls 1 kg Tragkraft, aber einer Reproduzierbarkeit von nur ± 1 mg eingesetzt werden kann. Der rel. Fehler bei Anwendung dieser Waage würde um eine Zehnerpotenz höher liegen, damit immer noch unter dem, der üblicherweise für eine gaschromatographische Analyse genannt wird.

In Tabelle I sind einige derartige Eichgemische im Vergleich zu Analysenergebnissen wiedergegeben, für die die Eichung nach dem Verfahren von VAN DE CRAATS[2] vorgenommen wurde.

TABELLE I

		Gegeben %	*Gefunden %*	*Rel. Fehler*
Gemisch 1				
(Flüssig in Stahlfl.)	Propan	88.3	88.4	+0.11%
40 g Kohlenwasserstoffe	Butadien-1,3	21.7	21.6	—0.46%
Gemisch 2				
(Flüssig in Stahlfl.)	*n*-Butan	87.6	87.8	+0.23%
40 g Kohlenwasserstoffe	Butadien-1,3	22.4	22.2	—0.89%
Gemisch 3				
(Gasförmig in Gaspipette)	Buten-1	36.5	36.1	—1.1%
2–3 g Kohlenwasserstoffe	2-M-Propen	63.5	63.9	+0.6%

Das beschriebene Verfahren der gravimetrischen Einstellung der Gemische hat gegenüber der vielfach üblichen volumetrischen Herstellung von Eichgemischen ausser der genaueren Messung den Vorteil, dass bei Vorliegen von Dampf- und Flüssigphase in den Stahlgefässen (Probeflasche C nach DIN 51 610) nebeneinander durch einfaches intensives Schütteln schnell eine homogene Mischung erhalten werden kann.

Die angegebenen Füllmengen von bis zu 40 g erscheinen für die meisten Laboratorien angemessen und reichen sogar in den meisten Fällen als Eichgemisch über einen grösseren Zeitraum. Sie können ohne Bedenken gelagert werden, wenn sich die Gemische in V2A- bzw. V4A-Stahlgefässen befinden.

Das angegebene Verfahren hat sich als geeignete Methode im Falle von Analysenvergleichen zwischen verschiedenen Laboratorien bewährt und lässt sich ausser auf Gaschromatographiegeräte auch auf andere physikalische Gasanalysengeräte, wie z.B. Spektrometriegeräte, anwenden.

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Eingegangen den 5. Oktober 1962

Electromigration in fused salts in the study of complex ions

The existence of complex ions in molten salt solutions has frequently been postulated, and direct evidence is available in a number of cases. Paper electromigration can be used to determine the sign of the charge on an ion, and is a particularly simple way of proving that anionic complexes of metal ions exist in solution. Electromigration in asbestos or glass fiber paper with a fused salt as background electrolyte has been described by ARNIKAR[1] and by ALBERTI *et al.*[2]. We have used this technique to show the presence of a number of transition-metal thiocyanate complexes in solution in molten KCNS, and to compare the behavior of these metals here with that in molten $LiNO_3$–KNO_3 eutectic mixture where essentially no complexing is expected.

The electromigration was carried out in strips of Whatman GF/A glass fiber paper, 30 cm long and 1 cm broad. The strip was saturated with the salt by moistening with an aqueous solution of it, and drying. The experiments were performed in a tubular Vycor furnace kept at the desired temperature. The strip was supported on a Pyrex bridge between Pyrex boats filled with excess salt. Steel rods were used as electrodes with KCNS, while a steel cathode and platinum anode proved suitable with the nitrate melt. They were inserted through holes in the furnace and dipped into the boats.

Results are reported in Table I. The anionic behavior of Zn(II), Co(II), Cr(III), and Re(V) shows that these ions form thiocyanate complexes in molten KCNS solution. In the case of Co(II) and Cr(III), this confirms the spectroscopic evidence of HARRINGTON AND SUNDHEIM[3]. The rhenium (V) thiocyanate complex ($K_3[ReO_2(SCN)_4]$)[4] was prepared by reduction of $KReO_4$ in the presence of KSCN in aqueous

TABLE I

Ion	*$LiNO_3$–KNO_3 eutectic, 255°*		*KSCN, 210°*	
	Time (h)	*Movement (cm)*[a]	*Time (h)*	*Movement (cm)*[a]
Ag(I)	3	+2.5	2	+2.5
Tl(I)	11/2	+5.5	21/4	+7.5
Co(II)	3	0[b]	3	—1.5
Cu(II)	3	0[b]	3	+2.5[c]
Zn(II)	2	+1.5	31/2	—0.5
Cd(II)	2	*ca.* +4	31/2	0[b]
Pb(II)	2	+4.5	2	+1.0
Sn(II)	11/2	+5.5	2	0
Cr(III)	2	+3.0	31/2	—2.0
Bi(III)	11/2	+4.5	21/4	0
Re(V)	—	—	21/2	—2.0

Average voltage gradient for each run *ca.* 10 V/cm).

[a] +: movement towards cathode; —: movement towards anode. Values are from center of initial spot to center of zone after moving.

[b] Insoluble precipitate formed.

[c] May have been converted to Cu(I).

solution, and this orange solution was used as the sample. The anionic motion of the orange zone is evidence that the complex ion is stable under these conditions.

Several other ions which might form thiocyanate complexes, such as Fe(III), and Ni(II), gave an apparently insoluble product, which may have been oxide, in both electrolytes.

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[1] H. J. ARNIKAR, *Compt. Rend.*, 244 (1957) 2241.
[2] G. ALBERTI, G. GRASSINI AND R. TRUCCO, *J. Electroanal. Chem.*, 3 (1962) 283.
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A simple device for cleaning and coating capillary columns

A simple device is described which can be used to remove the substrate from gas chromatographic capillary columns and replace it in 15 min. The unit was constructed of readily available materials* and assembly required a minimum of skill. Several units have been described in the literature[1-3], however, the present device is simpler, faster, and more convenient.

A reservoir was constructed of 1 in. × 6 in. pipe (A), capped at one end with a pipe cap as shown in Fig. 1. The other end of the pipe was fitted with a 1 in. coupling and 1 in. to $^1/_4$ in. pipe bushing. Swagelok tube fittings were used in the assembly on all tubing. A tee (B) having two $^1/_4$ in. tube fittings and a $^1/_4$ in. pipe fitting was drilled out so that a $^1/_4$ in. tube (E) could be inserted through the pipe end extending

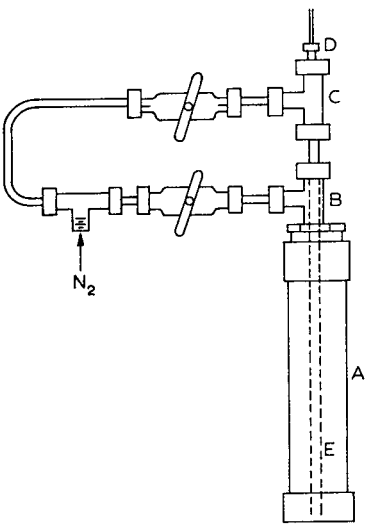

Fig. 1. Device for coating capillary columns.

to the bottom of the reservoir. The bottom of this tube was notched to allow the liquid to enter the tube. The close proximity of tube (E) to the bottom of the reservoir facilitated draining of the reservoir when cleaning. The tube was sealed by tightening the upper tube nut on tee (B). To the end of the tube was fastened another tee (C) to which ultimately the capillary column will be fastened through a $^1/_4$ in. to $^1/_{16}$ in. tube reducer (D). A shut-off valve was fitted to the center fitting of each tee and connected by tubing to a source of nitrogen gas. The entire assembly was constructed of stainless steel and all pipe threads were covered with Teflon tape prior to assembly.

New capillary columns were cleaned or the substrate removed from old columns by flushing with approximately 20 ml of solvent. The solvent was poured into the reservoir through tee (C) after removal of reducer (D). It was necessary to disconnect the nitrogen inlet fitting and open the lower valve to permit venting. The capillary

* References to specific products of commercial manufacture are for illustration and do not constitute endorsement by the U.S. Department of Agriculture.

column was attached at (D) and the solvent forced through the system with nitrogen. Nitrogen flow was continued until the entire system was dry.

Columns were coated in a similar manner except that a solution of the stationary phase was introduced instead of the pure solvent. In coating a 250-ft. column of 0.020 in. inside diameter, 50 ml of a 20 % solution of the stationary phase provided sufficient material and a nitrogen pressure of 80–100 lbs./sq. in. was adequate. The column was coated in stages to prevent substrate plugging of the capillary. The lower valve was opened for 2 min followed by opening of the upper valve facilitating distribution of the material through the column. The process was repeated until substrate appeared at the open end. Nitrogen gas was permitted to flow through the column for several minutes to evaporate the solvent. The column described has been cleaned and coated several times and each time a total of the 15 min was required for the entire procedure. If it is desired, the column can be equilibrated by allowing nitrogen to flow through it for a longer period of time.

The device was cleaned without disassembly of the reservoir. The nitrogen inlet was removed, the reservoir filled with solvent as described above and flushed using laboratory air pressure. The process was repeated at least 3 times to give satisfactory results.

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[1] G. DIJKSTRA AND J. DEGOEY, in D. H. DESTY, *Gas Chromatography*, Academic Press, New York, 1958, p. 60.
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* One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Apparatus for extraction of compounds from paper chromatograms

It is frequently necessary to extract the compounds separated on a paper chromatogram for further examination, for example by ultra-violet or infra-red spectrophotometry.

The apparatus described in this note (see Fig. 1) is simpler than those given by WYATT[1] and DENT[2], and can be used with volatile extracting solvents. A number may be compactly mounted in a rack for simultaneous extractions.

It is readily assembled from standard interchangeable glass joints, *i.e.*: Quickfit and Quartz Ltd. B19 test tube MF/24/2/6, a B19/B24 connecting bend (stillhead) SH1/23 and a B24 socket SRB/24 which has been drawn out and sealed as shown in the diagram.

The spot, with a small part of the surrounding paper, is cut out of the chromatogram, shaped to a point and attached to a paper wick (see Fig. 2). The paper is then

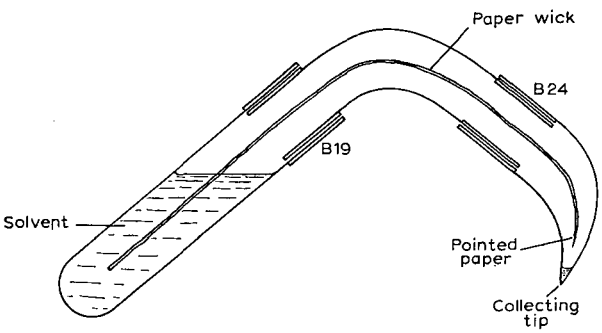

Fig. 1.

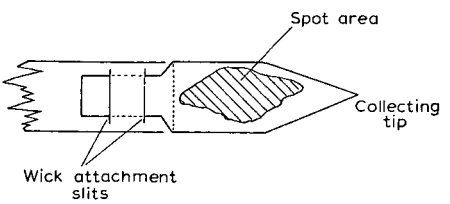

Fig. 2.

inserted into the apparatus and left to elute, the wick dipping into solvent in the B19 test tube and the paper point directed towards the tip of the B24 receiver. After two or three drops of solvent have fallen into the collecting tip, the spot should have been extracted and the receiver can be removed and stoppered until required.

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Quantitative Bestimmung von Saccharose mit Triphenyltetrazoliumchlorid mittels Papierchromatographie

Es ist eine ganze Reihe von Verfahren zur quantitativen Bestimmung der Saccharose mittels Papierchromatographie bekannt. Die Quantität wird entweder *in situ*, also nach Detektion direkt am chromatographischen Papier gewöhnlich mit dem Densitometer bestimmt, oder erst nach Elution der Saccharose aus dem chromatographischen Papier ausgewertet. Die eluierte Saccharose wird dann entweder titrimetrisch oder kolorimetrisch bestimmt.

Die quantitative Auswertung *in situ* ist zwar schnell, aber weniger genau und, was die Ausstattung an Geräten anbelangt ziemlich anspruchsvoll. Von den titrimetrischen Methoden wird am häufigsten die Perjodatmethode[1], von den kolorimetrischen die Methode nach SOMOGYI–NELSON[2] angewendet nach sauerer Hydrolyse der Saccharose.

Beide Methoden zeichnen sich durch hohe Genauigkeit aus, sind aber an Zeit und Arbeit sehr anspruchsvoll. Diese Nachteile der angeführten Methoden gaben uns den Anlass, die sehr elegante und schnelle Methode von FISCHER UND DÖRFEL[3] nach der die reduzierenden Zucker mit Hilfe von Triphenyltetrazoliumchlorid (TTC) bestimmt werden, auch auf die quantitative Bestimmung der Saccharose anzuwenden.

Methodisches

Der einzige gangbare Weg zur Bestimmung der Saccharose mittels TTC war ihre Hydrolyse mit dem Enzym Invertase, genauer β-h-Fruktosidase*. Zur Hydrolyse der Saccharose verwendeten wir das genannte Präparat, das nach vorhergehender Aufbereitung keinerlei reduzierende Eigenschaften auf TTC aufwies und den reinen Präparaten von Spitzenfirmen (Merck) gleichkam.

Die Aktivität des Enzyms war 6.55 Min./g des Präparats (die Zeit, die zur Hydrolyse von 4 g Saccharose in 25 cm³ Lösung bei einer Temperatur von 15.5° nötig ist, bis zur Herabsetzung des optischen Drehungsvermögens auf Null).

Die Enzymlösung wurde aus 0.1 g enzymatischen Präparats in 50 cm³ 0.5 % KH_2PO_4-Lösung hergestellt. Nach gründlicher Durchmischung wurde die Lösung bei 2,000 *g* zentrifugiert. Zur Besprühung wurde das klare Supernatant verwendet. Das chromatographische Papier wurde in seiner Gänze gleichmässig mit der Enzymlösung besprüht und in der feuchten Kammer bei 18–20° für 40 Min. inkubiert. Die Verlängerung der Inkubierung bis auf 60 Min. hat keinen Einfluss auf die quantitative Auswertung (siehe Fig. 1). Nach Beendigung der Inkubation wird das Chromatogramm bei 60–70° ausgetrocknet. Das weitere Verfahren wird nach den Vorschriften von FISCHER UND DÖRFEL ausgeführt.

Das chromatographische Papier wird durch eine frische Lösung von TTC (1 Teil 1 *N* NaOH in Methanol und 1 Teil 4 % TTC in Methanol) gezogen und frei getrocknet. Es empfiehlt sich die angeführten Operationen bei rotem Licht, oder wenigstens in Dämmerlicht auszuführen, damit der Hintergrund des Papiers lichtrosa verbleibt. Das chromatographische Papier wird dann 30 Min. bei 70° getrocknet. Die roten

* Das enzymatische Präparat bekamen wir aus dem Nährmittelforschungsinstitut in Bratislava (Výskumný ústav potravinársky), wo sie es durch Dehydrierung biologisch aktiver Hefe mittels Alkylglykoläther nach einer von ihnen patentierten Methode präparierten[4].

Flecke der "Saccharose" werden ausgeschnitten und in 10 cm^3 Mischung von Methanol und Essigsäure (10:1) eluiert. Die Elution wird in 10 Min. bei Zimmertemperatur ausgeführt.

Die Auswertung erfolgte auf einem Lange-Kolorimeter IV (blaues Filter) auf Grund einer Kalibrationskurve, die für jeden Papierbogen resp. für jede Versuchsserie eigens angefertigt wird.

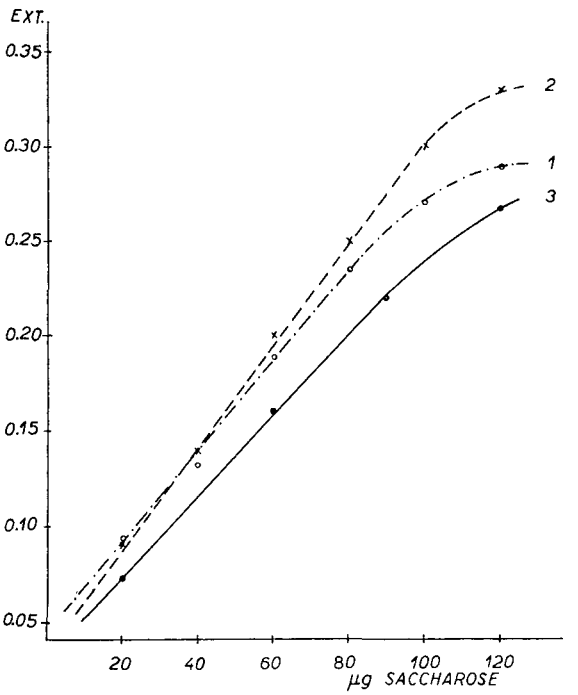

Fig. 1. Kalibrationskurve der Saccharose nach 20 (Kurve 1), 40 (Kurve 2), und 60 Min. (Kurve 3) enzymatischer Hydrolyse.

Diskussion

Die angeführte Methode zur Bestimmung der Saccharose hat sich bei unseren Serienanalysen von Pflanzenmaterial sehr bewährt. Um die Genauigkeit und Verlässlichkeit der Methode zu prüfen, haben wir eine ganze Reihe von Bestimmungen durchgeführt. Mit Rücksicht darauf, dass wir ein technisches enzymatisches Präparat verwendeten, haben wir seinen Einfluss auf die quantitative Bestimmung von Trauben- und Fruchtzucker durchgeführt. Die Ergebnisse haben gezeigt, dass es nach 60 Min. Inkubationszeit nur zu sehr geringen Verschiebungen der Kalibrationskurven der mit dem Enzym besprühten und nicht besprühten Flecke des Frucht- und Traubenzuckers kommt (Fig. 3 und 4). Auf die Genauigkeit der quantitativen Auswertung hat die Besprühung mit dem Enzympräparat überhaupt keinen Einfluss, da seine Veränderungen in der Kalibrierungskurve eingeschlossen sind. Um die optimale Zeitdauer der enzymatischen Hydrolyse festzustellen, haben wir ganze Serien von Proben nach 20, 40 und 60 Min. Hydrolyse ausgewertet. Die Ergebnisse zeigen, dass eine enzymatische Hydrolyse von 40 Min. Dauer am vorteilhaftesten ist (siehe Fig. 1).

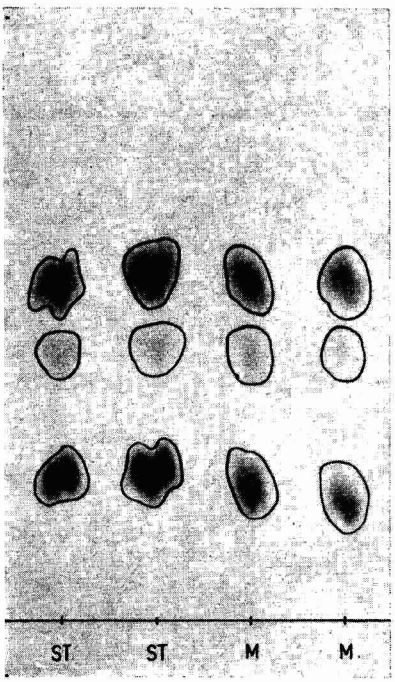

Fig. 2. Chromatogramm der Zucker nach enzymatischer Hydrolyse detegiert mit TTC. Entwicklungssystem Butanol–Essigsäure–Wasser (4:1:5); entwickelt 48 Stunden mit Durchlauf auf Whatman No. 1 Papier.

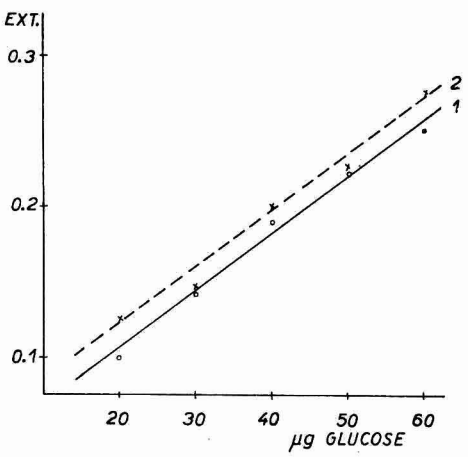

Fig. 3. Einfluss der Invertase auf die Kalibrationskurve der Glukose nach 60 Min. Inkubationszeit. (1) Kalibrationskurve nach Besprühung mit Invertase. (2) Kalibrationskurve ohne Besprühung mit Invertase.

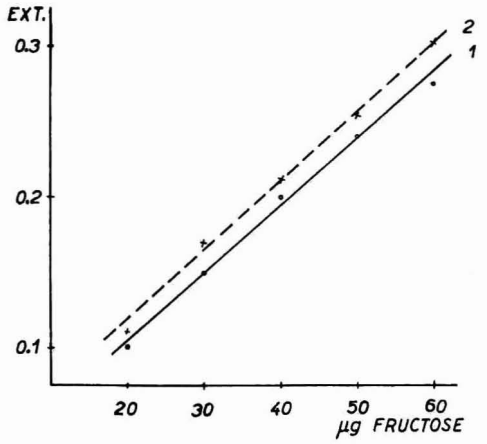

Fig. 4. Einfluss der Invertase auf die Kalibrationskurve der Fructose nach 60 Min. Inkubationszeit. (1) Kalibrationskurve nach Besprühung mit Invertase. (2) Kalibrationskurve ohne Besprühung mit Invertase.

Die Genauigkeit und Reproduzierbarkeit der Ergebnisse haben wir sowohl mit einer Standard-Saccharoselösung als auch direkt in Gerstenblättern überprüft, wo die angeführten Werte mit den nach der Methode von SOMOGYI–NELSON festgestellten Werten verglichen wurden (siehe Tabelle I).

TABELLE I

	n	*Ermittlung der Saccharose nach Somogyi–Nelson mg/1 g Trockensubstanz*	*Ermittlung von Saccharose mittels TTC mg/1 g Trockensubstanz*
30 µg	6	—	31 µg ± 2.1
80 µg	4	—	78.5 µg ± 3.3
I Blatt	4	33.6 ± 1.08	34.0 ± 0.99
II Blatt	4	29.4 ± 0.91	27.1 ± 0.92
III Blatt	4	15.6 ± 1.01	13.8 ± 0.87

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Book Reviews

Thin-layer chromatography

Dünnschicht-Chromatographie, Ein Laboratoriumshandbuch, edited by EGON STAHL, Springer Verlag, Berlin, 1962, xvi + 534 pages, price DM 56.—.

Dünnschicht-Chromatographie, by KURT RANDERATH, Verlag Chemie GMBH, Weinheim/Bergstr., 1962, 243 pages, price DM 22.—.

Both these books deal with a technique which has aroused considerable interest in the last few years. It permits separations on adsorbent layers utilising a technique very similar to and hardly more complicated than paper chromatography. Some authors have also found it advantageous to effect partition and ion-exchange separation on thin layers rather than on paper or in columns. This trend towards thin layers has also instigated their use in electrophoresis, and thus a technique, which is already widely used in the clinical laboratory in work with immunological reactions, has been "rediscovered" for the chemical laboratory.

Both books here reviewed were planned as handbooks giving detailed descriptions of apparatus and techniques, followed by chapters dealing with the separations of various groups of compounds. Both give numerous tables of R_F values, tables of reagents and some previously unpublished results. The book by RANDERATH is shorter, hence less detailed in some respects and on some topics fewer references are given. This should not, however, be counted as a serious defect since much of the more recent work has not been included in either book. Each of the two monographs would equally well serve the reader as a handbook for work in thin-layer chromatography.

The appearance of two books and the announcement of a third (in English) dealing with a method which is only a few years old and yet has already superseded the scarcely older paper techniques, gives food for thought.

The advantages of thin-layer chromatography over paper chromatography evidently lie in the use of a more finely dispersed solid phase, with which equilibria are more rapidly reached and hence more efficient separations are achieved with shorter development times and smaller samples. Its disadvantages are quite considerable: the thin layer must first be prepared and thus time is lost and, according to some authors, expensive equipment must be procured. It is also certain that the glass–adsorbent interface is an undesirable factor and interference due to it will be observed sooner or later in some separations. These considerations lead the reviewer to the conclusion that the general interest shown in thin-layer chromatography indicates rather a lack of interest (on the side of the industry concerned) in the development of suitable chromatographic papers. There is no thin layer which could not be replaced by adsorbent-impregnated glass fibre paper, providing sufficient attention is paid to the capillary qualities etc. of the paper. Similarly there appears to be really no reason why chromatographic papers are still made solely with fibres that are obviously too

coarse and are only retained due to industrial traditions. Perhaps simple incorporation of powdered cellulose into normal papers could already produce a "factory-made" product with the properties of cellulose thin layers.

However, if we look at the reluctance of industry to prepare ion-exchange papers, which, when finally produced, came "too little, too late", we may as well settle down to thin layers for a while.

MICHAEL LEDERER (Rome).

J. Chromatog., 11 (1963) 131–132

Paper chromatography

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The bibliography of paper chromatography from 1943 (*i.e.* from the beginning) to 1956 has been collected and published in the *Handbuch der Papierchromatographie* by I. M. HAIS AND K. MACEK. It contains the titles and complete references of 10,290 publications. From 1961 onwards the bibliography of paper chromatography compiled under the direction of Dr. K. MACEK is published in the *Journal of Chromatography* (starting with Vol. 8). The gap, namely from 1957 to 1960, has been now filled by the volume under review, which contains 8,292 references. There is also an author index (84 pages) and a "List of substances chromatographed" (117 pages). This book will be indispensable for research workers employing paper chromatography. It is attractively produced and no printer's errors could be found on glancing through the pages.

J. Chromatog., 11 (1963) 132

NEW BOOKS

Die technischen Anwendungen der Radioaktivität, Band I, by E. BRODA AND T. SCHÖNFELD, VEB Deutscher Verlag für Grundstoffindustrie, Leipzig, 1962, 372 pages.

Third revised and greatly enlarged edition.

Gas-Chromatographie, by E. BAYER, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1962, x + 324 pages, price DM 49.80.

Second, completely revised and enlarged edition.

Chromatography and Electrophoresis on Paper, A teaching level manual, by J. G. FEINBERG AND IVOR SMITH, Shandon Scientific Co., Ltd., London, 1962, xii + 130 pages, price 21 s.

Gas Chromatography 1962, Editor M. VAN SWAAY, Butterworths Sci. Publ., London, 1962, lii + 411 pages, price 100 s.

Proceedings of the fourth symposium organised by the Fachgruppe Analytische Chemie of the Gesellschaft Deutscher Chemiker and the Gas Chromatography Discussion Group of the Institute of Petroleum, Hamburg 13–16 June, 1962.

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GAS–LIQUID CHROMATOGRAPHY

A CONTRIBUTION TO THE THEORY OF SEPARATION IN OPEN HOLE TUBES

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INTRODUCTION

The degree of separation obtained in a chromatographic column depends upon the overlap of the elution curves of the components emerging successively from the column. The separation performance will be introduced as a measure of the degree of separation between two components irrespective of their relative volatility. The proposed measure will fully describe the influence of the physical properties of the components, the column properties and the operating conditions upon the separation. It has become common practice to consider the number of theoretical plates (n), or the length of a column equivalent to a theoretical plate (H.E.T.P.), as a measure of the separating capacity of gas chromatographic columns. The latter concept originates from the theory of distillation, where it has proved its practical value. It is, however, an empirical quantity and the theory, especially in packed columns, does not deal with the mechanisms that determine this quantity.

In chromatography, when comparing a coated capillary column and a packed column, both columns having an equal number of theoretical plates for a given component, it appears that with the latter the degree of separation is, as a rule, appreciably higher. This effect will be more pronounced at high vapour pressures of the components to be separated. From this it follows that the theoretical plate concept is only to a limited extent suitable to describe column resolution. It will be shown that the height equivalent to a transfer unit, H_{OG}, is a true measure of what a column can do in separating a given pair of components, independent of their relative volatility.

PRACTICAL ASPECTS OF THE TRANSFER UNIT

The degree of separation, S, of two neighbouring components will be simply defined as:

$$S = \frac{t_2 - t_1}{w} \tag{1}$$

In this expression the retention times t_1 and t_2 are obtained by dividing the column length l by v_1, and v_2, the velocities of components 1 and 2 respectively; w is the mean width of the two components (in time units) at the baseline, between the tangents to

their elution curves. The number of theoretical plates, n, is related to t and w by:

$$w = \frac{4\,t}{\sqrt{n}} \tag{2}$$

If w is small as compared to t, for S may be written:

$$S = \left(\frac{v_1}{v_2} - 1\right)\frac{\sqrt{n}}{4} \tag{3}$$

The elution velocity of a component is equal to the gas velocity v_0 times its fractional occurrence in the gas phase. If y and x are the mole fractions of a component in the gas phase and in the stationary phase respectively, the number N_g of moles in the gas phase can be expressed by:

$$N_g = y\,\frac{V_g p \times 273}{T \times 22.4 \cdot 10^3} = y\,\frac{V_g p}{82\,T} \tag{4}$$

and the number of moles N_s in the stationary phase by:

$$N_s = x\,\frac{V_s \rho_s}{M_s} \tag{5}$$

The relation between x and y at phase equilibrium reads (for ideal gases):

$$\frac{x}{y} = \frac{p}{\gamma P} \tag{6}$$

The fractional occurrence of a component in the gas phase is equal to:

$$\frac{N_g}{N_g + N_s}$$

which by applying eqns. (4), (5) and (6) leads to:

$$v = v_0\left(1 + \frac{82\,V_s \rho_s T}{\gamma P V_g M_s}\right)^{-1} = \frac{v_0}{1 + k} \tag{7}$$

where k, the capacity ratio is:

$$k = \frac{82\,V_s \rho_s T}{\gamma P V_g M_s} \tag{8}$$

The relative elution velocity v_1/v_2 can now be written as:

$$\frac{v_1}{v_2} = \frac{1 + k_2}{1 + k_1} \tag{9}$$

The relative volatility of two components is defined as:

$$\alpha_{1,2} = \frac{\gamma_1 P_1}{\gamma_2 P_2} = \frac{k_2}{k_1} \tag{10}$$

Substituting eqns. (9) and (10) in eqn. (3):

$$S = \frac{k_1}{1 + k_1} (\alpha_{1,2} - 1) \frac{\sqrt{n}}{4} \tag{11}$$

(For closely neighbouring components, $k_1 \cong k_2$, k_1 in eqn. (11) may be replaced by k.)

From eqn. (11) it will be evident that the degree of separation, as defined by eqn. (1), is not determined only by $\alpha_{1,2}$ and n, but also by the value of k. The factor $k/(1 + k)$ becomes of importance if 1 is not small as compared to k, that is for small values of k (say < 5). From eqn. (8) it follows that k will be small if V_s/V_g is small (thin liquid layers) and P, the vapour pressure of the pure component is large (high temperatures).

The number of transfer units N_{OG}

CHILTON AND COLBURN[1] defined the number of transfer units N_{OG} on overall gas base as:

$$N_{OG} = \int_{c_1}^{c_2} \frac{dc}{c^* - c} \tag{12}$$

and the height of a transfer unit as:

$$H_{OG} = \frac{l}{N_{OG}} \tag{13}$$

VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[2] have shown the relation between N_{OG} and n to be:

$$N_{OG} = 2 \left(\frac{k}{1 + k}\right)^2 n \tag{14}$$

or:

$$\sqrt{0.5\, N_{OG}} = \frac{k}{1 + k} \sqrt{n}$$

by substituting N_{OG} for n in eqn. (11):

$$S = (\alpha_{1,2} - 1) \frac{\sqrt{0.5\, N_{OG}}}{4} \tag{15}$$

According to this equation the degree of separation of two components is governed only by their relative volatility and the number of transfer units. Whereas the number of theoretical plates is a measure of column performance for large values of k only, the number of transfer units is a true measure of the separation performance irrespective of the value of k*.

The number of transfer units can be readily obtained from the chromatogram:

$$N_{OG} = 32 \left(\frac{t - t_0}{w}\right)^2 \tag{16}$$

* The problem of the imperfection of the theoretical plate concept has among others also been recognized by HALASZ[3], who introduced the concept of a modified theoretical plate containing the same parameters.

where t is the retention time of the component and t_0 that of the carrier gas (non-retained component).

To illustrate eqn. (15) the separation data are given of the system methyl ethyl ketone–methyl isopropyl ketone in a capillary column coated with dinonyl phthalate (see Table I).

TABLE I

SEPARATION OF METHYL ETHYL KETONE-METHYL ISOPROPYL KETONE IN A COPPER CAPILLARY COLUMN

Length of the capillary 15 m, inner diameter 0.25 mm. Coating: dinonyl phthalate, coating thickness 1.5 μ; nitrogen velocity 16.7 cm/sec at 22.5°; inlet pressure 1.28 kg/cm^2; outlet pressure 1.00 kg/cm^2.

Separation data	*Temperature (°C)*				
	22.5	*50*	*75*	*100*	*125*
P_1	0.117	0.385	0.860	1.87	3.79
P_2	0.034	0.166	0.550	1.51	3.54
t_1	20.9	9.5	6.8	5.4	4.95
w_1	0.90	0.35	0.21	0.15	0.125
t_2	35.5	13.5	8.7	6.25	5.47
w_2	1.50	0.45	0.27	0.175	0.135
t_0	3.00	3.26	3.47	3.64	3.84
$\bar{n}$	8,800	13,100	16,700	20,600	25,600
$N_{OG,1}$	12,680	10,140	8,040	4,580	2,520
$N_{OG,2}$	15,040	16,600	12,000	7,100	4,680
$\alpha_{1\cdot2}$	1.82	1.64	1.57	1.48	1.46
k_1	5.95	1.90	0.96	0.48	0.29
k_2	10.8	3.14	1.50	0.72	0.43
S	12.2	10.0	7.9	5.2	4.0

The variation of the number of transfer units and of the number of theoretical plates with temperature and with degree of component separation are presented in Figs. 1 and 2 respectively.

THEORY OF THE TRANSFER UNIT

The mass transfer in a column is governed by: (a) rate of mass transfer in the gas phase, (b) rate of mass transfer in the stationary phase, and (c) longitudinal and radial diffusion in the gas phase.

Mass transfer in the gas phase

JAKOB[4] derived an exact equation describing the heat transfer in a round tube for constant wall temperature and laminar gas flow. By analogy between heat and mass transfer his equation can be rewritten as:

$$\frac{c_0 - c_\theta}{c_0 - c_i} = 1 - 0.820\, e^{-m_0 l} - 0.097\, e^{-m_1 l} - 0.0135\, e^{-m_2 l} \qquad (17)$$

where:

$$m_0 = \frac{3.66\, D_g}{r^2 v_0}\,;\; m_1 = \frac{22.1\, D_g}{r^2 v_0} \text{ and } m_2 = \frac{53.0\, D_g}{r^2 v_0}$$

The distribution of a component in a capillary tube can be approximated by a Gaussian curve. Consequently the integrated driving force over the length of the

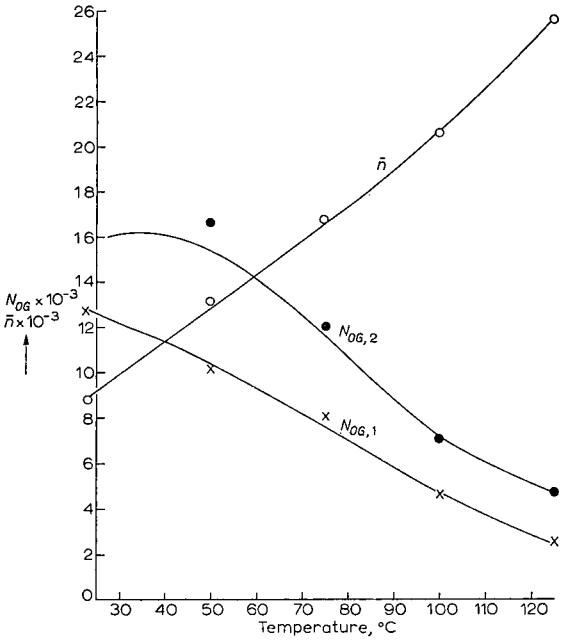

Fig. 1. Effect of temperature upon number of theoretical plates and number of transfer units.

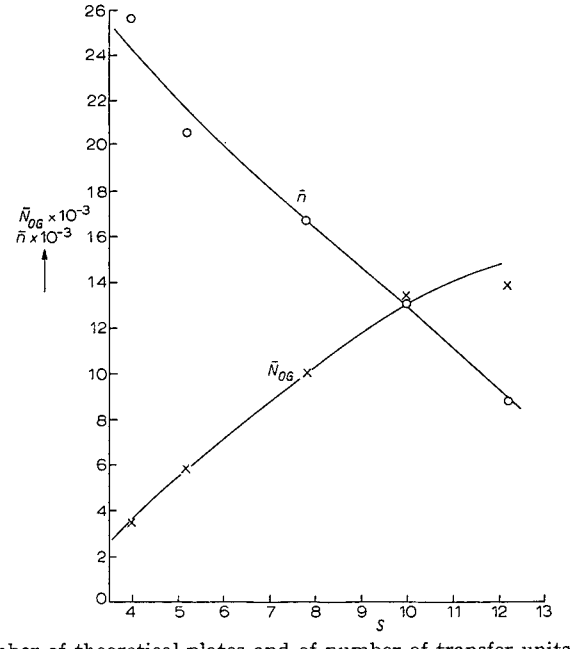

Fig. 2. Effect of number of theoretical plates and of number of transfer units on degree of component separation.

capillary is zero. In JAKOB's model a zero driving force is equivalent to $\theta \to \infty$ ($l \to \infty$).

For large values of l eqn. (17) reduces to:

$$\frac{c_0 - c_\theta}{c_0 - c_i} = 1 - 0.820\, e^{-\frac{3.66\, D_g l}{r^2 v_0}} \tag{18}$$

The amount of mass N, transferred during a time θ is given by:

$$N = V_g\,(c_\theta - c_0) \tag{19}$$

From eqns. (18) and (19) it follows that:

$$-N = V_g\,(c_0 - c_i)\left(1 - 0.820\, e^{-\frac{3.66\, D_g l}{r^2 v_0}}\right) \tag{20}$$

Replacing l/v_0 by θ and differentiating eqn. (20) with respect to θ:

$$\frac{dN}{d\theta} = -V_g\,(c_0 - c_i)\, 0.820\, e^{-\frac{3.66\, D_g l}{r^2 v_0}} \cdot \frac{3.66\, D_g}{r^2} \tag{21}$$

The mass transfer coefficient K_g, in the gas phase is defined as:

$$\frac{dN}{d\theta} = -K_g\,(c_\theta - c_i) \tag{22}$$

and eqn. (21) may be modified to:

$$K_g = \frac{c_0 - c_i}{c_\theta - c_i} \frac{3.66\, D_g}{r^2}\, 0.820\, V_g\, e^{-\frac{3.66\, D_g l}{r^2 v_0}} \tag{23}$$

From eqns. (23) and (18) it follows that:

$$K_g = \frac{3.66\, D_g}{r^2} V_g \tag{24}$$

Mass transfer coefficient in the stationary phase

For a constant interfacial concentration NEWMAN[5] derived for the mass transfer in a slab:

$$\frac{c_\theta - c_i}{c_0 - c_i} = \frac{8}{\pi^2}\left(e^{-\frac{D_s \theta \pi^2}{4z^2}} + \frac{1}{9} e^{-\frac{9 D_s \theta \pi^2}{4z^2}} + \frac{1}{25} e^{-\frac{25 D_s \theta \pi^2}{4z^2}} + \cdots\right) \tag{25}$$

For the same reasons as apply to the gas phase the relation reduces to:

$$\frac{c_\theta - c_i}{c_0 - c_i} = \frac{8}{\pi^2} e^{-\frac{D_s \theta \pi^2}{4z^2}} \tag{26}$$

The mass N transferred in time θ is:

$$N = V_s\,(c_\theta - c_0) \tag{27}$$

Substitution and differentiation to θ gives:

$$\frac{dN}{d\theta} = -V_s (c_0 - c_i) \frac{8}{\pi^2} \frac{D_s \pi^2}{4 z^2} e^{-\frac{D_s \theta \pi^2}{4 z^2}} \tag{28}$$

Again the coefficient of mass transfer K_s in the stationary phase is:

$$\frac{dN}{d\theta} = -K_s (c_\theta - c_i) \tag{29}$$

which leads to:

$$K_s = V_s \frac{\pi^2 D_s}{4 z^2} \tag{30}$$

OVERALL NUMBER OF TRANSFER UNITS IN THE GAS PHASE

According to the double film theory:

$$\frac{1}{K_{OG}} = \frac{1}{K_g} + \frac{K}{K_s} \tag{31}$$

where K_{OG} represents the overall coefficient of mass transfer on gas base and

$$K = \frac{c_g}{c_s} \text{ (equilibrium constant).}$$

From:

$$N_{OG} = \int_{c_1}^{c_2} \frac{dc}{c^* - c} = \frac{K_{OG} O}{G} \tag{32}$$

and:

$$H_{OG} = \frac{l}{N_{OG}} = \frac{lG}{K_{OG} O} \tag{33}$$

for a capillary column it is derived:

$$H_{OG} = \frac{v_0 V_g}{K_{OG}} \tag{34}$$

After substituting eqns. (24), (30) and (31) into eqn. (34):

$$H_{OG} = \frac{v_0 r^2}{3.66 D_g} + K \frac{4 z^2 v_0 V_g}{\pi^2 D_s V_s} = \frac{v_0 r^2}{3.66 D_g} + \frac{1}{k} \frac{4 z^2 v_0}{\pi^2 D_s} \tag{35}$$

Radial and longitudinal diffusion

From a study by WESTHAVER[6] the effect of radial and longitudinal diffusion on the H.E.T.P. can be obtained:

$$\text{H.E.T.P.} = \frac{v_0 r^2}{24 D_g} + \frac{2 D_g}{v_0} \tag{36}$$

or in terms of transfer units:

$$H_{OG} = \frac{(1 + k)^2}{k^2}\left(\frac{v_0 r^2}{48\, D_g} + \frac{D_g}{v_0}\right) \tag{37}$$

The overall transport in the capillary column is obtained by adding the heights of the transfer units for the different transport phenomena:

$$H_{OG} = \frac{(1 + k)^2}{k^2}\frac{D_g}{v_0} + \frac{1 + 2k + 14k^2}{48\, k_2}\frac{v_0 r^2}{D_g} + \frac{1}{k}\frac{4\, v_0 z^2}{\pi^2 D_s} \tag{38}$$

The degree of separation of two components is then:

$$S = (\alpha_{1,2} - 1)\left[\frac{(1 + k)^2}{k^2}\frac{D_g}{v_0} + \frac{1 + 2k + 14k^2}{48\, k^2}\frac{v_0 r^2}{D_g} + \frac{1}{k}\frac{4\, v_0 z^2}{\pi^2 D_s}\right]^{-\frac{1}{2}}\left(\frac{l}{32}\right)^{\frac{1}{2}} \tag{39}$$

CONCLUSION

The H.E.T.P. concept in gas–liquid chromatography might be misleading. The example represented in Fig. 2, clearly demonstrates that the operating conditions leading to an increase of the number of theoretical plates result in a decrease of the separation. The number of transfer units, however, is the exact measure of separation performance.

From eqn. (38) it can be deduced that for low values of k ($k < 5$) the number of transfer units decreases sharply with a decrease of k. The effect upon k, by the coating thickness, capillary diameter, vapour pressure of component, temperature and properties of the stationary phase follows from eqn. (8).

The height of a transfer unit, for most practical purposes, will be controlled by the rate of mass transfer in the stationary phase. The relevant term in eqn. (38) reads in physical units:

$$0.005\,\frac{\gamma P}{T}\,\frac{M_s}{\rho_s D_s}\, r v_0 z \tag{40}$$

This expression shows the significance of small capillary diameters, low molecular weight of the stationary phase, low gas velocity and thin coating layers. The first derivatives with respect to T of $\gamma P/T$ and of $1/D_s$ are positive and negative respectively and not of the same order of magnitude. Roughly at values of γP between 0.5 and 1.0 the ratio $\gamma P/TD_s$ will pass through a minimum.

In eqn. (38) the diffusion coefficient in the gas phase, D_g, is the only parameter that is pressure dependent. Low pressures result in high values of D_g. At very low pressures, the height of a transfer unit according to eqn. (38) will be controlled by the first term, representing the longitudinal diffusion.

LIST OF SYMBOLS

c_0 = concentration after time zero (g/cm^3)
c_θ = concentration after time θ (g/cm^3)
c_i = concentration at interphase gas–stationary phase (g/cm^3)
c^* = concentration in one phase in equilibrium with concentration in the other phase (g/cm^3)

e = base of natural logarithms
D_g = diffusion coefficient in the gas phase (cm^2/sec)
D_s = diffusion coefficient in the stationary phase (cm^2/sec)
G = gas rate (cm^3/sec)
H_{OG} = height of a transfer unit, based on gas-film resistance (cm)
H.E.T.P. = height equivalent to a theoretical plate (cm)
K_g = mass transfer coefficient in the gas phase (cm^2/sec)
K_s = mass transfer coefficient in the stationary phase (cm^2/sec)
K_{OG} = overall gas phase mass transfer coefficient (cm^2/sec)
$K = c_g/c_s$, equilibrium constant
k = ratio of quantity of component in stationary phase and in gas phase
l = length of column (cm)
M_s = molecular weight of stationary phase (g/mole)
N_{OG} = number of transfer units
n = number of theoretical plates
N = mass transferred (g/cm^2)
O = area of interphase gas phase–stationary phase
p = pressure (atm)
P = vapour pressure of component (atm)
r = inner radius of capillary (cm)
$S = (t_1 - t_2)/w$ degree of separation
T = temperature (°K)
t = retention time of a component emerging from column (sec)
t_0 = retention time of air peak (sec)
v = velocity of component in column (cm/sec)
v_0 = average velocity of gas stream in column (cm/sec)
V_g = volume of gas phase/unit interphase (cm)
V_s = volume of stationary phase/unit interphase (cm)
w = basewidth of component (sec)
x = mole fraction of component in stationary phase
y = mole fraction of component in gas phase
z = thickness of stationary phase (cm)
$\alpha_{1,2} = \gamma_1 P_1/\gamma_2 P_2$ relative volatility
γ = activity coefficient of component
ρ_s = density of stationary phase (g/cm^3).

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SUMMARY

The height equivalent to a transfer unit (H_{OG}) is, as opposed to the height equivalent to a theoretical plate (H.E.T.P.), an exact measure of the separation performance of gas–liquid chromatography columns.

A relation between physical properties of the stationary phase, physical properties of components to be separated, dimensions and operating conditions of capillary columns on the one hand and the H_{OG} on the other hand is derived. It is shown that a decrease of k, the capacity ratio of stationary phase to gas phase for a given component, results in an increase of the H_{OG} and a decrease in the H.E.T.P.

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EIN NEUARTIGER PROBENGEBER FÜR DIE KAPILLAR-CHROMATOGRAPHIE

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EINLEITUNG

Mit Hilfe der in jüngster Zeit entwickelten kapillar-chromatographischen Methode konnte die Trennung ganz geringer, bei Raumtemperatur gasförmiger oder flüssiger Substanzmengen (von 0.1 bis 100 μg) erzielt werden. Die sprunghafte Erhöhung der Trennleistung ist vorwiegend dem geistreichen Verfahren selbst zu verdanken, zur vollständigen Ausnützung der prinzipiellen Möglichkeiten tragen jedoch auch weitere wichtige Faktoren bei. Da die Trennfähigkeit der Kapillarsäulen nur bei μg oder noch kleineren Substanzmengen vollständig ausgenützt werden kann, kommt der reproduzierbaren Probendosierung in diesem Bereich eine grosse Bedeutung zu. Hinsichtlich der quantitativen Auswertung der Chromatogramme und der Trennschärfe, verfügt, unserer Meinung nach, der im weiteren zu beschreibende Probengeber, im Vergleich zu anderen bekannten Typen, über beachtliche Vorteile. Bei mehreren, in der Praxis verwendeten Probengebern kann es noch vor der Probengabe zu einer Fraktionierung der verschiedenartig flüchtigen Komponenten kommen, ausserdem vermischt sich der Dampf unreproduzierbar mit dem Trägergas, was eine vom Erwarteten quantitativ, wie auch qualitativ abweichende Dosierung zur Folge haben kann. Ein weiterer Faktor, der auf die Trennschärfe Einfluss ausübt, ist die zeitliche (oder räumliche) Ausdehnung des aufgegebenen Stoffimpulses. Der an die üblichen Probengeber gestellten Forderung, die Probe in höchstkonzentrierter Form zu dosieren, können die bisher beschriebenen Typen in der Mehrzahl der Fälle nicht entsprechen.

APPARATUR UND VERFAHREN

Die im vorhergehenden aufgeworfenen Probleme erklären, warum unser Bestreben dahinging, ein Verfahren auszuarbeiten, bei dem die Dosierung in zwei gesonderte Prozesse getrennt wurde.

Bei der ersten Stufe wird die Probe mittels einer beliebigen, auch bei den handelsüblichen Gas-Chromatographen angewendeten Methode, in ein vorhergehend evakuiertes und mit grosser Genauigkeit kalibriertes Volumen eingebracht. Nachdem die flüssige Probe restlos verdampft worden ist, was man durch entsprechendes Temperieren des Verdampfers erreicht, wird als zweite Stufe (auf deren Einzelheiten später noch eingegangen wird) ein entsprechender Teil der im Dampf befindlichen gesamten Substanzmenge in das Schleppgas dosiert.

Beschreibung der Probengeber

Der Probengeber ist in Fig. 1 schematisch dargestellt. Er besteht aus den folgenden Hauptteilen: dem Verdampferraum (1), der Halbmikrodosier- (2) und Mikrodosiervorrichtung (3) und dem Nadelventil (4). Mit Ausnahme der Handgriffe der Dosierstäbe und des Nadelventils ist der Probengeber gänzlich im Luftthermostat des Chroma-

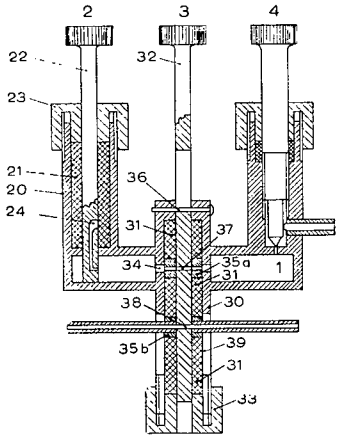

Fig. 1. Schematische Darstellung des Probengebers.

tographen untergebracht. Seine Aufheizung erfolgt mit einer von aussen angebrachten und thermisch isolierten Heizspirale, mit deren Hilfe seine Temperatur (zum Teil unabhängig von der des Luftthermostaten) bis etwa 230° beliebig eingestellt werden kann.

In der Abbildung haben wir die Halbmikrodosiervorrichtung, die im wesentlichen eine Spezialmikropipette ist, in ihrer meist verwendeten Form dargestellt. Beide Mündungen der längsgerichteten kalibrierten Bohrung (24) am unteren Teil des Dosierstabes (22) befinden sich an seiner Seitenwandung. Das Volumen der höher liegenden Querbohrung ist zumindest so gross wie das der darunter befindlichen, kalibrierten Längsbohrung. Der Dosierstab kann in einer entsprechend langen, eng angepassten Ringdichtung aus Teflon (21) bewegt werden. Eine unten verschlossene Metallröhre (20) umkleidet den Teflonring. Am unteren Teil der Röhre befindet sich eine Bohrung, deren Durchmesser den des Dosierstabes kaum übertrifft. Um Undichtigkeiten zu verhüten, wird der Teflonring mit einer Schraube (23) zusammengepresst. Diese Vorrichtung eignet sich zur Eingabe von 1 bis 20 μl Flüssigkeit in den Verdampferraum.

Die eigentliche Probendosierung geschieht mit Hilfe der Mikrodosiervorrichtung. Im senkrecht bewegbaren Dosierstab (32) sind zwei Querbohrungen angebracht, deren Lage durch die folgenden zwei Forderungen bestimmt ist: kommt die obere Bohrung mit dem Verdampferraum in Berührung, dann soll die untere Bohrung die ungestörte Strömung des Trägergases nach der Kolonne ermöglichen. Die Bohrungen des Dosierstabes kommunizieren durch je eine Metallscheibe (35) mit dem Gasraum, bzw. mit dem Schleppgasstrom. In beiden Scheiben ist eine vertikale Bohrung, deren Durchmesser dem Dosierstab entspricht und eine weitere quergerichtete Bohrung von der

gleichen Grösse, wie die des Stabes, untergebracht. An die Querbohrungen der unteren Metallscheibe schliessen sich die Leitungen der Schleppgaszuführung und Abführung an, während die der oberen Scheibe mit den nach dem Verdampferraum hinausgehenden grösseren Bohrungen (34) des Metallrohres (30) zusammenfallen, das die ganze Mikrodosiervorrichtung beherbergt. Die metallenen Scheiben sind zwecks Abdichtung mit drei Teflonringen (31) versehen. Mit Hilfe der Schraube (33) lassen sich alle drei Teflonhülsen fest zusammenpressen. Um dazu die Scheiben in vertikaler Richtung bewegen zu können, wurde am unteren Teil des Metallrohres, die die Scheiben und die Teflonringe häust, ein Schlitz (39) verfertigt, dessen Breite dem Durchmesser der zur Aus- und Einführung des Schleppgases dienenden Leitungen entspricht. Eine Hemme (36) schränkt die Bewegung des Dosierstabes in der Weise ein, dass in beiden Endstellungen entweder die eine, oder die andere Bohrung dem Schleppgas den Weg in die Säule freilässt. Zu gleicher Zeit verhindert die Hemme eine eventuelle Verdrehung des Stabes, die eine Verschliessung der Bohrungen zur Folge haben könnte.

Dosierung

Die Dosierung geht folgendermassen vor sich: Wird der Dosierstab hochgezogen, dann strömt das Schleppgas durch seine untere Bohrung (38) durch, während die obere Bohrung (37) mit dem Verdampferraum in Berührung kommt. Durch entsprechende Einstellung der Heizung kann dafür gesorgt werden, dass die Temperatur des Probengebers stets über dem Taupunkt der höchstsiedenden Probenkomponente zu liegen kommt. Man taucht die untere Öffnung des Halbmikrodosierstabes in die zu untersuchende Flüssigkeit und wartet ab, bis die kalibrierte Bohrung der Kapillarität zufolge sich mit Flüssigkeit füllt. Der dem Dosierstab anhaftende Flüssigkeitsüberschuss muss entfernt werden. Nachher wird der Stab (22) in die Teflonhülse des Halbmikrodosierers derart eingeführt, dass beide Mündungen der Bohrung durch die Ringdichtung (21) verschlossen werden. Nun wird das Nadelventil (4) geöffnet und der Verdampferraum (1) evakuiert. Inzwischen findet (noch in der Bohrung selbst) eine partielle Verdampfung der Probe durch die Erwärmung statt. Die obere Bohrung von grösserem Durchmesser, die Luft enthält, sichert die ungestörte Ausdehnung der sich erwärmenden Flüssigkeit. Nach Verschliessen des Nadelventils wird der Dosierstab bis zum Anstoss hinuntergedrückt, wodurch der untere Ausgang der Bohrung in den Verdampfer gelangt. Die im oberen Teil der Bohrung sich ausdehnende Luft spritzt die Probe in den Verdampferraum ein, wo sie restlos verdampft. Nach einigen Minuten Wartezeit wird durch Abdrücken des Mikrodosierstabes (32) die in seiner oberen Bohrung befindliche Dampfmenge in den Weg des Schleppgases gebracht. Wenn erwünscht, kann die Probengabe wiederholt werden, indem der Mikrodosierstab zurückgezogen und kurz danach wieder nach unten geschoben wird.

Die beschriebene Probendosierungsmethode hat den Nachteil dass auch kleine Luftmengen in den Probengeber gelangen. Das zu vermeiden wird aber praktisch nur in ganz seltenen Fällen nötig sein. Die Halbmikrodosiervorrichtung bedarf nur einer geringen Abwandlung, sollte sie für mit anderen Systemen (Injektionsspritze, Ampulle, Kapillarenbrecher) durchgeführten Dosierungen, oder für Probengaben aus der Gasphase eingesetzt werden. In diesem Falle wird lediglich der Dosierstab (22) durch entsprechend ausgebildete Dosierröhre ersetzt, von deren Varianten einige in Fig. 2 gezeigt werden. Es sei darauf hingewiesen, dass die dritte Variante der dargestellten

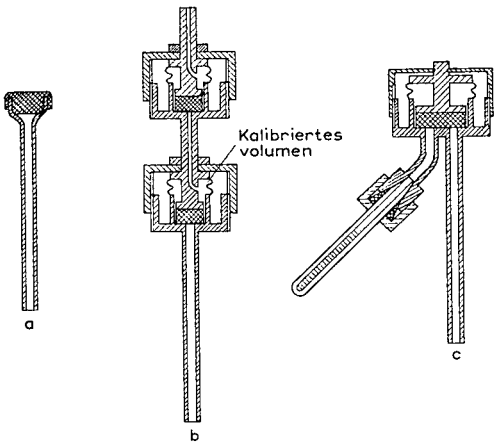

Fig. 2. Einige Varianten der Halbmikrodosierstäbe. (a) Metallröhre, abgeschlossen mit einer passenden Gummikappe, für die Dosierung mit der Mikrospritze. (b) Halbmikrodosierstab für Gase, mit Teflonventilen; gegen die Atmosphäre gedichtet mit ringförmigen Kupfermembranen. (c) Präzisionshalbmikrodosierstab für Flüssigkeiten, mit kalibriertem Behälter aus Glas, und Teflondichtungen.

Dosierstäbe auch für gleichzeitige Dosierung von Gasen und Dämpfen eingesetzt werden kann, da die Probe mit Hilfe eines entsprechenden Kältebades in der angesetzten Glaskapillare im tiefgekühlten Zustand aufbewahrt werden kann, bevor es zur effektiven Probengabe käme.

Sollte der Probengeber ausschliesslich für die Dosierung von Gasproben eingesetzt werden, erübrigt sich die Anwendung des Verdampfers. Wir haben auch einen Probengeber hergestellt, der für die schnelle, in festgesetzten Zeitintervallen vor sich gehende Probenahme aus einem kontinuierlichen Gasstrom zur Anwendung kommt. Dieser besteht lediglich aus einer etwas abgewandelten Mikrodosiervorrichtung, bei der die Probengasleitung an die Bohrung der oberen Scheibe befestigt ist und am Dosierstab auch eine dritte Bohrung angebracht wurde. Diese war nötig, da der Dosierstab in diesem Fall in beiden Endstellungen, in zwei voneinander vollständig unabhängigen Systemen die Kontinuität des Gasstromes zu sichern hat.

Der Verdampfer des in Fig. 1 dargestellten Probengebers ist ein zylindrischer Raum (70 mm Dmr., 5 mm hoch) in dem die Röhre (30 mm Aussendmr.) der Mikrodosiervorrichtung (30) untergebracht ist. Das Volumen des Verdampfers beträgt also etwa 16 ml. Der Durchmesser des Dosierstabes ist 5 mm, der der oberen Bohrung 2 mm, sodass das Volumen der Bohrung $1.6 \cdot 10^{-2}$ ml beträgt. Demnach dosiert die Mikrodosiervorrichtung ungefähr den tausendsten Teil der in den Verdampferraum eingebrachten Probe. Bei 20° betrug das an Hand einer geeichten Gasbürette, mit hoher Genauigkeit manometrisch bestimmte Volumen des Verdampferraumes 15.68 ml. Das genaue Volumen der oberen Bohrung wurde mit Quecksilber kalibriert, aus dem Durchschnittswert mehrerer Messungen wurde dafür $1.723 \cdot 10^{-2}$ ml berechnet; das effektive Teilungsverhältnis des Probengebers ist also 1:916.

Druckproben

Um die Gasdichtheit der Teflonhülsen zu kontrollieren, haben wir mit dem Probengeber auch Druckproben durchgeführt. Im Verdampfer wurde ein Überdruck von 5,

in den Schleppgasleitungen ein Überdruck von 8 atü hergestellt und die Ringdichtungen mit Hilfe der Schrauben so lange zusammengepresst bis kein Gasaustritt mehr zu beobachten war. Unter diesen Bedingungen waren die Dosierstäbe noch leicht bewegbar, besonders wenn sie aus Molybdän, mit polierter Oberfläche hergestellt worden sind. Von der tadellosen Arbeit des Probengebers überzeugten wir uns durch Dosierung von Proben in der Grössenordnung von 10 und 0.1 μg.

ERGEBNISSE

Wenn in den Verdampfer 10 μl Flüssigkeitsgemisch eingebracht wurde, war die Menge der in die Säule eingespeisten Probe, dem Teilungsverhältnis 1:916 entsprechend, von der Grössenordnung von 10 μg. Die zur Dosierung kommenden Flüssigkeitsgemische haben wir absichtlich aus den in der Praxis am häufigsten vorkommenden Verbindungstypen, — wie Äther, Ester, Ketone, aromatische und aliphatische Kohlenwasserstoffe (über diese Verbindungsgruppe siehe die zweite Versuchsreihe) und chlorierte Kohlenwasserstoffe — zusammengestellt, um auch über die eventuelle Löslichkeit der Komponenten im Dichtungsmaterial ein klares Bild zu bekommen. Zur Trennung sämtlicher Flüssigkeitsproben wurde (in Tabelle I sind diese Messungen

TABELLE I

DOSIERUNGSVERSUCHE MIT GAS- UND FLÜSSIGKEITSGEMISCHEN

Probenmenge (μg)	*Komponente des Gemisches*	*Berghöhe (mm)*					*Mittlere Höhe*	*Maximale Abweichung (%)*
	Diäthyläther*	33.1	33.5	32.4	32.5	32.8	32.9	± 1.7
9.0	Aceton	145.4	147.5	144.3	145.6	145.3	145.6	± 1.1
	Äthylalkohol	111.2	112.3	111.7	112.7	111.6	111.9	± 0.7
	Äthylacetat	14.3	15.0	14.5	15.0	14.8	14.7	± 2.4
9.5	Benzol*	190.4	191.6	192.0	193.0	192.6	191.9	± 0.7
	Toluol	39.2	40.1	40.0	39.5	39.0	39.6	± 1.4
14.1	Chloroform*	159.2	158.0	157.6	157.8	158.3	158.2	± 0.5
	Tetrachlorkohlenstoff	32.4	32.1	31.8	31.9	32.2	32.1	± 0.9
	Aceton	26.0	25.7	25.4	25.5	25.6	25.2	± 1.2
0.21	Äthan	52.5	52.1	52.4	53.6	53.4	53.0	± 1.4
	Propan	nicht auswertbar						
	Isobutan	91.0	90.5	90.7	90.9	90.8	90.8	± 0.3
	n-Butan	72.5	72.3	72.4	72.2	72.3	72.3	± 0.2

* Bedeutung der Sterne siehe im Text.

mit* bezeichnet worden) eine Säule angewendet, deren Länge 1.5 m, der innere Durchmesser 6 mm betrug und die mit 15 gew.-% Carbowachs-2000 benetzten feinkörnigen Sterchamol enthielt. Die Trennung wurde in allen Fällen bei 60° durchgeführt. Als Schleppgas wurde Wasserstoff bei einer Strömungsgeschwindigkeit von 50 ml/min, als Detektor ein Flammendetektor eingesetzt. Die Temperatur des Probengebers war auf 120° eingestellt. (Als Beispiel, haben wir in Fig. 3 die Dosierversuche mit dem Chloroform–Tetrachlorkohlenstoff–Aceton-Gemisch angegeben.)

Zur Trennung geringerer Substanzmengen (0.1 μg) haben wir eine 30 m lange,

mit Squalan benetzte Kapillarsäule bei Raumtemperatur verwendet. Die Strömungsgeschwindigkeit des Stickstoff-Schleppgases war 5 ml/min, die des in den Flammenionisationsdetektor eingeführten Wasserstoffes, 30 ml/min. Dosiert wurde ein Äthan-, Propan-, Isobutan- und *n*-Butan-Gemisch (Chromatogramme siehe Fig. 4). Zur Probeneingabe ist hier derselbe Mikrodosierstab verwendet worden der zur Dosierung von Substanzmengen im Bereich von 10 μg diente und so musste die Menge der in den

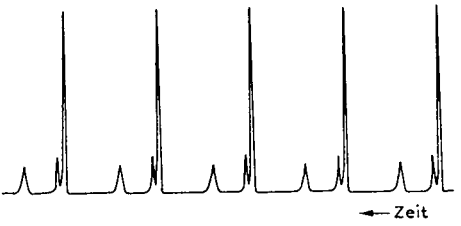

Fig. 3. Chromatogramme einer Flüssigkeitsprobe; die Komponenten sind der Reihe nach: Chloroform, Tetrachlorkohlenstoff und Aceton.

"Verdampfer" eingeführten Probe um zwei Zehnerpotenzen verringert werden. Dies konnten wir dadurch erreichen, dass wir 100 μl Gasgemisch mit einer Präzisionsmikrospritze in den "Verdampferraum" injizierten, der vorhergehend mit Luft auf Atmosphärendruck gefüllt worden war. Durch die Anwendung einer Dosierröhre mit Serumkappe wurde das Volumen des "Verdampferraumes" auf 16.27 ml erhöht, wobei sich das Teilungsverhältnis zu 1:944 änderte.

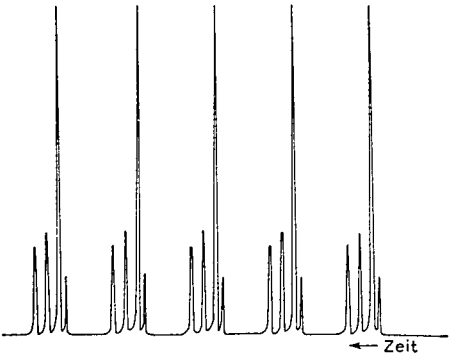

Fig. 4. Chromatogramme einer Gasprobe; die Komponenten sind der Reihe nach: Äthan, Propan, Isobutan und *n*-Butan.

Aus jeder einzelnen Probe wurden unter analogen Bedingungen fünf Analysen nacheinander durchgeführt*. Zum Vergleich der so erhaltenen Chromatogramme wurden die Berghöhen der entsprechenden Komponenten herangezogen. Die Ergebnisse sind in Tabelle I zusammengefasst. Wie aus diesen Angaben ersichtlich, beträgt die maximale Abweichung der Berghöhen in der Mehrzahl der Fälle weniger als $\pm$ 1 % und nur dort, wo die Berge kleiner sind (wo also die Ungenauigkeit der Aus-

* Mit dem Halbmikrodosierer wurde in allen Fällen nur eine Probe aufgegeben. Die in Hinsicht auf die Reproduzierbarkeit mitgeteilten Angaben beziehen sich daher auf den *Mikrodosierer*. Die Reproduzierbarkeit war bei dem Halbmikrodosierer unter $\pm$ 1 %.

wertung auch sonst grössere prozentuale Fehler ergibt), erreicht sie den Wert von $\pm$ 2 %.

Ausser der guten Reproduzierbarkeit der Dosierung, besitzt der Probengeber den weiteren Vorteil, dass er einen scharf "abgegrenzten" Stoffimpuls in die Säule bringt. Dies hat auch eine erhöhte Trennfähigkeit zur Folge, zu deren numerischen Auswertung aber weitere, mit einem Probengeber anderen Typs durchzuführende, vergleichende Untersuchungen erforderlich sind.

Zusammenfassend kann also festgestellt werden, dass der beschriebene Probengeber über die folgenden Vorteile verfügt:

1. Er ist für die Dosierung von Flüssigkeiten ebenso geeignet wie für Gase.
2. Vor der Dosierung wird die Flüssigkeitsprobe im Verdampferraum restlos verdampft, so dass die Zusammensetzung des in die Säule eingeführten Dampfes der Zusammensetzung der Flüssigkeit entspricht.
3. Die zu dosierende Substanzmenge kann durch Einsatz von Dosierstäben mit verschiedenen Bohrungen innerhalb weiterer Grenzen variiert werden.
4. Die Reproduzierbarkeit der aufgegebenen Probenmenge liegt innerhalb von $\pm$ 2 %.
5. Der in die Säule eingeführte, scharf "abgegrenzte" Stoffimpuls sichert eine gute Trennleistung.
6. Die Vorrichtung arbeitet einfach, das Verfahren ist im Prinzip automatisierbar.

ZUSAMMENFASSUNG

Es wird ein neuartiger Probengeber zur Dosierung von Gas- und Flüssigkeitsproben in der Grössenordnung von 10^{-2} bis 10^{2} μg, beschrieben. Die Reproduzierbarkeit beträgt etwa $\pm$ 1 %. Der Dosierer arbeitet nach dem folgenden Prinzip: Vorerst wird die flüssige Probe unter Anwendung eines handelsüblichen Probengebers (Mikrodipper, Injektionsspritze etc.) in einen vorhergehend evakuierten Verdampfer eingebracht, wo sie restlos verdampft. Nachher wird ein entsprechender Teil der im Dampf befindlichen gesamten Substanzmenge, mit Hilfe eines zweckmässig ausgestalteten Dosierstabes in das Trägergas eingebracht. Der totalen Verdampfung zufolge tritt keine Fraktionierung auf. Die Dosierung erfolgt in der Form eines scharf abgegrenzten Stoffimpulses. Auf Grund dieser Eigenschaften kann der Probengeber insbesondere auf dem Gebiet der Kapillar-Chromatographie mit Erfolg eingesetzt werden.

SUMMARY

A new sampling device for gases and liquids in amounts of the order of 10^{-2} to 10^{2} μg is described. The reproducibility of sampling is $\pm$ 1 %. The device is based on the following principle: by means of a common sampler (microdipper, hypodermic syringe, etc.) the liquid sample is brought into a previously evacuated chamber in which it evaporates completely. With the help of a suitably shaped sampling rod, a small fraction of the whole vapour sample is introduced into the carrier gas. Because the sample evaporates completely there is no danger of fractionation. In this method of introduction the sample has the form of a highly concentrated "plug". Owing to these features the sampler can be used successfully in capillary chromatography.

OPERATIONAL MECHANISM AND PERFORMANCE OF A SUBSIDIARY DISCHARGE ARGON IONIZATION DETECTOR

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It has been shown in a work[1] previously reported that a subsidiary discharge argon ionization detector in conjunction with an electrometer of high gain can be used to measure the amounts of components present in the effluent of a gas chromatographic column. The principle of operation of the detector, *i.e.* the mechanism of ionization of the sample, might be regarded as the same as that of LOVELOCK's detector[2], but it has an outstanding feature in that the radioactive source is replaced by a D.C. subsidiary discharge. The detector, consisting of a concentric diode with discharge electrodes mounted in the scavenging gas flow (discharge gas flow), was said to operate in such a way that primary electrons generated by the discharge were carried into the sensing chamber by the flow of the discharge gas. A complete description of this mechanism was not given in the previous paper, however, and the way in which primary electrons are generated was not at all well understood. The primary purpose of this work was to obtain more information on this aspect of the device.

It is known that the limiting noise in an argon ionization detector is determined by the uniformity with which primary electrons are generated. Therefore, in this detector the steadiness of the subsidiary discharge is of great importance. In the previous paper attention was drawn to the fact that the same arrangement could give more satisfactory operation if helium were used as the discharge gas. The use of helium permits a relatively low voltage breakdown to be started and a steady low-noise discharge to be maintained at atmospheric pressure.

The present investigation was conducted with a glass-housed detector of small volume, helium being used as the discharge gas and argon as the carrier gas. As a preliminary test, the discharge in the helium flow was studied with a simple discharge tube to prove its suitability as an electron source. Next, the characteristics of the background current were investigated in detail with a view to clarifying the generation mechanism of primary electrons. Finally, the response of the detector was tested. The performance studies proved to be quite satisfactory. The results of these measurements, together with a discussion of these experimental results, are given in the present paper.

APPARATUS

The experimental setup, which is shown in Fig. 1, is similar to the arrangement already described in the previous paper. The only significant difference is the incorporation of a helium flow line, so that the discharge may be excited in pure helium flow. Argon is used as the carrier gas, in order to maintain the high sensitivity of the argon

ionization detector. The two gases are supplied from normal commercial cylinders, and the purities are both listed as being above 99.9 %.

The most important part of the apparatus is the detector, of which the electrode geometry must be carefully chosen, if good signal-to-noise performance is to be

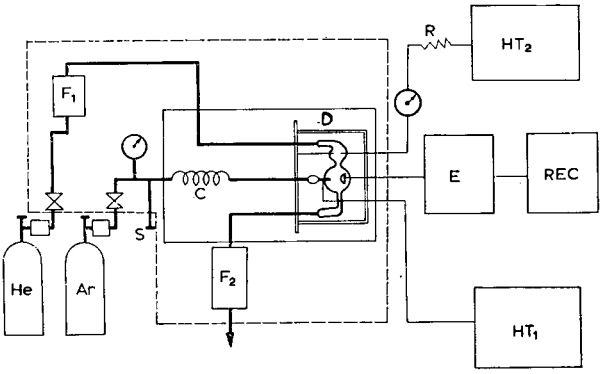

Fig. 1. Diagram of apparatus. Ar = argon cylinder; He = helium cylinder; F_1, F_2 = flow meters; S = sample introducing device; C = column; D = detector; HT_1 = high voltage supply for the detector; HT_2 = high voltage supply for the discharge; R = current limiting resistor; E = electrometer amplifier; REC = recorder.

realized. The detector used in this investigation is shown schematically in Fig. 2. It consists of two chambers: one of which is the discharge chamber and the other the sensing chamber. The discharge chamber is made of a glass tube with an O.D. of 10 mm and a length of about 20 mm, into which is mounted a pair of 0.5 mm Kovar wires, the electrodes for the subsidiary discharge. The ends of the electrodes are bent towards each other, so that a steady discharge may be fixed there. Helium is led through a Kovar spring pipe (1.5 mm O.D. and 1 mm I.D.) into the discharge chamber, whence it flows into the sensing chamber through a narrow passage. The sensing chamber is also made of a 20 mm O.D. spherical glass tube. The inlet for the effluent gas, which acts as an anode, is made of a Kovar tube with an O.D. of 1.5 mm, an I.D. of

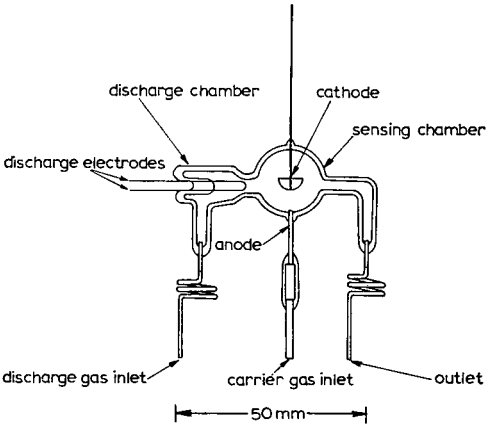

Fig. 2. Subsidiary discharge argon ionization detector.

1 mm and a length of 15 mm. The cathode is a semi-spherical nickel dish, and is located at the center of the sensing chamber.

EXPERIMENTAL RESULTS AND DISCUSSION

Subsidiary discharge

Many experimenters have studied the electrical discharge of helium at various pressures and in various different geometrical arrangements[3]. Since most workers have obtained data for pure helium with seal-off tubes, much of the available data cannot be applied to the actual operation of the detector, where the handling of helium from commercial cylinders can introduce small quantities of impurities, and occasional exposure to the air may change the surface condition of the electrodes.

A preliminary investigation on the characteristics of the subsidiary discharge in the flow of helium was made, using a discharge tube shown in Fig. 3. Electrodes of

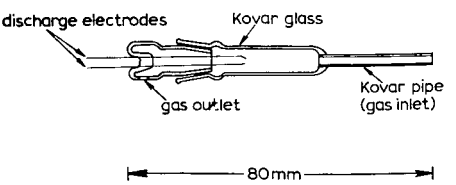

Fig. 3. Discharge tube.

0.5 mm Kovar wires were mounted, and made to be demountable from the tube by means of a ground glass joint, so that the spacing between the electrodes could be varied. The spacing was measured at an accuracy of about 0.01 mm with an optical projector.

In Fig. 4 is shown the breakdown voltage V_s plotted against the electrode spacing d. The curve represents the average of many series of measurement. The maximum breakdown voltage was usually obtained in the first run, and after the first breakdown was passed, V_s assumed a value which was appreciably less than the first one, and as the measurements were repeated, it decreased slightly. This gradual decrease is probably due to the cleaning up of the electrodes. The spread in the breakdown voltage, however, was less than about 50 V.

The results of the second investigation, designed to obtain the relation between the discharge current i_d and the maintaining voltage V_m, are shown in Fig. 5. The curves obtained at various gap distances all exhibit constant voltage characteristics. In view of the fact that the noise current to the sensing electrode depends on the stability of the discharge, such a constant voltage characteristic necessitates a stabilized voltage supply. The fluctuation of the discharge current Δi due to the instability of the voltage supply is given by $\Delta i = \Delta V/R$, where R is the current limiting resistance in the discharge circuit.

Background current

(*a*) *Effect of the polarity of voltage supply for the discharge.* Fig. 6 shows the typical background current curve which was obtained with a circuit shown in the inset

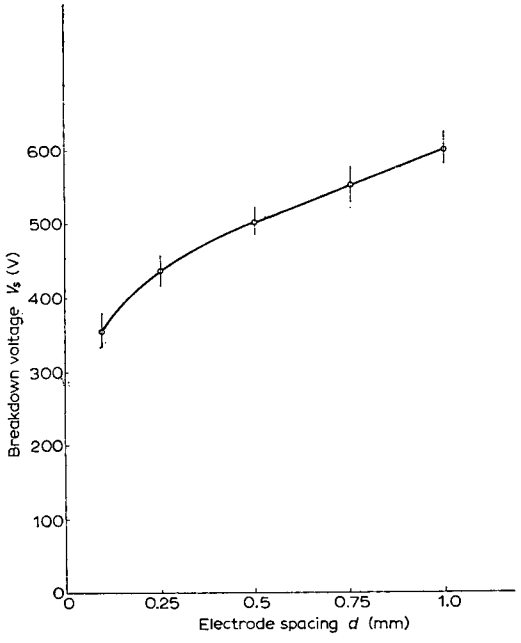

Fig. 4. Breakdown voltage in the helium flow as a function of electrode spacing.

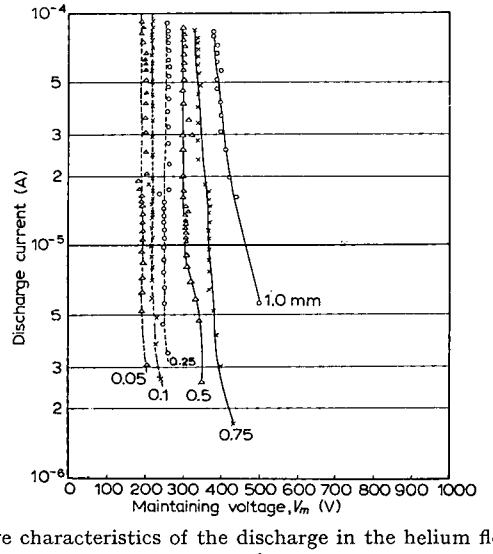

Fig. 5. Current–voltage characteristics of the discharge in the helium flow at different electrode spacings.

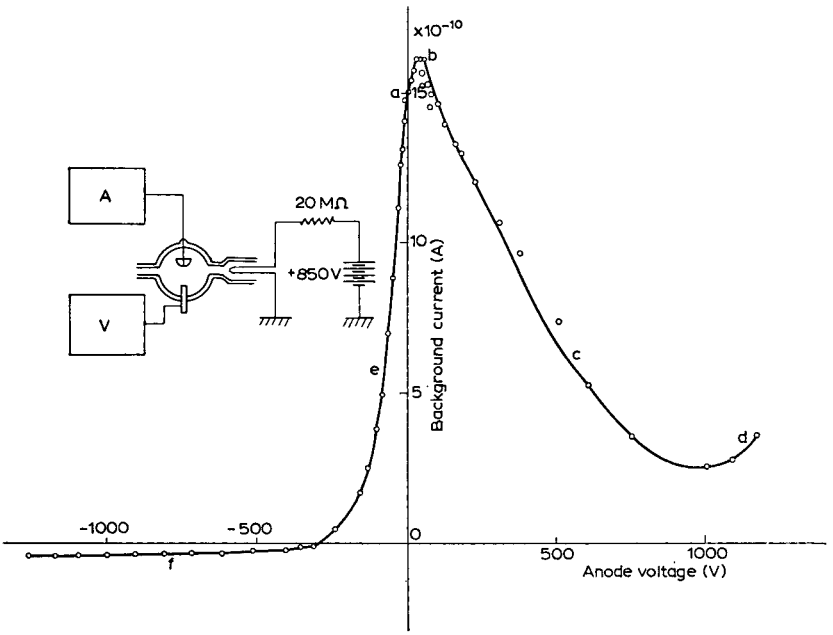

Fig. 6. Typical background current as a function of anode voltage, discharge being excited by a positive high voltage supply. Helium flow = 60 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

figure of Fig. 6. It should be noted here that the subsidiary discharge is excited by a positive high voltage supply. Measurement was made under the following conditions:

Argon carrier gas: 60 ml/min
Helium discharge gas: 60 ml/min
Discharge current: 30 μA

The curve exhibits a rather complicated shape: at negative high anode voltages a weak electron current flows to the cathode, and as the anode voltage is increased, there is a steep rise at voltages ranging from —200 V to 50 V with a maximum at 50 V, followed by a decline at higher anode voltages. It is now essential to offer some physical explanation for these characteristics. The explanation is, however, not too difficult, as will be apparent later.

In Fig. 7 is shown the background current to the cathode which was measured with a circuit shown in the inset figure of Fig. 7. In this case, the subsidiary discharge was excited by a negative high voltage supply. It can be seen that, although the other operational conditions are exactly the same as those mentioned above, the curve is very different from that of Fig. 6; it has such a shape as would be described if the curve of Fig. 6 were turned around the origin by 180°. This difference can be ascribed to the polarity of the high voltage supply for the discharge, and it is clear that the background current depends on the potential of the discharge chamber referred to that of the sensing chamber. An interpretation of the trend of the curves of Fig. 6 and Fig. 7, which, at the same time, explains the operational mechanism of this detector, is offered below.

(1) In the first case, when the discharge is excited by a positive high voltage supply (Fig. 6), the potential of the discharge chamber is positive because one electrode is at the earth potential and the other at about 250 V. When the anode voltage is 0 V, the potential of the sensing chamber is the earth potential and is negative with

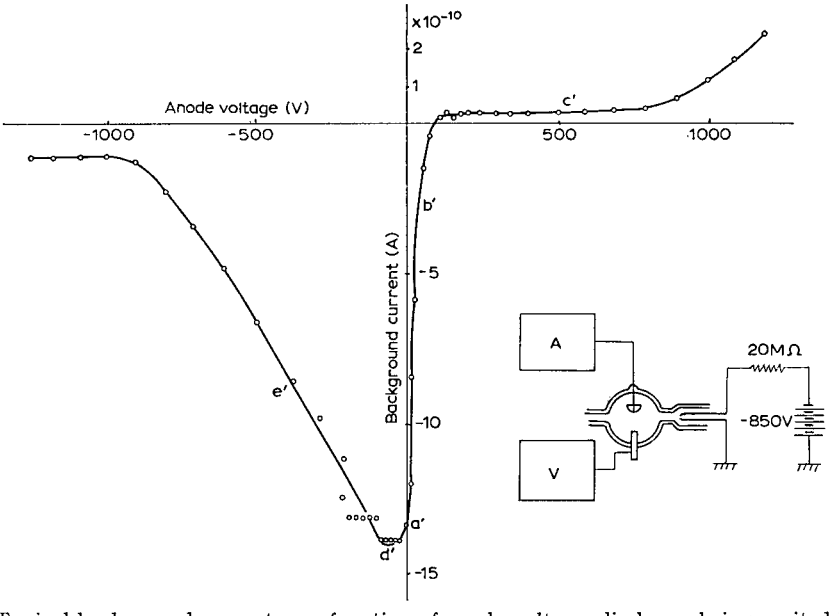

Fig. 7. Typical background current as a function of anode voltage, discharge being excited by a negative high voltage supply. Helium flow = 60 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

respect to that of the discharge chamber. Thus, ions produced by the discharge are drawn to the sensing chamber by an electrical force acting between the two chambers. These ions are captured by both the anode and the cathode, so that positive current is read by the electrometer (point a in Fig. 6). When the anode voltage is 50 V, positive ions are still drawn to the sensing chamber, but, repelled by the anode, most of them are collected by the cathode. At this voltage maximum ion current is measured by the electrometer (point b in Fig. 6). As the anode voltage is increased further, the potential of the sensing chamber rises, which starts inhibiting the flow of positive ions from the discharge and pulling out electrons gradually. This accounts for the negative characteristics of the curve (point c in Fig. 6). At voltages higher than 1000 V, ionization by collision of electrons sets in, thus again giving rise to an increase in the background current (point d in Fig. 6).

Next, let us analyze the current curve in the region below 0 V. When we apply negative voltage to the anode, the potential of the sensing chamber becomes negative, causing many positive ions to be drawn to the sensing chamber. Since most of these ions are collected by the anode, the share taken by the cathode is reduced, resulting in a decrease in the background current (point e in Fig. 6). In the region below —300 V, a small amount of saturated negative current is observed, which indicates that there is a flow of electrons to the cathode. Perhaps these electrons are produced

by photoionization, because at these anode voltages it is less likely for electrons to enter the sensing chamber from the discharge (point f in Fig. 6).

(2) When the discharge is excited by a negative high voltage supply (Fig. 7), the potential of the discharge chamber is negative. If the anode voltage is 0 V, the potential of the sensing chamber is the earth potential and positive with respect to that of the discharge chamber. This condition is favourable for electrons to be drawn to the sensing chamber by the electric field between the two chambers. Electrons thus drawn to the sensing chamber are captured by both the anode and the cathode. Thus, negative current is read by the electrometer (point a′ in Fig. 7). As the anode voltage is increased, more electrons are drawn to the sensing chamber. However, with a larger share of these electrons taken up by the anode, the electron current to the cathode decreases (point b′ in Fig. 7). At higher anode voltages a small amount of saturated positive ion current is seen to flow to the cathode. It appears that this positive current may be ascribed to photoionization within the sensing chamber. It should be noted here that the potential of the sensing chamber is now so highly positive with respect to that of the discharge chamber that a larger electron current flows to the anode (point c′ in Fig. 7).

When we apply negative voltage to the anode, the minimum background current is obtained at —50 V. The reason for this is that electrons still drawn to the sensing chamber are all collected by the cathode (point d′ in Fig. 7). Further increase in the negative value of the anode voltage causes the potential of the sensing chamber to be lowered, which starts inhibiting the flow of electrons to the sensing chamber. This, in turn, results in a gradual reduction in the electron current to the cathode (point e′ in Fig. 7).

Now it can be concluded from the qualitative considerations above that the background current to the cathode is predominantly due to the transfer of charged particles between the two chambers. It is clear that charged particles also flow to the anode, and of these particles the electrons are effective in ionizing sample gases. Therefore, it can be predicted that under the operational condition of c′ of Fig. 7, where an abundant supply of electrons is available in spite of the low background current, an advantageous performance of the detector may be realized.

(*b*) *Effect of the circuit condition of the discharge.* The presentation of the data which follows is mainly for the purpose of confirming the preceding considerations and illustrating a more effective way to use the detector. As stated earlier, the transfer of charged particles between the two chambers is dependent upon the potential of the discharge chamber relative to that of the sensing chamber. Since an abundant supply of free electrons would give an increased efficiency in actual practice, it would definitely be more satisfying if the discharge could be fired in such a manner that its potential becomes highly negative with respect to that of the sensing chamber. Such an adjustment of the discharge potential is easily achieved by a simple modification of the discharge circuit, *e.g.* by changing the position of the current limiting resistor in the circuit.

Fig. 8 illustrates the effect of the potential drop across the resistor on the background current. Measurements were made by exciting a discharge by a negative high voltage supply and with circuits shown in the inset figures of Fig. 8. The result of these measurements is what might be expected: lowering the discharge potential by the drop across the resistor causes many more electrons to be drawn to the sensing

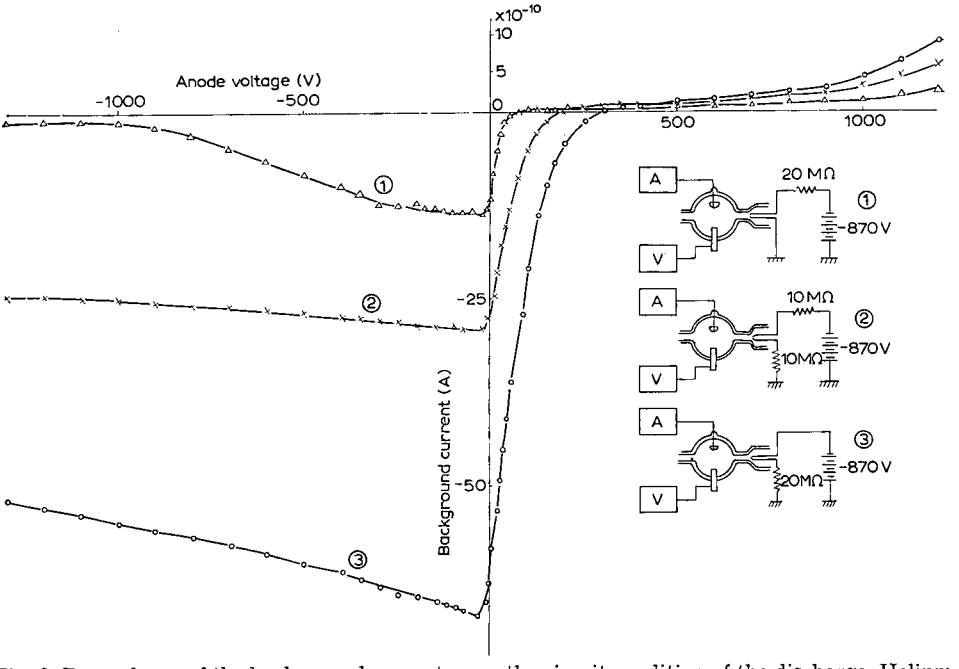

Fig. 8. Dependence of the background current upon the circuit condition of the discharge. Helium flow = 60 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

chamber, as illustrated by an increased electron current in the region of negative anode voltages. It is seen, moreover, that positive background current at positive anode voltages, which may be attributable to photoionization, is much less than the electron current, independent of the modification of the circuit. The control of the detector by the circuit modification is one of the particular advantages of this system.

(c) *Effect of the geometrical arrangement of the electrodes.* Thus far, we have dealt primarily with the mechanism by which primary electrons (background current) are generated, and made clear that the electrical force plays a predominant role. This fact leads us at once to the belief that the character of the detector must be governed to a great extent by the geometrical configuration of the electrodes, particularly by the position of the discharge electrodes relative to the sensing chamber.

Fig. 9 shows the background current for three different values of the anode position, the cathode being fixed at the center of the sensing chamber. It can be seen that the curves show no pronounced difference except that the slope marked b′ becomes less steep for a detector with widely spaced internal electrodes. Of importance in this study is the anode voltage at which the cathode collects zero net background current from the discharge. An advantage of operation at this point is that the input resistor of the electrometer (input sensitivity) can be switched without any noticeable shift in the baseline of the chromatogram.

In Fig. 10 are shown the background currents illustrating the effect of the position of the discharge electrodes relative to the sensing chamber. As would be expected, the intensity varies to a great extent with their position: it is easy to provide an abundant electron current, say in excess of 10^{-8} A.

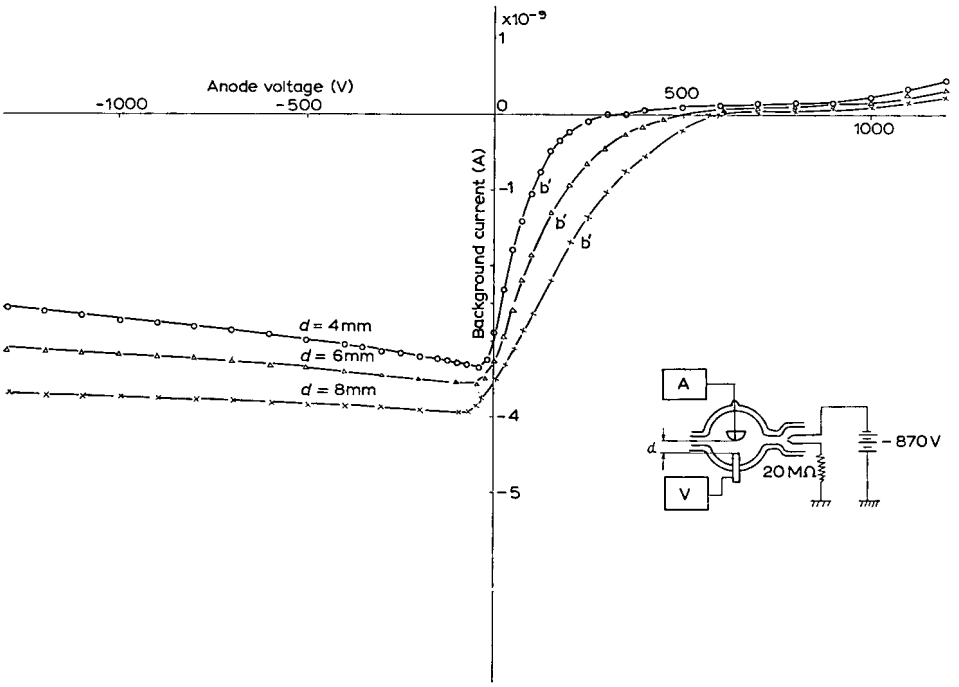

Fig. 9. Dependence of the background current upon the anode position. Helium flow = 20 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

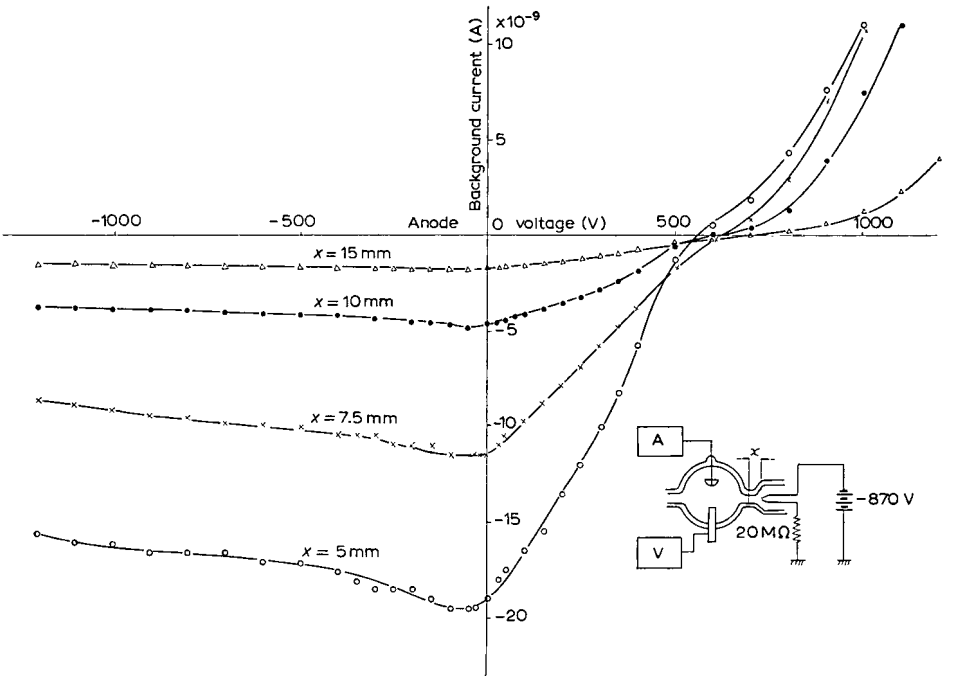

Fig. 10. Dependence of the background current upon the position of the discharge electrodes relative to the sensing chamber. Helium flow = 60 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

(*d*) *Effect of the flow rate of the discharge gas.* Fig. 11 shows the background current as a function of the helium flow at different anode voltages. It can be seen that the background current increases continually as the flow rate increases. This result seems to indicate that charged particles are entrained by the discharge gas into the sensing chamber, and it was on the basis of this observation that we postulated the "entrainment" theory in the previous paper.

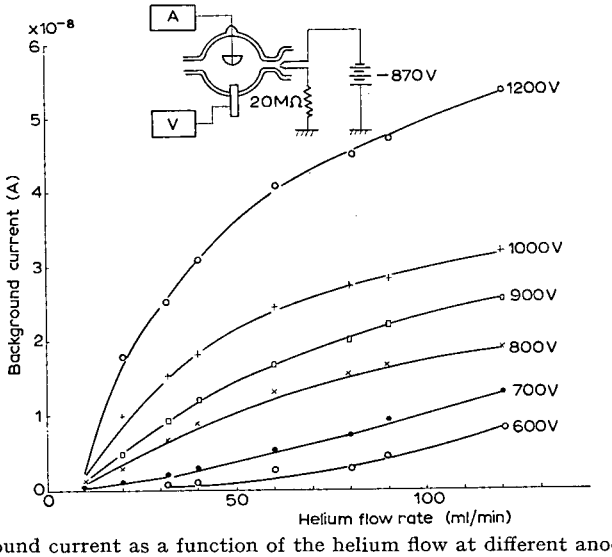

Fig. 11. Background current as a function of the helium flow at different anode voltages. Argon flow = 60 ml/min; discharge current = 30 μA.

Very recently the problem has been studied by NOREM[4] of the Perkin-Elmer Corporation with the same discharge detector. He measured the background current as a function of the helium flow in both the normal and the reverse direction, with no argon flow, and found that even in the case of the reverse flow, the current increased with the helium flow in the same way as in the normal-flow case. This disproves clearly the "entrainment" theory.

It might be expected, however, that at higher flow rates charged particles would have a much greater chance of separating from the discharge due to a turbulent action of the helium flow. These escaping charged particles, while negligibly small in number compared with those existing in the discharge path, would be of importance in determining the intensity of the background current.

Response for propane

The response of the detector varies to a great extent with the mode of its operation, *i.e.*, with the discharge circuit, the discharge current, the operating voltage and the flow rates of the carrier and the discharge gas. Measurements were made to investigate the effect of these factors on the response for propane.

(*a*) *Effect of the discharge circuit.* It has been shown that it is possible to increase the primary electron current by making the discharge potential highly negative with respect to the sensing chamber. This should result in more efficient ionization of the

sample and thus increase the sensitivity of the detector. As a test of this premise, the peak current of 0.0022 ml propane (gas volume) was measured, making simple alterations to the discharge circuit, while keeping other operational conditions unchanged.

The results of these measurements are summarized in Tables I and II. These tables are of interest in that they show the relative merit of the various discharge circuits. It can be seen that, for the case of the positive discharge (Table I), the background current is appreciably larger than the peak current, and that the higher the discharge potential, the larger the background current and the smaller the response. Thus, the positive discharge cannot be used advantageously in most circumstances. On the other hand, the negative discharge gives a peak current much larger than the background current (Table II). Furthermore, it is noted that the response increases as the discharge potential becomes more negative.

(*b*) *Effect of the helium flow.* According to Fig. 11, the number of electrons entering into the sensing chamber varies with the helium flow. This, together with the change of the gas composition in the sensing chamber, would affect the response of the detector.

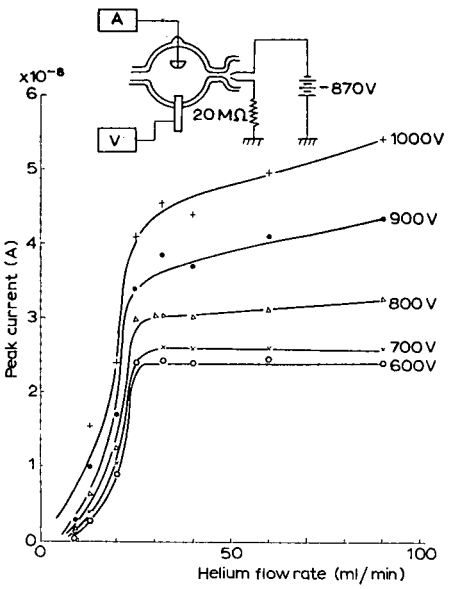

Fig. 12. Peak current as a function of the helium flow at different anode voltages. Sample = $4.5 \cdot 10^{-7}$ g/sec of propane; argon flow = 60 ml/min; discharge current = 30 μA.

To clarify this effect, measurements were made for a negative discharge of 30 μA at different anode voltages. The result obtained is given in Fig. 12. It can be seen that for the helium flow below 30 ml/min, there is a steep rise in the response, while above 30 ml/min this ceases to depend on the helium flow. The initial increase is attributable solely to the increase in the primary electrons, since, according to LOVELOCK[5], the change of the gas composition in the region of lower helium flow (the region of lower proportion of helium in argon) has no appreciable effect on the ionization efficiency. At helium flow higher than 30 ml/min, where the primary

TABLE I

EFFECT OF THE DISCHARGE CIRCUIT ON THE RESPONSE FOR PROPANE

(Discharge is excited by a positive high voltage supply)

Sample: propane 0.0022 ml. Temperature: 20°. Column: squalane 5 m. Carrier gas: argon, 60 ml/min. Discharge gas: helium, 60 ml/min. Discharge current: 30 μA.

*Discharge circuit**	*Anode voltage* *V*	*Background current* *A* × 10^9	*Peak current*** *A* × 10^9
1	600	3.1	1.1
	700	2.9	2.3
2	600	9.0	1.2
	700	8.8	1.9
3	600	16.5	0.9
	700	16.0	1.3

* 1. A current limiting resistor of 20 MΩ is inserted between the high voltage supply and one electrode. The other electrode is at the earth potential. The discharge potential is positive and low.

2. A 10 MΩ resistor is inserted between the high voltage supply and one electrode. Also between the other electrode and the earth is inserted a resistor of 10 MΩ. The discharge potential is positive and medium.

3. One electrode is connected directly to the high voltage supply. Between the other electrode and the earth is inserted a 20 MΩ resistor. The discharge potential is positive and high.

** Peak current is the net deflection above the background current.

TABLE II

EFFECT OF THE DISCHARGE CIRCUIT ON THE RESPONSE FOR PROPANE

(Discharge is excited by a negative high voltage supply)

Sample: propane 0.0022 ml. Temperature: 20°. Column: squalane 5 m. Carrier gas: argon, 60 ml/min. Discharge gas: helium, 60 ml/min. Discharge current: 30 μA.

*Discharge circuit**	*Anode voltage* *V*	*Background current* *A* × 10^9	*Peak current*** *A* × 10^9
1	600	0.74	13.0
	700	1.20	14.5
2	600	1.55	17.5
	700	2.2	19.0
3	600	2.8	23.0
	700	5.4	25.5

* 1. A current limiting resistor of 20 MΩ is inserted between the high voltage supply and one of the electrodes. The other is at the earth potential. The discharge potential is negative and low.

2. A 10 MΩ resistor is inserted between the high voltage supply and one of the electrodes. A resistor of 10 MΩ is also inserted between the other electrode and the earth. The discharge potential is negative and medium.

3. One of the electrodes is directly connected to the high voltage supply, and a 20 MΩ resistor is inserted between the other electrode and the earth. The discharge potential is negative and high.

** Peak current is the net deflection above the background current.

electron current still increases with the helium flow, increasing proportion of helium in argon starts impairing the ionization efficiency. These compensating effects give a flat region in the response-flow curves.

(*c*) *Linearity and limit of detection.* Fig. 13 shows the response of the detector as a function of the mass flow rate of propane at different anode voltages. As has been observed with the conventional argon ionization detector, a linear relationship was not obtained over the entire range of the mass flow.

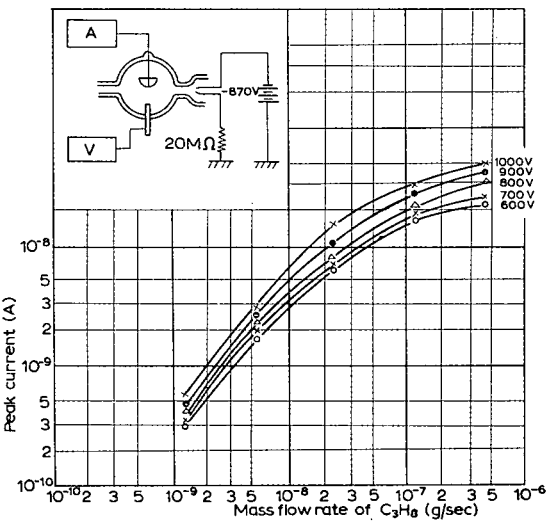

Fig. 13. The relationship between the peak current and the mass flow rate of propane at different anode voltages. Helium flow = 60 ml/min; argon flow = 60 ml/min; discharge current = 30 μA.

In order to obtain figures of the limit of detection, additional measurements were made, operating the detector at that anode voltage at which the cathode collects zero net background current. This condition allows us to make an accurate determination of the noise level of the detector. The results obtained are given in Table III. The table is arranged as follows: In the first column is given the helium flow rate, at

TABLE III

THE PERFORMANCE CHARACTERISTICS OF THE DETECTOR UNDER THE CONDITION OF ZERO NET BACKGROUND CURRENT

Sample: propane 0.0022 ml (mass flow rate: $4.5 \cdot 10^{-7}$ g/sec). Column: squalane 5 m. Temperature: 20°. Carrier gas: argon 60 ml/min. Discharge gas: helium. Discharge current: 30 μA.

Flow rate ml/min	*Background current* ($V = o$) A	*Anode voltage* ($i_0 = o$) V	*Noise current* A	*Peak current* A	Q_{min} g/sec	C_{min} g/ml
5	$-3.3 \cdot 10^{-10}$	580	$3 \cdot 10^{-13}$	$4.0 \cdot 10^{-10}$	$6.75 \cdot 10^{-10}$	$6.75 \cdot 10^{-10}$
10	$-1.25 \cdot 10^{-9}$	575	$1.1 \cdot 10^{-12}$	$1.5 \cdot 10^{-9}$	$6.6 \cdot 10^{-10}$	$6.6 \cdot 10^{-10}$
20	$-9.0 \cdot 10^{-9}$	570	$6.0 \cdot 10^{-12}$	$8.5 \cdot 10^{-9}$	$6.4 \cdot 10^{-10}$	$6.4 \cdot 10^{-10}$
30	$-1.7 \cdot 10^{-8}$	560	$1.6 \cdot 10^{-11}$	$2.1 \cdot 10^{-8}$	$6.8 \cdot 10^{-10}$	$6.8 \cdot 10^{-10}$
40	$-2.8 \cdot 10^{-8}$	550	$4.5 \cdot 10^{-11}$	$2.1 \cdot 10^{-8}$	$2.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$
60	$-5.1 \cdot 10^{-8}$	535	$2.0 \cdot 10^{-10}$	$2.05 \cdot 10^{-8}$	$8.8 \cdot 10^{-9}$	$8.8 \cdot 10^{-9}$

which measurement was made, and in the second column the background current at zero anode voltage. This quantity represents a measure of the primary electrons drawn to the sensing chamber. The third column gives the operating anode voltage at which the background current disappears. The fourth column gives the noise current at the operating voltage. In the fifth is given the peak current obtained on 0.0022 ml propane at a mass flow rate of $4.5 \cdot 10^{-7}$ g/sec. Columns six and seven give values of the minimum detectable quantity, Q_{min}, and of the minimum detectable concentration, C_{min}, which are calculated from the relations given by CONDON *et al.*[6].

From these data, it must be recognized that the experimental values of the limit of detection depend critically upon the flow rate of helium. At lower helium flow, $Q_{min} \approx 6.5 \cdot 10^{-10}$ g/sec and $C_{min} \approx 6.5 \cdot 10^{-10}$ g/ml, while at flow rates above 30 ml/min, Q_{min} and C_{min} increase with the flow rate.

It is difficult to say how much significance should be attached to the values of this table, because the noise current should depend on the conditions of measurement, *i.e.* on the stability of the high voltage supply for the discharge, the stability of the anode voltage, the uniformity of the helium flow and the bandwidth of the electrometer. Furthermore, the operating conditions of these measurements do not represent those giving the best values for Q_{min} and C_{min}, since at higher anode voltages the multiplication effects would improve the S/N ratio up to some optimum point, as in the LOVELOCK's detector.

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SUMMARY

A subsidiary discharge argon ionization detector, which uses helium as the discharge gas and argon as the carrier gas, is described. The properties of the discharge in the helium flow were first studied from the point of view of its suitability as a source of primary electrons. Next, the generation mechanism of primary electrons was investigated by measurements of the background current to the cathode. The background current measured as a function of the anode voltage was found to depend on the discharge potential with respect to the potential of the sensing chamber, which indicates that the transfer of charged particles from the discharge to the sensing chamber is due to an electrical force acting between the two chambers. From these results, a new technique of operation was developed. Finally the response was tested for propane, and the relationships between the response and the background current were discussed.

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DETERMINATION OF OXYGEN, NITROGEN AND CARBON DIOXIDE IN AIR SAMPLES

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Studies connected with life systems in enclosed capsules and space suits require accurate measurements of the concentrations of atmospheric gases. Of these gases, nitrogen, oxygen, and carbon dioxide are of paramount importance. The determination of such gases, based on the gas chromatographic principle, shows great promise. Molecular sieves have been used with great success as column packing materials for the separation of oxygen and nitrogen[1]. Silica gel has been used for the gas chromatographic analysis of carbon dioxide[2]. However, neither of these solid stationary phases completely resolves all three gases. Molecular sieve 5A, while resolving oxygen and nitrogen very efficiently, retains carbon dioxide permanently under the conditions of analysis; silica gel, on the other hand, while separating carbon dioxide, does not separate nitrogen from oxygen.

An attempt was made to resolve oxygen, nitrogen, and carbon dioxide from a single analytical sample, using a parallel dual column gas chromatograph[3]. Molecular sieve 5A and silica gel-packed columns were used in the initial experiments. The parallel dual column arrangement permitted splitting of the injected sample into two columns, molecular sieve and silica gel, where the gases were separated simultaneously. A 5-ft., 1/4-in. diameter column packed with molecular sieve 5A, 60–80 mesh, was used for the separation of oxygen and nitrogen. A 2-ft., 1/4-in. diameter column packed with silica gel, 20–200 mesh, was used for the separation of carbon dioxide from nitrogen and oxygen.

One injection of an air sample into this system was found to produce four peaks: an oxygen peak and a nitrogen peak, as they elute from the molecular sieve column, a peak for carbon dioxide, and a combined peak for oxygen and nitrogen as they elute from the silica gel column. The conditions of analysis may be adjusted in such a way that each peak appears at different retention times. With this arrangement, complete analysis of nitrogen, oxygen, and carbon dioxide is possible from one sample.

A slight modification permits determination of carbon dioxide at the p.p.m. level. Two valves were incorporated at the entry side of each column, permitting use of the columns in separate operations.

APPARATUS

The design of the instrument is shown in Fig. 1. A Gow-Mac pretzel-type thermoconductivity cell with four tungsten hot wires connected in a Wheatstone bridge arrange-

ment is used for detection. A Leeds and Northrup model G, 0–1 mV recorder and a Perkin-Elmer integrator are used in the readout portion of the instrument. The sample injection system incorporates a modified Beckman two-way gas sampling valve, a

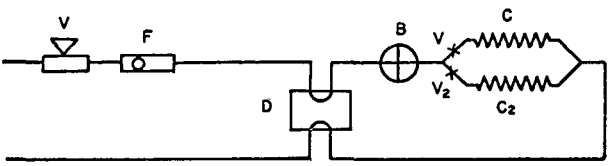

Fig. 1. Gas chromatograph. V = Needle valve; F = Flow meter; D = Detector; B = Beckman sampling valve; V_1, V_2 = Valves; C_1 = Molecular sieve column; C_2 = Silica gel column.

vacuum pump, and a manometer. The conventional spiral loops of the Beckman valve were replaced by 4-in., 1/4-in. diameter stainless steel U-tubes. This arrangement permits the application of a cold bath over the sampling loops.

EXPERIMENTAL

For the simultaneous determination of all three components, a sample of air is introduced into the loop of the Beckman valve and injected in the helium stream. The following operating conditions are used in the analysis.

Gas: He, inlet pressure $2^3/_4$ p.s.i.g.
Columns: (A) 5-ft., 1/4-in. diameter tube packed with molecular sieve 5A 60–80 mesh,
(B) 2-ft., 1/4-in. diameter tube packed with silica gel 20–200 mesh,
The columns are connected in parallel
Temperature: 26°
Bridge current: 150 mA,
Recorder: 0–1 mV model G, L and N,
Speed: 1/2 in. per min.

The areas under the four peaks are integrated, and from the absolute values of the nitrogen-oxygen peak and of the carbon dioxide peak, concentration of carbon dioxide is calculated. The peak area for oxygen and nitrogen resolved on the molecular sieve columns provides a ratio of oxygen to nitrogen. The concentrations of nitrogen and oxygen are calculated after correction is made for the concentration of carbon dioxide in the sample.

For the analysis at the p.p.m. level of air samples containing carbon dioxide, the following procedure is used. An evacuated sample gas bulb of 10–250 ml volume is used for procuring the sample. With the valve to the molecular sieve column open, and the valve to the silica gel column closed, 1–2 ml of air are introduced into the helium stream and resolved on the molecular sieve column. A flowrate of 165 ml/min of helium is used with this column. The areas under the nitrogen and oxygen peaks are integrated, and the ratio is calculated in the conventional manner. The pressure of the remaining sample in the gas bulb is measured with a manometer. A Dewar Flask containing liquid nitrogen is placed over the collecting loop of the Beckman valve, and the sample is transferred into it by pumping. The carbon dioxide is con-

densed in the collecting tube of the Beckman valve while non-condensables are removed by vacuum pumping. The valve to the molecular sieve column is closed, the valve to the silica gel columns is opened, and the rate of helium flow is adjusted to 77 ml/min.

At this point, the collection tube containing carbon dioxide is pivoted into the helium stream; the liquid nitrogen trap is removed and a beaker containing hot water placed over the U-tube to facilitate evaporation of the condensed gases. The area of the carbon dioxide peak is integrated as it is eluted from the silica gel column. For calibration purposes, a known volume of carbon dioxide is introduced in the gas chromatograph and analyzed under the conditions described above.

Calculation:

$$O_2 = \frac{c(O_2) \times (100 - \%\ CO_2)}{c(O_2 + N_2) \times 100}\ \%$$

$$N_2 = \frac{c(N_2) \times (100 - \%\ CO_2)}{c(O_2 + N_2) \times 100}\ \%$$

$$CO_2 = \frac{C_S \times P_R \times V_R}{C_R \times P_S \times V_S} \times 100\ \%$$

where c = number of integral counts,
C_S = number of integral counts for the peak of carbon dioxide (sample),
C_R = number of integral counts of the peak of carbon dioxide (reference),
P_R = pressure of the CO_2 reference,
V_R = volume of the CO_2 reference,
P_S = pressure of sample,
V_S = volume of sample.

RESULTS

The retention times obtained under the described conditions of analysis are given in Table I.

The sensitivity of the method, with respect to carbon dioxide, was calculated by using the results of eight determinations of standard samples for carbon dioxide content. One cc volume of carbon dioxide at 1 mm mercury pressure, using the

TABLE I

RETENTION TIMES OF OXYGEN, NITROGEN AND CARBON DIOXIDE

Gas	*Column*	*Retention time (min)*	
		*Simultaneous technique**	*Separate technique***
Oxygen and nitrogen	Silica gel	1	0.7
Oxygen	Molecular sieve	2	1.2
Nitrogen	Molecular sieve	6	2.7
Carbon dioxide	Silica gel	26	8.4

* Inlet pressure, 2 3/4 p.s.i.g.
** Inlet pressure, 4 p.s.i.g.

maximum sensitivity of the instrument and the 0–1 mV range of the recorder produced an average of 29.5 integral counts. Under the conditions of analysis, quantities of carbon dioxide corresponding to 5 integral counts are detectable. Using sample sizes of 1 cc magnitude, 200 p.p.m. of carbon dioxide can be detected. With 10 cc samples, the lower level of detectability for carbon dioxide will be 20 p.p.m., and with 100 cc samples, 2 p.p.m. The sensitivity of the method can be increased replacing hot-wire detection with thermistor detection.

The argon and oxygen appears as one peak using this procedure; however, since the ratio of argon to oxygen in atmospheric air is known, necessary corrections for concentration of oxygen and argon can easily be made.

SUMMARY

A gas chromatographic system for analysis of nitrogen, oxygen, and carbon dioxide in air samples is described. The system utilizes a parallel dual column arrangement for simultaneous determination of all three components. The lower limit of detection for carbon dioxide is 2 p.p.m.

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COMPOSITION ANALYSIS OF POLYBUTENES*

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INTRODUCTION

Polymer pyrolysis may proceed by two paths: (a) chain unzipping to yield monomer and (b) random cleavage. The chain fracture process proceeds by free radical propagation along the polymer chain at thermodynamically weak bonds or sites favoring free radical formation. The random cleavage process proceeds by simultaneous random free radical attack at points along the chain and by multiple thermal homolytic chain cleavage. This latter pyrolysis process gives rise to low monomer yields and an apparently random product distribution. The two processes can operate in competition[1]. The ultrahigh temperature pyrolysis work of BARLOW, LEHRLE AND ROBB[2] has demonstrated that at greater than 900° pyrolysis process *b* supersedes *a*. This has been demonstrated for polymethacrylates, polymethylstyrenes, and several other polymers which can give high monomer yields[2].

The saturated hydrocarbon polymer "rate of evaporation" studies of WALL AND STRAUS[3] indicate that polyisobutylene and polypropylene decompose principally by random cleavage of the polymer chain. However, the exceptionally high yield of monomer (20%) from polyisobutylene reported in their studies suggests a non-statistical distribution of pyrolysis products.

Information from polymer degradation studies is derived from two sources: (1) rate of pyrolysis studies and (2) an examination of polymer degradation products. The first class of information has provided data for much of the current theoretical work in the field of polyolefin degradation[3–6].

The techniques and method of pyrolysis/gas chromatography which give the second type of information have been treated by various workers[7–9] but have not found wide application in the field of polymer pyrolysis mechanism studies. This has been due largely to lack of sufficient theoretical information needed to effect more than an empirical relationship between pyrolysis products and polymer structure.

In consideration of this previous work, it is now possible to investigate the pyrolysis behavior of polybutenes by means of pyrolysis product analysis. The polybutenes are currently of great interest to the petroleum industry due to their wide application.

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EXPERIMENTAL

Polymer samples were prepared by the ionic polymerization of various mixtures of propylene, 1-butene, *cis*- and *trans*-2-butene, and isobutylene. Isobutylene content ranged from 10 % to 100 %. The nature of the polymerization caused the average molecular weight of the copolymers to depend directly on the isobutylene content of the reaction mixture. These low molecular weight polymers are hereafter referred to as polybutenes.

Two of the polybutene samples, A and C, were segregated into narrow molecular weight range fractions by the solvent-thermal-gradient column methods of BAKER AND WILLIAMS[10] and of CANTOW, PORTER AND JOHNSON[11]. The solvent–nonsolvent system was benzene–acetone, and the thermal gradient was 60–28° down the column. Molecular weights were determined by intrinsic viscometry. Studies were made on polybutene fractions which differed in composition but had the same molecular weight.

Nuclear magnetic resonance (NMR) spectra were determined on the unfractionated polybutene samples diluted to 12.5 % with carbon tetrachloride, see Fig. 1.

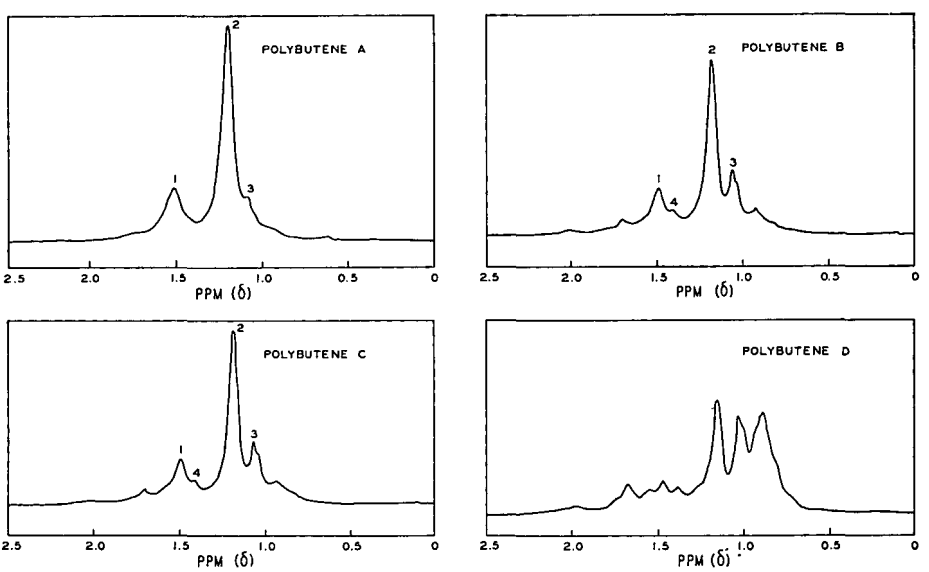


Fig. 1. Nuclear magnetic resonance spectra of polyisobutene containing various amounts of impurities. A = Pure isobutylene feed stock. B = 70 % Isobutylene, 20 % propylene, 10 % butene mixture. C = 58 % Isobutylene, 30 % propylene, and 12 % butene mixture. D = 30 % Isobutylene, 40 % propylene, and 30 % butene mixture. The butene mixture consists of butene-1 and butene-2 in isomeric proportion.

The NMR proton peaks were assigned on the basis of reported resonance values[12] and laboratory working standards. The instrument used was a Varian A-60.

The apparatus used for pyrolysis studies has been described previously[13,14]. The time required for the sample to reach furnace temperature was 15 sec under the conditions used in this study.

The chromatograph was of conventional design using 8000 Ω (nominal) thermistor

detectors and two columns in series. The first column was a 10-ft. 40/60 mesh Johns-Manville C-22 firebrick column coated with 28.6 % Carbowax 100. The second column was a 50-ft. 40/60 mesh Johns-Manville C-22 firebrick column coated with 23 % propylene carbonate. All columns were constructed from 1/4-in. copper tubing. The Carbowax 100 column was operated at 100° and the propylene carbonate column at 25°. Helium carrier gas flow was maintained at 35 cc/min.

Polymer samples were pretreated for 3 h at 4 mm pressure and 50° after being weighed into pyrolysis cups. Degassing was necessary to effect a relatively complete removal of sorbed oxygen, thus insuring conditions of anaerobic pyrolysis.

The identity of the major components of pyrolysis (propylene, isobutylene, and 2,4,4-trimethylpentene-2) was established by freezing out the gas under the chromatographic peak followed by infrared and mass spectrometric identification. Other components were identified from elution times.

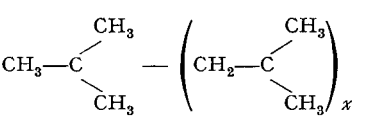
Gas chromatographic response curves were constructed for isobutylene, propylene, and 1-pentene using API standards. These curves were used in the calculation of the weight per cent yields of the principal components.

The amount of chromatographable material produced by the anaerobic pyrolysis of Sample A was determined by trapping all gases leaving the column in a weighed molecular sieve (5A) tube, equipped with stopcocks, similar to that used in carbon-hydrogen analyses. This molecular sieve tube was cooled in boiling liquid nitrogen. At —40° molecular sieve retains all hydrocarbons. On the basis of elution times from the Carbowax column, the sieve contained materials from C_1 to branched chain C_{16}. The amount of material on the sieve was obtained from the weight change in the dry, helium-filled sieve before and after pyrolysis. The weighing error, calculated from three blank runs, was ± 0.001 g.

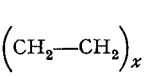
RESULTS

Nuclear magnetic resonance

The NMR spectra shown in Fig. 1 indicate large differences in the proton types and relative concentrations for four polybutenes. Polybutene A, made from relatively pure isobutylene, exhibits only three proton types in large concentration. Peak 1 is assigned to protons in chain methylene. Peak 2 is due to the side chain methyl protons. Peak 3 is due to terminal protons. The ratio of the areas of Peak 1 to Peak 2 is 2:6. The small size of Peak 3 indicates a relatively high molecular weight polymer. The NMR spectra and methyl–methylene proton ratios indicate a structure which would be expected of a pure, regular head-to-tail polyisobutylene of high molecular weight:



The NMR spectra of polybutenes B and C indicate a composition similar to polybutene A but of lower isobutylene content. Two additional peaks appear in the spectra. Peak 4 is thought to be due to a methylene proton paired with a second methylene:



The split in the terminal methyl proton Peak 3 indicates two types of chain end. The area ratio of Peak 1 to Peak 2 is near 2:6 which indicates that the polymers are essentially polyisobutylene with occasional methylene irregularities.

The NMR spectrum of polybutene D is quite different from polybutenes A, B and C. At least five different types of chain protons are present as well as two types of side chain protons. The terminal proton peak is broadened into a shoulder suggesting a low molecular weight material containing both a large number of terminal protons and proton environments. The number of methyl branches on the chain is low. The assignments above are tentative in view of limited reference compounds available. Further NMR work aimed at verifying these assignments is in progress.

Pyrolysis

Fig. 2 illustrates the general pyrolysis/gas chromatographic pattern of Sample A. In general, polyisobutylene pyrolyzes in the temperature range 430° to 600° to yield, in varying ratios, methane, ethane, propane, butane, ethylene, propylene, neopentane, 1-butene, isobutylene, *trans*- and *cis*-2-butene, 2-methyl-1-butene, and *trans*- and *cis*-2-pentenes. In addition to these lighter products, the dimer of isobutylene, 2,4,4-trimethyl-2-pentene, is produced. Isobutylene and propylene make up over 30 % of the pyrolysis products and, as such, constitute the principal pyrolysis products. Isobutylene trimer and tetramer materials are also found. In the pyrolysis temperature

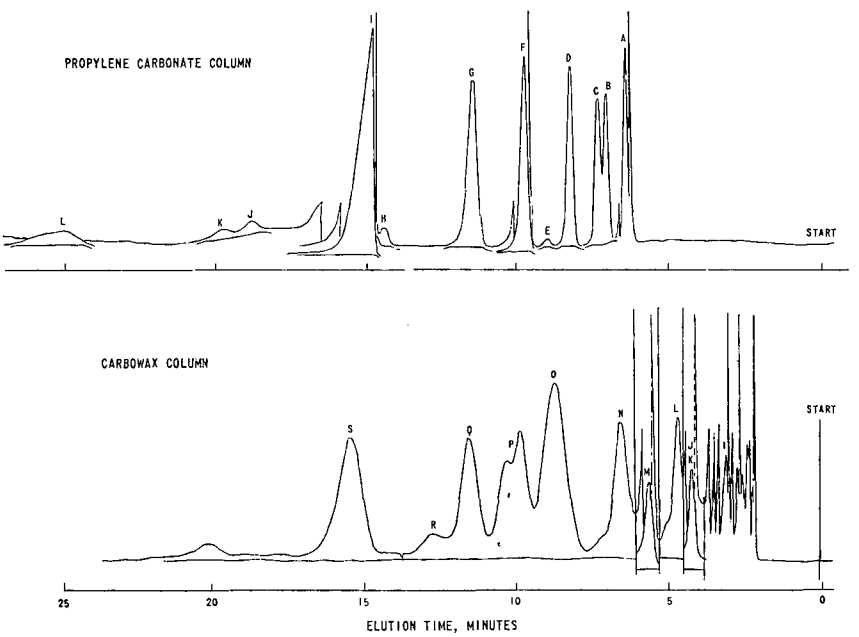


Fig. 2. Pyrolysis/gas chromatograms of polyisobutylene A. A sample equivalent to 0.0198 g of polymer was pyrolyzed at 485° in a 35 cc/min helium carrier gas stream. The Carbowax column record was recorded at four times the base sensitivity of the propylene carbonate column. The chromatographic peaks are identified as follows: A = Air and methane. B = Ethane. C = Ethylene. D = Propane. E = Butane. F = Propene. G = Isobutane. H = Butene-1. I = Isobutylene. J = *trans*-2-Butene. K = *cis*-2-Butene. L = 2-Methyl-1-butene. M = 2,2,4-Trimethyl-2-pentene. N, O = Dimer olefins. P, Q, R = Trimer olefins. S = Tetramer olefin.

range 400° to 500°, 80–86 % of the polymer sample is pyrolyzed to materials in the carbon range C_1 to C_{16}. The remaining 14–20 % is not found in the pyrolysis cup and is, therefore, trapped on the column. This non-chromatographable material must be relatively long polymer chain fragments which are swept from the pyrolysis chamber before further pyrolytic cleavage occurs. The reproducibility of pyrolysis product yields is ± 5 % as is shown in Table I.

TABLE I

PRINCIPAL PRODUCT YIELD OF POLYBUTENES PYROLYZED AT 525°

Sample	*Average molecular weight*	*Weight % propylene**	*Weight % isobutylene**	*Weight % 2,4,4-trimethyl-2-pentene**	*Weight % trimers and tetramers**
A	5300	6.61	30.9	2.37	25.9
		6.52	31.0	2.20	24.6
B	1400	6.35	26.3	2.77	30.0
		6.05	24.8	2.40	31.0
C	1200	5.84	23.4	2.80	32.0
		5.63	21.0	2.75	31.8
D	450	3.93	13.8	3.09	30.0
		3.50	13.6	2.89	29.6

* Weight % based on initial sample weight.

In order to isolate the variables present in any detailed pyrolytic study, experiments were performed on the effects of temperature, flow rate, and sample size on pyrolysis results. The rate of decomposition of polyisobutylene below 420° is too slow to give chromatograms with properly resolved peaks. For all polybutene samples, the production of isobutylene and propylene rises sharply from 420° to 485° and then decreases slowly from 485° to 550°. The point of maximum production of isobutylene was unique for each of the four polybutenes as shown in Fig. 3.

The temperature of initial pyrolysis as well as the temperature at which maximum isobutylene production occurred was found to increase with increasing molecular weight and increasing degree of polymer chain homogeneity. The polybutene samples show an increasing yield of isobutylene in the order A, B, C, D.

Isobutylene production was found to increase by 15 % when the helium flow rate was decreased from 35 cc to 20 cc/min at 550°. Production of the dimer, 2,4,4-trimethyl-2-pentene, decreased under the same conditions as did the peaks due to higher molecular weight chain fragments. This indicates that the decomposition of polymer under the dynamic conditions of pyrolysis/gas chromatography is incomplete and that a higher isobutylene yield can be effected by further action on high molecular weight fragments. With careful control of carrier gas flow rate, isobutylene yield is kept within ± 5 %.

Sample sizes from 0.01 to 0.05 g showed a small, 4.8 %, variation in the weight per cent of isobutylene and propylene produced. This variation is within the observed reproducibility of 5 % and was not considered further.

The effect of molecular weight on the weight per cent yield of isobutylene was

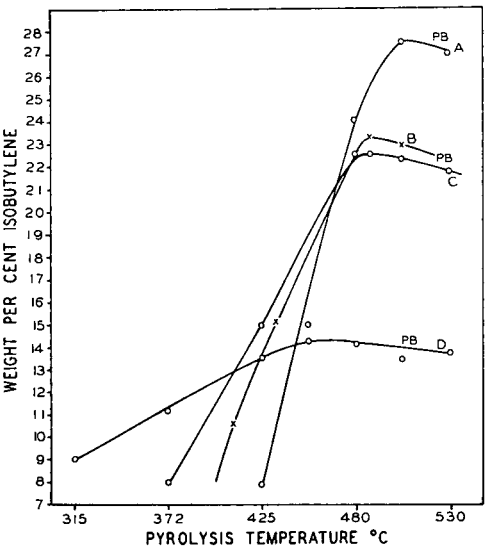


Fig. 3. Isobutylene yield at various pyrolysis temperatures.

studied under uniform conditions of flow (35 cc/min), sample size (0.02 g), and temperature 485°. The weight per cent yield of isobutylene increased with increasing molecular weight as shown in Fig. 4. However, the data from Samples A and C formed two experimental groups. The increase of isobutylene production with increasing molecular weight fractions of pure polyisobutylene, Sample A, was small. Conversely, the increase in isobutylene production from Sample C was large with the increasing molecular weight of the fraction.

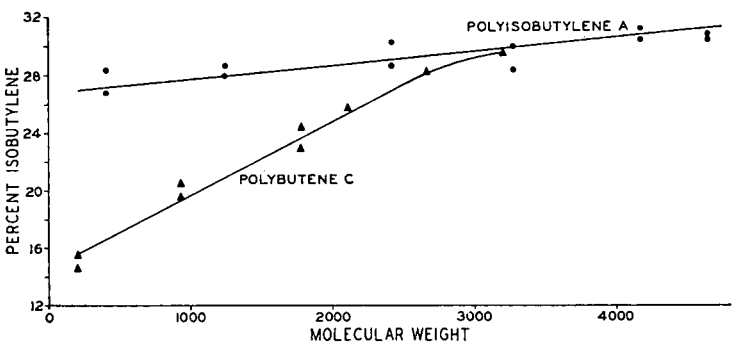


Fig. 4. Isobutylene yield of polybutenes A and C at different molecular weights.

DISCUSSION

The 80 % to 86 % yield of chromatographable material indicates that the degree and rate of degradation are high for polyisobutylene under the conditions used in this study. The 22 % to 27 % average yield of isobutylene from Sample A agrees well with

the mass spectrometric data of WALL AND STRAUS[3] and is comparable, therefore with other experimental studies done by the "rate of evaporation" techniques.

The high monomer yield from pure polyisobutylene is unusual for the pyrolysis of polyolefins. The 5 % yield of propylene and 13 % yield of 2,4,4-trimethyl-2-pentene also do not fit with the expected statistical product distribution of a system undergoing random cleavage. SIMHA's general therory[6] predicts a wider distribution of products. Since all kinetic data[3,6] indicate that the thermal decomposition proceeds by the simultaneous cleavage of the chain at many locations, the polymer chains must contain weak points at relatively even intervals which gives rise to the restricted product distribution.

This weak bond, at a quaternary carbon atom, agrees with the work of GRANT AND GRASSIE[15] and GRASSIE[16] who noted that polymethacrylic acid produced large amounts of monomer whereas polyacrylic acid did not. Polypropylene has a structure equivalent to polyisobutylene but lacking a quaternary carbon atom. Correspondingly, the pyrolysis product distribution of polypropylene is large; and the monomer yield, 2 %, is low[17]. The higher decomposition temperature of polypropylene, as opposed to polyisobutylene, 380° and 340°, respectively[18], further indicates a large difference in chain stability towards thermal cleavage.

The pyrolysis temperature of maximum isobutylene production increases with increasing isobutylene content, see Fig. 3. This is probably caused by the addition of new but uncharacterized weak bond sites present in polymers B, C and D. This decrease in thermal stability of a polymer chain with increase in number of pendent groups is consistent with other pyrolysis studies[17].

The increase of the isobutylene yield and the decrease in dimer yield with decreased flow rate indicate incomplete decomposition of dimer fragments at higher flow rates; *i.e.*, shorter residence time in the pyrolysis chamber. This effect necessitates accounting for the dimer production when considering monomer yield.

The molecular weight effects observed with fractionated polyisobutylenes is in agreement with the statements of SIMHA[19] and WALL[3] concerning the role of molecular weight in a randomly cleaved system. Molecular weight has very little effect on monomer yield. A large effect is noted, Sample B, when the final molecular weight of the polymer is determined by the initial isobutylene content of the reactants. The distribution of fragments above the dimer, 2,4,4-trimethyl-2-pentene, follows a decreasing exponential curve which is further evidence of a random cleavage of the chain[17]. If unzipping via radical propagation down the length of the chain were operating to any extent, a sharp cutoff of pyrolysis fragments would have been observed[6].

CONCLUSION

This work confirms that polyisobutylene decomposes primarily by a random cleavage of the polymer chain. Due to the instability of the quaternary carbon atom in the chain, cleavage at these sites is favored. The high thermal stability of the isobutylene fragment over that of possible higher cleavage products favors the apparent high monomer yield. The effect of impurities in the polyisobutylene chain is to lower the thermal stability of the material by the introduction of irregular chain branching. These additional branched sites result in cleavage products other than isobutylene or 2,4,4-trimethyl-2-pentene.

The structure of polybutenes is defined by combined pyrolysis/gas chromatography and NMR. The pyrolysis chromatograms give a direct analysis of polybutene type, and NMR spectra define chain structure according to branching. The approximate molecular weight of the polymer is also reflected by NMR spectra and pyrolysis chromatograms where original reactant composition controls the product molecular weight.

SUMMARY

The structure and composition of polyolefins made from pure isobutylene and from isobutylene diluted with various amounts of other light olefins have been investigated. The techniques used were nuclear magnetic resonance and combined anaerobic pyrolysis and gas chromatography. Nuclear magnetic resonance gave a measure of molecular weight and of isobutylene polymerized into the polymers. Pyrolysis chromatograms of pure polyisobutylene indicated a broad range of volatile hydrocarbon products, principal of which were propylene and isobutylene. For three mixed olefin polymers, the yields of propylene and isobutylene were directly proportional to the amount of isobutylene polymerized into the copolymer. Each polymer composition exhibited a pyrolysis temperature for maximum isobutylene yield. This temperature increased with the isobutylene content of the polymer.

Pyrolysis chromatograms were found to be virtually identical for a molecular weight series of pure polyisobutylenes. Fractionated olefin copolymers did show a distinct increase in isobutylene production from pyrolysis with increasing molecular weight. This is because the molecular weight of butene copolymers generally increases with the isobutylene content of the copolymerized mixture.

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A PROPOSED BASIS FOR THE SYSTEMATIC IDENTIFICATION OF UNSATURATED FATTY ACID ESTERS THROUGH GAS–LIQUID CHROMATOGRAPHY ON POLYESTER SUBSTRATES

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INTRODUCTION

The linear relation obtained when the log retention times for methyl esters of certain unsaturated fatty acids possessing the same number of double bonds, but different chain lengths, are plotted against the number of carbon atoms in the fatty acid chain has been shown[1,2] to be correlated with the end carbon chain (the number of carbon atoms from the center of the double bond farthest removed from the carboxyl group to and including the terminal methyl group*). This relationship may depend on the contribution made by the end carbon chain to some form of modification of the vapour pressure, since saturated esters having the same overall chain length display increasing retention times as either the alcohol or acid moiety is shortened[3], and this correlates with vapour pressure changes known for this type of ester[4]. In octadecenoic acid esters this also appears to be the case, since as the double bond moves from the central 9-position towards the carboxyl end[5] or towards the terminal methyl end[6,7] the retention time increases. It is therefore instructive to compare the effect of the end carbon chain with that of what we may call the carboxyl end chain (the number of carbon atoms from the carboxyl group to the first carbon atom of the first double bond, inclusive), and to examine the variables which may affect these relationships.

LINEAR LOG PLOT RELATIONSHIP

On the basis of the proposed linear relation it would at first appear that the role of the carboxyl end chain is a neutral one, the addition of two carbon atoms (for pairs of commonly occurring fatty acids of even chain lengths) contributing the same effect independently of the actual size of the carboxyl end chain. This appears to be the case for the range covered by the linearly related pairs with carboxyl end chains as short as 5 and 7, or as long as 9 and 11, or even 11 and 13 if the unknown listed by FARQUHAR *et al.*[8] with a retention time relative to stearate of 4.38 is the 13,16-docosadienoic ester. Although for esters of saturated acids the log of properly corrected retention times when plotted against the number of carbon atoms in the chain usually gives a straight line over a wide range of chain lengths[9,10], there is some indication that for the shorter chain lengths this line may inflect upwards as the chain length decreases[11,12]. In

* One more carbon atom than as originally defined[1].

the latter instance[12] the average line for the points representing dodecanoic, tetradecanoic and hexadecanoic esters is parallel to the lines joining the unsaturated ester points, and both converge with the line joining the points for octadecanoic, eicosanoic and docosanoic esters as the chain length increases. On the other hand in the data of FARQUHAR *et al.*[8] at three temperatures all the saturated ester points from dodecanoic to eicosanoic fall on a straight line, while the unsaturated lines and the saturated line become more convergent as the operating temperature decreases. Depending on operating conditions, it is therefore possible that the contribution of the carboxyl end chain may reflect a modification of retention time paralleling that observed with certain of the shorter chain saturated acids, the precise range of chain lengths and slope of the line of these acids being influenced by many operating variables. This could explain why the unsaturated ester lines appear to be parallel to each other as the range of saturated acids corresponding to the carboxyl end chains is fairly narrow and therefore would give a nearly linear log plot. Moreover since the linear log plot usually relates only pairs of acids the error associated with any slight change in the slope of the log plot of the lower saturated fatty acids will not seriously affect the apparent parallel linear relationship between such pairs as the carboxyl end chains vary in length. Unless this range is co-linear with the higher saturated fatty esters the latter line will not be parallel with the unsaturated acid lines.

SEPARATION FACTORS

In a study of the separation factors of acids of the same chain length three main types of separation factors have been established[13]. In type I separation factors the end carbon chains are fixed and the separation factors may be grouped on the basis of the three-carbon ratios of the carboxyl end chains commonly occurring in natural lipid systems (Table I). These separation factors may not be completely independent of the length of the end carbon chain, with some indication that as the latter increases, for a given fatty acid chain length, the type I separation factors also increase for those pairs with the same carboxyl end chain ratio. This also corresponds to fewer double bonds, but the type II separation factors (see below) indicate that the number of double bonds should be relatively unimportant.

In comparing the separation factors for the pairs of acids which may be linearly related by the log plot relationship it is seen that as the fatty acid chain length increases (or the respective carboxyl end chains are each increased) there is some evidence of diminution in the separation factors, particularly in the case of the more reliable EGA data. This supports the contention that the magnitude of the carboxyl end chains may affect the parallel linearity of the log plot relationship.

In addition to the separation factors for the adjacent pairs of acids listed in Table I very good agreement is observed in those based on pairs differing by two double bonds but having the same end carbon chains. Thus for a 4/10 carboxyl end chain ratio the EGA values are 1.21 and 1.22. For the 5/11 carboxyl end chain ratios the EGA values are 1.24 and 1.24, and the EGS values are 1.31 and 1.31.

In type II separation factors (Table II) the chain length and carboxyl end chain are fixed and the separation factors may be grouped on the basis of the end carbon chain ratios. These factors are of a higher order of magnitude than the type I factors and correspondingly more accurate. The correlation is very good, independent of the

TABLE I

TYPE I SEPARATION FACTORS BASED ON THE RATIOS OF CARBOXYL END CHAINS WITH FIXED FATTY ACID CHAIN LENGTH AND END CARBON CHAINS

Carboxyl end chain ratio	*Fatty acid*		*End carbon chain*	*Polyester*			
				EGA (197°)		*EGS (205°)*	
				$r_{18:0}$	*Separation factor*	$r_{18:1}$	*Separation factor*
	22:6	4, 7, 10, 13, 16, 19	3	7.75		6.20	
4/7	22:5	7, 10, 13, 16, 19	3	7.00	1.10	5.47	1.13
	22:5	*4, 7, 10, 13, 16*	6	6.09		—	
4/7	22:4	*7, 10, 13, 16*	6	5.50	1.11	—	—
	20:5	5, 8, 11, 14, 17	3	3.85		3.34	
5/8	20:4	*8, 11, 14, 17*	3	3.51	1.10	3.05	1.10
	20:4	5, 8, 11, 14	6	3.04		2.58	
5/8	20:3	8, 11, 14	6	2.76	1.10	2.32	1.11
	18:4	6, 9, 12, 15	3	1.97		1.83	
6/9	18:3	9, 12, 15	3	1.72	1.14	1.56	1.17
	18:3	*6, 9, 12*	6	1.54		1.41	
6/9	18:2	9, 12	6	1.34	1.15	1.21	1.17
	18:2	*6, 9*	9	*1.29*		1.18	
6/9	18:1	9	9	1.12	1.15	1.00	1.18
	16:3	6, 9, 12	4	—		0.925	
6/9	16:2	9, 12	4	—	—	0.773	1.19
	16:2	6, 9	7	—		0.730	
6/9	16:1	9	7	—	—	0.612	1.19
	22:5	7, 10, 13, 16, 19	3	7.00		—	
7/10	22:4	*10, 13, 16, 19*	3	*6.40*	1.09	—	—
	22:4	*7, 10, 13, 16*	6	5.50		—	
7/10	22:3	*10, 13, 16*	6	5.00	1.10	—	—
	20:4	*8, 11, 14, 17*	3	3.51		3.05	
8/11	20:3	*11, 14, 17*	3	*3.10*	1.13	2.54	1.20
	20:3	8, 11, 14	6	2.76		2.32	
8/11	20:2	11, 14	6	2.45	1.13	1.97	1.18
	20:2	8, 11	9	2.32		—	
8/11	20:1	11	9	2.02	1.15	—	—

chain length and number of double bonds, and the types follow closely those found in naturally occurring lipid mixtures.

The type III separation factors (Table III) are based on pairs of acids with the same chain length and the same number of double bonds. The correlation between these factors apparently depends on the respective pairs of acids being linearly related by the log plot system. The type III factors can be obtained by dividing the applicable type II factor by the appropriate average type I factor (inverting the carboxyl end chain ratio, but not the numerical value). The most highly centralized systems of polyethylenic unsaturation have the lowest type III separation factors (*cf.* ref. 14),

TABLE II

TYPE II SEPARATION FACTORS BASED ON THE RATIOS OF END CARBON CHAINS WITH FIXED FATTY ACID CHAIN LENGTH AND CARBOXYL END CHAINS

Fatty acid		End carbon chain	Polyester								
			EGA				EGS				
			$r_{18:0}$	Separation factors			$r_{18:1}$	Separation factors			
				3/6	6/9	3/9		3/6	6/9	3/9	4/7
22:6	4, 7, 10, 13, 16, 19	3	7.75				6.20				
22:5	*4, 7, 10, 13, 16*	6	6.09	1.27			4.64	1.33			
22:4	*4, 7, 10, 13*	9	*5.10*		1.19	1.52	—		—	—	
22:5	7, 10, 13, 16, 19	3	7.00				—				
22:4	*7, 10, 13, 16*	6	5.50	1.27			—	—			
22:4	*10, 13, 16, 19*	3	6.40				—				
22:3	*10, 13, 16*	6	5.00	1.28			—	—			
20:5	5, 8, 11, 14, 17	3	3.85				3.34				
20:4	5, 8, 11, 14	6	3.04	1.27			2.58	1.30			
20:3	*5, 8, 11*	9	*2.53*		1.20	1.52	2.11		1.22	1.58	
20:4	*8, 11, 14, 17*	3	3.51				3.05				
20:3	8, 11, 14	6	2.76	1.27			2.32	1.31			
20:2	8, 11	9	2.32		1.19	1.51	—		—	—	
18:4	6, 9, 12, 15	3	1.97				1.83				
18:3	*6, 9, 12*	6	1.54	1.28			1.41	1.30			
18:2	*6, 9*	9	*1.29*		1.19	1.53	1.18		1.20	1.55	
18:3	9, 12, 15	3	1.72				1.56				
18:2	9, 12	6	1.34	1.28			1.21	1.29			
18:1	9	9	1.12		1.19	1.54	1.00		1.21	1.56	
16:3	6, 9, 12	4	—				0.925				
16:2	6, 9	7	—				0.730				1.27
16:2	9, 12	4	—				0.773				
16:1	9	7	—				0.612				1.26

particularly evident in the case of the 6,9- and 9,12-octadecadienoates. In this case although the numerical index ratios of the two types of end chains are the same the greater influence of the end carbon chains gives the necessary change in volatility to ensure some separation. It must be presumed that the carboxyl end chain ratio contribution is reduced in effect by the ester linkage and alcohol moiety.

The gas–liquid chromatography of complex lipid mixtures such as marine oils is greatly assisted by the use of these separation factors, supplementing in a more precise way the linear log plot system. Thus a suspected component may be identified by its various relations to any of several different acids which have been identified. Alternatively the retention time may be predicted and the location on the chromatogram examined for a component. When even less information is available trial and error procedures including several components may be necessary.

In the tabulated data drawn from that of FARQUHAR *et al.*[8] a number of component fatty acids (in italics) have been identified through the use of these systematic separation factors[13], as well as by the linear log plot relationship[2]. In addition a few others

TABLE III

TYPE III SEPARATION FACTORS WITH FIXED FATTY ACID CHAIN LENGTH AND NUMBER OF DOUBLE BONDS, COMPARING FOUND AND CALCULATED VALUES

Fatty acid		EGA Polyester							EGS Polyester						
		$r_{18:0}$	Type III (found)	Type II		Type I		Type III (calc.)	$r_{18:1}$	Type III (found)	Type II		Type I		Type III (calc.)
22:5	7, 10, 13, 16, 19	7.00							5.47						
22:5	*4, 7, 10, 13, 16*	6.09	1.15	3/6	1.27	7/4	1.10	1.15	4.64	1.18	3/6	1.31	7/4	1.13	1.16
22:4	*10, 13, 16, 19*	*6.40*							—						
22:4	*7, 10, 13, 16*	5.50	1.16	3/6	1.27	10/7	1.10	1.15	—	—	—	—	—	—	—
20:4	*8, 11, 14, 17*	3.51							3.05						
20:4	5, 8, 11, 14	3.04	1.15	3/6	1.27	8/5	1.10	1.15	2.58	1.18	3/6	1.31	8/5	1.11	1.18
20:3	*11, 14, 17*	3.10							2.54						
20:3	8, 11, 14	2.76	1.12	3/6	1.27	11/8	1.13	1.12	2.32	1.09	3/6	1.31	11/8	1.19	1.10
18:3	9, 12, 15	1.72							1.56						
18:3	*6, 9, 12*	1.54	1.12	3/6	1.27	9/6	1.15	1.10	1.41	1.11	3/6	1.31	9/6	1.18	1.11
22:4	*7, 10, 13, 16*	5.50							—						
22:4	*4, 7, 10, 13*	*5.10*	1.08	6/9	1.19	7/4	1.10	1.08	—	—	—	—	—	—	—
20:2	11, 14	2.45							—						
20:2	8, 11	2.32	1.05	6/9	1.19	11/8	1.13	1.05	—	—	—	—	—	—	—
18:2	9, 12	1.34							1.21						
18:2	*6, 9*	*1.29*	1.04	6/9	1.19	9/6	1.15	1.03	1.18	1.02	6/9	1.21	9/6	1.18	1.02
18:2	*11, 14*	*1.45*							—						
18:2	*8, 11*	*1.33*	1.09	4/7	1.23	11/8	1.13	1.09	—	—	—	—	—	—	—
16:2	9, 12	—							0.773						
16:2	6, 9	—	—	—	—	—	—	—	0.730	1.06	4/7	1.26	9/6	1.18	1.06

have been listed with the retention time and structure both in italics, indicating hypothetical acids with predicted retention times. These are included to complement certain fatty acids of known structure which could not otherwise be included in a particular table. The excellent correlation of all of these acids and retention times in the various types of separation factors indicates the reliability of this systematic approach to fatty acid identification. The data for the EGS polyester are drawn from a preliminary identification study[12] on the component fatty acids of seal blubber and virtually all of the acids were tentatively identified by both the linear log plot and separation factors. All the fatty acids employed in the EGS data (Tables, I, II and III) have been associated with peaks excepting 6,9-octadecadienoic. An Aerograph A-90 was employed in these studies, still air bath temperature 205°, injection port temperature 250°. Columns were copper tubing, $^1/_4$ in. O.D. and 10 ft. in length, packed with 20% commercial EGS on GC-22 "Super-Support". Helium at 90 ml/min was employed as the carrier gas.

The type I and type III separation factors are particularly susceptible to small errors in retention time. Thus the data of FARQUHAR *et al.*[8] giving the temperature variation of retention time for 9,12,15-octadecatrienoic acid, in comparison for that for 6,9,12,15-octadecatetraenoic acid, suggests that 1.72 at 197.5° is slightly high, and a value of 1.70 improves the correlation for all three types of separation factors. The retention time of 5.10 for 4,7,10,13-docosatetraenoate has been adopted instead of the previous tentative identification[13] of this acid with a component with the value of 5.30, since the latter obviously does not fit the separation factors, and also since there is reason to believe that this may be a heneicosapentaenoate tentatively identified in seal oil[12]. In the EGS series reasonable correlation is obtained excepting with the 4,7,10,13,16,19-docosahexaenoate, for which it is difficult to determine a retention time accurately owing to limitations of the apparatus. A value of 6.10 for the retention time of this ester would give very satisfactory results with all three types of separation factors.

The above data are all based on the normally occurring fatty acids related to 9-hexadecenoic, 9-octadecenoic, 11-eicosenoic and 13-docosenoic acids. An opportunity to extend this to other C_{18} monoethylenic acids and acids related to the latter is provided by some hitherto unpublished information[7] provided through the courtesy of Dr. C. R. SCHOLFIELD. The relative retention times in Table IV represent composite

TABLE IV

TYPE I AND TYPE II SEPARATION FACTORS FOR FATTY ACIDS RELATED TO 9-, 12- AND 15-OCTADECENOIC ACIDS ON AN EGS POLYESTER

Type I						*Type II*					
Fatty acid		$r_{18:1}$	*Separation factors*			*Fatty acid*		$r_{18:1}$	*Separation factors*		
			9/12	*12/15*	*9/15*				*3/6*	*6/9*	*3/9*
18:3	9, 12, 15	1.71				18:3	9, 12, 15	1.71			
18:2	12, 15	1.43	1.20			18:2	9, 12	1.28	1.34		
18:1	15	1.10		1.30	1.56	18:1	9	1.00		1.28	1.71
18:2	9, 12	1.28				18:2	12, 15	1.43			
18:1	12	1.04	1.23			18:1	12	1.04	1.37		

data from different analyses on an EGS polyester and therefore must be regarded as only approximately correct, but general agreement is indicated between the pairs in the type I and type II factors, while the type III separation factor for the 9,12- and 12,15-octadecadienes calculated from the average of each (1.12) agrees with the value found from the retention times (1.12). Although no direct comparison may be made, this type III separation factor is much higher than the corresponding factor (Table III) obtained on EGS polyester for the highly centralized 6,9- and 9,12-octadecadienes, agreeing with the view that the most highly centralized unsaturated fatty acid ester structures have the shortest retention time and the lowest type III separation factors.

VARIABLES AFFECTING SEPARATION FACTORS

In general the separation factors between unsaturated fatty acids are improved slightly by a reduction in operating temperatures. This conclusion is based on the temperature dependent data listed by FARQUHAR *et al.*[8] and other work[15] (see Table V).

TABLE V

COMPARISON OF TYPE II SEPARATION FACTOR PROPORTIONS OBTAINED BY DIFFERENT AUTHORS

Separation factors			*Temp.*	*Polyester*	*%*	*Support*	*Mesh*	*Wash*		*Gas*	*Ref.*
3/9	*3/6*	*6/9*						*Acid*	*Base*		
1.45	1.22	1.18	205	BDS	5	Celite 545	40–60	Yes	No	He	16
1.45	1.24	1.17	203	DEGS	20	Celite 545	60–80	Yes	No	He	17
1.46	1.24	1.18	205	EGS	5	Chromosorb W	40–60			He	16
1.46	1.23	1.18	203	DEGS	20	Celite 545	60–80	Yes	Yes	He	18
1.48	1.26	1.18	221	LAC-2R-446	25	Chromosorb W	30–60			He	19
1.48	1.24	1.19	200	Reoplex 400	30	Celite 545	60–80	Yes	Yes	He	9
1.49	1.24	1.20	205	BDS	7	Chromosorb W	40–60			He	16
1.50	1.26	1.19	197	Reoplex 400	18	Celite 545	100–120	Yes	Yes	Ar	8
1.52	1.27	1.20	177	EGA	2.3	Embacel	60–100			Ar	20
1.52	1.27	1.20	196	EGA	15	Chromosorb W	80–100			Ar	14
1.53	1.26	1.21	205	EGS	20	GC-22	60–80			He	12
1.54	1.28	1.20	197	DEGS	20	Chromosorb	35–80			He	11
1.54	1.28	1.20	197	EGA	18	Celite 545	100–120	Yes	Yes	Ar	8
1.54	1.28	1.20	184	EGA	18	Celite 545	100–120	Yes	Yes	Ar	8
1.57	1.30	1.21	173	EGA	18	Celite 545	100–120	Yes	Yes	Ar	8
1.58	1.31	1.21	193	LAC-2R-728	30	Chromosorb	48–65			Ar	21
1.58	1.29	1.23	200	DEGS	20	Celite 545	48–85	Yes	Yes	H	10
1.59	1.30	1.22	180	EGA	25	Celite 545	100–210			Ar	5
1.60	1.30	1.22	200	DEGS	20	GC-22	60–80			He	15
1.64	1.33	1.24	180	DEGS	20	GC-22	60–80			He	15

This is the reverse of the usual criterion of the efficiency of polyester substrates, the separation of the corresponding saturated and monounsaturated acid esters, which normally improves as the operating temperature is increased.

The type II separation factors are independent of carrier gas flow rate[15,16] but for a particular polyester on a particular support the variation with the ratio of support to substrate is an important factor in most cases[16]. This is shown in Fig. 1 where it is apparent that for the type II 3/9 separation factor, under otherwise identical conditions, a BDS polyester is less sensitive to concentration change on Chromosorb W than on C-22 firebrick, and on both is less sensitive to change than is

EGS polyester. The other type II separation factors appear to be roughly proportional to the 3/9 ratio factor.

An attempt was made to determine the general influence of operational variables on the type II separation factors by surveying the literature separations reported for the series 9-octadecenoate, 9,12-octadecadienoate, and 9,12,15-octadecatrienoate (Table V). Since the 3/9 end carbon chain ratio has the largest numerical value the

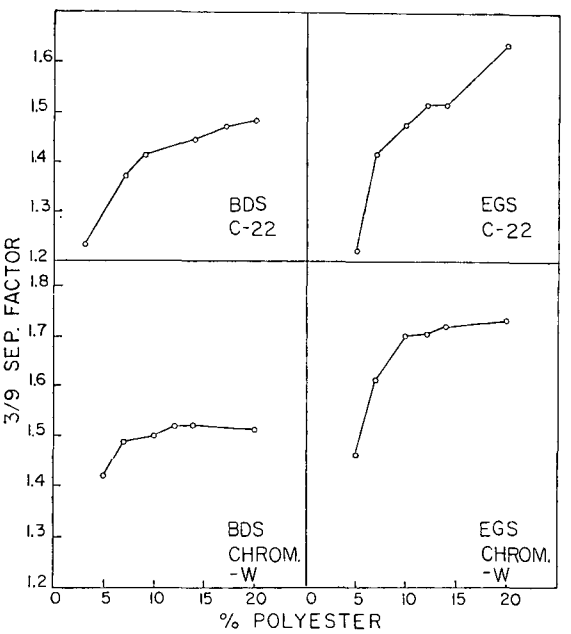

Fig. 1. Variation of type II separation factors (3/9 end carbon chain ratio) with concentration of polyester for different polyesters and supports.

results have been arranged in order of increase of this value. The dependent values for 3/6 and 6/9 end carbon chain ratios seem to increase in proportion, and when plotted an apparently linear relation is obtained (Fig. 2). Owing to the scale needed to express the small quantities involved there is considerable scatter of points, especially for the 6/9 values, but the 3/6 values are moderately consistent. It is very difficult to assess the merits of the data chosen, since there is little indication in the literature in many cases of whether the data are based on corrected or adjusted retention times, or whether the retention times were determined by the frontal tangent or half-base width systems, or by the less desirable method of dropping a perpendicular from the peak top. However, where several groups of ratios are taken from the data of one author[8,15,16] the agreement with the plotted lines is very good. In general, however, it appears that there is no general correlation in terms of polyester, percentage polyester, support, support size, temperature or carrier gas. In the absence of any correlating factor, the graph might be used as an approximate guide to determine any two of the separation factors if the other is known, but this should be restricted to the range of values plotted, since it has not been shown that a true linear relation exists and extrapolation would be dangerous.

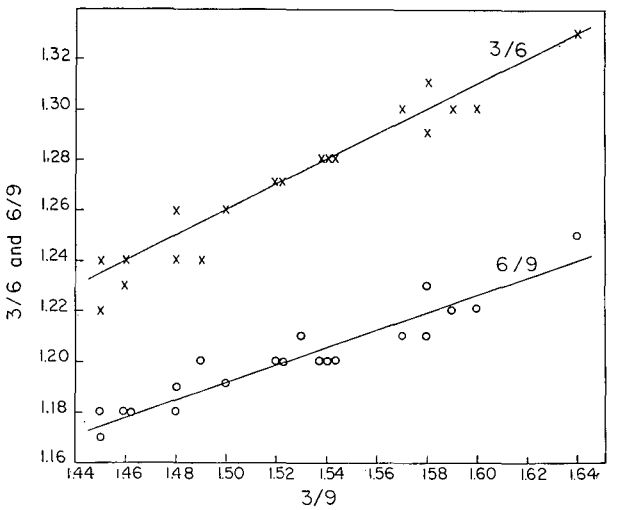

Fig. 2. Plot of data (Table V) relating type II separation factors for methyl 9-octadecenoate, 9,12-octadecadienoate and 9,12,15-octadecatrienoate.

The above discussion applies only to the type II separation factors, but clearly shows that the complex interchange of variables, while affecting the magnitude of these separation factors, has a lesser effect on the proportional differences between separation factors of this type. This is in agreement with the proposed source of this particular relationship, that of differences in volatility induced by the size of the end carbon chain. The latter should not be influenced by polarity of the support for example, yet this would have some effect on the carboxyl end chain, hence modifying the relationship between the carboxyl end chain and the end carbon chain. This would affect the magnitude of the type II separation factors, but would not necessarily have a serious effect on the proportional differences which are due solely to the end carbon chains.

It is therefore probable that derivatives of the unsaturated acids produced by modifying the carboxyl group will have the same type II separation factors as the corresponding methyl esters analysed on the identical column. The data for such a comparison are not immediately available, but the separation factors for the dimethyl acetals of the aldehydes corresponding to 9-octadecenoic, 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids[22], respectively 1.25, 1.19 and 1.50 for end carbon chain ratios of 3/6, 6/9, and 3/9, fit the plot (Fig. 2) derived from methyl esters very well. Moreover the separation factor for 9,12-octadecadienyl acetate and 9-octadecenyl acetate[23] on EGA at 184.8° is 1.21, the same as the value reported by the same author[8] for the corresponding methyl esters on EGA at 184.5° under presumably comparable conditions.

Considering the three types of separation factors in general as applied to fatty acid ester separations, it is obvious that improvement in one separation between particular acids may hinder separation in other cases. Thus the type I and type II separations are obviously slightly better with the EGS polyester on GC-22 (Tables I and II), but the type III separations (Table III) for certain critical pairs such as the 6,9- and 9,12-octadecadienoates are apparently superior with the EGA polyester on Celite 545.

SUMMARY

Systematic separation factors may be established among various unsaturated fatty acid methyl esters analysed by gas–liquid chromatography on polyester substrates. These are apparently dependent on differences in the magnitude of the carboxyl end chain and end carbon chain parts of the fatty acid chain.

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LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

V. SILICA AS ADSORBENT. ADSORBENT STANDARDIZATION

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INTRODUCTION

Silica (virtually synonymous with silica gel, silicic acid[1,2]) has probably been used more frequently in liquid-adsorption chromatographic separation than any other single adsorbent. Its properties as an adsorbent and the mechanism whereby it functions in this capacity form the subject of an extensive literature[2]. As early as 1950 TRUEBLOOD AND MALMBERG[3] noted that many chromatographic systems employing silicic acid as adsorbent show linearity in the adsorption isotherm over a wide range of solute concentrations. Despite this observation and the general recognition[4,5] of the desirability of restricting systematic studies of the chromatographic process to linear isotherm systems, the only systematic study of linear adsorption on silica to date is that of SPORER AND TRUEBLOOD[4] for a silicic acid–celite mixture. This investigation was limited to the effect of solute molecular structure on separation, only one eluent (benzene) and one adsorbent activity being used.

Parts II–IV[6–8] in this series of papers have presented a physical model of linear elution adsorption chromatography (LEAC) on alumina, and have related adsorption affinity or solute linear retention volumes, $\underline{R}^\circ$, to solute molecular structure, eluent type, and adsorbent activity through the correlational eqn. (1):

$$\log \underline{R}^\circ = \log V_a + \alpha \left[\sum^{i} Q^\circ_i - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i + \sum^{j} q^\circ_j - \varepsilon^\circ \sum^{i} \delta_i\right] \qquad (1)$$

V_a and α are adsorbent parameters; V_a is proportional to the surface area of the adsorbent, and α is a measure of the average strength of adsorbent sites. ε° is an eluent parameter, increasing in value for more strongly adsorbing solvents. $\sum^{i} \delta_i$ is proportional to the size or area of the solute, $\sum^{i} Q^\circ_i$ is the sum of the adsorption energies Q°_i of individual solute groups i, $\sum^{j} q^\circ_j$ includes the effect of various solute geometries j which influence adsorption, and the term $f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i$ arises from the localization of a strong solute group k on a particular adsorbent site.

The general chemical and physical similarity of the adsorbents alumina and silica, as well as the considerable practical interest and theoretical background associated with the latter adsorbent, make silica a logical choice for study in the context of our preceding work on alumina. The present communication represents a

preliminary effort at the understanding of the factors which determine adsorption affinity on silica, with special emphasis on the similarities and differences in the adsorptive properties of it and alumina.

EXPERIMENTAL

The acquisition of experimental LEAC retention volume data ($\underline{R}°$ values in ml/g) was carried out as described in Part III[7] for alumina. Davison Code Number 12 and 62 silica gels were used as adsorbents. The manufacturer's product was first calcined at 400° for 16 h, following which adsorbent samples of varying activity were prepared by adding liquid water to the calcined material and permitting it to equilibrate for at least 24 h. KLEIN[9] has suggested that the addition of liquid water rather than water vapor may have an adverse effect upon the separation efficiency (*i.e.*, HETP) of deactivated silica. While this *may* be a consideration in the preparation of adsorbent for practical separations, KLEIN'S data do not show any significant differences in adsorption affinity (*i.e.*, $\underline{R}°$ values) arising from the method of adsorbent preparation. In related studies on alumina, we find the adsorptive properties of deactivated aluminas to be independent of the method of adding water. The purification of solutes and eluents has been previously described in Part II[6].

Table I gives some of the physical properties of the two silica samples used in the present study. These figures include our values on calcined adsorbent and data for the original adsorbents from the manufacturer's specifications and from the study by KLEIN[10] of the same two adsorbents. The thermal processing of silica at temperatures above 300° has been shown[11] to reduce surface area (through sintering) and, eventually, to destroy adsorptive properties[12]. Little if any surface area appears to have been lost in the two calcined samples of Table I.

TABLE I

PHYSICAL PROPERTIES OF ADSORBENTS USED IN PRESENT STUDY

Adsorbent	*Surface area (BET) (m^2/g)*	*Pore volume (ml/g)*	*Average pore diameter (Å)*
Davison Code No. 12			
calcined 400°	778	0.40	20
original manufacturer	830	0.45	22
Klein	805	0.41	20
Davison Code No. 62			
calcined 400°	287	1.28	178
original manufacturer	340	1.16	140
Klein	274	1.16	170

The equivalence of LEAC retention volume data and equilibrium distribution coefficients has been demonstrated for alumina as adsorbent[6]. A similar comparison of equilibrium and chromatographic data for Code 12 silica deactivated with 4.6 % added water is given in Table II. The linearity of both equilibrium and chromatographic systems is evident, but the chromatographic retention volumes are lower

TABLE II

EQUILIBRIUM SOLUTE DISTRIBUTION COEFFICIENTS AND CHROMATOGRAPHIC EQUIVALENT RETENTION VOLUMES AS A FUNCTION OF SOLUTE CONCENTRATIONS FOR THE SYSTEM NAPHTHALENE (SOLUTE)–*n*-PENTANE–CODE 12 4.6 % H_2O–SiO_2

Equilibrium data			*Chromatographic data*	
Solute concentrations				
Non-sorbed phase (g/ml) × 10^6	*Adsorbed phase* (g/g) × 10^6	$\underline{K}$ (ml/g)	*Column loading* (g/g) × 10^6	$\underline{R}$ (ml/g)
161	459	8.2	620	7.8
52.5	153	8.4	263	8.0
13.4	38.0	8.3	240	7.9
5.36	15.3	8.4	68	7.7
1.32	3.82	8.5	31	7.7
	Average	8.4	8	7.8
			Average	7.8

than the equilibrium coefficients by 7 %. As seen in Table III for several other systems, this phenomenon is rather general. The magnitude of the discrepancy will not seriously affect the significance or the interpretation of the chromatographic data we will describe. It will be shown in a forthcoming publication that the differences in chromatographic and equilibrium $\underline{R}°$ values for Code 12 silica result partially from the smaller equivalent theoretical plate numbers associated with columns of this adsorbent. Using silicic acid of unspecified pore diameter, SPORER AND TRUEBLOOD[4] observed equivalence of chromatographic and equilibrium data at flow rates of 0.1–0.2 ml/min. For columns of similar diameter, our flow rates were considerably larger, 2–5 ml/min for experimental convenience.

An additional experimental difference between adsorption on alumina and silica was noted in the present study. The elution of solutes containing basic nitrogen from silica results in incomplete recovery of sample from the column. The effect is most pronounced at small column loadings, and in some cases no sample elutes from a column from which elution occurs readily (but with incomplete recovery) at higher sample concentrations. Table IV presents some equilibrium isotherm data for the adsorption of aniline from benzene onto 16 % H_2O–SiO_2 (Code 12). The equilibrium distribution coefficients $\underline{K}$ show a singular dependence upon solute concentration. Some of these adsorption data are plotted (adsorbed *versus* solution concentrations)

TABLE III

COMPARISON OF EQUILIBRIUM LINEAR DISTRIBUTION COEFFICIENTS WITH LINEAR EQUIVALENT RETENTION VOLUMES FOR SEVERAL ADSORPTION SYSTEMS; CODE 12 SILICA

Solute	% H_2O *on silica**	*Eluent*	$\underline{R}°$	$\underline{K}°$	$\underline{R}°/\underline{K}°$
Naphthalene	4.6	Pentane	7.8	8.4	0.93
Dibenzyl	4.6	Pentane	27.5	34.6	0.79
Phenanthrene	4.6	Pentane	16.2	17.6	0.92
Naphthalene	8.0	Pentane	4.8	5.3	0.91

* Chromatographic activity.

TABLE IV

EQUILIBRIUM SOLUTE DISTRIBUTION COEFFICIENTS AS A FUNCTION OF SOLUTE CONCENTRATION FOR THE SYSTEM ANILINE (SOLUTE)–BENZENE–CODE 12 16% H_2O–SiO_2

Solute concentrations		$\underline{K}$	$\underline{K}'$
Non-sorbed phase (g/ml) × 10^6	*Adsorbed phase* (g/ml) × 10^6	(ml/g)	(ml/g)
0.0	29	290	—
0.5	73	146	—
4.5	220	49	—
22.9	536	23.4	8.8
37.5	694	18.5	9.2
222	2370	10.7	9.1
519	4830	9.3	8.6
1030	9510	9.2	8.9
2270	17200	7.6	7.4
2760	20000	7.3	7.1
4470	27700	6.2	6.1
9900	44500	4.5	4.5
16400	57800	3.5	3.5

in Fig. 1, and it is noted that the straight line through the values at high aniline concentrations intersects the vertical axis at a concentration corresponding to $350 \cdot 10^{-6}$ g aniline/g of adsorbent, rather than passing through the origin as in "normal" adsorption. It is believed that this behavior reflects the presence of a small number of sites on the silica surface (probably strong acid groups) upon which chemisorption (or very strong physical adsorption) can occur. If the extrapolated value of aniline uptake by the adsorbent at zero solution concentration is assumed to correspond to the concentration of these chemisorption sites in terms of their capacity for aniline, "normal" adsorption of this solute on silica can be calculated by subtracting $350 \cdot 10^{-6}$ g/g from the total adsorbed phase concentrations. Distribution coefficients $\underline{K}'$ corrected

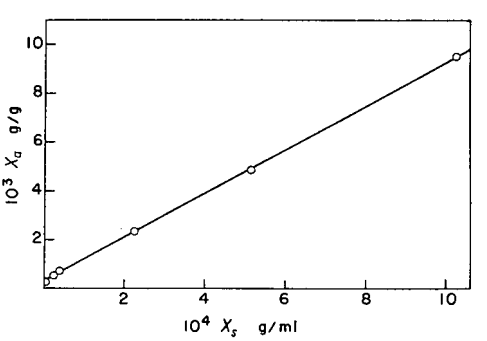

Fig. 1. Equilibrium adsorption data for aniline (solute), benzene (eluent), and 16% Code 12 H_2O–SiO_2.

in this manner for chemisorption are listed in Table IV, and are seen to show a rather normal dependence upon adsorbent loading. For adsorbent loadings less than 0.01 g/g, values of $\underline{K}'$ are constant (linear) within experimental error, and for greater adsorbent loadings the values of $\underline{K}'$ decline as for other systems with a linear capacity[5] of about

0.01 g/g. The occurrence of chemisorption during a chromatographic elution should theoretically have little, if any, effect upon the "normal" adsorption and elution of the solute in excess of that chemisorbed. In confirmation of this expectation in the system of Table IV, $\underline{R}°$ for aniline eluted by benzene from 16% H_2O–SiO_2 (Code 12) was found equal to 10.0 ml/g, within experimental error of the corrected linear distribution coefficient $\underline{K}'$ (8.9 ml/g).

Although the effects of chemisorption in chromatographic systems may be unimportant in theoretical studies such as the present, chemisorption seriously limits the usefulness of an adsorbent in practical separations of chemisorbed species. Chemisorbed substances will normally be smeared over the entire column, rather than concentrated in the usual narrow elution band. Under such circumstances, it is also possible for the same solute to give two apparently distinct and widely separated elution bands if after collection of the physically adsorbed solute (in a normal elution band) and several intermediate fractions, a new eluent sufficiently strong to displace the chemisorbed solute is used.

The experimental measurement of large retention volumes ($\underline{R}° > 100$) is frequently tedious and time consuming, while the pronounced spreading of the elution band in such cases makes the detection of solute in the eluate impossible in some cases. If the retention volume ($\underline{R}°_1$) is desired for eluent 1 and a particular adsorbent, and it is anticipated that $\underline{R}°_1$ will be inconveniently large, the quantity may be obtained indirectly as follows. Assume the measurement of a retention volume of convenient size for the same solute and adsorbent, but using a stronger eluent 2. $\underline{R}°_2$ should be larger than 5 ml/g. Finally, charge the solute to its column in the usual manner, and elute with some volume V_1 (ml/g) of eluent 1, where V_1 is less than $\underline{R}°_1$. This will cause the movement of the solute a fractional distance ($V_1/\underline{R}°_1$) up the column. Then change eluents from 1 to 2, cleaning eluent 1 out of the lines to the column, and elute with eluent 2 to determine a new retention volume $\underline{R}'_2$ for this eluent. $\underline{R}°_1$ is now given as:

$$\underline{R}°_1 = (V_1\underline{R}°_2)/(\underline{R}°_2 - \underline{R}'_2) \qquad (2)$$

Eqn. (2) and the related technique have been used to advantage on several occasions in the present study.

THE PREDICTION OF LEAC RETENTION VOLUME DATA

Relative to the adsorbent alumina, chromatographic separation over silica is characterized by both pronounced similarities and differences. It is shown in the following sections that eqn. (1) for alumina as adsorbent must be modified when silica is used, because of fundamental differences in the mode of adsorption on these two adsorbents. Eqn. (3) predicts LEAC solute retention volumes on silica:

$$\log \underline{R}° = \log V_a + \alpha\{\sum^{i} Q°_i - 0.113\,(n - 6\,r) + \sum^{j} q°_j - \varepsilon° \sum^{i} [\delta_i + 14.5\,f\,(Q°_i)]\} \qquad (3)$$

Here, V_a and α have the same significance as in eqn. (1) for alumina. As illustrated by the data of Table I, commercial samples of silica come in a wide range of surface areas. This generally necessitates the consideration of *both* adsorbent surface volume V_a

and surface energy α in the standardization of adsorbent, as discussed in some detail in a following section. For a particular grade of adsorbent, where the surface area and hence V_a are reasonably constant, a single measurement of $\underline{R}°$ for a standard solute (*e.g.*, naphthalene) eluted by a standard solvent (*e.g.*, pentane) suffices to determine both V_a and α. While both V_a and α are independently variable for alumina as well as silica, the latter procedure was used (Part II[6]) for the standardization of Alcoa F-20 alumina. Table V offers a similar tabulation of values of V_a, α, and the

TABLE V

CODE 12 SILICA GEL ACTIVITY FUNCTION AND ADSORBED VOLUME AS A FUNCTION OF PERCENT ADSORBED WATER

% H_2O-SiO_2	*Activity function* α	*Adsorbed volume*		*Standard solute retention volume**	
		V_a	$\log V_a$	$\underline{R}°$ (ml/g)	$\underline{R}°/V_a$
0.0	1.00	0.30	—0.52	32	107
1.0	0.94	0.29	—0.54	21	72
2.0	0.85	0.28	—0.55	14	50
4.0	0.75	0.26	—0.58	9.0	35
7.5	0.68	0.22	—0.65	5.1	23
10.0	0.64	0.20	—0.70	4.0	20
15.0	0.61	0.15	—0.82	2.6	17
20.0	0.58	0.10	—1.00	1.5	15

* Elution of naphthalene by pentane.

standard solute $\underline{R}°$ value for samples of Davison Code 12 silica of varying water content. For this particular adsorbent, activity standardization and the determination of V_a and α values are carried out exactly as for Alcoa F-20 alumina in Part II.

For other silica samples (generally of lower surface area than Davison Code 12), V_a is given by eqn. (4), which is theoretically justified in a latter section.

$$V_a = 0.00035 \text{ (surface area of calcined adsorbent in } m^2/g) - 0.01 \text{ (\% water added to calcined sample)} \quad (4)$$

The standard $\underline{R}°$ value (for naphthalene, pentane) is obtained, and the ratio $(\underline{R}°/V_a)$ calculated. The adsorbent surface energy α may be interpolated from the second and fifth columns of Table V.

The term $\sum_i Q°_i$ of eqn. (3) plays the same role as in eqn. (1), and in general has virtually the same value. Table VI lists some specific experimental $Q°_i$ values for silica. For the adsorption of other solute groups i on silica, the alumina $Q°_i$ values of Part IV[8] are believed interchangeable with an accuracy of ± 20 %. If the $Q°_i$ values of Table VI are compared with previous values for alumina in the case of *aliphatic carbon* groups, it is seen that these latter values for silica include the $q°_j$ terms for substitution on aromatic carbon, terms that were reported separately for alumina in Part II[6]. Similarly, a distinction is now made between methyl aliphatic carbon groups, and other saturated carbon atoms. These changes in classification are made for purposes of convenience and consistency.

The term 0.113 $(n - 6r)$ of eqn. (3) is used only for solutes possessing aromatic ring systems. r refers to the number of such uncondensed ring systems (*e.g.*, one in benzene, naphthalene, two in dibenzyl), while n is the total number of unsaturated carbon atoms in the solute.

TABLE VI

SOLUTE GROUP ADSORPTION FACTORS Q°_i FOR SILICA AS ADSORBENT

Group	*Attachment*	
	Aromatic carbon	*Aliphatic carbon*
$-CH_2-$, $-CH$	+0.01	−0.05
$-CH_3$	+0.13	+0.07
$-CH=$	+0.25	+0.25
$-S-R$	+1.29	+2.94
$-O-R$	+1.83	
$-NO_2$	+2.77	
$-CO_2-R$	+3.45	
$-CHO$	(3.9)*	
$-CO-R$	+4.69	
$-OH$	(4.2)*	(5.6)*
$-NH_2$	+5.1	(7.8)*
$-NH-$ (carbazole)	+3.00	
$-COOH$	(6.1)*	

* Estimated values from data of SPORER AND TRUEBLOOD[4] (see following section).

The term $\sum_i q^\circ_j$ of eqn. (3) is equivalent to the same term in eqn. (1), and it is assumed that its value is in general comparable for both adsorbents.

The eluent term $\varepsilon^\circ \sum_i [\delta_i + 14.6\, f(Q^\circ_i)]$ of eqn. (3) is quite similar to the term $\varepsilon^\circ \sum_i \delta_i$ for alumina (eqn. (1)). This is more readily seen if we equate the term $[\delta_i + 14.6\, f(Q^\circ_i)]$ for each solute group i to an *effective* solute group area δ_i', comparable to δ_i. Values of the eluent strength parameter ε° have been measured for three pure solvents (in addition to pentane), and these values are given in Table VII. As shown, these

TABLE VII

ELUENT PARAMETERS FOR ADSORPTION ON SILICA

Solvent	ε°	
	$_cSiO_2$	Al_2O_3
n-Pentane	0.000	0.000
Carbon tetrachloride	0.11*	0.18
Benzene	0.25	0.32
Methylene chloride	0.32	0.42

* Approximate value for limited data on CCl_4–pentane binaries and Code 12 silica; pure CCl_4 does not have a unique value of ε° for elution from Code 12 silica.

values are in general slightly lower than the corresponding alumina ε° values, but it is not known if this effect is general. Values of ε° for several binary solvents were also measured, and it was found that the previous relationship for alumina (eqn. (2) of

Part III[7]) between pure solvent ε° values and the ε° values of binaries holds for silica as well:

$$\varepsilon^\circ_{AB} = \varepsilon^\circ_A + \frac{\log \left[X_B \, 10^{\alpha \, n_b \, (\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B\right]}{n_b \, \alpha} \tag{5}$$

n_b refers to the value of $\sum_i \delta_i$ for the stronger solvent B. ε°_A and ε°_B are the eluent strengths of eluents A and B, respectively. ε°_{AB} is the corresponding value of the resulting binary eluent.

The values of δ_i in eqn. (3) are the same as previously tabulated values for alumina (Part III[7]). The increase in the effective size δ'_i of these groups on silica is due to a localization phenomenon, and the localization function $f(Q^\circ_i)$ for silica is the same as the corresponding function $f(Q^\circ_k)$ described in Part IV[8] for alumina.

As is the case for eqn. (1) and adsorption on alumina, the application of eqn. (3) has varying levels of accuracy according to what is calculated. For example, eqn. (3), or (1) for alumina, may be re-written to predict the effect of adsorbent activity change on retention volume:

$$\log \underline{R}^\circ = \log V_a + \alpha \, (\overline{S, E}) \tag{6}$$

The term $(\overline{S, E})$ is readily identified with the solute and eluent terms of eqns. (1) and (3), and is hence *constant* for a particular solute, eluent combination. Measurement of a solute retention volume using an adsorbent sample of defined activity (V_a and α known) permits the calculation of $(\overline{S, E})$ from eqn. (6), following which $\underline{R}^\circ$ for the same solute and eluent can be calculated for any other adsorbent whose values of V_a and α are specified. The accuracy of eqn. (6) is generally greater than for eqn. (3), as expected from its greater simplicity. It should also be noted that while eqn. (6) applies to elution from both alumina and silica, the value of the parameter $(\overline{S, E})$ will be different for the two adsorbents.

The effect on solute retention volume of changing both adsorbent activity and eluent type is given by eqn. (7):

$$\log \underline{R}^\circ = \log V_a + \alpha \, (S^\circ - \varepsilon^\circ \sum_i \delta_i') \tag{7}$$

where for alumina,

$$\delta_i' = \delta_i$$

and for silica,

$$\delta_i' = \delta_i + 14.5 \, f(Q^\circ_i)$$

The solute parameter S° is measurable from a single value of $\underline{R}^\circ$ using any adsorbent–eluent combination. It is assumed that the adsorbent and eluent parameters have been evaluated for the combination in question. Values of $\underline{R}^\circ$ for the same solute and any other adsorbent–eluent combination can then be calculated from eqn. (7). The accuracy of eqn. (7) is intermediate between that of eqns. (3) and (6). The following paragraphs provide some examples of the applications of these correlational equations in predicting $\underline{R}^\circ$ values for silica as adsorbent, and through comparison with experimental data give some insight into the accuracy associated with each type of calculation.

The first example demonstrates the application of eqn. (6) to the prediction of $\underline{R}^\circ$ values for different adsorbent activities. Consider the elution of the hydrocarbon

acenaphthylene by *n*-pentane from a variety of silica adsorbents. It is assumed that for the large surface area Davison Code 12 (D-12) silica samples an *effective* rather than "nominal" (% water actually added to calcined adsorbent) water content has been *chromatographically measured*, using the standard solute retention volume data of Table V (same procedure as for alumina in Parts II–IV[6–8]). Further assume that $\underline{R}°$ has been measured for acenaphthylene eluted by pentane from D-12 4.6 % H_2O–SiO_2 (actual value of log $\underline{R}°$ found equals 1.10). It is desired to predict log $\underline{R}°$ for the pentane elution of acenaphthylene from three other adsorbent samples: (a) D-12 1.0 % H_2O-SiO_2, (b) D-12 8.0 % H_2O–SiO_2, and (c) Davison Code 62 (D-62) silica which has had 1 % water added to calcined adsorbent. It is necessary first to calculate the value of the parameter $(\overline{S, E})$ in eqn. (6) for the elution of acenaphthylene by pentane. From the experimental value of $\underline{R}°$ for the D-12 4.6 % H_2O–SiO_2, we have:

$$1.10 = -0.59 + 0.74\ (\overline{S, E})$$

since log V_a and α are, respectively, —0.59 and 0.74 from Table V. From this, $(\overline{S, E})$ is calculated at 2.28. Similarly, values of log V_a and α for adsorbents (a) and (b) may also be obtained from Table V, and log $\underline{R}°$ for the elution of acenaphthylene by pentane from these two adsorbents calculated from eqn. (6).

Adsorbent	*log V_a*	*α*	*Calc. log $\underline{R}°$*
(a) 1.0 % D-12	—0.54	0.94	1.60
(b) 8.0 % D-12	—0.66	0.67	0.87

The experimental values found were (a) 1.56 and (b) 0.88. For the D-62 1 % H_2O–SiO_2, the surface area of the calcined adsorbent is 287 m^2/g, and V_a is calculated as 0.090 from eqn. (4). $\underline{R}°$ for the standard solute (naphthalene–pentane) elution was found equal to 2.6, so that $(\underline{R}°/V_a)$ is 29. α is interpolated as 0.715 in Table V. Finally, from eqn. (6) for this adsorbent,

$$\log R° = -1.05 + 0.715\ (2.28) = 0.58$$

An experimental value of 0.56 was found.

In a second application of eqn. (6), consider the elution of the solute phenetole (ethoxybenzene) by *n*-pentane from a number of different silica samples. Assume that an experimental value of $\underline{R}°$ is available for the elution of phenetole by pentane from D-12 6.9 % H_2O–SiO_2 (log $\underline{R}°$ equal 1.66), and that values of $\underline{R}°$ are desired for the adsorbents (a) D-12 4.6 % H_2O–SiO_2, (b) D-12 16.0 % H_2O–SiO_2, and (c) D-62 1 %

Adsorbent	*log V_a*	*α*	*Calc. log $\underline{R}°$*
D-12 6.9 % H_2O–SiO_2	—0.64	0.69	$(\overline{S, E})$ equal 3.33
(a) D-12 4.6 %	—0.53	0.74	1.93
(b) D-12 16 %	—0.86	0.60	1.14
(c) D-62 1 %	—1.05	0.715	1.33

H_2O–SiO_2 (the adsorbent used in the preceding example). Values of log V_a and α for these four adsorbents may again be obtained as in the preceding example, a value of $(\overline{S,E})$ calculated, and values of log $\underline{R}°$ predicted for the latter three adsorbents. The experimental values of log $\underline{R}°$ observed for adsorbents (a–c) were, respectively, 1.92, 1.13 and 1.46.

A third example illustrates the application of eqn. (7) in predicting the effect of both eluent and adsorbent activity change on $\underline{R}°$. Consider the elution of the solute methyl benzoate by a variety of eluents from three adsorbents: (a) D-12 1.6% H_2O–SiO_2, (b) D-12 16% H_2O–SiO_2, and (c) the same D-62 1% H_2O–SiO_2 of preceding examples. Assume that an experimental $\underline{R}°$ value for methyl benzoate has been measured for the elution by pentane from D-12 16% H_2O–SiO_2 (log $\underline{R}°$ equal 2.24). It is desired to calculate $\underline{R}°$ in the following chromatographic systems (I–V).

System	*Adsorbent*	*Eluent*	*log V_a*	*α*	*$\varepsilon°$*
—	(b) D-12 16%	*n*-Pentane	—0.86	0.60	0.00
I	(a) D-12 1.6%	50% v Benzene–pentane	—0.55	0.89	0.207
II	(b) D-12 16%	10% v Benzene–pentane	—0.86	0.60	0.077
III	(b) D-12 16%	25% v Benzene–pentane	—0.86	0.60	0.137
IV	(b) D-12 16%	Benzene	—0.86	0.60	0.250
V	(c) D-62 1%	*n*-Pentane	—1.05	0.715	0.000

Values of log V_a and α for each chromatographic system are listed, as previously. The $\varepsilon°$ values for the pure eluents are those of Table VII. For the binary eluents, eqn. (5) was used to evaluate $\varepsilon°$. It is first necessary to calculate $\sum_i \delta_i'$ for methyl benzoate. The value of δ_i for each aromatic carbon atom in this solute is 1.0, and for the $-CO_2CH_3$ group, 3.0, from Part III[7]. $f(Q°_i)$ from Part IV[8] is 0.00 for the aromatic carbon atoms ($Q°_i$ equal 0.25 from Table VI). Similarly, $f(Q°_i)$ is 0.43 for the ester group ($Q°_i$ equal 3.45). δ_i' is therefore 1.0 for each aromatic carbon atom in methyl benzoate, and $(3 + 14.5 \times 0.41)$ or 8.9 for the ester group. The sum of these contributions to $\sum_i \delta_i'$ equals 14.9. The solute parameter $S°$ is calculated for methyl benzoate from the experimental $\underline{R}°$ value for this solute, using eqn. (7):

$$2.24 = -0.86 + 0.60\,[S° - (0.00 \times 14.9)]$$

or $S°$ equals 5.17. Values of $\underline{R}°$ can be calculated for each of the above chromatographic systems I–V.

$$\text{(I)}\quad \log \underline{R}° = -0.55 + 0.89\,[5.17 - (0.207 \times 14.9)]$$
$$= 1.31$$

The experimental value was 1.33.

$$\text{(II)}\quad \log \underline{R}° = -0.86 + 0.60\,[5.17 - (0.077 \times 14.9)]$$
$$= 1.55$$

The experimental value was 1.52.

$$\text{(III)}\quad \log \underline{R}^\circ = -0.86 + 0.60\,[5.17 - (0.137 \times 14.9)] = 1.02$$

The experimental value was 1.04.

$$\text{(IV)}\quad \log \underline{R}^\circ = -0.86 + 0.60\,[5.17 - (0.25 \times 14.9)] = 0.00$$

The experimental value was —0.08.

$$\text{(V)}\quad \log \underline{R}^\circ = -1.05 + 0.715\,[5.17 - (0.00 \times 14.9)] = 2.65$$

The experimental value was 2.34.

The following five examples will illustrate the calculation of $\underline{R}^\circ$ values from eqn. (3). First, consider the elution of *n*-butylbenzene by pentane from D-12 1.0 % H_2O–SiO_2. Log V_a and α are —0.54 and 0.94, respectively. $\sum_i Q^\circ_i$ is the sum of contributions from six aromatic carbon atoms (6 × 0.25), one methylene carbon attached to an aromatic ring (1 × 0.01), two methylene groups attached to aliphatic carbon [2 × (—0.05)], and one methyl group attached to aliphatic carbon (0.07) for a total of 1.48. The number of aromatic carbon atoms (n) is six, and the number of aromatic rings (r) equals one. No specific geometry terms are known for this solute, so $\sum_j q^\circ_j$ equals zero. Finally, the eluent strength ε° for pentane equals zero and

$$\log \underline{R}^\circ = -0.54 + 0.94\ [1.48 - 0.113\,(\,6 - 6\,) + 0 - 0] = 0.85$$

The experimental value was 0.86.

Second, consider the elution of phenanthrene by pentane from D-62 1 % H_2O–SiO_2. Log V_a and α for this adsorbent (which is the same as in previous examples) are —1.05 and 0.715, respectively. Fourteen aromatic carbon atoms make a total contribution to $\sum_i Q^\circ_i$ of 3.50, and n equals 14. There is only one uncondensed ring system (r equal 1). There are no geometry terms q°_j, and the eluent parameter is again zero. Therefore,

$$\log \underline{R}^\circ = -1.05 + 0.715\,[3.50 - 0.113\,(14 - 6\,) + 0 - 0] = 0.81$$

The experimental value was 0.70.

Third, to calculate $\underline{R}^\circ$ for the elution of 1,2-diphenylethane (dibenzyl) from D-12 8.0 % H_2O–SiO_2 by pentane. The values of log V_a and α are —0.66 and 0.67, respectively. $\sum_i Q^\circ_i$ is the sum of contributions from 12 aromatic carbon atoms (12 × 0.25) and two methylene groups attached to aromatic rings (2 × 0.01), for a total of 3.02. The values of n and r are 12 and 2, respectively. $\sum_i q^\circ_j$ and ε° are zero, so

$$\log \underline{R}^\circ = -0.66 + 0.67\ [3.02 - 0.113\ (12 - 12) + 0 - 0]$$
$$= 1.36$$

The experimental value was 1.30.

Fourth, consider the elution of 1,2,4-tricarbomethoxybenzene from D-12 16 % H_2O–SiO_2 by methylene chloride. The values of log V_a and α are —0.86 and 0.60, respectively. The contributions to $\sum_i Q^\circ_i$ include 6 aromatic carbon atoms (6×0.25) and three aromatic ester groups (3×3.45) for a total of 11.85. n and r are 6 and 1, respectively. An *ortho* ester grouping contributes a q°_j value of 0.48 (Table III, Part IV[8]), and ε° is equal to 0.32 (Table VII). The δ_i values of aromatic carbon and $-CO_2CH_3$ groups are 1.0 and 3.0, as before. The localization functions $f(Q^\circ_i)$ for these two groups are 0.00 and 0.41, respectively. Therefore,

$$\log \underline{R}^\circ = -0.86 + 0.60\{11.85 - 0.113\ (6 - 6) + 0.48 - 0.32\ [6 + 3\ (3 + 14.5 \times 0.41)]\}$$
$$= 0.24$$

The experimental value was 0.30.

In a final example of the application of eqn. (3), consider the elution of carbazole by 15 % v methylene chloride–pentane from D-12 16 % H_2O–SiO_2. Log V_a and α are given in the preceding example, $\sum_i Q^\circ_i$ equals ($12 \times 0.25 + 3.00$), or 6.00, and n and r are 12 and 1, respectively. $\sum_j q^\circ_j$ is zero, and ε° can be calculated from eqn. (5) as 0.123. δ_i for each of the first six carbon atoms is 1.0, and for the second six and the nitrogen atom, 0.5. $f(Q^\circ_i)$ is 0.00 for aromatic carbons and 0.32 for the nitrogen group (Q°_i equal 3.00). Therefore,

$$\log \underline{R}^\circ = -0.86 + 0.60\ [6.00 - 0.113\ (12 - 6) + 0 - 1.23\ (9.5 + 14.5 \times 32)]$$
$$= 1.29$$

The experimental value was 1.04.

EFFECT OF ADSORBENT ACTIVITY IN SEPARATIONS ON SILICA

The derivation of the parameters V_a and α for alumina as adsorbent has been discussed in Part II[6]. As will be evident from the discussion in the latter part of this communication, the same procedure is inapplicable for the measurement of silica V_a values. Eqn. (4) provides a satisfactory alternative to the problem of deriving V_a for silica of varying water content. The adsorbent parameters for Code 12 silica as a function of added water are listed in Table V; these values were obtained from eqns. (4) and (6), and a number of chromatographic measurements. Fig. 2 compares the adsorbent activity functions of Alcoa F-20 alumina and D-12 silica for various fractional coverages by added water (ml added water/ml water for monolayer coverage). Both adsorbent activity functions decline with increasing water coverage, as expected, with the effect more pronounced for silica as adsorbent.

The validity of eqn. (6) permits the construction for any adsorbent of a family of "master curves" as in Part II for alumina. Fig. 3 shows such a plot for D-12 silica.

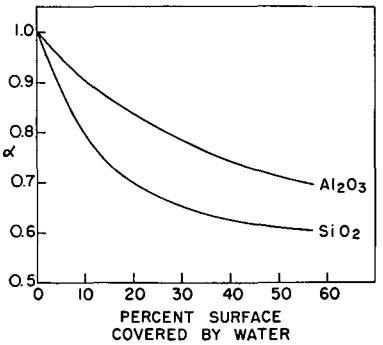

Fig. 2. Adsorbent activity function for silica and alumina *versus* water deactivation.

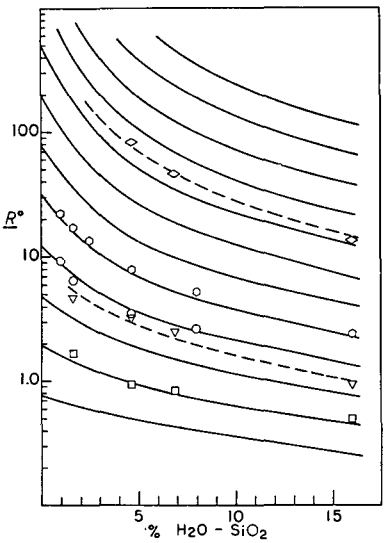

Fig. 3. Dependence of retention volume on adsorbent activity; Code 12 silica gel. ○ isopropylbenzene, pentane; □ nitrobenzene, benzene; ▽ 2-acetonaphthone, benzene; ◇ phenetole, pentane; ⬡ naphthalene, pentane; ····· theoretical curves.

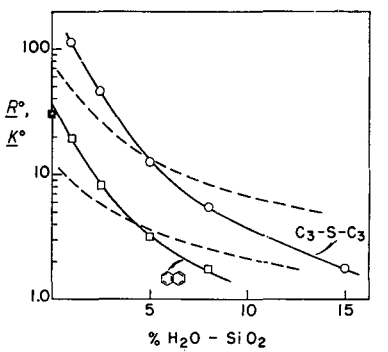

Fig. 4. Equilibrium and chromatographic data for naphthalene and propyl sulfide (solutes), carbon tetrachloride (eluent), and Code 12 silica of varying activity. □ $\underline{K}°$, naphthalene; ■ $\underline{R}°$, naphthalene; ○ $\underline{K}°$, propyl sulfide; – – – eqn. (6).

The applicability of eqn. (6) requires that a series of $\underline{R}°$ values for the same solute and eluent (changing adsorbent activity) fall on a single master curve. Several experimental tests of this requirement are shown in Fig. 3, with the adherence of data to the master plots, and to eqn. (6), being demonstrated. Additional tests of eqn. (6) and of the D-12 adsorbent parameters of Table V are offered in Tables VIII and IX. In

TABLE VIII

TEST OF EQUATION (6) IN CORRELATION OF SOLUTE RETENTION VOLUMES WITH ADSORBENT ACTIVITY; PENTANE ELUTION OF HYDROCARBONS

Solute	*log $\underline{R}°$ for various adsorbent water contents*						*Av. dev.*	$\overline{S, E}$
	1.0%	*1.6%*	*2.5%*	*4.6%*	*8.0%*	*16.0%*		
Isopropylbenzene								
(Exptl.)	0.97	0.81		0.54	0.42		±0.03	1.61
(Calc.)	0.97	0.76		0.60	0.42			
Naphthalene								
(Exptl.)	1.33	1.22	1.13	0.90	0.72	0.38	±0.02	2.02
(Calc.)	1.36	1.25	1.10	0.90	0.69	0.35		
Acenaphthylene								
(Exptl.)	1.56			1.10	0.88		±0.02	2.28
(Calc.)	1.60			1.10	0.87			
Dibenzyl								
(Exptl.)		2.08			1.30	0.87	±0.02	2.92
(Calc.)		2.05			1.30	0.89		
Perylene								
(Exptl.)				1.76	1.52	1.10	±0.03	3.23
(Calc.)				1.80	1.50	1.08		

all cases, the retention volumes calculated from average ($\overline{S, E}$) values are in reasonable agreement (± 0.02 log units in Table VIII, ± 0.05 log units in Table IX) with values predicted from eqn. (6).

One exception to the applicability of eqn. (6) to Code 12 silica has been noted. Elution with CCl_4, which is "regular" in the case of alumina, does not give the predicted dependence of $\underline{R}°$ values on adsorbent activity (as in Fig. 3). This is illustrated in Fig. 4 for the elution of both naphthalene and propyl sulfide from D-12 silica of varying activity. The dashed lines superimposed on the experimental plots are the theoretical curves predicted by eqn. (6) and Fig. 3. This "irregularity" of the eluent CCl_4 for the present adsorbent is believed to arise from the combination of a small average pore diameter in Code 12 silica with a large eluent diameter. As an immediate result, it is assumed that certain of the smaller adsorbent pores in Code 12 silica are inaccessible to CCl_4 and other eluents of comparable cross section. In the case of calcined adsorbent, there is no possibility of the redistribution of adsorbed water to cover these "bare" pores, so that there will exist adsorption regions uncovered by any adsorbed species. Solute adsorption energies in these uncovered regions will be very much larger than for "normal" adsorption on covered regions, and this will increase the *average* adsorption energy of solute in all regions and, in turn $\underline{R}°$. This is observed, CCl_4 being a strong eluent relative to pentane for alumina as adsorbent ("normal" behavior), but *apparently* weaker than pentane in the elution of naphthalene from calcined Code 12 silica ($\underline{R}°$ is 27.5 and 30.0 ml/g, respectively, for elution of naphtha-

TABLE IX

TEST OF EQUATION (6) IN CORRELATION OF SOLUTE RETENTION VOLUMES WITH ADSORBENT ACTIVITY; ELUTION OF NON-HYDROCARBONS FROM CODE 12 SILICA

Solute	*Eluent*	*log $\underline{R}°$ for various water contents*				*Av. dev.*	$\overline{S,E}$
		1.6%	*4.6%*	*6.9%*	*16.0%*		
Propyl sulfide	Pentane						
(Exptl.)		2.07	1.45	1.39	0.88	±0.01	2.94
(Calc.)		2.06	1.44	1.39	0.90		
Nitrobenzene	Benzene						
(Exptl.)		0.22	—0.03	—0.08	—0.30	±0.04	0.84
(Calc.)		0.20	0.03	—0.10	—0.36		
p-Nitroanisole	Benzene						
(Exptl.)		0.67	0.52	0.40	—0.04	±0.05	1.44
(Calc.)		0.73	0.47	0.35	0.00		
2-Acetonaphthalene	Benzene						
(Exptl.)		1.18	0.88	0.86	0.55	±0.11	2.12
(Calc.)		1.34	0.98	0.82	0.41		
Dimethyl isophthalate	CH_2Cl_2						
(Exptl.)		1.43		0.87	0.34	±0.08	2.17
(Calc.)		1.38		0.79	0.44		
Phenyl ethyl sulfide	Pentane						
(Exptl.)			1.53	1.27	0.81	±0.03	2.80
(Calc.)			1.48	1.29	0.82		
Phenetole	Pentane						
(Exptl.)			1.92	1.66	1.13	±0.02	3.35
(Calc.)			1.89	1.67	1.15		

lene from Code 12 silica by pentane and CCl_4). If this explanation of these latter data is true, then a plot of eluent strength $\varepsilon°$ *versus* percentage CCl_4 for pentane–CCl_4 binary eluents should go through a maximum, and $\underline{R}°$ for naphthalene should pass through a minimum, because of the ability of pentane to cover adsorbent surfaces inaccessible to CCl_4, and the greater adsorption affinity of CCl_4 on remaining adsorption regions. This is in fact observed; $\underline{R}°$ for naphthalene eluted by 25 % v CCl_4–pentane from the same calcined Code 12 silica was found equal to 15.5 ml/g. It is assumed that elution by CCl_4 from water deactivated adsorbent permits the redistribution of adsorbed water into "bare" pores, with resulting cancellation of the efficiency of these regions for adsorption of solute. The overall effect is to produce the "irregular" dependence of $\underline{R}°$ on adsorbent activity which is shown in Fig. 4.

While only three eluents are represented in the data of Tables VI and VII, there is no reason to doubt the validity of eqn. (5) in the case of other eluents, except where small pore diameter adsorbents and eluents of large cross section are combined in a single chromatographic system.

EFFECT OF ELUENT TYPE IN SEPARATION OVER SILICA

The effect of eluent type on solute retention volume can be obtained from eqn. (1) for alumina or eqn. (3) for silica by combining certain of the solute terms into a solute adsorption energy factor $S°$ as in eqn. (7). For alumina, $S°$ in eqn. (7) is defined by:

$$S° = \sum^{i} Q°_i - f(Q°_k) \sum^{i \neq k} Q°_i + \sum^{j} q°_j$$

The area of the solute $\sum_i \delta_i$ is assumed to determine the number of eluent molecules displaced from the adsorbent surface by a solute molecule, and this in combination with the relative adsorption energy of the eluent ε° determines the effect of eluent on $\underline{R}^\circ$. In Part III[7], eqn. (7) was tested for a broad range of solute and eluent types in adsorption on alumina. The verification of its validity for alumina was accomplished largely with pure eluents as opposed to binaries. The similar test of eqn. (7) for silica as adsorbent using pure eluents is unreasonable for several reasons*.

TABLE X

EFFECT OF ELUENT IN ELUTION OF HYDROCARBONS FROM CODE 12 SILICA

Solute	$\sum_i \delta_i$	Adsorbent*	log $\underline{R}^\circ$						Av. dev.	S°
			1.6%		16%					
		Eluent**	10% B-P	50% B-P	10% B-P	25% B-P	5% M-P	15% M-P		
		$\alpha\varepsilon^\circ$***	0.099	0.195	0.042	0.076	0.0333	0.075		
Naphthalene	8									
(Exptl.)			0.38						±0.07	2.02
(Calc.)			0.45							
Phenanthrene	10									
(Exptl.)			0.76	—0.21	0.20		0.35		±0.03	2.55
(Calc.)			0.73	—0.23	0.25		0.34			
Chrysene	12									
(Exptl.)			1.05	—0.17	0.51		0.52	0.04	±0.04	3.09
(Calc.)			1.01	—0.14	0.49		0.59	0.09		
Perylene	13									
(Exptl.)			1.05		0.49	0.12	0.69	0.16	±0.03	3.23
(Calc.)			1.04		0.53	0.09	0.65	0.11		
Picene	14									
(Exptl.)					0.84	0.26	0.90	0.28	±0.04	3.67
(Calc.)					0.75	0.28	0.88	0.29		

* % Water (chromatographic) on Code 12 silica.
** Symbols defined as following: P, pentane; B, benzene; M, methylene chloride; % refers to volume percent.
*** Measured from above data (best fit).

Experimental retention volume data are presented in Table X for the elution of several hydrocarbons from Code 12 silica of two activities by several *binary* eluents. Values of S° for these various solutes had been previously obtained from eqn. (7) and retention volume data for pentane elution (see Table XV). Given the values of

* First, as noted in the previous section, eqn. (6) does not apply to Code 12 silica and the eluent CCl_4, and, for the same reasons, neither does eqn. (7). The testing of eqn. (7) is most accurately and conveniently done with eluent pairs whose strengths are not widely different. With the exception of CCl_4, there are no pure eluents with ε° values intermediate between the saturate solvents and solvents as strong or stronger than benzene. Consequently, if CCl_4 cannot be used, those solutes which are readily eluted by the saturates cannot be tested for Code 12 silica in eqn. (7). Second, as will be seen, the retention volumes of the aromatic hydrocarbons are essentially zero for elution by benzene and stronger solvents. These hydrocarbon solutes were used as standards in Part III for the evaluation of alumina ε° values, but cannot be similarly used for elution from Code 12 silica by pure solvents. Finally, as will also be seen the variation of solute retention volume with eluent strength is much more pronounced for the elution of non-hydrocarbons from any silica relative to alumina. This greatly increases the necessity of the similarity of ε° values in the eluents used to test eqn. (7) and to evaluate $\sum_i \delta_i'$ for these solutes. These various considerations make it mandatory to use binary eluents in testing the applicability of eqn. (7) for silica as adsorbent.

V_a and α for these two adsorbents (Table V), it is possible to calculate best values of the effective eluent strength $\alpha\varepsilon°$ for each binary by means of eqn. (7), if the $\sum_i \delta_i'$ values for each solute are assumed the same ($\sum_i \delta_i$) as for alumina. These values of $\alpha\varepsilon°$ are summarized at the top of Table X. Finally, the adequacy of eqn. (7) in correlating the eluent effects of Table X is shown in the comparison of experimental values with those calculated by means of eqn. (7) and the various associated parameters. Agreement is good (average deviation $\pm$ 0.04 log units). The fit of these hydrocarbon elution data to eqn. (7) is further illustrated in Fig. 5, where log $\underline{R}°$ values for the

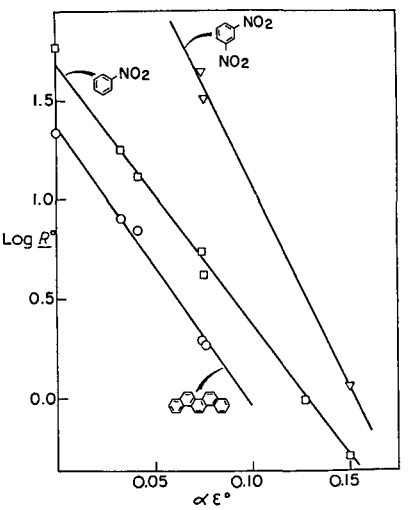

Fig. 5 Dependence of solute retention volume on eluent strength; elution from Code 12 16% H_2O–SiO_2.

hydrocarbon picene and the adsorbent D-12 16 % H_2O–SiO_2 are plotted *versus* $\alpha\varepsilon°$. The slope of the straight line through these points is the calculated value of $\sum_i \delta_i$ for picene (14).

Experimental data for the elution of several non-hydrocarbons from the same two adsorbents of Table X are presented in Table XI. The values of $\alpha\varepsilon°$ shown for each binary at the top of Table XI are either the values previously calculated from the data of Table X for the hydrocarbons, or are extrapolated values from eqn. (7) and the data of Table XI. Data for two of the solutes of Table XI, nitrobenzene (NB) and dinitrobenzene (DNB), eluted from 16 % H_2O–SiO_2 are plotted in Fig. 5. The predicted linear relationship between log $\underline{R}°$ and $\alpha\varepsilon°$ is again observed (as for picene), but the slopes of the plots for NB and DNB ($\sum_i \delta_i'$) are much larger than predicted from the δ_i values measured for elution from alumina (Part III[7]); *observed* $\Sigma\, \delta_i'$, NB, 13.4; DNB, 20.4; *predicted* $\Sigma\, \delta_i$, NB, 8.5; DNB, 11.0. The excellent correlation of the data of Table X suggests that δ_i' for the aromatic carbon atom is the same for both silica and alumina, as required by the equivalence of this quantity with the *area* of the aromatic carbon group (a quantity independent of adsorbent). For the adsorption of the nitro group on silica, however, δ_i' for this group is much larger than for ad-

TABLE XI

EFFECT OF ELUENT ON ELUTION OF NON-HYDROCARBONS FROM CODE 12 SILICA

Solute	$\sum_i^i \delta_i'$	Adsorbent*	log $\underline{R}^\circ$									Av. dev.	S°
			1.6%	16%									
		Eluent**	50% B-P	P	10% B-P	25% B-P	B	5% M-P	15% M-P	40% M-P	M		
		$\alpha\varepsilon^\circ$***	0.195	0.000	0.042	0.076	0.150	0.033	0.075	0.127	0.192		
Phenyl ethyl sulfide													
(Exptl.)	9.4		0.06	0.81	0.35			0.46				±0.02	2.75
(Calc.)	9.6		0.03	0.79	0.39			0.47					
Phenetole													
(Exptl.)	10.4		0.38	1.13	0.66	0.27		0.74				±0.03	3.29
(Calc.)	10.6		0.31	1.11	0.66	0.30		0.76					
Nitrobenzene													
(Exptl.)	13.4		0.72	1.76	1.11	0.61	—0.30	1.25	0.73	—0.01		±0.04	4.25
(Calc.)	13.2		0.66	1.69	1.14	0.69	—0.29	1.25	0.70	0.01			
Methyl benzoate													
(Exptl.)	14.1		1.33	2.13	1.52	1.04	—0.08	1.69	1.14			±0.06	4.93
(Calc.)	13.5		1.21	2.10	1.53	1.07	0.07	1.65	1.09				
2-(1-Thiapropyl)-naphthalene													
(Exptl.)	11.8		0.03	1.07	0.54	0.05						±0.05	3.13
(Calc.)	11.6		—0.02	1.02	0.53	0.14							

2-Methoxynaphthalene												
(Exptl.)	11.2	0.57	1.35	0.89	0.42						±0.06	3.77
(Calc.)	12.2	0.43	1.40	0.89	0.47							
2-Acetonaphthone												
(Exptl.)	17.2					0.55	2.61	1.78	0.94	—0.11	±0.06	6.58
(Calc.)	17.2					0.51	2.52	1.80	0.91	—0.21		
p-Diethoxybenzene												
(Exptl.)	16.5				1.46	0.09	1.96	1.20	0.36		±0.10	5.46
(Calc.)	15.2				1.26	0.14	1.92	1.28	0.49			
m-Dinitrobenzene												
(Exptl.)	20.0				1.50	0.08		1.64			±0.05	6.64
(Calc.)	20.2				1.57	0.06		1.59				
m-Nitroanisole												
(Exptl.)	17.1			1.49	1.03	—0.31		1.08			±0.05	5.30
(Calc.)	17.4			1.59	1.00	—0.29		1.01				
Carbazole												
(Exptl.)	17.9			1.47	0.88	—0.53	1.71	1.04	0.17		±0.08	5.25
(Calc.)	17.9			1.54	0.89	—0.40	1.70	0.95	0.02			
Dimethyl isophthalate												
(Exptl.)	(14.2)					0.91			1.26	0.34	±0.17	8.30
(Calc.)	21.0					0.97			1.46	0.09		

* % Water (chromatographic) on Code 12 silica.
** Symbols defined as follows: P, pentane; B, benzene; M, methylene chloride; % refers to volume percent.
*** Experimental value from hydrocarbon $\underline{R}^\circ$ values.

TABLE XII

SUMMARY OF SOLUTE PARAMETERS FOR SILICA AS ADSORBENT
(EXPERIMENTAL DATA FOR CODE 12)

Group	δ_i'	$(\delta_i' - \delta_i)$		Q°_i		
				SiO_2		Al_2O_3 Ref.[3]
		Exptl.	Calc.*	This study	Ref.[4]	
-CH=	0.5–1	0.0	0.0	0.25	—	0 31
Ar–S–R	3.6	1.1	0.4	1.29	—	1.32
Ar–O–R	4.6	2.6	1.9	1.83	2.3	1.77
Ar–NO_2	7.2	4.7	4.4	2.77	2.5	2.75
Ar–CO_2–R	7.5	4.5	5.9	3.45	—	3.32
Ar–CO–R	9.2	7.2	6.5	4.69	4.9	3.74
Ar–NH_2			6.5	5.1**	4.7	4.41
=NH (carbazole)	8.9	8.4	4.6	3.00	—	4.1
R–S–R				2.94	—	2.65

* $14.5 f(Q^\circ_k)$.
** Assumed δ_i' for $-NH_2$ equal $14.5 f(Q^\circ_k) + \delta_i$.

sorption on alumina: 2.5 on alumina, 7.2–7.4 on silica (from plots of Fig. 5). This increase in the *apparent area* δ_i' of non-hydrocarbon adsorbing groups on silica relative to alumina is rather general, as shown by the experimental (least squares) $\sum_i \delta_i'$ values of the solutes in Table XI, and the summary of best δ_i' values in Table XII. These latter values were derived from the data of Table XI, just as the δ_i' value of a nitro group can be derived from Fig. 5. Using these δ_i' values of Table XII in eqn. (7), the experimental data of Table XI can be calculated with satisfactory accuracy (average deviation $\pm$ 0.06 log units). The S° values used are the average values calculated from the data of Table XI by means of eqn. (7).

For elution from alumina, the δ_i' (or δ_i) values of various groups i closely approximate the relative area of i. In the case of elution from silica, it is apparent that this is no longer true, a group such as aceto (δ_i' equal 9.2) appearing larger than an entire naphthalene molecule ($\sum_i \delta_i'$ equal 8.0), in regard to the number of eluent molecules that appear to be displaced by the aceto group. If the nominal area δ_i (from adsorption on alumina) is subtracted from δ_i' for each group i, the difference may be taken as the excess area required by i in adsorption on silica. Interestingly, this excess area appears to correlate with the localization function $f(Q^\circ_k)$ described in Part IV[8]

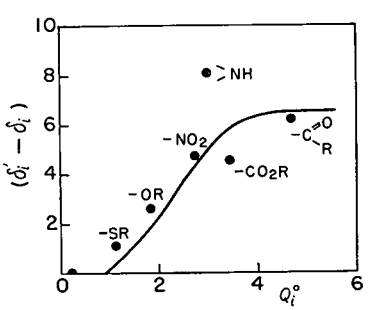

Fig. 6. Dependence of solute group excess area on adsorption strength of group (Code 12 silica).

for alumina. This is demonstrated in Fig. 6, where $(\delta_i'-\delta_i)$ is plotted *versus* Q°_i, the relative adsorption energy of the group i in silica (see next section for derivation of these latter values). Superimposed on this plot is the curve $14.5\, f(Q^\circ_i)$. This correlation of $(\delta_i'-\delta_i)$ and $f(Q^\circ_i)$ is also shown by the comparison of experimental and calculated values of $(\delta_i' - \delta_i)$ in Table XII, an average deviation of only $\pm$ 0.7 units being observed for the *exocyclic* substituents. The excess area of the nitrogen group which forms part of the carbazole nucleus is somewhat larger than predicted by the expression $14.5\, f(Q^\circ_i)$, and this may be a general property of strongly adsorbed groups forming part of an aromatic nucleus.

Eqn. (7) may be made specific for adsorption upon silica by substituting the expression: $[\delta_i + 14.5\, f(Q^\circ_i)]$ for δ_i':

$$\log R^\circ = \log V_a + \alpha \{ S^\circ - \varepsilon^\circ \sum^{i} [\delta_i + 14.5\, f(Q^\circ_i)]\} \qquad (8)$$

The correlation of Fig. 6 suggests that the large *apparent* areas δ_i' of strongly adsorbing groups arises from a localization phenomenon similar to that observed in adsorption on alumina, but which takes a different manifestation in adsorption on silica. This possibility will be further explored in a forthcoming publication.

The eluent strength parameters for benzene and methylene chloride which were evaluated from the data of Tables X and XI are summarized in Table VII. Table XIII

TABLE XIII

COMPARISON OF EXPERIMENTAL AND CALCULATED $\alpha\varepsilon^\circ$ VALUES FOR BINARY ELUENTS AND CODE 12 SILICA

Eluent	*Adsorbent activity* (% H_2O - SiO_2)	$\alpha\varepsilon^\circ_{AB}$	
		Exptl.	*Calc.**
10 % v Benzene–pentane	1.6	0.099	0.093
50 % v Benzene–pentane	1.6	0.195	0.184
10 % v Benzene–pentane	16.0	0.042	0.046
25 % v Benzene–pentane	16.0	0.076	0.082
5 % v Methylene chloride–pentane	16.0	0.033	0.032
15 % v Methylene chloride–pentane	16.0	0.075	0.074
40 % v Methylene chloride–pentane	16.0	0.127	0.134

* From eqn. (5).

compares experimental values of $\alpha\varepsilon^\circ_{AB}$ for the various binaries of Tables X and XI with values calculated from eqn. (5) and the eluent parameters of Table VII. The agreement is good (average deviation $\pm$ 0.005).

EFFECT OF SOLUTE STRUCTURE IN ADSORPTION ON SILICA

The effect of solute structure on adsorption affinity is included in the solute parameter S°, previously defined for adsorption on alumina, and experimentally measurable for adsorption on silica through eqn. (7). Elution of a large number of solutes from silica in the present study has provided experimental S° values for a wide range in solute

structures. Some of these $S°$ values have already been presented in Tables X and XI. The following discussion of these $S°$ values in terms of our previous correlations for alumina has been broken down into two parts, hydrocarbons and non-hydrocarbons.

Hydrocarbons

Values of $\underline{R}°$ for the elution of a number of alkyl substituted hydrocarbons from Code 12 silica by pentane are summarized in Table XIV. These data permit the derivation of $S°$ values for each compound (listed as "experimental" $S°$ values) from eqn. (7). The assumption that the localization term $f(Q°_k)\sum_{i\neq k} Q°_i$ does not contribute to $S°$ for the hydrocarbons (because of their small group adsorption energies) permits

TABLE XIV

RETENTION VOLUMES FOR THE ALKYL-SUBSTITUTED OLEFINS AND BENZENES; PENTANE ELUTION FROM CODE 12 1.0% H_2O–SiO_2

Solute	$\underline{R}°$ *(ml/g)*	$S°$	
		Exptl.	*Calc.*
cis-Pentene-2	1.34	0.71	0.74
2-Methylbutene-2	2.11	0.91	0.91
2,3-Dimethylbutene-2	2.50	1.00	1.11
Pentadiene-1,3	3.50	1.15	1.11
Benzene	7.5	1.51	1.48
Toluene	9.8	1.63	1.61
o-Xylene	14.5	1.81	1.81
p-Xylene	13.2	1.77	1.77
1,2,3-Trimethylbenzene	20.3	1.97	1.97
1,3,5-Trimethylbenzene	17.4	1.89	1.89
Pentamethylbenzene	23.8	2.04	2.31
Hexamethylbenzene	24.5	2.05	2.52
n-Butylbenzene	7.2	1.49	1.46
n-Octylbenzene	4.9	1.31	1.37
n-Decylbenzene	3.6	1.17	1.17
n-Eicosylbenzene	1.84	0.85	0.68
Isopropylbenzene	9.0	1.60	1.66
tert.-Butylbenzene	8.8	1.59	1.73

the reduction of these $S°$ values into values of $Q°_i$ and $q°_j$ corresponding to each different structural group and those specific solute geometries which affect adsorption. The solute parameters for silica are summarized in Table XV, along with corresponding values for alumina from Part II for comparison. In some cases the alumina $Q°_i$ values appear slightly different from those reported in Part II[6], since no distinction between methyl and methylene carbon types was made in Part II. The parameters of Table IV include this distinction, their derivation using the value of $\varepsilon°$ for pentane (see Part III[7]). The most pronounced difference in these solute parameters of Table XV between the two adsorbents is in the preference for adsorption of methyl relative to methylene carbon groups on silica, and the reverse on alumina. The other parameters

TABLE XV

COMPARISON OF HYDROCARBON SOLUTE PARAMETERS FOR ALUMINA AND SILICA

Group or geometry	$Q°_i$		$q°_j$	
	SiO_2	Al_2O_3	SiO_2	Al_2O_3
$-CH_2-$, $-CH<$	−0.049	+0.02		
$-CH_3$	+0.074	−0.03		
$-CH=$	+0.247	+0.31		
Methyl substitution on aromatic carbon			+0.055	+0.09
Alkyl substitution on aromatic carbon			+0.055	+0.05
Vicinal alkyl substitution			+0.039	+0.09

of Table XV all show similar contributions to $S°$ from different solute groups and geometries. The silica values tend to be lower than the alumina values, probably reflecting generally weaker bonding of the hydrocarbons to the former adsorbent. The only $S°$ values of Table XIV which are not well correlated by the parameters of Table XIV are for pentamethyl- and hexamethylbenzene.

Table XVI summarizes $S°$ values for a number of *unsubstituted* olefins and aromatic hydrocarbons. The $S°$ values of ethylene and butadiene could not be conveniently

TABLE XVI

SOLUTE ADSORPTION FACTORS $S°$ FOR THE UNSUBSTITUTED OLEFINS AND AROMATIC HYDROCARBONS; PENTANE ELUTION FROM CODE 12 SILICA

Solute	$S°$	*No. of $S°$ values*	*Av. dev.**	*Adsorbent activities***
Ethylene	0.44***	3	±0.04	1.0
Butadiene	1.02***	1	—	1.0
Benzene	1.48	2	0.00	1.0 ,8.0
Styrene	1.71	1	—	1.6
Naphthalene	2.02	6	0.03	See Table IV
Azulene	2.35	2	0.06	1.0, 8.0
Acenaphthylene	2.28	3	0.03	See Table IV
Phenanthrene	2.55	2	0.02	4.6, 8.0
Anthracene	2.60	1	—	8.0
Fluoranthene	2.79	1	—	8.0
Pyrene	2.57	2	0.02	4.6, 8.0
Triphenylene	3.15	2	0.03	4.6, 8.0
Chrysene	3.09	2	0.01	8.0, 16.0
1,2-Benzanthracene	3.09	1	—	8.0
Perylene	3.23	3	0.03	4.6, 8.0, 16.0
1,2-Benzpyrene	3.28	1	—	16.0
Picene	3.67	1	—	16.0
3,4-Benztetraphene	3.68	1	—	16.0
Dibenzyl	2.92	3	0.03	1.6, 6.9, 16.0

* Between experimental $S°$ values for same solute.
** Defined by silica water content (%).
*** Substituted olefin values corrected for effect of substituents.

measured for the compounds themselves, so the values for methyl-substituted olefins (Table XIV) were corrected to zero substituents using the parameters of Table XV. The $S°$ values of Table XVI are plotted *versus* the number of aromatic plus olefinic carbon atoms n in Fig. 7. $S°$ equals 0.246 n for $n \leqslant 6$, and (0.246 × 6) +

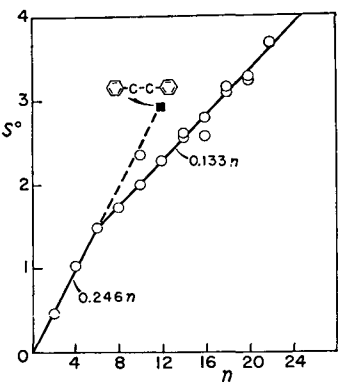

Fig. 7. Plot of $S°$ for unsubstituted aromatics *versus* aromatic carbon number n; Code 12 silica gel.

0.133 (n — 6) for n larger than 6. In the case of the solute dibenzyl, where the two phenyl groups are not fused together, and where the effect of alkyl substitution on the two aromatic rings (2 × 0.055) is just canceled by the adsorption of the two methylene carbons [2 × (—0.049)], $S°$ is given by virtually the same expression (0.243 n) as for the solutes no larger than benzene ($n \leqslant 6$). It thus appears that the contribution to $S°$ from each group of fused aromatic or unsaturated carbon atoms is given as 0.246 n for the first six carbon atoms, and 0.133 n for additional carbon atoms in the fused cluster. The physical interpretation of this relationship will be discussed in the final section of this paper.

Non-hydrocarbons

Table XVII summarizes $S°$ values for 21 non-hydrocarbons. The monofunctional benzene derivatives were used to derive values of $Q°_i$ for each functional group represented, assuming that $Q°_i$ equals $S°$ for a group i substituted benzene, minus $S°$ for benzene (1.48). These values of $Q°_i$ are listed in Table XII, along with corresponding alumina values for comparison. Values of $Q°_i$ for aliphatic sulfur and carbazole nitrogen, which were derived in a somewhat different but consistent fashion, are also included. The solute group adsorption parameters $Q°_i$ for both adsorbents are quite similar, as shown in the plot of these values for silica *versus* those for alumina in Fig. 8. The values of $S°$ for the polysubstituted benzenes and substituted naphthalenes of Table XVII appear equal to $S°$ for the unsubstituted hydrocarbon plus the sum of $Q°_i$ values for all substituents. This relationship can be stated:

$$S° = \sum^{i} Q°_i - 0.113\,(n - 6r) + \sum^{i} q°_j \qquad (9)$$

The term 0.113 (n — 6r) corrects for the lower adsorption energy of carbon atoms past the first six in each of r unfused ring systems comprising the solute. The compar-

TABLE XVII

SOLUTE ADSORPTION FACTORS S° FOR NON-HYDROCARBONS ELUTED FROM CODE 12 SILICA

Solute	S° *Exptl.*	S° *Calc.**	*No. of* S° *values*	*Av. dev.***	*Chromatographic system****
Phenyl ethyl sulfide	2.77	—	6	±0.06	4.6%, pentane; 6.9%, pentane; see Table XI
Phenetole	3.31	—	7	0.04	Same as above
Nitrobenzene	4.25	—	8	0.06	See Table XI
Methyl benzoate	4.93	—	7	0.09	See Table XI
Acetophenone	6.17	—	1	—	16.0%, benzene
Aniline	6.6§	—	1	—	16.0%, benzene
2-(1-Thiapropyl)-naphthalene	3.13	3.31	4	0.07	See Table XI
1-Methoxynaphthalene	3.12	3.85	1	—	16.0%, pentane
2-Methoxynaphthalene	3.77	3.85	4	0.09	See Table XI
Methyl 2-naphthoate	5.32	5.47	1	—	16.0%, benzene
2-Acetonaphthone	6.58	6.71	5	0.16	See Table XI
o-Diethoxybenzene	5.75	5.14	1	—	16.0%, benzene
p-Diethoxybenzene	5.46	5.14	5	0.17	See Table XI
m-Nitroanisole	5.30	6.08	4	0.09	See Table XI
p-Nitroanisole	5.80	6.08	1	—	16.0%, benzene
m-Dinitrobenzene	6.64	7.02	3	0.08	See Table XI
Dimethyl isophthalate	8.55	8.38	4	0.37	See Tables IX, XI
2-Nitroaniline	8.1§	9.35	1	—	16.0%, methylene chloride
1,2,4-Tricarbomethoxybenzene	11.1	11.83	1	—	16.0%, methylene chloride
2,4-Dinitroaniline	11.3§	12.12	1	—	16.0%, methylene chloride
Carbazole	5.25	—	7	0.11	See Table XI

* From eqn. (9).
** Between experimental S° values for same solute.
*** Defined by silica water content and eluent.
§ Assumes δ_i' for $-NH_2$ equal $14.5\,f(Q^\circ_i)$.

ison of experimental and calculated S° values in Table XVII shows an average deviation of ± 0.3 log units, corresponding to an average deviation between calculated and experimental R° values of about ± 0.2 log units. Eqn. (9) is seen not to include the localization term which is part of S° for adsorption on alumina. The localization term in alumina S° values results in a lowering of experimental S° values, with the effect being largest at large values of S°. The experimental S° values for the "poly-

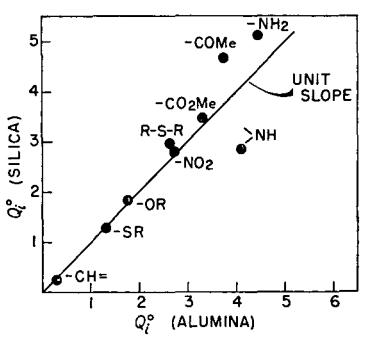

Fig. 8. Group adsorption factors Q°_i; Code 12 silica *versus* Alcoa F-20 alumina.

TABLE XVIII

SOLUTE RETENTION VOLUMES FOR CODE 62 1% H_2O-SiO_2; PENTANE ELUTION

Solute	log $\underline{R}°$	
	Exptl.	*Calc.**
Benzene	0.16	0.01
Naphthalene	0.42	0.39
Azulene	0.66	0.63
Acenaphthylene	0.56	0.58
Phenanthrene	0.70	0.77
Anthracene	0.70	0.81
Fluoranthene	0.78	0.94
Pyrene	0.66	0.79
Triphenylene	0.95	1.20
Chrysene	0.96	1.16
1,2-Benzanthracene	0.97	1.16
Perylene	0.98	1.26
1,2-Benzpyrene	0.96	1.30
Dibenzyl	1.10	1.04
Phenyl ethyl sulfide	1.07	0.93
Phenetole	1.46	1.33
Nitrobenzene	1.61	1.99
2-(1-Thiapropyl)-naphthalene	1.30	1.19
Methyl benzoate	2.34	2.47

* From eqn. (7), assumes V_a equal 0.09 and α equal 0.715; $S°$ data of Tables XVI and XVII.

functional" solutes of Table XVIII (substituted naphthalenes and polysubstituted benzenes) are plotted *versus* calculated values $\sum_i Q°_i$ from eqn. (9) in Fig. 9, while a similar plot for elution of these same solutes from alumina is offered for comparison in Fig. 10 (data of Part IV[8]). It appears that localization of the type exhibited in adsorption on alumina (Fig. 10) is absent for adsorption on silica (Fig. 9). This conclusion

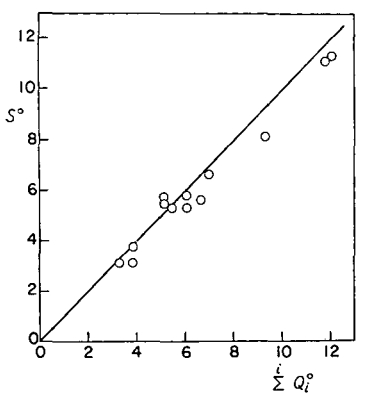

Fig. 9. Experimental *versus* calculated $S°$ values for polyfunctional non-hydrocarbons; Code 12 silica.

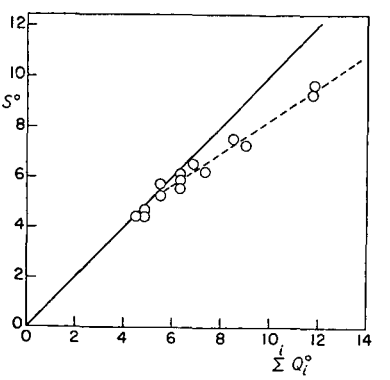

Fig. 10. Experimental *versus* calculated $S°$ values for polyfunctional non-hydrocarbons; Alcoa F-20 alumina.

is confirmed by the observations of SPORER AND TRUEBLOOD[4] on adsorption from benzene onto a silicic acid–celite mixture; these authors found that the adsorption energy ($S°$) of polysubstituted benzenes was given as the sum of group adsorption energy values.

ADSORBENT STANDARDIZATION

Adsorbent standardization can refer either to the characterization of the adsorptive properties of a solid, or to the selection and/or modification of such a solid so as to obtain an adsorbent which exactly duplicates some standard. The well-known procedure of BROCKMANN[14] for the classification of the adsorbent activity of water-deactivated aluminas was the first attempt at adsorbent standardization by means of a chromatographic test, and is still in use. A similar classification for water-deactivated silica has been proposed recently[15]. The activity grades reported by either procedure[14,15] represent ranges in adsorbent acitivities, and are intended to be qualitative rather than quantitative designations. Since these activity tests do not distinguish between the separate contributions of surface area and surface energy to adsorbent properties, it is likely that they will fail when applied to solids of widely varying surface area. It has been postulated (Part II[6]) that an adsorbent such as alumina can be completely characterized by its surface area (or V_a) and average surface energy (or α). The present section will be devoted to the further testing of this postulate in the case of silica, and the examination of some of its chromatographic consequences.

The experimental measurement of V_a and α for the adsorbent Alcoa F-20 alumina of varying water content was described in Part II. Eqn. (1) was used in conjunction with the elution of the unsubstituted aromatic hydrocarbons by pentane. For these solutes and this eluent, eqn. (1) simplifies to:

$$\log \underline{R}° = \log V_a + 0.31\,\alpha n \qquad (10)$$

where n is the number of carbon atoms. Extrapolation of the linear plot of $\log \underline{R}°$ *versus* n for a particular adsorbent sample to n equal zero gives V_a, while the slope of the plot divided by 0.31 gives α. This procedure is somewhat less suited for application to silica because of the non-linear form of such an experimental plot (as in Fig. 7). It was also shown in Part II for alumina that the value of V_a for calcined alumina is close to that predicted by the saturation uptake of tetramethylbenzene and by the BET surface area (nitrogen adsorption). Furthermore, the facile elution of adsorbed water from deactivated alumina which contains more than V_a ml/g of adsorbed water suggests that the volumes of a monolayer of water, nitrogen, and tetramethylbenzene are all approximately equal. This suggests that the adsorbent surface volume is calculable from the value corresponding to calcined adsorbent, minus the volume (ml/g) of added water. With V_a for calcined adsorbent calculable from the surface area, eqn. (4) follows. This relationship is tested below for the previously reported (Part II) data for V_a as a function of Alcoa F-20 alumina water content (Table XIX).

For all except possibly the 4.9 % H_2O–Al_2O_3 sample, the two columns of V_a values in Table XIX agree within the experimental uncertainty of the first column. This suggests, as is theoretically reasonable in the light of our preceding remarks, that eqn. (4) will apply to other adsorbents as long as the amount of adsorbed water does not approach monolayer coverage.

TABLE XIX

SURFACE VOLUME OF WATER-DEACTIVATED ALUMINAS (ALCOA F-20)

Added water (%)	V_a	
	Exptl., eqn. (10)	*Calc., eqn. (4)*
0.0	0.040, 0.034*	0.054
1.0	0.029	0.044
4.0	0.015	0.014
4.9	0.011	0.005

* Benzene eluent.

Assuming the measurement of V_a by means of eqn. (4), the value of α can be obtained from a single standard solute elution by means of eqn. (6), or

$$\alpha = \log (\underline{R}^\circ/V_a)/(\overline{S,E})$$

We have defined α as 1.00 for calcined adsorbent in Part II[6] and the present paper. The value of $(\overline{S,E})$ can thus be evaluated from the standard solute $\underline{R}^\circ$ value for calcined adsorbent, following which α for any other adsorbent activity can be calculated from a standard solute retention volume through the above relationship. This procedure provides the basis of the tabulation and use of the values of α and $(\underline{R}^\circ/V_a)$ given in Table V.

Table XVIII presents some experimental data for the elution of several solutes from Code 62 1 % H_2O–SiO_2 by pentane. As in the first example of the preceding section on "The Prediction... etc.," V_a and α for this adsorbent were calculated as 0.090 and 0.715, respectively. With the adsorbent of Table XVIII thus characterized, the $\underline{R}^\circ$ values in Table XVIII can be calculated by means of eqn. (7) and the S° values of Tables XVI and XVII. The average deviation between these experimental and calculated values in Table XVIII is $\pm$ 0.15 log units, which is somewhat higher than expected for a Code 12 silica. The correlation for Code 62 silica would be improved moderately by assuming that the coefficient 0.113 in eqn. (3) is really 0.14 for Code 62 silica.

It is interesting to note that the adsorbent activity function α for a D-62 4 % H_2O–SiO_2 was measured as 0.70, relative to 0.71 for the same adsorbent with only 1 % added water. This implies that the surface energy of D-62 1 % H_2O–SiO_2 is reasonably constant, since further water deactivation gives insignificant reduction in average surface energy. For D-62 silica, 1 % and 4 % added water corresponds to 10 % and 40 % surface coverage by adsorbed water. By contrast, α for a D-12 silica deactivated with 10 % of a monolayer of adsorbed water (3 % adsorbed water) is 0.80, which declines to a value of 0.68 for deactivation by 40 % of a monolayer of adsorbed water (12 % H_2O).

SPORER AND TRUEBLOOD[4] have reported data for still another silica, a "silicic acid–celite" mixture. Their frontal analysis study of this adsorbent can be reduced to an equivalent LEAC basis by setting their values of K equal to $\underline{R}^\circ$. Only the substituted benzenes eluted by benzene were studied by SPORER AND TRUEBLOOD. Because

ε° for benzene (0.25) is equal to Q°_i for each of the aromatic carbons of the benzene ring, eqn. (3) takes the following form for these data:

$$\log \underline{R}^\circ = \log V_a + \alpha \left[\sum^{i} (Q^\circ_i - 0.25\ \delta_i')\right]$$

where the summation is over the substituents i on the benzene ring, exclusive of ring carbon atoms. SPORER AND TRUEBLOOD have fitted their experimental data to a mathematical expression which can be related to the above equation. From their value K_{ph}, we have V_a equal to 0.3 $\pm$ 0.1. Similarly, their values of K_i for each of the substituents i reported by them are equal to $10^{\alpha(Q^\circ_i - 0.25\ \delta_i')}$. For those substituents studied by both us and the latter authors, we have plotted values of log K_i *versus* $(Q^\circ_i - 0.25\ \delta_i')$ in Fig. 11. The slope (0.71) of this plot is equal to α for their adsorbent. The plot of Fig. 11 shows reasonable agreement between these two independent

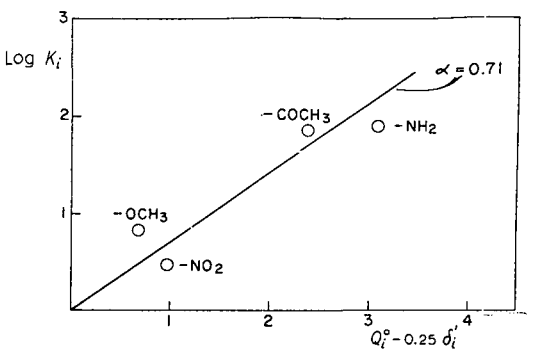

Fig. 11. Comparison of SPORER AND TRUEBLOOD solute group parameters with those of present study.

studies, particularly when it is realized that the use of benzene as eluent by SPORER AND TRUEBLOOD has substracted a large term $(\varepsilon^\circ \delta_i')$ from each Q°_i value. Table XII lists values of Q°_i calculated from the data of SPORER AND TRUEBLOOD with the assumption α equal 0.71 for their adsorbent. The average deviation of their Q°_i values from those reported in the present study is only $\pm$ 11 %, or 0.4 units out of values of Q°_i ranging from 1.8 to 5.4. SPORER AND TRUEBLOOD's values of K_i for substituents not included in the present study have been used in Table VI to extend our list of Q°_i values for silica. The values of V_a and α for their adsorbent (0.3, 0.71) suggest that it is comparable to a D-12 6 % H_2O–SiO_2 (V_a equal 0.24 and α equal 0.71).

A final point of interest relative to adsorbent standardization is the exact duplication of adsorbent columns for routine separation and analysis. The problem has been briefly discussed elsewhere[16]. There are three separate aspects of the adsorbent which require standardization in the general case: V_a, α, and the effect of adsorbent on separation efficiency (HETP or solute band width). The last variable has been treated at length elsewhere[9,12,13,16], and a further discussion will not be attempted at this time. If the surface area of starting calcined adsorbent is reasonably constant from one batch of adsorbent to another, then addition of a given quantity of water to the adsorbent will always give the same values of V_a and α as for the standard adsorbent containing the same amount of adsorbed water. Slight differences in starting surface

energies and areas may be compensated by corresponding changes in the amounts of water added. In any case, the final adsorbent is standardized by adjusting the total added water so that the final adsorbent has the same $\underline{R}°$ value for a standard solute and eluent as does the original standard adsorbent. This procedure has been followed for Alcoa F-20 alumina in Part II[6] and in several recently reported analytical procedures which use this adsorbent[17–19]. STRAUCH[20] has reported an essentially equivalent adsorbent standardization technique, using the measurement of equilibrium values of K rather than $\underline{R}$. Where control of the adsorbent starting surface area within narrow limits is impossible (a phenomenon difficult to imagine), it is possible to standardize the adsorbent with respect to α, and then to compensate variation in V_a by reciprocally varying the weight of adsorbent used in the column.

KLEIN[10] and others[20,21] have reported that adsorbent pore diameter also plays a role in determining adsorbent properties. Where this effect is important, the adsorbent must be similarly matched to a standard in terms of pore diameter. We have been unable to verify the major importance of this variable *per se* in the present studies, other than to confirm that separation efficiency increases with pore diameter as claimed by KLEIN[10]. The next section provides additional comment on this subject.

THE MECHANISM OF ADSORPTION ON SILICA

Some differences (as well as similarities) may be noted in the adsorption of various solutes on silica relative to alumina. These differences are empirically summarized in the differing forms of the correlational equations for alumina, eqn. (1) and for silica, eqn. (3). It remains to comment briefly on the fundamental basis of these differences, a more detailed interpretation being reserved to a latter communication.

The surface structure of silica and its role in the adsorption process have been discussed extensively. It has been claimed that the topography and particularly the pore diameter[10,21,22] of silica samples are important factors in determining the relative adsorption of a solute. This postulate assumes either that the access of a solute to certain parts of the adsorbent surface is limited, or that the effective surface energy of some adsorption regions is reduced by virtue of certain solute geometries. In this connection, consider the 0.113 (n — 6) term of eqn. (3) and the related experimental dependence of $S°$ on n in Fig. 7. For solutes no larger than benzene, the dependence of $S°$ on n is "normal" relative to adsorption on alumina in two respects. First, $S°$ increases linearly with n, implying that each additional aromatic carbon atom contributes an equal increment to the adsorption energy of the solute. Second, the value of this energy increment for silica ($Q°_i$ equal 0.25) is comparable to that for alumina ($Q°_i$ equal 0.31) as expected from the similarity of $Q°_i$ for other solute groups i on the two adsorbents (see Fig. 8). For larger solutes, $S°$ is smaller than expected by analogy with adsorption on alumina, and a previous communication[13] has attributed this phenomenon to the presence of pores of diameter small enough to exclude solutes larger than benzene. This explanation appears ruled out, however, by the behavior of Code 62 silica toward the larger aromatic hydrocarbons. The average pore diameter of this latter adsorbent (170 Å) is larger than that of either the Code 12 silica (22 Å) or the previously studied Alcoa F-20 alumina (79 Å), yet the curvature of the $S°$ *versus* n plot for Code 62 silica is if anything *more* severe than for Code 12; this is shown by the deviation of the experimental data (for Code 62 silica) in Fig. 12 from

the calculated curve (for Code 12 silica). It is now believed that the anomalous adsorption energies of the larger aromatic hydrocarbons on silica are related to a localization effect such as occurs in the adsorption of certain solutes on alumina[8]. Because of some as yet unexplained differences, however, in the two adsorbents, localization affects the $S°$ values of the larger aromatics on silica, and the $S°$ values of solutes with strongly adsorbing groups (large $Q°_i$ values) on alumina. Consequently, the $f(Q°_k) \sum_{i \neq k} Q°_i$ term in the correlation equation for alumina is absent from that for silica.

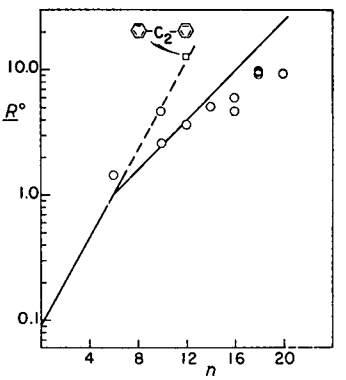

Fig. 12. Retention volumes of aromatic hydrocarbons as a function of carbon number; pentane, eluent; Code 62 1.0% H_2O–SiO_2.

Similarly, it has already been suggested that the large apparent areas δ' of strongly adsorbing groups when adsorbed on silica is related to the localization of these groups (see Fig. 6 and related discussion). This again suggests fundamental differences in the character of the adsorption sites on alumina and on silica. The further analysis of these differences is in progress.

GLOSSARY OF TERMS

D-12, D-62 = Refers to Davison Code Numbers 12 and 62.
$f(Q°_i), f(Q°_k)$ = Localization function for adsorbing group i and k.
$\underline{K}$ = Solute distribution coefficient between adsorbed and non-sorbed phases; $\underline{K} = X_a/X_s$.
$\underline{K}°$ = Value of $\underline{K}$ in linear isotherm region (low coverage).
$\underline{K}'$ = Value of $\underline{K}$ corrected for chemisorbed solute; see Table IV and related discussion.
K_i = Group adsorption factor as defined by SPORER AND TRUEBLOOD[4].
K_{ph} = Value of K_i for benzene ring.
n = Number of aromatic and unsaturated carbon atoms in a solute.
n_b = Value of $\sum_i \delta_i$ for an eluent B.
$Q°_i$ = Solute substituent adsorption parameter.
$q°_j$ = Solute geometry parameter.
r = The number of aromatic ring systems separated by alkyl groups in a solute.

$\underline{R}^\circ$ = Linear equivalent retention volume; ml/g.
$\underline{R}^\circ_1$, $\underline{R}^\circ_2$ = Values of $\underline{R}^\circ$ for a solute using eluents 1 and 2.
$\underline{R}'_2$ = Corrected equivalent retention volume after initial elution by a second eluent, 1; equal to total retention volume measured from point at which eluent 2 is used, minus column volume, divided by adsorbent weight (see experimental section).
S° = Total solute adsorption parameter.
SA = Surface area.
$(\overline{S, E})$ = Solute–eluent combined adsorption parameter.
V_a = Volume of adsorbent monolayer; ml/g.
X_s = Concentration of solute in solution phase; g/ml.
X_B = Mole fraction of stronger solvent B in eluent binary A–B.
α = Adsorbent activity function; adsorbent average surface energy.
δ_i = Substituent surface volume, proportional to surface area.
δ_i' = Apparent substituent surface volume; $\delta_i' = \delta_i$ for alumina; $\delta_i' = \delta_i + 14.6\, f(Q^\circ_i)$ for silica.
ε° = Eluent strength parameter.
ε°_A, ε°_B, ε°_{AB} = Values of ε° for solvent A, solvent B and eluent binary A–B.

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SUMMARY

Linear retention volume data have been obtained for several solutes of widely varied molecular structure, eluted by a number of different eluents, from both high and low surface area silica samples of varying water content. All of these data can be quantitatively correlated with a fundamental equation similar, but not identical, to that previously developed for adsorption on alumina. The major differences which exist between adsorption on alumina and silica appear to arise from fundamental differences in the way solutes *localize* (or orient) around adsorption sites. Adsorbent pore diameter appears relatively unimportant in affecting adsorption affinity and solute retention volume, apart from the general dependence of adsorbent surface area on pore diameter, and with the exception of the combination of large eluents (such as carbon tetrachloride) and small pore diameters. The standardization of adsorbents for routine separation purposes has been discussed in detail.

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DIE DÜNNSCHICHTCHROMATOGRAPHIE DER KETOSÄUREN

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EINLEITUNG

Zur Trennung der Ketosäuren wurden eine Anzahl von papierchromatographischen Methoden entwickelt[1]. Infolge der Labilität einiger Ketosäuren kommen die zur Trennung freier Säuren angewandten Methoden[2,3] nicht mehr stark in Frage, sondern die Ketosäuren werden zur Papierchromatographie in Abkömmlinge, üblicherweise in 2,4-Dinitrophenylhydrazone (DNP), umgewandelt. Als mobile Phasen werden entweder neutrale oder alkalische, alkoholhaltige Lösungsmittel angewendet[4–7]. Da die DNP-Abkömmlinge der Ketosäuren in zwei isomeren Formen auftreten können, die bei der Papierchromatographie als getrennte Flecken in Erscheinung treten, wirkt dies auf die Identifizierung der Verbindungen erschwerend. Einige Autoren haben aus den DNP-Abkömmlingen der Ketosäuren durch Hydrierung die entsprechenden Aminosäuren hergestellt und die so erhaltenen Aminosäuren identifiziert[1].

In den der vorliegenden Arbeit zugrunde liegenden Untersuchungen wurde die Entwicklung passender Lösungsmittelsysteme und Chromatographiermethoden für die dünnschichtchromatographische Trennung der DNP-Abkömmlinge folgender Ketosäuren angestrebt: α-Keto-β-methylvaleriansäure, α-Ketoisocapronsäure, α-Ketoisovaleriansäure, α-Ketobuttersäure, Brenztraubensäure, Lävulinsäure, α-Ketoglutarsäure und Oxalessigsäure.

EXPERIMENTELLES

Material

α-Keto-β-methylvaleriansäure wurde aus Isoleucin und α-Ketoisocapronsäure aus Leucin durch Oxydation dargestellt[8]. Die übrigen Ketosäuren wurden fertig bezogen und entsprachen dem geforderten Reinheitsgrad. Zur Herstellung der DNP-Abkömmlinge wurde zur wässerigen Lösung jeder Ketosäure 2,4-Dinitrophenylhydrazinlösung (DN-Lösung) im Überschuss zugefügt; die DN-Lösung enthielt 2.5 g DN/1000 ml 2 *N* HCl. Nach der Reagenszugabe liess man bei Zimmertemperatur über Nacht stehen. Der DNP-Niederschlag wurde abfiltriert, mit 2 *N* HCl und Wasser gewaschen und über Phosphorpentoxyd im Exsiccator getrocknet.

Aufarbeitung des Materials

Die Löslichkeit der DNP-Abkömmlinge der Ketosäuren in 2 *N* HCl ist derart gross, dass aus Lösungen mit nur kleinen Ketosäurekonzentrationen überhaupt kein DNP-Niederschlag erhalten werden kann. Zur Isolierung der Hydrazone der Ketosäuren wurden diverse Extraktionsverfahren entwickelt[9–11]. Da in den meisten Fällen die

Extraktionsverfahren besonders bei der Untersuchung der Ketosäuren von Naturprodukten eingesetzt werden müssen, wurde der DNP-Niederschlag jeder Ketosäure mit den gleichen Lösungsmitteln und nach dem gleichen Verfahren behandelt wie bei der Extraktion[11]. Aus den so behandelten DNP-Niederschlägen wurden Lösungen hergestellt, bei denen als Lösungsmittel ein Gemisch von Dioxan (E. Merck, pro anal.) und 0.067 *M* Phosphatpufferlösung (pH 7.2) im Volumenverhältnis 1:1 diente. Die Konzentration der Lösungen betrug 5 mg DNP-Niederschlag/5 ml Lösungsmittel. ALTMANN *et al.*[12] haben als Lösungsmittel für die DNP-Abkömmlinge der Ketosäuren — beim Auftragen der Startflecken auf das Papier — 0.2 *M* Phosphatpufferlösung (pH 7.2) gebraucht. Nach ihren Beobachtungen verursachen organische Lösungsmittel einen Abbau der DNP-Abkömmlinge der Ketosäuren, insbesondere ein Decarboxylieren derselben. Da jedoch die DNP-Abkömmlinge einiger Ketosäuren in blosser Phosphatpufferlösung schwer löslich sind, wurde von uns als Lösungsmittel für die Niederschläge das vorgenannte Gemisch von Dioxan und Phosphatpufferlösung angewandt.

Bei den DNP-Abkömmlingen der Ketosäuren können auch durch pH-Änderungen, die das Extrahieren mit sich bringt, gewisse Komplikationen verursacht werden, so z.B. das Isomerisieren bestimmter Komponenten[13]. Aus diesem Grunde wurden für vergleichende Untersuchungen auch Lösungen von nicht mit den Extraktionslösungsmitteln behandelten DNP-Niederschlägen angefertigt; als deren Lösungsmittel kam das früher erwähnte Dioxan–Phosphatpuffer-Gemisch zur Anwendung.

Die Dünnschichtmethode

Die Glasplatte (20 × 20 cm) wird mit Kieselgel G (E. Merck, für Dünnschichtchromatographie nach Stahl) beschichtet. Zur Beschichtung werden 30 Kieselgel G in 65 ml Wasser suspendiert. Bei einigen Versuchen wurde an Stelle von Wasser eine Lösung verwendet, die 5 ml Propionsäure und 60 ml Wasser enthielt. Bei der Beschichtung der Platten wurden zwei verschiedene Schichtdicken gewählt. Die beschichteten Platten wurden während ca. 30 Min. bei 110–120° aktiviert. In einem Abstand von 11.5 cm vom unteren Plattenrand ritzten wir in die Kieselgelschicht eine Querlinie. Die zu untersuchenden Verbindungen wurden auf die noch heissen Platten aufgetragen, die Platten liess man darauf abkühlen und während ca. 30 Min. in einer geschlossenen, mobile Phase enthaltenden, Kammer sättigen; während dieser Zeit ruhten die Platten auf speziellen gläsernen Unterlagen. Wenn darauf die Kammer schräg gestellt wurde, kam die Platte mit der mobilen Phase in Berührung und man liess diese bis zur Querlinie aufsteigen. Die Laufdauer betrug zwischen 35 und 40 Min. Bei einigen Versuchen, auf deren Platten keine Querlinien gezogen worden waren, liess man die mobile Phase bis zum oberen Plattenrand aufsteigen, die Laufzeit betrug dann beinahe zwei Stunden.

Lösungsmittelsysteme und Durchführung der Dünnschichtchromatographie

Von den erprobten Lösungsmitteln wurde das Gemisch aus Petroläther (BDH, Sdp. 60–80°) und Äthylformiat (Fluka, puriss.) im Volumenverhältnis von 13:7 einer näheren Untersuchung unterworfen. Mit diesem Lösungsmittelgemisch liefen alle zu untersuchenden Komponenten, doch zeigte es sich, dass der Gehalt des verwendeten Äthylformiates an freier Säure bei verschiedenen Lieferungen unterschiedlich war

und dies somit die Reproduzierbarkeit der Versuchsresultate ungünstig beeinflusste. Als das Lösungsmittelgemisch zwecks Neutralisierung mit $NaHCO_3$ geschüttelt wurde, hatte das zur Folge, dass die Komponenten überhaupt nicht mehr liefen. Das Lösungsmittel muss also freie Säure enthalten. Aus diesem Grunde wurde eine Versuchsserie angesetzt, bei der zu je 100 ml neutralisiertem Lösungsmittelgemisch vor der Dünnschichtchromatographie verschieden grosse Ameisensäuremengen (E. Merck, pro anal.) zugesetzt wurden. An Stelle von Ameisensäure nahmen wir auch Essigsäure (Fluka, puriss.), Buttersäure (Fluka, puriss.), Propionsäure (Fluka, puriss.) und Valeriansäure (Fluka, puriss.).

Da bei der Extraktion der Hydrazone oft kleine Mengen freies Dinitrophenylhydrazin mit extrahiert werden, wurde bei allen Dünnschichtentwicklungen als Vergleichssubstanz eine Lösung von 2,4-Dinitrophenylhydrazin in einem Gemisch von Dioxan und Phosphatpuffer (pH 7.2) im Verhältnis 1:1 verwendet. Bei den fertigen Chromatogrammen wurden die zurückgelegten Laufstrecken der Hydrazon- und der Hydrazinflecken ausgemessen. In Tabelle I werden die Verhältnisse der Laufstrecken — berechnet in Bezug auf diejenige des 2,4-Dinitrophenylhydrazins — oder R_B-Werte dargestellt. Beim Auftreten von Isomeren wurde nur die dominierende Komponente für die Berechnung in Betracht gezogen. In die Tabelle I sind ebenfalls die verwendeten mobilen Phasen und die mit einem Cord-Meter an sechs verschiedenen Stellen der Platte gemessenen Mittelwerte der Schichtdicke aufgenommen. Die durchschnittliche Abweichung von der mittleren Schichtdicke war bei jeder einzelnen Platte kleiner als $\pm$ 0.01 mm, jedoch war der Unterschied in der Schichtdicke erheblich grösser in Bezug auf die nach gleicher Art hergestellten Platten untereinander, wie aus Tabelle I hervorgeht. Die Fig. 1 und 2 zeigen zwei Chromatogramme der Tabelle I.

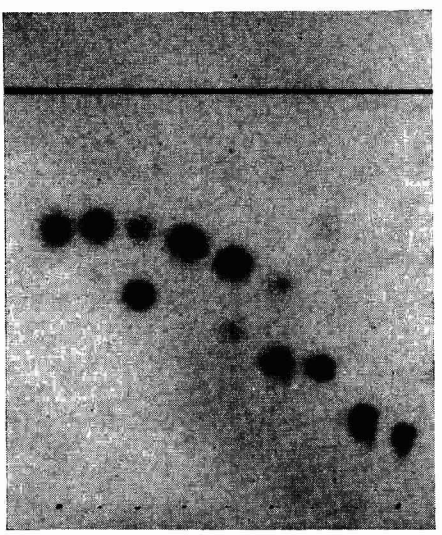


Fig. 1. Das mit Petroläther–Äthylformiat/Ameisensäure (100 ml/0.052 mol.) als mobile Phase von den DNP-Komponenten der Ketosäuren erhaltene Dünnschichtchromatogramm. Substanzmenge in den Startflecken 2 μg. Komponenten: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure. V: 2,4-Dinitrophenylhydrazin.

TABELLE I

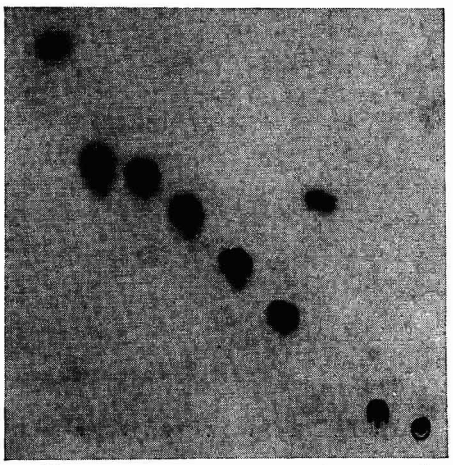
R_B-WERTE* DER DNP-KOMPONENTEN DER KETOSÄUREN ERHALTEN BEI DÜNNSCHICHTCHROMATOGRAPHIE MIT DER MOBILEN PHASE PETROLÄTHER–ÄTHYLFORMIAT/SÄURE

Komponenten: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure. Die Bezeichnung † nach dem R_B-Wert besagt, dass der Flecken des schwächeren Isomers *oberhalb* des dominierenden Fleckens und die Bezeichnung $^+$, dass der Flecken des schwächeren Isomers *unterhalb* des dominierenden Fleckens auftrat.

Lösungsmittel/Säure/Schichtdicke			R_B-*Werte*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
100 ml/0.013 mol.	Ameisensäure	/0.12	0.84	0.58†	0.74	0.64$^+$	0.34†	0.31	0.07	0.03
100 ml/0.026 mol.	Ameisensäure	/0.08	0.95	0.64†	0.85	0.75$^+$	0.39†	0.34	0.15	0.08
100 ml/0.052 mol.	Ameisensäure	/0.08	1.02	0.76†	0.95	0.88$^+$	0.52†	0.50	0.32	0.25
100 ml/0.104 mol.	Ameisensäure	/0.10	1.00$^+$	0.79†	0.95$^+$	0.89$^+$	0.55†	0.57	0.42	0.37
100 ml/0.026 mol.	Essigsäure	/0.10	0.51	0.49	0.43	0.34	0.24	0.43	0.03	0.01
100 ml/0.052 mol.	Essigsäure	/0.08	0.74	0.69†	0.65	0.56	0.41†	0.55	0.08	0.03
100 ml/0.103 mol.	Essigsäure	/0.09	0.83	0.81†	0.79	0.68$^+$	0.51†	0.66	0.20	0.05
100 ml/0.206 mol.	Essigsäure	/0.11	0.93	0.89†	0.83	0.76$^+$	0.57†	0.68	0.28	0.13
100 ml/0.026 mol.	Propionsäure	/0.10	0.26	0.30	0.20	0.15	0.13	0.36	0.02	0.01
100 ml/0.052 mol.	Propionsäure	/0.11	0.42	0.44	0.35	0.26	0.22	0.48	0.02	0.01
100 ml/0.104 mol.	Propionsäure	/0.07	0.70	0.67	0.57	0.44	0.30	0.58	0.06	0.02
100 ml/0.207 mol.	Propionsäure	/0.09	0.86	0.83†	0.75	0.66$^+$	0.50†	0.71	0.18	0.04
100 ml/0.104 mol.	Buttersäure	/0.09	0.48	0.49	0.41	0.32	0.24	0.53	0.03	0.02
100 ml/0 208 mol.	Buttersäure	/0.04	0.85	0.77†	0.71	0.59	0.45†	0.65	0.07	0.04
100 ml/0.416 mol.	Buttersäure	/0.10	0.86	0.78†	0.75	0.64	0.44†	0.73	0.18	0.07
100 ml/0.104 mol.	Valeriansäure	/0.12	0.44	0.44	0.35	0.25	0.18	0.48	0.03	0.02
100 ml/0.208 mol.	Valeriansäure	/0.06	0.65	0.59†	0.55	0.45$^+$	0.29†	0.61	0.06	0.02
100 ml/0.416 mol.	Valeriansäure	/0.11	0.64	0.68†	0.58	0.45$^+$	0.34†	0.68	0.11	0.04

* R_B = Laufstrecke des DNP-Fleckens der Ketosäure/Laufstrecke des Dinitrophenylhydrazinfleckens.

Bei den Dünnschichtchromatogrammen der Tabelle I wurde neben den einzelnen Komponenten immer auch ein Gemisch aller acht Komponenten gleichzeitig chromatographiert. Eine befriedigende Trennung konnte jedoch nicht erreicht werden. Aus diesem Grunde wurden Versuche angestellt, bei denen eine Änderung der Ad-



V 1 2 3 4 5 6 7 8

Fig. 2. Das mit Petroläther–Äthylformiat/Propionsäure (100 ml/0.104 mol.) als mobile Phase von den DNP-Komponenten der Ketosäuren erhaltene Dünnschichtchromatogramm. Substanzmenge in den Startflecken 2 μg. Komponenten: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure. V: 2,4-Dinitrophenylhydrazin.

sorbtions-Eigenschaften des Kieselgel G durch Veränderung der Schichtdicke und durch Zusatz von Säure zur Streichmasse angestrebt wurde. In Tabelle II werden die R_B-Werte der DNP-Komponenten der Ketosäuren — berechnet in Bezug auf das 2,4-Dinitrophenylhydrazin — der Versuche wiedergegeben, bei denen sowohl neutrale

TABELLE II

R_B-WERTE* DER DNP-KOMPONENTEN DER KETOSÄUREN ERHALTEN BEI DÜNNSCHICHTCHROMATOGRAPHIE MIT PETROLÄTHER–ÄTHYLFORMIAT/PROPIONSÄURE (100 ml/0.104 mol.) ALS MOBILE PHASE UND MIT NEUTRALER, SOWIE SAURER STREICHMASSE BEI VERSCHIEDENEN SCHICHTDICKEN

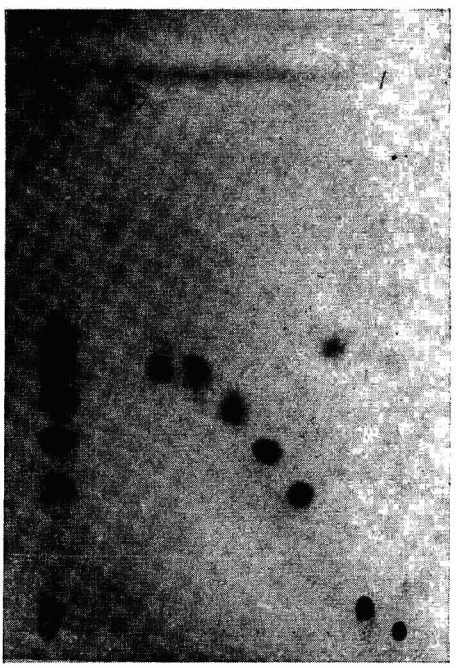
Komponenten: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure

Schichtdicke (mm)	*Streichmasse*	*R_B-Werte*							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
0.10	neutral	0.58	0.56	0.59	0.48	0.28	0.56	0.05	0.02
0.10	sauer	0.48	0.48	0.39	0.29	0.20	0.49	0.02	0.02
0.16	neutral	0.50	0.51	0.43	0.35	0.24	0.55	0.04	0.01
0.17	sauer	0.45	0.42	0.37	0.30	0.20	0.52	0.02	0.00

* R_B = Laufstrecke des DNP-Fleckens der Ketosäure/Laufstrecke des Dinitrophenylhydrazinfleckens.

(65 ml Wasser/30 g Kieselgel G) als auch saure Streichmasse (60 ml Wasser/5 ml Propionsäure/30 g Kieselgel G) bei zwei verschiedenen Schichtdicken verwendet wurden. Die Schichtdicken wurden an sechs verschiedenen Stellen jeder Platte gemessen. Die in Tabelle II mitgeteilten Schichtdicken stellen Mittelwerte der Messungen dar. Der mittlere Fehler des Schichtdickenmittelwertes bei den dünneren Schichten war kleiner als $\pm$ 0.01 mm, bei den dickeren Schichten jedoch bewegte er sich in der Grössenordnung von $\pm$ 0.01 mm. Als mobile Phase wurde das beim Chromatogramm der Fig. 2 verwendete Lösungsmittelgemisch gebraucht, da dabei das Fehlen von Isomeren die Trennung vereinfacht. Es sei in diesem Zusammenhang bemerkt, dass das erwähnte Lösungsmittelgemisch auch zur Trennung einiger anderer DNP-Komponenten angewendet werden kann und man z.B. von den DNP-Abkömmlingen des Furfurals bei Anwendung dieses Lösungsmittels als mobile Phase bei der Dünnschichtchromatographie nur einen einzigen Flecken erhält, während sich mit einigen anderen Lösungsmitteln sowohl bei der Dünnschicht-, wie auch bei der Papierchromatographie zwei zueinander isomere Flecken ausbilden[14].

Aus dem Gemisch der Komponenten 1–8 trennten sich die einzelnen Komponenten am besten bei der letzten Entwicklung der Tabelle II, bei der also die Schichtdicke vergrössert und die Streichmasse sauer war. Dabei liefen die Komponenten 1



M V 1 2 3 4 5 6 7 8

Fig. 3. Das mit Petroläther–Äthylformiat/Propionsäure (100 ml/0.104 mol.) als mobile Phase von den DNP-Komponenten der Ketosäuren erhaltene Dünnschichtchromatogramm. Die Substanzmenge jeder Komponente im Startflecken betrug 1 μg, die Streichmasse war sauer und die Schichtdicke betrug 0.17 $\pm$ 0.01 mm. Komponenten: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure. V: 2,4-Dinitrophenylhydrazin. M: Gemisch der Komponenten plus V.

und 2 gleichartig, jedoch trennte sich die einzige γ-Ketosäure des Gemisches, die Lävulinsäure, aus dem Gemisch als separater Flecken. Hingegen trennte sie sich bei den übrigen Entwicklungen der Tabelle II nicht als separater Flecken, obwohl auf Grund der R_B-Werte erwartet werden konnte, dass sie vor den anderen auch in der zweitletzten Entwicklung der Tabelle II laufen würde. Fig. 3 zeigt das der letzten Entwicklung der Tabelle II entsprechende Dünnschichtchromatogramm. Auf der Kieselgelschicht wurde keine Querlinie gezogen, sondern man liess das Lösungsmittel bis zum oberen Plattenrand aufsteigen.

Über den Abbau der DNP-Komponenten der Ketosäuren in Dioxan–Phosphatpuffer-Lösung

Bei den vorher beschriebenen Versuchen wurden die DNP-Niederschläge der Ketosäuren zuerst mit Extraktionslösungsmittel[11] behandelt und danach — zwecks Auftragung auf die Dünnschichtplatte — in einem 1:1-Gemisch von Dioxan und 0.067 *M* Phosphatpufferlösung (pH 7.2) aufgelöst. Wenn die Chromatographierung mit frischen, höchstens während zwei Tagen bei Raumtemperatur gestandenen Lösungen der DNP-Niederschläge durchgeführt wurde, konnte einzig oberhalb des Lävulinsäurefleckens ein sehr schwacher weiterer Flecken erkannt werden (in den Fig. 1 und 2 mit Kreisen markiert). Wenn der in Fig. 2 gezeigte Versuch mit Lösungen der DNP-Niederschläge, die während einer Woche bei Raumtemperatur gestanden hatten, wiederholt wurde, trat ein weiterer Flecken auch oberhalb des Oxalessigsäurefleckens auf, wie aus Fig. 4 ersehen werden kann. Es tritt offenbar eine Zersetzung der genannten Substanzen ein.

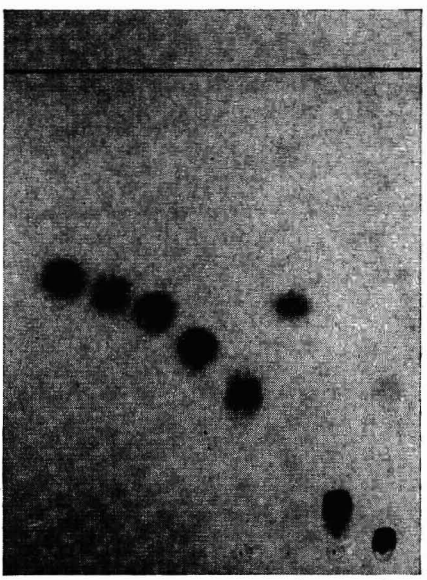


Fig. 4. Wiederholung des in Fig. 2 gezeigten Versuches, nachdem die Lösungen der DNP-Komponenten der Ketosäuren während einer Woche bei Raumtemperatur gestanden hatten. DNP-Komponenten der Ketosäuren: (1) α-Keto-β-methylvaleriansäure; (2) α-Ketoisocapronsäure; (3) α-Ketoisovaleriansäure; (4) α-Ketobuttersäure; (5) Brenztraubensäure; (6) Lävulinsäure; (7) α-Ketoglutarsäure; (8) Oxalessigsäure.

Aus Fig. 4 kann ersehen werden, dass das sich aus dem DNP-Abkömmling der Oxalessigsäure gebildete Abbauprodukt auf gleiche Weise wandert, wie der DNP-Abkömmling der Brenztraubensäure. Die gleiche Beobachtung konnte auch bei anderen, in Tabelle I erwähnten mobilen Phasen gemacht werden. Es ist demnach offensichtlich, dass der DNP-Abkömmling der Oxalessigsäure in der Dioxan–Phosphatpuffer-Lösung langsam decarboxyliert. Als vergleichende Versuche mit solchen DNP-Niederschlägen der Ketosäuren angestellt wurden, die nach der Fällung nicht mit Extraktionslösungsmittel behandelt worden waren, und als Lösungsmittel zur Auftragung der Niederschläge auf die Platten wiederum ein Gemisch von Dioxan und Phosphatpuffer zur Anwendung kam, zeigten sich keine — auch nach Stehenlassen der Lösung — der aus Fig. 4 ersichtlichen Abbauprodukte der Komponenten. Dies weist darauf hin, dass der Grund für den Abbau bei der Extraktionsbehandlung gesucht werden muss.

BESPRECHUNG DER RESULTATE

Mit neutralisiertem Gemisch von Petroläther–Äthylformiat (13:7) als mobile Phase blieben die DNP-Komponenten der Ketosäuren am Startflecken. Art und Menge der der mobilen Phase zugesetzten Säuren beeinflusst sowohl die in Bezug auf das 2,4-Dinitrophenylhydrazin berechneten R_B-Werte wie auch die sich während der Entwicklung aus den Komponenten bildenden Flecken (Tabelle I). Mit der Zunahme der Säurekonzentration der mobilen Phase wachsen auch die R_B-Werte, bei gleicher molarer Konzentration aber wachsendem Molgewicht der Säuren vermindern sich jedoch die R_B-Werte (Tabelle I). Wenn die mobile Phase auch nur geringe Mengen an Ameisensäure enthält, trennen sich von den meisten Komponenten während der Entwicklung zwei aufeinanderfolgende Flecken ab — sie stellen zwei einander isomere Verbindungen dar (Tabelle I, Fig. 1). Wenn an Stelle von Ameisensäure deren höhere Homologen verwendet wurden, geschah bei geringen Säurekonzentrationen keine Abtrennung von Isomeren (Tabelle I, Fig. 2). Im Allgemeinen kann beobachtet werden, dass mit der Erhöhung der Säurekonzentration der mobilen Phase auch die Anzahl der Komponenten zunimmt, aus denen sich Isomere bilden. Demnach ist es möglich, dass die in der mobilen Phase enthaltene Säure, sofern deren Konzentration und Stärke ausreichend sind, im Verlaufe der Entwicklung eine Isomerisierung einzelner Komponenten verursacht. Andrerseits besteht die Möglichkeit, dass die Extraktionsbehandlung der DNP-Abkömmlinge mit der damit verbundenen pH-Änderung — wie einige Autoren beobachten konnten[13] — oder das bei der Auflösung der Niederschläge gebrauchte Lösungsmittel eine Isomerisierung der Komponenten verursachen kann. Bei bestimmten Komponenten wären dabei beide Isomere schon im Startflecken vorhanden, aber mit mobilen Phasen, durch welche die Isomere nicht getrennt werden, bliebe ihre Wanderungsgeschwindigkeit die gleiche. Von den Isomeren ist immer eine Komponente die dominierende. Die dominierende Komponente der Brenztraubensäure und der α-Ketoisocapronsäure wandert langsamer, diejenige der α-Ketobuttersäure, α-Ketoisovaleriansäure und der α-Keto-β-methylvaleriansäure dagegen schneller als ihr Isomer (Tabelle I, Fig. 1).

Oberhalb des Lävulinsäurefleckens erscheint in den Fig. 1 und 2 ein sehr schwacher Flecken (in den Figuren mit Kreisen markiert). Da das Auftreten des Fleckens von der Art der mobilen Phase unabhängig ist, scheint nicht ein Flecken des Isomers, sondern der eines Abbauproduktes in Frage zu stehen. Wenn man die

Lösungen der DNP-Niederschläge bei Raumtemperatur während einer Woche stehen lässt, zeigt sich bei der Dünnschichtentwicklung ein Flecken eines Abbauproduktes ebenfalls oberhalb des Fleckens der Oxalessigsäure (Fig. 4). Dieses Abbauprodukt wandert bei der Dünnschichtentwicklung auf gleiche Weise wie der DNP-Abkömmling der Brenztraubensäure und wurde demnach als Folge der Decarboxylierung der Oxalessigsäure gebildet. Falls die DNP-Niederschläge der Ketosäuren nicht mit Extraktionslösungsmittel behandelt wurden und man zwecks Auftragung auf die Platte als Lösungsmittel für die Niederschläge, wie bei den anderen Versuchen, ein Gemisch von Dioxan und Phosphatpuffer anwendet, können bei der Dünnschichtentwicklung keine der vorher erwähnten Abbauprodukte festgestellt werden, woraus der Schluss gezogen werden kann, dass die Extraktionsbehandlung auf den Abbau der DNP-Komponenten der Lävulin- und der Oxalessigsäure einen bestimmten Einfluss ausübt.

Eine Vergrösserung der Schichtdicke verkleinert den R_B-Wert aller Komponenten. Der R_B-Wert der einzigen γ-Ketosäure, der Lävulinsäure, verändert sich bei der Vergrösserung der Schichtdicke prozentual am wenigsten (Tabelle II). Falls der Streichmasse Propionsäure zugegeben wird, wirkt dieser Zusatz auf die R_B-Werte verkleinernd. Der Vorteil eines Zusatzes von Propionsäure zur Streichmasse und einer grösseren Schichtdicke besteht darin, dass die einzelnen Komponenten aus dem Gemisch besser getrennt werden (Fig. 3). Dabei wandern nur die DNP-Abkömmlinge der α-Keto-β-methylvaleriansäure und der α-Ketoisocapronsäure zusammen, während die Trennung der anderen befriedigend verläuft.

ZUSAMMENFASSUNG

Zur Trennung der 2,4-Dinitrophenylhydrazone von acht Ketosäuren wurde eine dünnschichtchromatographische Methode entwickelt. Bei Verwendung der mobilen Phase Petroläther–Äthylformiat/Monocarbonsäure nahmen die R_B-Werte der Komponenten — berechnet in Bezug auf das 2,4-Dinitrophenylhydrazin — zu, wenn die Säurekonzentration der mobilen Phase grösser, oder das Molekulargewicht der der mobilen Phase zugesetzten Säure kleiner wurde. Falls die mobile Phase auch nur in geringem Masse Ameisensäure enthielt, trennten sich bei einigen Komponenten im Verlaufe der Entwicklung zwei aufeinanderfolgende Isomerenflecken ab. Mit den höheren Homologen der Ameisensäure konnte bei geringen Säurekonzentrationen aus den Komponenten keine Abtrennung von Isomeren konstatiert werden. Bei Vergrösserung der Säurekonzentration der mobilen Phase nahm auch die Anzahl der Komponenten zu, die zwei Flecken lieferten. Als Folge der Extraktionsbehandlung der Niederschläge waren einige Komponenten in ihren Lösungen nicht stabil. Die Vergrösserung der Schichtdicke wie auch ein Zusatz von Säure zur Streichmasse haben ein Absinken der R_B-Werte zur Folge. Durch Ansäuren der Streichmasse und mit einer dickeren Schicht von Kieselgel G konnte die Trennung der einzelnen Komponenten aus dem Gemisch verbessert werden.

SUMMARY

A method of thin-layer chromatography was developed for the separation of the 2,4-dinitrophenylhydrazones of eight keto-acids. When petroleum ether–ethyl formate/

monocarboxylic acid was used as the solvent system higher R_B values of the components—measured with reference to 2,4-dinitrophenylhydrazine—were obtained with increasing acid concentration or with decreasing molecular weight of the acid in the solvent system. If the mobile phase contained even small amounts of formic acid, some of the compounds gave two successive spots due to isomers. With higher homologues of formic acid no separation of isomers could be observed when the acid concentration was low. When the concentration was raised, the number of components that gave two spots also increased. Owing to the extraction procedure to which the precipitates were subjected some of the compounds were not stable in solution. A thicker layer of Kieselgel G and also addition of acid to the adsorbent–water mixture before coating caused the R_B values to decrease. The separation of the individual keto-acids from the mixture could be improved by acidifying the spreading mixture and using a thicker layer.

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THE SEPARATION OF ALKALOIDS ON PAPER IMPREGNATED WITH ZIRCONIUM PHOSPHATE

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Chromatography on ion-exchange resins has been used comparatively rarely for the separation of alkaloids. A number of separations, concentrations and purifications have been carried out (for a review see ref. 1), but in most cases the adsorption of the alkaloids is so strong that organic solvents are necessary for eluting them from the resin.

It was shown recently by CATELLI[2] that amino acids may be separated on paper impregnated with zirconium phosphate in a manner analogous to separations effected on cellulose phosphate paper. Since zirconium phosphate has exchangeable phosphoric acid groups like an organic resin but lacks the organic network of the resin, we thought that novel separation effects might be possible on zirconium phosphate, *i.e.* separations which cannot be obtained on organic resins where adsorption is a contributing factor or on inorganic exchangers, such as Florisil, which are unstable in acid solutions.

As shown in the experimental part below, the study of 35 alkaloids revealed numerous possibilities of separation, and it was found that paper impregnated with zirconium phosphate may also be used as a novel technique for the chromatographic identification of a number of alkaloids.

EXPERIMENTAL

Preparation of paper impregnated with zirconium phosphate

The method of preparation proposed by ALBERTI AND GRASSINI[3] was used with two modifications. In order to obtain a very uniform loading with zirconium oxychloride the solution was allowed to enter the paper by ascending chromatographic development instead of dipping the paper into a solution. The second modification was brought about by the desirability of examining papers of different exchange capacities. Thus not only a 30% zirconyl chloride solution in 4*N* HCl was used to impregnate the paper, but also 5%, 10%, 15%, 20% and 25% solutions, giving papers with relative capacities varying as to the amount of zirconyl phosphate in the solution employed. Subsequent drying, dipping into phosphoric acid, washing and drying were then performed as described by ALBERTI AND GRASSINI[3].

Chromatography of alkaloids

Table I shows the R_F values of some alkaloids on papers of different capacities and using aqueous acetic acid and hydrochloric acid as solvents. The R_F values on pure cellulose paper (Whatman No. 1) are also given for comparison. Development was carried out by the ascending method after equilibration for 24 hours.

The alkaloids were spotted on the paper as bases or chlorides in ethanol or aqueous solution. After development the papers were sprayed with Dragendorff reagent yielding orange spots on a yellow background.

TABLE I

R_F VALUES OF ALKALOIDS ON PAPER IMPREGNATED WITH ZIRCONIUM PHOSPHATE

Conditions: ascending development in containers equilibrated for 24 h at 28° ± 1°. The alkaloids were spotted on the paper as 1 % solutions in ethanol (when a salt was employed, the type is indicated in the table). Reagent: the papers were dipped into Dragendorff's reagent. Paper: T = Whatman No. 1 paper run as control. 5 %, 10 % refers to the degree of impregnation. For details see text.

Alkaloid	*R_F values*										
	10 % Acetic acid							*1 N Hydrochloric acid*			
	T	*5 %*	*10 %*	*15 %*	*20 %*	*25 %*	*30 %*	*T*	*5 %*	*15 %*	*30 %*
Morphine	0.90	0.36	0.24	0.22	0.20	0.12	0.13	0.81	0.80	0.73	0.58
Apomorphine	0.64	0.12	0.06	0.10	0.10	0.04	0.03	0.36	0.32	0.30	0.26
Heroine	0.95	0.33	0.22	0.20	0.22	0.11	0.15	0.86	0.82	0.73	0.62
Papaverine	0.87	0.27	0.16	0.13	0.09	0.04	0.08	0.71	0.60	0.44	0.30
Hydrastine	0.88	0.15	0.10	0.06	0.07	0.00	0.00	0.81	0.62	0.58	0.43
Quinine	0.88	0.03	0.00	0.00	0.00	0.00	0.00	0.88	0.73	0.56	0.38
Cinchonine	0.90	0.03	0.00	0.00	0.00	0.00	0.00	0.89	0.73	0.54	0.37
Cinchonidine	0.91	0.04	0.00	0.00	0.00	0.00	0.00	0.90	0.73	0.58	0.37
Tropine	0.90	0.55	0.40	0.22	0.19	0.18	0.17	0.92	0.91	0.80	0.64
Atropine	0.92	0.42	0.25	0.22	0.19	0.18	0.16	0.87	0.88	0.79	0.59
Homatropine	0.91	0.42	0.25	0.24	0.19	0.18	0.16	0.90	0.87	0.83	0.64
Cocaine	0.88	0.58	0.34	0.33	0.29	0.26	0.20	0.84	0.83	0.76	0.65
Tropacocaine HCl	0.89	0.36	0.27	0.18	0.16	0.17	0.13	0.83	0.78	0.73	0.68
Hyoscyamine	0.90	0.42	0.30	0.25	0.19	0.25	0.14	0.89	0.88	0.84	0.66
Veratrine	0.87	0.80	0.61	0.68	0.58	0.49	0.48	0.79	0.80	0.76	0.70
Protoveratrine	0.94	0.89	(1) 0.71 (2) 0.90	(1) 0.78 (2) 0.92	(1) 0.64 (2) 0.94	(1) 0.58 (2) 0.78	(1) 0.60 (2) 0.92	0.84	0.91	0.92	0.79
Mescaline HCl	0.92	0.55		0.41			0.22	0.58	0.90	0.81	0.80
Ephedrine	0.77	0.63		0.47			0.30	0.60	0.91	0.80	0.72
Eserine	0.93	0.41		0.27			0.13	0.59	0.93	0.77	0.51
Ergotamine tartrate	0.64	0.03		0.02			0.00	0.31	0.27	0.16	0.17
Yohimbine HCl	0.74	0.40		0.26			0.15	0.60	0.62	0.55	0.53
Colchicine	0.83	0.83		0.72			0.67	0.80	0.71	0.73	0.58
Harmine	0.62	0.04		0.03			0.02	0.28	0.21	0.19	0.11
β-Erythroidine HCl	0.90	0.21		0.16			0.06	0.92	0.81	0.75	0.55
Caffeine	0.84	0.73		0.69			0.51	0.83	0.67	0.64	0.48
Theophylline	0.77	0.71		0.70			0.50	0.71	0.69	0.61	0.55
Emetine	0.90	0.05		0.02			0.00	0.83	0.77	0.80	0.64
Solanine	0.88	0.84		0.69			0.53	0.81	0.82	0.70	
Arecoline	0.92	0.45		0.26			0.16	0.92	0.92	0.86	0.76
Aspidospermine	0.88	0.59		0.28			0.14	0.84	0.80	0.75	0.72
Diaboline	0.86	0.38		0.17			0.15	0.85	0.84	0.74	0.66
Brucine	0.81	0.17		0.07			0.05	0.69	0.68	0.54	0.49
Vomicine	0.76	0.52		0.20			0.19	0.63	0.65	0.57	0.53
Strychnine nitrate	0.80	0.13		0.08			0.04	0.74	0.66	0.57	0.53
Jervine	0.76	0.28		0.18			0.12	0.96	0.95	0.87	

DISCUSSION

Most of the alkaloids show a negligible adsorption on cellulose (especially from acetic acid), thus the adsorption can in most cases be credited to the effect of the zirconium phosphate. Depending on the capacity of the paper, the R_F values may be varied over a wide range, thus indicating excellent possibilities both for column and thin-layer separations with zirconium phosphate–cellulose mixtures.

The method permits separation of several alkaloids belonging to the same group, *e.g.* atropine, cocaine, tropacocaine, hyoscyamine (especially with 5 % paper) the separations depending on the pK values of the substances.

In the group of strychnine alkaloids the separation of strychnine and its dimethyl-derivative brucine is rather poor because the two alkaloids have the same fundamental structure of the non-indolic nitrogen group, whereas a good separation of vomicine and diaboline is obtained. The latter, which are separated only with difficulty by paper chromatography with the common solvents, possess different structures as reported by MARINI-BETTÒLO[4] and CAGGIANO AND MARINI-BETTÒLO[5].

Better results are generally obtained with the 5 % paper than with other papers, and acetic acid generally gives better resolution than N HCl.

In the case of quinine, cinchonine and cinchonidine no displacement was observed on zirconium phosphate in acetic acid. This fact can be attributed to the tendency of quinine salts to form insoluble complexes in acetic acid with several metal ions whereas these are unstable in HCl. The same effect is also obtained in acetic acid with harmine, emetine and ergotamine.

SUMMARY

The chromatographic separation has been studied of 35 alkaloids on paper impregnated with zirconium phosphate. It has been shown that in comparison to ordinary Whatman paper the R_F values of the various alkaloids can be varied over a wide range, thus enabling the identification of several closely related alkaloids.

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PAPER CHROMATOGRAPHY OF CHOLESTEROL ESTERS

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INTRODUCTION

Few methods are available for the study of the individual cholesterol esters. Alkali isomerization of the fatty acids and, more recently, gas chromatography of the methyl esters of the fatty acids have been used. Some chromatographic separations have also been reported. LABARRERE *et al.*[1] described a method for separating some saturated and unsaturated cholesterol esters by ascending reverse-phase paper chromatography using two solvent systems consecutively on the same paper strip. MARTIN[2] has described a method of separating some of the short chain saturated cholesterol esters. KLEIN AND JANSSEN[3] employed a silicic acid column for the fractionation of mixtures of cholesterol esters using a benzene–hexane solvent. Recently HAMILTON *et al.*[4] have described the separation of cholesterol esters on silica gel impregnated glass fiber paper. A silicic acid paper chromatographic method using benzene–hexane is described here.

METHODS

Preparation of standard cholesterol esters

Cholesterol stearate and cholesterol oleate were obtained from the British Drug Houses, Toronto, Canada; cholesterol palmitate and cholesterol oleate were obtained from the Aldrich Chemical Company. Myristic, acharidonic, linoleic, and linolenic acids were obtained from the California Corporation for Biochemical Research and the acid chlorides were prepared by the oxalyl chloride method of WOOD *et al.*[5] The cholesterol esters were then prepared by the esterification procedure of SWELL AND TREADWELL[6]. Purification of the saturated cholesterol esters was accomplished by repeated recrystallization.

Silicic acid bath chromatography was used to purify cholesterol linoleate and cholesterol linolenate. The esterified reaction mixture was dissolved in 100 ml hexane and filtered. Twenty grams of silicic acid (Mallinckrodt 100 mesh) were then added to the hexane solution in a beaker and the contents stirred for ten minutes. The suspension was allowed to settle and the hexane decanted. The cholesterol ester was then eluted from the silicic acid with a total of 1000 ml of 13 % benzene in hexane and crystallized in cold acetone. This procedure increased the yield over the recrystallization method

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and provided a purer compound. The melting points agreed closely and the infrared spectra were identical with those published by LABARRERE *et al.*[1].

Total lipid extracts of human and rat plasma were prepared by the method of FOLCH *et al.*[7]. The cholesterol esters were then separated as a group by silicic acid column chromatography as described by MARINETTI *et al.*[8].

Silicic acid paper chromatography

Whatman No. 1 papers, 19 × 21 cm and 19 × 44 cm, were impregnated with silicic acid as described by MARINETTI *et al.*[8]. The papers were stored in a glass cylinder containing anhydrous $CaSO_4$. The samples were applied to the papers on a line 1.5 in. from one edge. A maximum of 10–15 μg of each cholesterol ester in 20–50 μl of hexane was applied to the small chromatograms, while a maximum of 20–25 μg of each was applied to the large chromatogram. It was possible to apply up to 70–80 μg of a mixture per spot in the large chromatogram. The paper edges were then positioned to form a cylinder which was held together with stainless steel wire. Ascending chromatography was then carried out. The final procedure adopted was as follows: The small chromatograms were run in Mason jars containing 50–60 ml of 8–10% benzene in hexane (Eastman Organic, practical grade) at room temperature (usually 27–30°). The chromatographic run required about 1.5 hours. The larger chromatograms were run in cylinders measuring 6 in. internal diameter and 18 in. in height. The chromatographic solvent was 5% benzene in hexane (250 ml) and the usual time for the chromatographic run was 7–11 hours.

At the end of the chromatographic run, the papers were allowed to dry and then were lightly sprayed with a solvent mixture of diisobutyl ketone–acetic acid–water (40:20:3 v/v). The papers were allowed to dry again in a hood. The chromatograms were then stained in a 0.001% aqueous solution of Rhodamine 6G for 2 min and observed while wet under an ultraviolet lamp of 256 mμ wavelength (mineralight, Ultra-Violet Products, Inc., San Gabriel, Calif., Model V41). Iodine vapor staining was used to test for unsaturation. A phosphomolybdic acid stain was used to test for cholesterol.

RESULTS

Factors affecting chromatography and results with known standards

Various concentrations of benzene in hexane from 2% to 20% were tested. It was found that using the Mason Jar system and a benzene concentration of 20%, a mixture of the cholesterol esters travelled to the front as one spot. Conversely, at a benzene concentration of 2%, the cholesterol esters stayed at or streaked slightly from the origin. The best separation was achieved with 10% benzene in hexane. For the large cylinder, a 5% benzene concentration was found to be the best solvent if a filter paper liner was used. It was necessary to prepare fresh solvents after every five to six runs when the benzene concentration tended to fall below 5%. The system described allows good separation, with minor fluctuations, in room temperature. Humidity was controlled satisfactorily by storing the papers in a desiccator and minimizing the exposure to room atmosphere. The large cylinders, with the advantages of better separation and greater load capacity, were useful for quantitative work. The Mason Jars were useful for rapid scanning.

The results with known cholesterol esters are shown in Fig. 1, column 3. The

order of resolution (from the front to the origin) was: saturated cholesterol esters, cholesterol oleate, cholesterol linoleate, and cholesterol linolenate. Free cholesterol stayed near the origin. There was no practical separation among the saturated cholesterol esters.

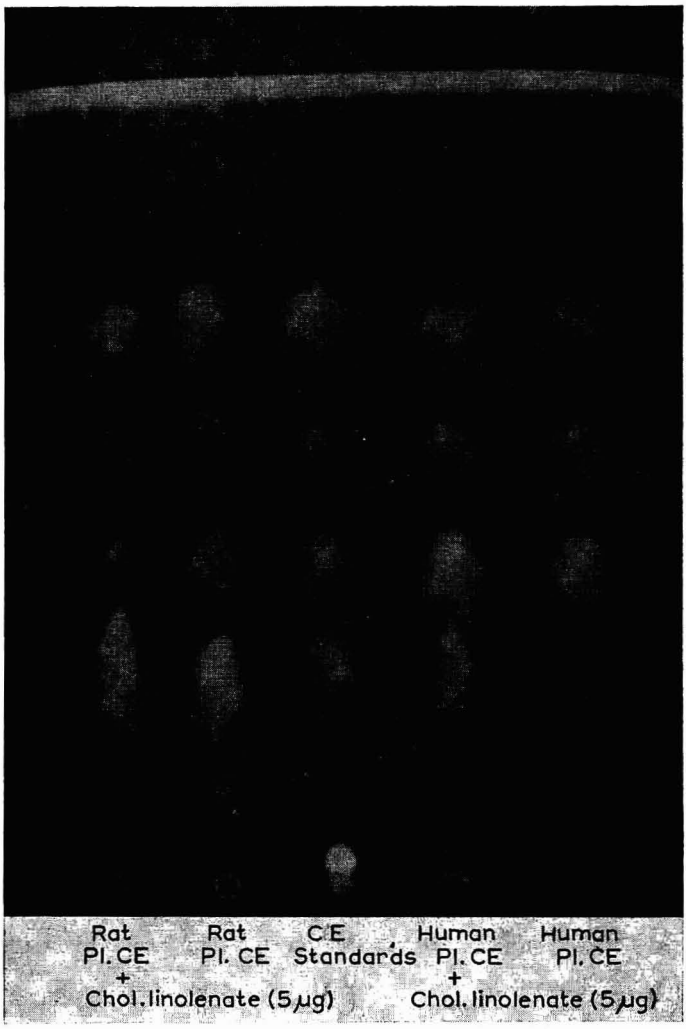

Fig. 1. Paper chromatography of rat, 40 µg, and human, 30 µg, plasma cholesterol esters. Cholesterol linolenate has been added in columns 1 and 4 in an attempt to determine whether or not cholesterol linolenate could be separated from cholesterol arachidonate in this system. Cholesterol ester standards were: cholesterol palmitate 10 µg, cholesterol stearate 10 µg, cholesterol oleate 5 µg, cholesterol linoleate 5 µg, cholesterol linolenate 5 µg, cholesterol 10 µg.

Identification of paper chromatography spots by column chromatography and gas chromatographic analysis

It was felt necessary to isolate the naturally occurring cholesterol esters of plasma by column chromatography in order to identify with certainty the ester portion by

gas chromatography. Rat plasma cholesterol esters generally resolved into four major overlapping fractions on silicic acid chromatography by the method of KLEIN[3]. Fig. 2 demonstrates the combined column and paper chromatographic analyses of a successful column run. Those tubes which contained more than one cholesterol ester were discarded and the tubes containing the same single ester were combined.

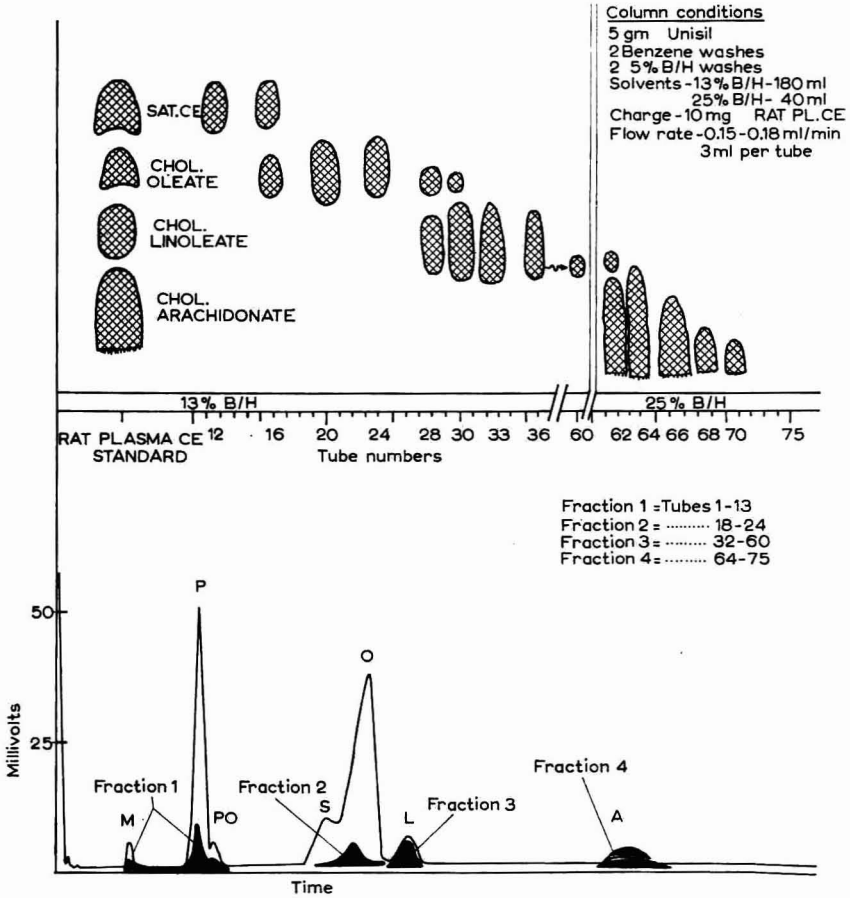

Fig. 2. Top: paper chromatographic analysis of tube fractions obtained by column chromatography of rat plasma cholesterol esters. Bottom: gas chromatographic analysis of the fatty acid methyl esters of the four major fractions obtained from the silicic acid column. M = methyl myristate; P = methyl palmitate; PO = methyl palmitoleate; S = methyl stearate; O = methyl oleate; L = methyl linoleate; A = methyl arachidonate. The line represents the fatty acid methyl ester standards.

Fraction I (tubes 1–13) on paper chromatography corresponded to saturated cholesterol esters. Gas chromatography of the fatty acid methyl esters revealed predominantly methyl palmitate and a small amount of methyl myristate and methyl palmitoleate. Fraction II (tubes 18–24) corresponded to cholesterol oleate. Gas chromatography of the fatty acid esters demonstrated predominantly methyl oleate with a minute amount of palmitate and palmitoleate. Fraction III (tubes 32–60) corresponded to cholesterol linoleate. Gas chromatography of the fatty acid esters showed essentially

only methyl linoleate. Fraction IV (tubes 64–75) on paper chromatography corresponded to the fourth spot. Gas chromatography of the fatty acid esters demonstrated predominantly methyl arachidonate.

Frequently, the fourth fraction, cholesterol arachidonate, could not be eluted from the column and in such instances a more polar 25 % benzene in hexane solvent was used to yield this ester.

Paper chromatography of human and rat plasma cholesterol esters

As indicated above, human and rat plasma cholesterol esters resolved into four spots (Fig. 1). The first spot corresponded primarily to saturated cholesterol esters. (This spot may also contain cholesterol palmitoleate.) The second spot corresponded to cholesterol oleate and the third spot was cholesterol linoleate. The fourth spot was cholesterol arachidonate as identified by gas chromatography on rat plasma.

All of the spots obtained by paper chromatography of human and rat plasma cholesterol esters stained for cholesterol with the phosphomolybdic acid reagent; and with the exception of the first spot (saturated cholesterol esters) also reacted with iodine vapors. It was found that the largest spot in human plasma cholesterol esters was cholesterol linoleate, while the largest spot in rat plasma cholesterol was cholesterol arachidonate.

Occasionally, when the chromatograms were overloaded with rat plasma cholesterol esters, it was possible to detect a fifth spot just below cholesterol arachidonate. This spot was positive for cholesterol with phosphomolybdic acid reagent, and stained with iodine vapors. However, this compound was present in very small amounts and was not identified. It may represent a more highly unsaturated cholesterol ester.

As a known pure cholesterol arachidonate standard was unavailable, it was not possible to test directly the separation of cholesterol linolenate from cholesterol arachidonate. However, the presence of cholesterol arachidonate in both human and rat plasma cholesterol esters allowed the addition of known cholesterol linolenate to both human and rat plasma cholesterol esters to determine if separation occurs. As can be seen (Fig. 1), there is no separation of these two cholesterol esters under these conditions. Smaller amounts were sometimes partially resolved.

DISCUSSION

It is evident that the various factors affecting the paper chromatography of cholesterol esters are essentially the same as those described by MARINETTI *et al.*[8]. However, they can be sufficiently controlled to allow reproducible results. It appears necessary to use known pure cholesterol ester standards or known naturally-occurring cholesterol ester mixtures for identification purposes.

The paper chromatographic analysis of human and rat plasma cholesterol esters as done by this method confirms other reports in the literature. KLEIN *et al.*[3] by their column method report cholesterol linoleate to be about 60 % of the total human plasma cholesterol esters, and SWELL *et al.*[9] by gas chromatography analysis, report that cholesterol arachidonate constitutes 50 % of the total rat plasma cholesterol esters.

The migration and separation of cholesterol esters is dependent on several factors, two of which are: (1) the chain length of the fatty acid moiety and (2) the degree of

unsaturation. The migration is based on the polarity of the esters. Increase in chain length of the fatty acid enhances mobility while increasing unsaturation decreases it and the balance of these two factors determines the mobility of a particular cholesterol ester. The less polar cholesterol esters (*i.e.* C_{14} to C_{20} saturated cholesterol esters) migrate faster than the more polar unsaturated esters. Cholesterol linoleate ($C_{18:2}$) moves below cholesterol oleate, and cholesterol linolenate ($C_{18:3}$) moves below cholesterol linoleate. Cholesterol arachidonate ($C_{20:4}$), because of the additional two carbon atoms of the fatty acid chain, moves with cholesterol linolenate.

It is hoped that this system will be helpful in: (a) identification of individual and mixtures of cholesterol esters and checking on the purity of a particular cholesterol ester, (b) the piloting and developing of column methods for the separation of individual cholesterol esters in quantity for further studies and (c) a semiquantitative estimate of the relative composition of a mixture of cholesterol esters.

ACKNOWLEDGEMENTS

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The authors wish to express their gratitude for the valuable advice of Dr. GUIDO MARINETTI of the Department of Biochemistry at the University of Rochester, for his help with the methods used in this work and for his assistance in the preparation of the manuscript. The authors are also endebted to Dr. LEWIS GIDEZ of the Albert Einstein College of Medicine for the gas chromatographic analyses and for help in the preparation of the cholesterol esters.

SUMMARY

A silicic acid paper chromatographic method for the separation of four major classes of cholesterol esters, namely saturated cholesterol esters, cholesterol oleate, cholesterol linoleate and cholesterol linolenate plus cholesterol arachidonate is described. This method uses a benzene–hexane solvent. The use of combined column and paper chromatography as well as gas chromatographic analysis is presented for definitive identification of the various spots. The predominance of cholesterol linoleate in human plasma, and cholesterol arachidonate in rat plasma is confirmed.

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QUANTITATIVE PAPER RADIOCHROMATOGRAPHY USING TOLLENS REAGENT

II. AMMONIACAL SILVER NITRATE REAGENTS CONTAINING LESS THAN EQUIMOLAR PROPORTIONS OF AMMONIA FOR THE OXIDATION OF SACCHARIDES ON PAPER CHROMATOGRAMS

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INTRODUCTION

The application of Tollens reagent to radiometric determination of saccharides on paper chromatograms was described in a previous paper[1]. According to that method, silver deposited in spots on paper as a result of the oxidation of separated substances is converted into $Ag^{131}I$ and determined radiometrically.

Oxidations carried out on paper with silver compounds are also accompanied by oxidation of the cellulose of the support and elementary silver is deposited in the background of the chromatogram. It was shown that this effect does not interfere in principal with radiochromatographic determinations, it is, nevertheless, the critical factor limiting the sensitivity of the method.

Because of the solubility of sugars aqueous solutions of Tollens reagent in common use can be added to paper chromatograms of sugars only by spraying. This fact involves further difficulties, as the background formed after spraying exhibits a non-uniformity which not only decreases the sensitivity of the determination, but also, in many cases, appears to be the source of serious errors.

Since attempts to improve the spraying techniques have been unsuccessful, our attention was turned to other methods of oxidation of sugars, especially methods employing the technique of adding silver reagents to the chromatograms by dipping.

The appropriate experiments were performed to test the methods described by TREVELYAN, PROCTOR AND HARRISON[2] and DEDONDER[3]. It was found that the strongly alkaline reaction conditions prevalent in the first method accelerated the oxidation very markedly but, on the other hand, caused very serious difficulties in the course of the removal of the excess reagent. This did not occur in DEDONDER'S method, but the rate of the reaction under the conditions described by him was very slow.

During these experiments a very marked positive effect of minute amounts of ammonia on sugar oxidations with silver nitrate was observed. This observation led to the development of a new type of ammoniacal silver nitrate reagent, the preparation and properties of which will be described in the present paper.

EXPERIMENTAL

Reagents

Saccharides. Glucose, fructose and inositol, commersial preparations, analytical grade were used in all experiments.

Silver reagents. Silver nitrate solution (SNS): 1 ml of saturated aqueous $AgNO_3$ solution was added to 200 ml of acetone. The precipitated salt was dissolved by dropwise addition of a minimal amount of water (about 5 ml)[2].

Ammoniacal silver nitrate solution (ASNS): 1 ml of saturated aqueous $AgNO_3$ solution was converted into ammoniacal silver nitrate with concentrated ammonia (about 1.6 ml). This solution was then added to 197.4 ml of acetone and the precipitated salt was dissolved with water as above.

Both solutions were used for the preparation of the thirteen reagents listed in Table I.

TABLE I

SILVER NITRATE REAGENTS CONTAINING DIFFERENT AMOUNTS OF AMMONIA

Reagent	$\frac{V_{ASNS}}{V_{ASNS} + V_{SNS}} \times 100\%$
SNS	0
R 8	8.4
R 17	16.7
R 25	25.0
R 33	33.3
R 42	41.7
R 50	50.0
R 58	58.3
R 67	66.7
R 75	75.0
R 83	83.4
R 92	91.7
ASNS	100.0

Note. Stock solutions of SNS and ASNS can be stored in a dark place over a period of several days. All reagents containing deficient amounts of ammonia are unstable and must be freshly prepared before use.

Chromatography

Whatman No. 1 paper strip chromatograms, 350 mm long and 15 mm wide, were prepared. The substances to be tested in amounts of 4.10^{-2} μmoles were spotted on the starting point located 120 mm from the lower end of the strip. The chromatograms were developed by the ascending technique for 18 h at room temperature, using the system *n*-butanol–acetic acid–water (6:1:2)[4]. The developed chromatograms were air dried for 72 h before further operations.

Oxidation reaction

Chromatograms were drawn quickly through the reagents and air dried. This manipulation was repeated three times, but at the third time the drying was omitted and the wet chromatograms were placed horizontally[5] in an oven. The reaction was carried out at 50° in a humid atmosphere produced by placing flat vessels filled with water on the bottom of the oven. A fan mounted inside the oven provided adequate circulation of the air.

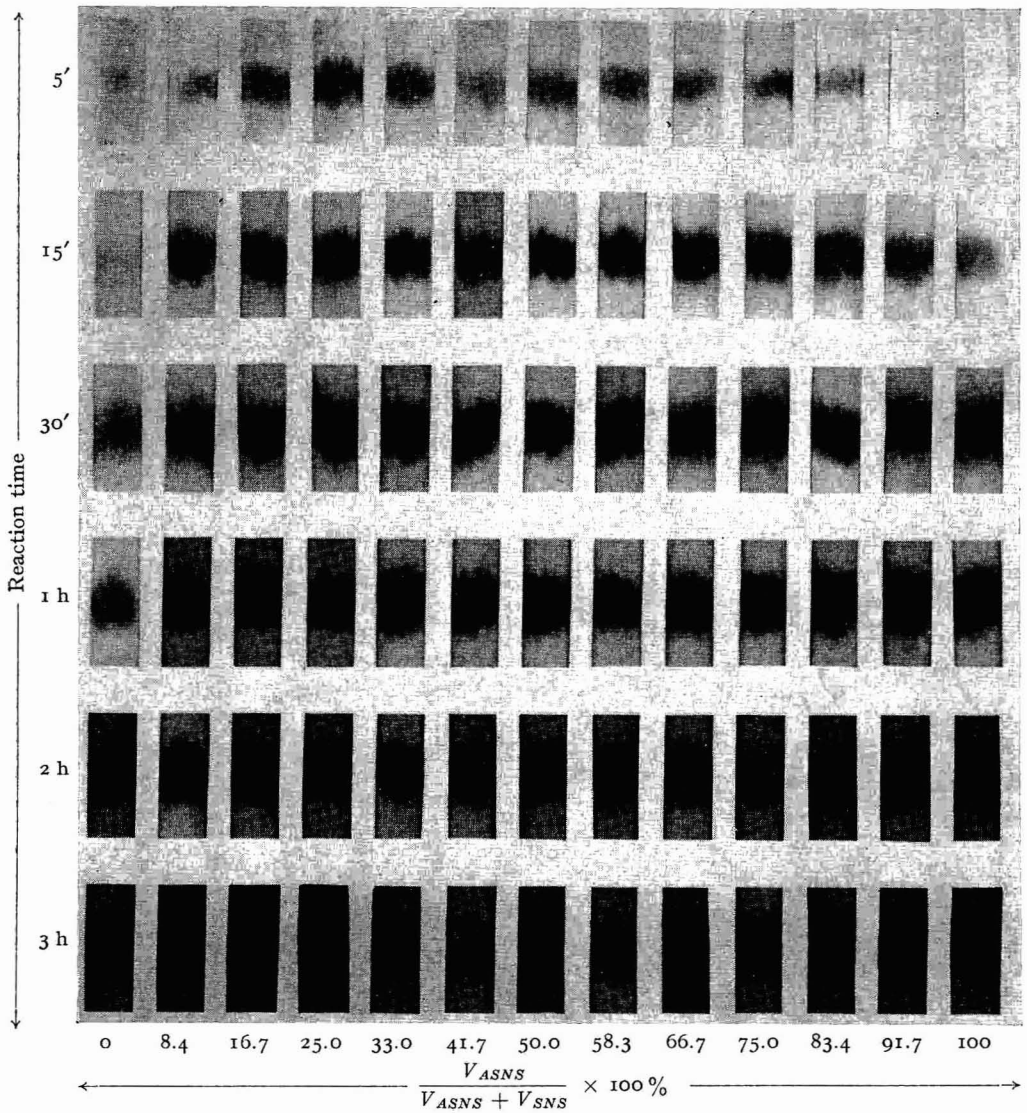

Fig. 1. Oxidation of glucose with ammoniacal silver nitrate reagents containing less than equimolar proportions of ammonia.

Immediately after the reaction, excess reagents were removed from the chromatograms by washing for 2 min with 10 % sodium thiosulphate followed by three washings for 5 min with water.

RESULTS AND DISCUSSION

The oxidizing properties of silver nitrate reagents containing different amounts of ammonia were tested in reactions with glucose, fructose and inositol. The thirteen reagents listed in Table I were used. The reactions were interrupted at intervals of 5, 15 and 30 min, 1, 2 and 3 h. The results of the experiments are presented in Figs. 1–3.

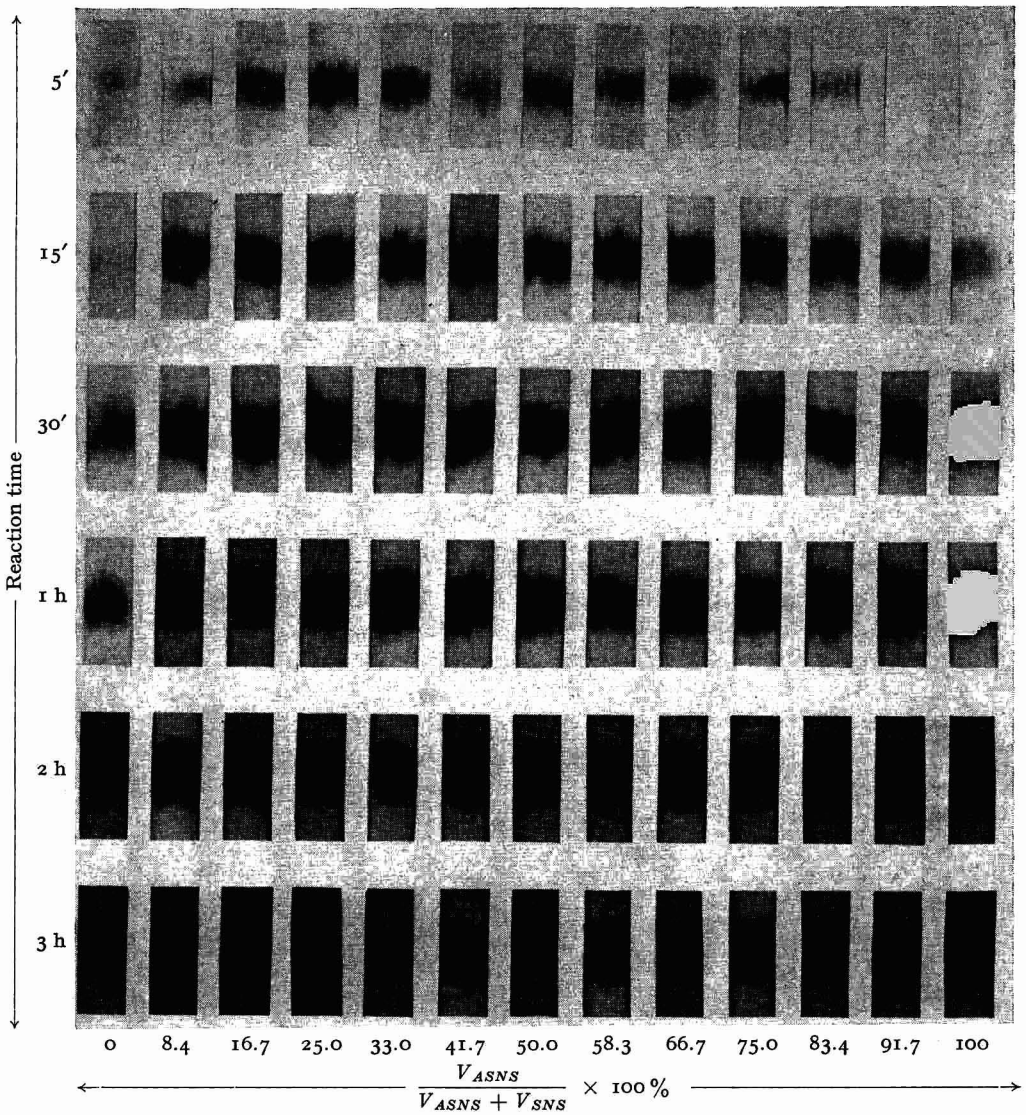

Fig. 2. Oxidation of fructose with ammoniacal silver nitrate reagents containing less than equimolar proportions of ammonia.

It can be seen that all ammoniacal silver nitrate solutions containing less than equimolar proportions of ammonia show higher reactivity towards the tested compounds than do SNS or ASNS. The early stages of the reaction may be used to determine the most reactive mixtures. These were reagents R25–R58 in the case of glucose and reagents R42–R58 in the case of fructose and inositol.

In evaluating the reagents tested, the most important criterion is the comparison of the results obtained with glucose and inositol—compounds representing the highest and the lowest susceptibility towards oxidizing agents among low-molecular sugar compounds. Distinct silver spots could be seen with glucose after only 5 min reaction.

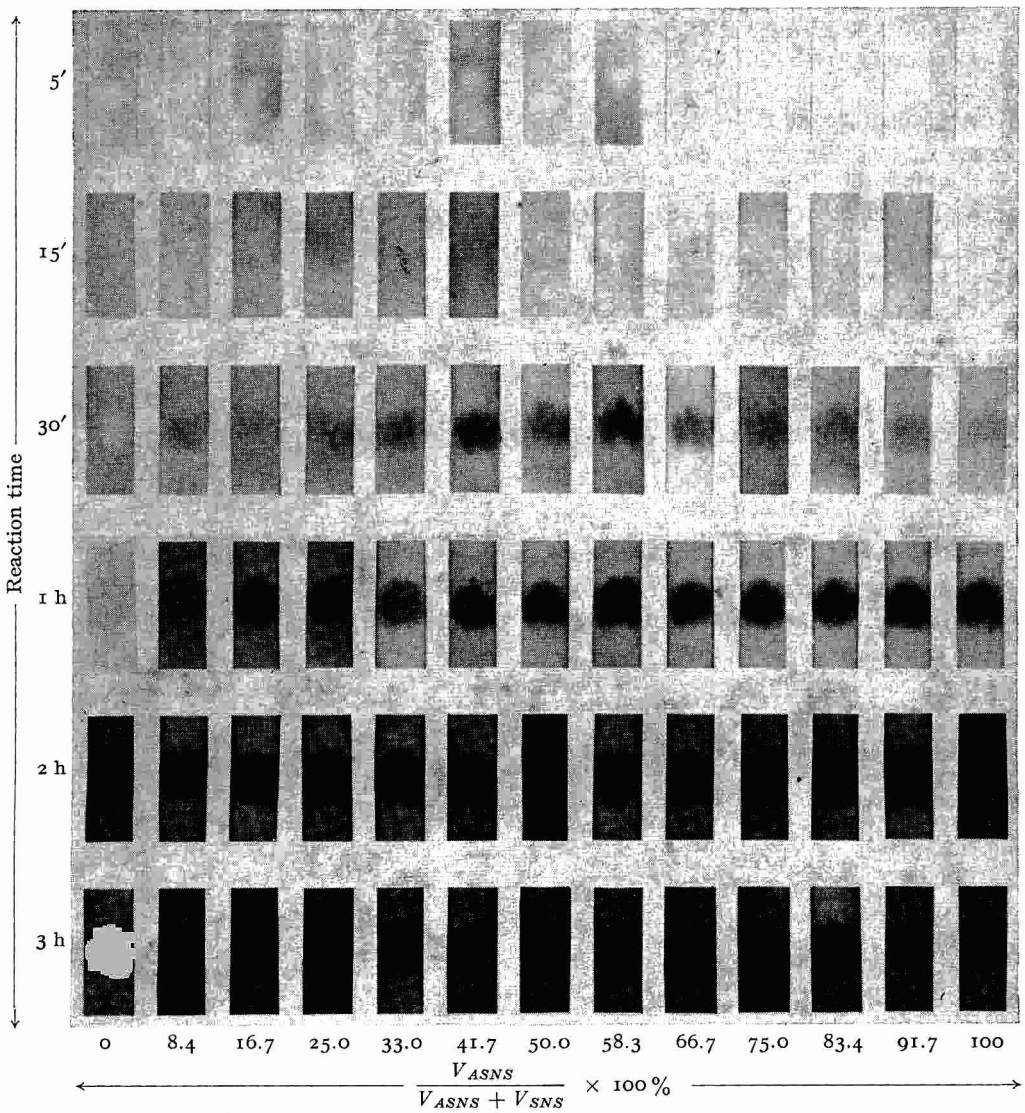

Fig. 3. Oxidation of inositol with ammoniacal silver nitrate reagents containing less than equimolar proportions of ammonia.

A similar picture was obtained with inositol after 30 min. The ratio, however, of the times needed to carry the reactions to completion was much less than six to one. In the case of glucose 1 h was needed whereas for inositol the limit was in the range of 2 h. The behaviour of inositol shows that the conditions described are suitable for analysis of the whole range of monosaccharides and related compounds.

The higher reactivity of mixtures containing smaller amounts of ammonia than is necessary to form the diamminesilver complex—$[Ag(NH_3)_2]^+$— may be connected with the existence in solution of a dynamic equilibrium of the kind

$$Ag^+ + [Ag(NH_3)_2]^+ \rightleftharpoons 2[Ag(NH_3)]^+$$

and the higher reactivity of the monoamminesilver cation. This hypothesis is supported by the fact that maximal reactivity of the tested reagents occurred in the case of solutions containing ammonia in the range of 50 % compared to that calculated on the basis of the diamminesilver complex.

It should be mentioned that the inhibiting influences of an excess of ammonia or ammonium salts on the oxidizing ability of Tollens reagent was observed by DREYER[6]. The same effect seems to occur with DEDONDER's method[3].

Further studies on the application of the modified ammoniacal silver nitrate reagents in radiochromatography of sugars are in progress.

ACKNOWLEDGEMENT

The skilful technical assistence of Miss ALICJA PANKIEWICZ is gratefully acknowledged.

SUMMARY

Ammoniacal silver nitrate reagents possessing higher reactivity towards saccharides than Tollens reagent are described. The highest reactivity is shown with unstable reagents containing about equimolar amounts of silver nitrate and ammonia. Acetone–water solutions of the reagents can be added to paper chromatograms of saccharides by dipping.

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PAPER CHROMATOGRAPHY OF CARBOHYDRATES AND RELATED COMPOUNDS IN THE PRESENCE OF BENZENEBORONIC ACID

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The well-known reaction of polyhydroxy-compounds with borate ions to form anionic complexes has been used extensively for the separation of carbohydrates and related compounds by paper electrophoresis in borate solution[1] and chromatography on columns of anion exchange resins[2] and charcoal[3]. The presence of boric acid has also been shown to affect the paper chromatographic behaviour of carbohydrates[4], the increase or decrease in R_F value being dependent on the pH of the solvent[5]. We now report the paper chromatographic behaviour of carbohydrates and related compounds in the presence of benzeneboronic acid.

EXPERIMENTAL

Solvents

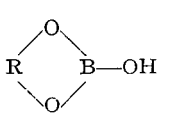
The solvents used for descending chromatography on Whatman No. 1 paper were (a) ethyl acetate–acetic acid–water (9:2:2 v/v) and (b) 0.55 % solution of benzeneboronic acid in ethyl acetate–acetic acid–water (9:2:2 v/v). The solvent front moves about 30 cm in 4–5 h.

Spray reagent

The compounds were detected on paper chromatograms with potassium periodatocuprate and rosaniline[6].

RESULTS AND DISCUSSION

The products of the reactions between boric acid or borate ions and polyhydroxy-compounds have structures of the types I–IV.



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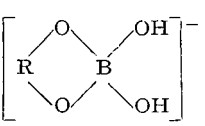

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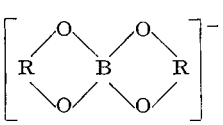

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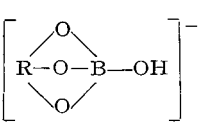
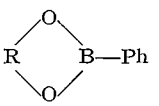


(IV)

Since boric acid, $B(OH)_3$, does not act as a proton donor but a Lewis acid[7], accepting the electron pair of the base, *e.g.* OH^-, to form the anion $B(OH)_4^-$, the compounds formed at acidic pH values are neutral esters (I) whereas those formed under alkaline conditions are anionic complexes (II–IV). Compounds with structure I should have higher R_F values in solvents with a stationary aqueous phase than those with structures II–IV. This is indeed confirmed by the chromatographic behaviour of D-glucitol in solvents containing (i) boric acid and acetic acid (R_G 2.2; movement with respect to glucose) and (ii) boric acid and pyridine (R_G 0.3)[5].

Benzeneboronic acid, $Ph \cdot B(OH)_2$, is known to react with several polyhydroxy-compounds to give esters with structure V[8–10]. The detailed structures of some of these have been elucidated[11,12].



(V)

The replacement of the hydroxyl group of I by a phenyl group should increase the affinity of the ester for the organic solvent and thus result in an increase in R_F value. The results (Table I) show that this is indeed the case; in solvent (b), in which the boric acid has been replaced by benzeneboronic acid, D-glucitol moves with an R_G value of 5.6.

TABLE I

R_F VALUES OF CARBOHYDRATES AND RELATED COMPOUNDS IN SOLVENTS (a) AND (b)

Compound	*R_F value*	
	Solvent (a)	*Solvent (b)*
Glycerol	0.32	0.35
Erythritol	0.23	0.31
D-Arabitol	0.14	0.50
1-deoxy-	0.45	0.71
5-deoxy-	0.46	0.85
Ribitol	0.14	0.48
2-deoxy-D-	0.32	0.46
Xylitol	0.14	0.45
Allitol	0.17	0.49
D-Altritol	0.16	0.51
1-deoxy-	0.36	0.85
1,6-dideoxy-	0.57	0.97
Galactitol	0.07	0.47
1-deoxy-D-	0.31	0.68
1,6-dideoxy-	0.58	0.85
D-Glucitol	0.08	0.45
2-deoxy-	0.22	0.60
3-O-methyl-	0.19	0.44
4-O-methyl-	0.30	0.40
D-Mannitol	0.08	0.43
1,6-dideoxy-	0.58	0.96
2-O-methyl-	0.22	0.70
1,2-di-O-methyl-	0.46	0.82

(continued on p. 255)

TABLE I (*continued*)

Compound	R_F value	
	Solvent (a)	Solvent (b)
DL-Glycerose	0.38	0.40
D-Erythrose	0.31	0.84
L-Threose	0.31	0.53
D-Arabinose	0.12	0.11
D-Lyxose	0.18	0.18
D-Ribose	0.25	0.50
2-deoxy-	0.40	0.41
D-Xylose	0.15	0.15
D-Altrose		
1,6-anhydro-β-pyranose	0.20	0.19
D-Galactose	0.06	0.08
6-deoxy-	0.19	0.18
1,6-anhydro-β-pyranose	0.33	0.38
D-Glucose	0.08	0.08
3-O-methyl-	0.21	0.23
5-deoxy-	0.28	0.27
methyl α-pyranoside	0.20	0.21
1,6-anhydro-β-pyranose	0.33	0.31
D-Gulose	0.13	0.27
1,6-anhydro-β-pyranose	0.31	0.30
L-Idose	0.09	0.16
D-Mannose	0.08	0.09
6-deoxy-	0.22	0.25
3,4-di-O-methyl-	0.55	0.59
methyl α-pyranoside	0.42	0.42
1,6-anhydro-β-pyranose	0.33	0.39
D-Fructose	0.11	0.12
L-Sorbose	0.10	0.16
allo-Inositol	0.04	0.11
dextro-Inositol		
3-O-methyl-	0.09	0.08
epi-Inositol	0.01	0.04
levo-Inositol	0.03	0.02
2-O-methyl-	0.07	0.07
muco-Inositol	0.05	0.05
1-deoxy-	0.08	0.08
myo-Inositol	0.02	0.02
1-deoxy-	0.07	0.06
scyllo-Inositol	0	0

Table I shows the R_F values of some carbohydrates and related compounds in the solvent containing the benzeneboronic acid [solvent (b)]. In all cases comparison was made with a solvent from which benzeneboronic acid was omitted [solvent (a)]. It can be seen that a number of useful separations are obtained, *e.g.* most aldoses and ketoses are well separated from their reduction products within 4 to 5 hours.

The isolated benzeneboronates of many polyhydroxy-compounds are easily hydrolysed, even during chromatography, with a solvent containing water, *e.g.* solvent (a). In this solvent the benzeneboronic acid, which can be detected under U.V. light, moves almost with the solvent front and hence is easily separated from the polyhydroxy-compounds. Thus, solvent (b) offers an advantage for separations on a preparative scale. Normally, boric acid is removed from an eluate by repeated distillation with methanol. However, the benzeneboronic acid can be separated from the polyhydroxy-

compounds by re-chromatography of the eluate in solvent (a), avoiding any destruction of the polyhydroxy-compounds which might occur when boric acid is removed by repeated distillation with methanol.

It seems reasonable to assume that only compounds which have at least two hydroxyl groups in an appropriate spatial arrangement to react with benzeneboronic acid will have significantly higher R_F values in solvent (b) than in solvent (a). However, comparison of the R_F values in the two solvents cannot be regarded as a satisfactory method to detect such an arrangement in a compound, since *e.g.*, the R_F values of glycerol and D-glucose are not appreciably altered by the presence of benzeneboronic acid, although crystalline benzeneboronates of these have been obtained[11,12]. It is likely that, under the conditions of the chromatography, the equilibrium does not favour the formation of certain benzeneboronates, which will of course have differing relative stabilities according to ring size, substituents, etc. On the other hand, Table I shows that the aldoses and cyclitols, the R_F values of which are markedly affected by the presence of benzeneboronic acid, have in their more stable conformation a 1 (*ax*), 3 (*ax*)-diol grouping. *muco*-Inositol and 1,6-anhydro-β-D-glucopyranose also possess such a diol grouping, but as mentioned earlier the conditions of the chromatography might not favour the formation of their benzeneboronates. 2-Deoxy-D-ribose, the R_F value of which is the same in both solvents, possesses such a diol grouping only in the C1 conformation (REEVES' nomenclature)[13] of its α-anomer. It is not possible to decide which anomer and conformation of D-ribose reacts with benzeneboronic acid.

During the course of this work GAREGG AND LINDBERG[14] reported the paper electrophoretic behaviour of carbohydrates in solutions of sulphonated benzeneboronic acid. Presumably, under the conditions used, the esters formed migrate due to the ionisation of the sulphonic acid group.

ACKNOWLEDGEMENTS

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SUMMARY

The R_F values of a number of polyhydroxy-compounds are markedly increased by the addition of benzeneboronic acid to the solvent. The increase is due to the formation of esters between benzeneboronic acid and the polyhydroxy-compounds. For certain carbohydrates and cyclitols the increase has been related to their structures. Acyclic polyhydroxy-compounds have, in general, much higher R_F values in the solvent containing benzeneboronic acid than the aldoses or ketoses from which they derive. This provides a rapid method for the separation of pairs of such compounds.

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Short Communication

Cation exchange separation of calcium and magnesium at high ionic strength*

In the course of a general investigation of the cation exchange behavior of the elements in very high ionic strength media, a marked difference was found in the adsorbability of Mg(II) and Ca(II) which may be utilized for rapid and simple separation of these elements from each other.

Adsorbability of Mg(II) in HCl and $HClO_4$ solutions decreases with ionic strength qualitatively as expected from the usual mass action expressions. In 9 *M* $HClO_4$ and 9 *M* HCl, the distribution coefficients *D* (amount per kg dry resin/amount per liter solution) were *ca.* 4.0 and 0.65 respectively, for Dowex 50-X4 at 25°. These results are in general agreement with those published recently by MANN[1].

Adsorbability of Ca(II) in perchloric acid solutions first decreases with *M* $HClO_4$ as expected, shows a minimum distribution coefficient, $D = ca.\ 7$ near 4 *M* $HClO_4$ and then increases rapidly to $D = ca.\ 10^3$ in 11 *M* $HClO_4$. While strong adsorption of Ca(II) was also observed by CHOPPIN AND DINIUS[2], our results differ from theirs in that we find a very much steeper rise of distribution coefficients above 6 *M* $HClO_4$.

In hydrochloric acid, adsorbability of Ca(II) goes through a minimum near 5 *M* HCl ($D = ca.\ 2$); it then increases moderately to $D = ca.\ 30$ in 12 *M* HCl. These results are in substantial agreement with earlier observations by DIAMOND[3].

The differences in adsorbabilities and hence the separation factors between Mg(II) and Ca(II) are thus very much larger in $HClO_4$ than in HCl solutions of equal but high concentration. In concentrated perchloric acid solutions the difference is large enough and adsorption of Mg(II) small enough to permit concentration and isolation of trace amounts of Ca(II) from moderately large volumes of Mg(II) solutions.

Adsorbabilities of Ca(II) and Mg(II) in mixtures of HCl and $HClO_4$ of constant total acidity are intermediate between those in the pure acids as demonstrated in Fig. 1, a plot of log *D* *vs.* the fraction $HClO_4$ (F_{HClO_4}) in 9 *M* acid mixtures. The separation factor, D_{Ca}/D_{Mg}, computed from the data of Fig. 1, increases gradually from 8.2 in 9 *M* HCl to 75 in 9 *M* $HClO_4$ solution.

In the chromatographic application of high ionic strength cation exchange, difficulties are encountered from the slow exchange rates. However, sharp elution bands can be achieved with reasonable flow rates by use of very fine mesh resins and occasional operation at elevated temperatures. We have also observed that HCl–$HClO_4$ mixtures have somewhat more favorable rates than $HClO_4$ solutions of the same ionic strength.

* This document is based on work performed for the U.S. Atomic Energy Commission and for the Office of Saline Water, U.S. Department of the Interior, at the Oak Ridge National Laboratory, Oak Ridge, Tenn., operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

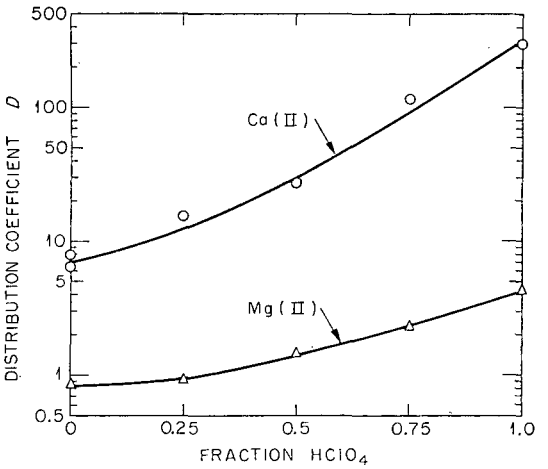

Fig. 1. Adsorption of Mg(II) and Ca(II) from 9 *M* HCl–$HClO_4$ mixtures (Dowex 50-X4, 25°).

A typical separation illustrating the use of high ionic strength media is shown in Fig. 2. A small aliquot of a mixture of tracers of Mg(II) and Ca(II) (^{28}Mg, $T_{1/2}$ = 21 h; ^{47}Ca–^{47}Sc, $T_{1/2}$ = 4.7 d–3.4 d) in 6.0 *M* $HClO_4$–2.6 *M* HCl was added to a small column (0.25 cm^2 × 6 cm) of Dowex 50-X4 at 25°; resin of mesh size 270 to 325 was used. Effluents were analyzed by gamma spectrometry.

On elution with 6.4 *M* $HClO_4$–2.6 *M* HCl, Mg(II) appeared rapidly in the effluent with maximum concentration near 1 column volume. The band was free of calcium (and scandium) activity and was symmetrical at the flow rate used (0.3 cm/min). Ca(II) can readily be eluted with *ca.* 5 or 6 *M* HCl, *i.e.*, a medium in which it shows minimum adsorption. In the experiment portrayed in Fig. 2, elution was carried out with 5 *M* HCl. Ca(II) appeared in maximum concentration after *ca.* 1.5 column volumes; the daughter activity ^{47}Sc was slightly more retained than Ca(II) under these conditions and its elution band followed that of Ca(II) with some overlapping.

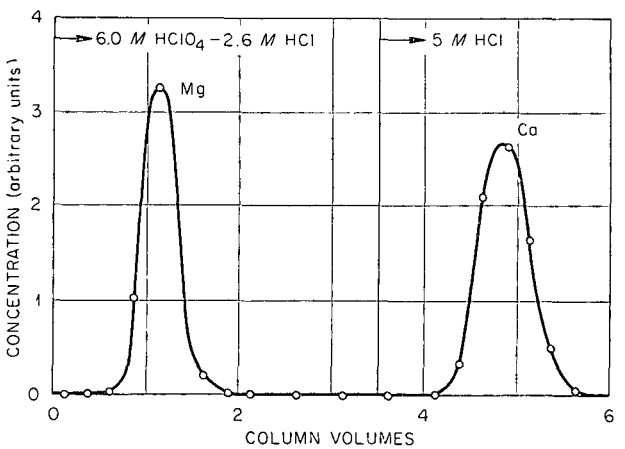

Fig. 2. High ionic strength cation exchange separation of Mg(II) and Ca(II); Dowex 50-X4, 25°, 6 cm column.

Although this separation was carried out with trace concentrations of Ca(II) and Mg(II), the method has been applied equally successfully to macro amounts (0.1 *M*) of these elements. The method should also be readily adaptable to the analysis of natural waters containing reasonable amounts of sodium chloride as well as such other ions as carbonates and sulfates.

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Notes

Chromatographic separation of *Rauwolfia serpentina* and opium alkaloids on thin layers of alumina

The chromatographic separation of alkaloids constitutes a problem that has been studied extensively. This is due above all to the wide distribution of these substances in natural materials, in which they mostly occur in low concentration accompanied by large amounts of impurities. Use has been made of paper chromatography for the separation of alkaloids, but one of the drawbacks of this method is that separation is only obtained after 5 to 12 h[1]. Moreover, the separation is not so clear specially in the case of *Rauwolfia* alkaloids. These alkaloids are located by means of ultra violet light, and ajmaline becomes visible only after spraying the developed chromatogram with sodium acetate solution[1]. In studies on the composition of complexes or crude products as regards their individual basic constituents, specially in the case of *Rauwolfia* alkaloids a need was felt for a suitable and quick method for their chromatographic analysis.

We have succeeded in achieving further progress in the chromatography of *Rauwolfia* and opium alkaloids by applying the method described by MOTTIER AND POTTERAT[2] for the separation of some synthetic fat-soluble pigments.

Materials and methods

0.5 % solutions of the following alkaloids were prepared: reserpine, serpentinine, serpentine, ajmaline, ajmalicine, morphine, narcotine, codeine and papaverine.

The *Rauwolfia* alkaloid solutions were dissolved in chloroform, narcotine in acetone, codeine, papaverine and morphine in ethyl alcohol.

Aluminium oxide for chromatography (E. Merck & Co., according to Brockmann) was used as the adsorption medium for the *Rauwolfia* alkaloids and aluminium oxide anhyd. (E. Merck & Co.) for the opium alkaloids.

Detection was carried out by means of the modified Dragendorff's reagent. Spraying has to be done very carefully and from a distance to avoid the absorbent layer being blown away by the aerosol stream.

Preparation of chromatographic plates with a thin layer of alumina

Plates were prepared according to the method of DAVIDEK *et al.*[3] by applying alumina in a dry state to glass plates of 90 × 350 mm. The alumina was subsequently smoothed by means of a roller made from a glass rod. The plates thus prepared are ready to be used directly for the chromatographic separation.

Experimental arrangement

The solution of the individual alkaloid or of a mixture is applied at a distance of about 2–3 cm from the edge of the plates. After allowing the solvent to dry, the plate is placed in an inclined position (at an angle of 20–30°) in the chromatographic chamber together with the solvent. A glass solvent trough 18 in. long × 6 in. wide × 5 in. high is used as the chromatographic chamber. Development with the solvent is carried out by the ascending technique until the solvent has reached the end (about 40–60 min). The plate is then removed from the chamber and allowed to dry. Finally it is sprayed very carefully with Dragendorff's reagent.

Discussion

Model experiments were performed with some of the *Rauwolfia* and opium alkaloids. The dependence of the R_F values of the individual substances on the solvent system as well as the best solvent system for their separation were studied.

Table I shows the R_F values obtained for *Rauwolfia* alkaloids using different solvent mixtures. It has been found that ethyl alcohol is essential for the separation of serpentine and ajmaline. On the other hand reserpine and ajmalicine can only be separated in the absence of alcohol. Consequently two chromatograms must be run

TABLE I

SEPARATION OF *Rauwolfia* ALKALOIDS BY THIN-LAYER CHROMATOGRAPHY

No.	*Alkaloids*	*Solvents*	*R_F values*	*Remarks*
1	Serpentine	Chloroform–acetone (85:15)	0.024	Serpentine and ajmaline gave only one spot
	Ajmaline			
	Reserpine		0.60	
	Ajmalicine		0.77	
2	Serpentine	Absolute ethyl alcohol	0.75	
	Serpentinine		0.86	
	Ajmaline		0.87	
3	Serpentine	Chloroform–ethyl	0.34	
	Ajmaline	alcohol–acetone (90:5:5)	0.51	
	Serpentinine		0.73	
	Reserpine		0.89	

TABLE II

SEPARATION OF OPIUM ALKALOIDS BY THIN-LAYER CHROMATOGRAPHY

No.	*Alkaloids*	*Solvents*	R_F *values*
1	Morphine	Dry acetone	0.033
	Codeine		0.77
	Papaverine		0.89
	Narcotine		0.92
2	Morphine	Chloroform	0.034
	Codeine		0.38
	Narcotine		0.77
	Papaverine		0.88
3	Morphine	Benzene–chloroform–	0.06
	Codeine	–acetone (70:15:15)	0.33
	Papaverine		0.73
	Narcotine		0.77

with the solvents chloroform–ethyl alcohol–acetone (90:5:5) and chloroform–acetone (85:15) to obtain a complete separation of all the alkaloids.

Table II shows the data obtained for opium alkaloids. In this case chloroform, acetone or a mixture of benzene–chloroform–acetone (70:15:15) were found to be most suitable for the separation of the opium alkaloids morphine, codeine, narcotine and papaverine. If chloroform alone is used as the irrigating solvent the spot of

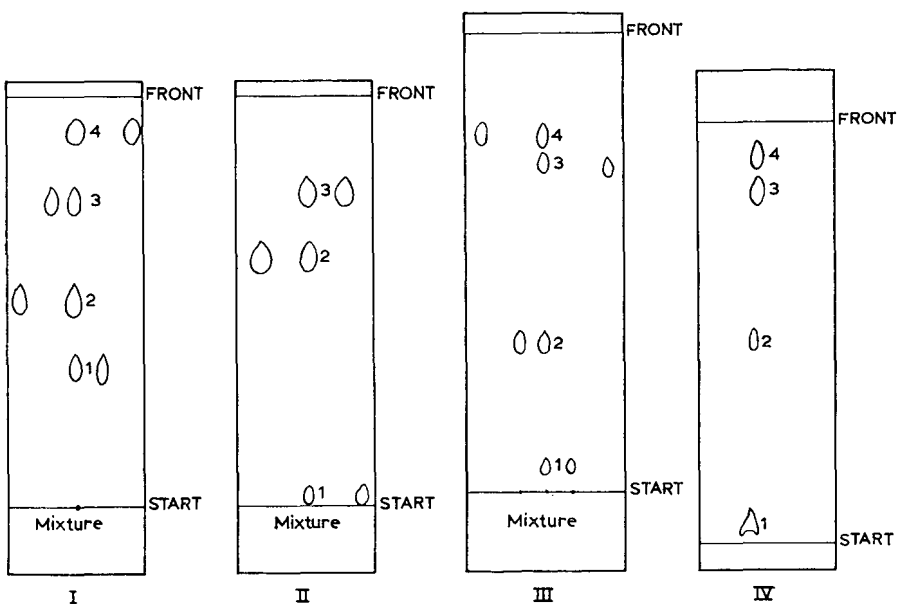

Fig. 1. I. Chromatogram of *Rauwolfia serpentina* alkaloids. Solvent: chloroform–ethyl alcohol–acetone (90:5:5). 1 = serpentine; 2 = serpentinine; 3 = ajmaline; 4 = reserpine. II. Chromatogram of *Rauwolfia serpentina* alkaloids. Solvent: chloroform–acetone (85:15). 1 = serpentine; 2 = reserpine; 3 = ajmalicine. III. Chromatogram of opium alkaloids. Solvent: benzene–chloroform–acetone (70:15:15). 1 = morphine; 2 = codeine; 3 = papaverine; 4 = narcotine. IV. Chromatogram of opium alkaloids. Solvent: pure chloroform. 1 = morphine; 2 = codeine; 3 = narcotine; 4 = papaverine.

papaverine is located at the end of the chromatogram, while with acetone or a mixture of benzene, chloroform and acetone narcotine is found at the top.

Typical chromatograms obtained in this way are shown in Fig. 1, I–IV.

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Use of sequentially applied location reagents and multiple transparent overlays in thin-layer chromatography*

Studies now in progress in this laboratory, utilizing thin-layer chromatography for the separation of tissue lipids[1], have demonstrated a need for a simple, rapid method for permanently recording the positions and total areas of separated lipid classes on the developed chromatoplate prior to quantitation. The separated lipids have differed widely in amount under various experimental conditions, producing a degree of variability as well as interference in the final separations that is not completely standardized by comparison with known lipids. This has necessitated the frequent use of multiple, sequentially applied location reagents. Difficulties have occurred when spots previously demonstrated would be hidden by location reagents applied later in the sequence. It became impossible to relate these sequentially visualized areas unless a permanent record was made after the use of each location reagent. Recent notes by GETZ AND LAWSON[2] and HILTON AND HALL[3] have suggested the feasibility of utilizing standard office photocopiers. The overlay technique described in this communication requires no special equipment and has the added feature that it permits the superpositioning of the series of overlays outlined during sequential spot localization. Direct comparisons can thus be made of the various spots developed with the individual location reagents, positive identifications can be made, and—most important—any incomplete separations can be rapidly determined.

The developed chromatoplate, after being sprayed with the first location reagent, is placed under a plate of either clear glass or plastic. This plate is elevated from the surface of the table enough to allow the chromatoplate to be slipped under-

* This investigation was supported in part by a research contract, Project Number 6X99-26-001-09, from the Medical Research and Development Command, U. S. Army, Washington 25, D. C.

neath without touching the overlying glass or plastic. The spotting guide provided with the Camag* apparatus, inverted, works very well as this stand. A piece of thin, clear, flexible plastic, *e.g.*, 0.005-gauge polyethylene, cut to the size of the glass plates used for the thin-layer chromatography, is then placed on the transparent stand above the developed chromatoplate and the area of each spot outlined. A standard ink glass-marking pen with a fine point works well for this purpose. Notes may be inked in on the overlay plastic, if desired, at this time. Other spots can then be located with other reagents, another overlay being traced each time. The transparent overlays can then be compared by direct apposition. After the spots have been identified, the individual overlays are placed underneath the chromatoplate; with transillumination the total areas containing the particular metabolites desired are easily located.

This method is being applied in this laboratory in a study of the alterations induced by hepatotoxic agents in lipid metabolism of the rat liver. Dichlorofluorescein is first sprayed onto the developed chromatoplate. This visualizes the pH gradient of the plate with different solvent systems[1] and spots the organic acids, all of which are recorded on the first overlay. The chromatoplate is then placed under U.V. light, and the fluorescent areas, which include almost all the lipid fractions in sufficiently high concentration, are outlined on the second overlay. Iodine vapor is then used for detection of unsaturated lipids, a third overlay being made. Finally a fourth overlay is made of the spots located with the Liebermann–Burchard reagent. Direct apposition of these four overlays identifies the various lipids by their relative positions and types of reactions observed with the various location reagents. Incomplete separations are noted. This is particularly important in experiments with shorter chain fatty acids, which usually remain much closer to the origin than the longer chain fatty acids, and are thus incompletely separated from other relatively polar lipids. When ^{14}C-labelled fatty acids are used, radioactivity would be found with these more polar lipids due to the ^{14}C in the incompletely separated fatty acid. In such a case, the separation is repeated, using a different solvent system. The overlays are then placed beneath the glass plate, both chromatoplate and overlay transilluminated, and the silica gel above the desired outline removed for quantitation. Finally, the overlays are stapled together and taped into a loose-leaf notebook with the experimental notes. The data accumulated with the quantitative studies are later summarized on a composite fifth overlay.

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Separation of isomeric methylated deoxyguanosines on thin cellulose layers prepared with a glass rod applicator*

The separation of nucleic acid derivatives by thin layer chromatography has already been demonstrated by RANDERATH[1-3], who used cellulose powder and Ecteola cellulose[4] for the preparation of thin layers and demonstrated the advantages of this method over the more classical paper chromatography[5]. We report here an application of thin-layer chromatography to the separation of methylated isomeric deoxyguanosines obtained by the action of diazomethane on deoxyguanosine. These products, otherwise separated by paper chromatography and isolated by column chromatography on cellulose powder, have been identified as principally 1-methyl-deoxyguanosine (MGDR-I) and O^6-methyl deoxyguanosine (MGDR-II), R_F values 0.70 and 0.78 (isopropanol–water, 70:30) and 0.72 and 0.80 (isopropanol–water–ammonia, 70:25:5), respectively[6].

In the present study the chromatoplates were prepared by applying cellulose layers with a simple glass rod applicator, as well as by use of a conventional applicator. Plates prepared by both methods gave equally effective separation of MGDR-I and MGDR-II. Glass rod applicator has been used previously by LEES AND DEMURIA[7] and by DUNCAN[8], who successfully applied silica gel G and Kiesel gel G slurries to glass plates.

Experimental

Preparation of chromatoplates. A mixture of 15 g of cellulose powder MN 300 G (Macherey, Nagel and Co., Düren, Germany) having particle size less than 10 mμ and containing plaster of Paris as the binder, and 90 ml of water was vigorously stirred by a mechanical stirrer for about 2 min. The slurry formed was then applied to one side of well-cleaned glass plates (200 mm × 50 mm in size) arranged in a row on a template. In place of a template a slab of glass plate of appropriate size may be used. A thick uniform glass rod, preferably with a ground surface (10 mm in diameter), around which Scotch tape was wound to the desired thickness, 250 mμ, at two positions less than the width of the glass plate apart, was drawn over the cellulose slurry in the manner described by LEES AND DEMURIA[7]. The uniform thin layer of cellulose obtained was dried and heated in the usual manner[9,10]. LEES AND DEMURIA applied Scotch tape to the sides of the glass plates. Their method also gave satisfactory results although it is less simple than the present method.

Glass plates coated with cellulose layers to a thickness of 250 mμ by a conventional adjustable applicator were prepared for purpose of comparison.

Comparison of chromatoplates. A mixture of the methylated products, MGDR-I and MGDR-II[6], in methanol (5 μg in 4 μl) was applied to plates prepared by the two methods, at a distance of 1/2 in. from one end on as small a spot as possible. The plates were developed simultaneously in the same bath, using a mixture of isopropanol and water (70:30) as a solvent, by the ascending technique. After 2 h when the solvent had risen to an appreciable height the plates were taken out, dried and examined under short wave ultraviolet light.

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There was good reproducible resolution of the mixture in both cases. Two distinct spots, one dark and the other brightly fluorescent in ultraviolet light, separated in each case. These spots, scraped from the glass plates and eluted with $N/10$ hydrochloric acid or $N/100$ sodium hydroxide, gave products with ultraviolet spectra dentical to those of the corresponding compounds isolated earlier[6]. R_F values are listed in Table I.

TABLE I

No. of Expt.	*Spots*	*R_F values on cellulose plates prepared with applicator*	*R_F values on cellulose plates prepared with glass rod*
1	Dark spot (MGDR-I)	0.72	0.76
	Bright spot (MGDR-II)	0.86	0.88
2	Dark spot (MGDR-I)	0.74	0.76
	Bright spot (MGDR-II)	0.88	0.87

Although the small differences in R_F values in the two cases are possibly attributable to differences in the thickness of the layers, it is clear that satisfactory thin layers of cellulose can be prepared simply and inexpensively by this glass rod technique. The rapidity and sensitivity of the method made it particularly useful for monitoring the fractions eluted from cellulose columns. This technique has also been extended with success to the preparation of plates with ion-exchange cellulose powder (MN 300 G/Ecteola). These latter plates have been used for separation of isomeric methylated deoxyribonucleotides to be reported later.

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Possibility for using a simple expression for peak symmetry characterization in gas chromatography

It is known that a Gaussian peak is described by the following equation (see Fig. 1):

$$y = y_0 \exp.-\left(\frac{x^2}{2\sigma^2}\right) \tag{1}$$

where y is the peak height (h), y_0 is the peak height at maximum (h_{max}), x is the distance from the ordinate, and σ is the variation coefficient of the peak. The Gaussian peak is symmetrical on both sides of the ordinate; thus, the peak width (w) at any point is:

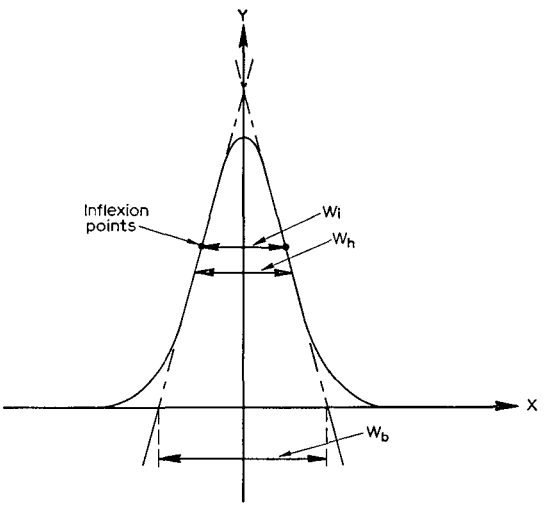

Fig. 1.

$$w = 2x \tag{2}$$

The peak width at two different positions is of primary importance: the width at the inflexion points (w_i) which is equal to 2σ:

$$w_i = 2\sigma \tag{3}$$

and the peak width at half height (w_h). Besides these two values, the so-called peak width at base or the band intercept (w_b) is of importance: it is equal to the length cut from the base line by the two tangents (drawn to the inflexion points) and is equal to 4σ:

$$w_b = 4\sigma \tag{4}$$

The value of w_h can be calculated as a function of sigma from equation (1) if $h_{max}/2$, h_{max}, and $w_h/2$ are substituted for y, y_0, and x respectively. The result of this calculation is:

$$w_h = 2\sigma\sqrt{2\ln 2} = 2.355\sigma \tag{5}$$

Dividing eqn. (4) by eqn. (5):

$$\frac{w_b}{w_h} = \frac{4\sigma}{2.355\sigma} = 1.698 \tag{6}$$

TABLE I

	Liquid phase (adsorbent)	Column Length (m)	Column O.D. (in.)	Support material[a]	Liquid phase concentr. (wt-%)	Temp. of analysis (°C)	He flow rate[b] (ml/min)	t_R[c] (mm)	w_b[c] (mm)	w_h[c] (mm)	HETP (mm)	w_b/w_h	Test substance
q	Diethylene glycol succinate	2	1/4	A	20	210	63	110	6.2	5.8	1.0	1.07	palmitate
q	UCON-Oil LB 550-X	2	1/4	A	20	75	92	59	5.5	3.8	2.0	1.45	ethanol
m	β,β'-Oxydipropionitrile	2	1/4	A	25	75	92	103	9.3	5.4	1.6	1.72	ethanol
m	7,8-Benzoquinoline	4	1/4	A	20	100	120	384	19.4	11.0	5.6	1.76	*m*-xylene
m	Carbowax 1500	2	1/4	B	10	70	66	53	7.1	3.9	3.6	1.82	ethanol
m	2,5-Hexanedione	2	1/4	A	35	25	52	66	7.0	3.8	1.9	1.84	*n*-pentane
m	Carbowax 1500	2	1/4	A	20	75	86	102	10.0	5.4	1.7	1.85	ethanol
m	Dimethyl sulfolane	2	1/4	A	35	25	55	21	2.6	1.4	2.4	1.86	*n*-pentane
m	Squalane	2	1/4	A	20	50	45	86	7.8	4.1	1.6	1.90	*n*-pentane
n	DC-200 Silicone oil	2	1/4	A	20	50	50	45	4.2	2.2	1.7	1.91	*n*-pentane
n	Diisodecyl phthalate	2	1/4	A	20	50	52	42	4.2	2.2	2.1	1.91	*n*-pentane
n	Di-2-ethylhexyl sebacate	2	1/4	A	20	50	50	54	4.8	2.5	1.5	1.92	*n*-pentane
p	Silica gel, 30/60 mesh	2	1/4	—	—	100	52	38	2.0	1.0	2.2	2.00	CO_2
o	Picric acid/fluorene	2	1/4	A	20	110	106	62	3.8	1.8	1.1	2.11	toluene
o	DC Silicone high vacuum grease	2	1/4	A	20	100	63	46	4.0	1.8	1.3	2.22	toluene
p	Activated charcoal, 30/60 mesh	2	1/4	—	—	100	70	35	2.8	1.2	1.4	2.33	methane
p	Molecular sieve 5A, 30/60 mesh	2	1/4	—	—	50	67	36	2.4	1.0	1.1	2.40	nitrogen
m	Butanediol succinate (BDS)	2	1/8	C	8	180	48	108	6.8	3.8	0.49	1.79	palmitate
								228	14.1	8.0	0.48	1.76	oleate

[a] Support material: A = GC-22 60/80 mesh; B = Teflon < 35 mesh; C = HMDS-treated Chromosorb W 60/80 mesh.
[b] Measured at column outlet at ambient temperature. Uncorrected value.
[c] 12.7 mm = 1 min.

This means that if the peak is truly Gaussian, the ratio of the peak width at base and at half height is constant.

In gas chromatography, both w_b and w_h are frequently used for the calculation of the number of theoretical plates. It is now suggested that their ratio may be used for the control and expression of peak symmetry.

Equation (6) showed that in case of a truly Gaussian peak, the ratio of w_b and w_h is equal to 1.698; thus, if at an actual chromatographic peak, the ratio differs from 1.698 ($\approx$ 1.70), it is an indication of peak unsymmetry.

Experimental

In order to test applicability of this method, we analyzed pure substances on eighteen different packed columns. Three of them were adsorption columns while the others were partition columns. Thirteen partition columns were made using GC-22, 60/80 mesh as support material; this is a conventional diatomaceous earth type support, a product of the Coast Engineering Laboratory, Hermosa Beach, California and is not completely inert. Thus, if a polar substance is analyzed, peak tailing may occur. One column was made using Teflon which is completely inert, as support material. Finally, one column was made by using hexamethyldisilazane (HMDS) treated Chromosorb W, 80/100 mesh which is after Teflon and glass beads, the next best inert material; it is a product of Johns-Manville Corporation, New York. All columns except one were 2 m long; the one longer column was 4 m and made with 7,8-benzoquinoline liquid phase. The columns with GC-22 support and Teflon, and the adsorption columns were made of tubing with 1/4 in. outside diameter; the HMDS-treated Chromosorb W column had 1/8 in. O.D. tubing. Table I gives the column dimensions, the concentration of the liquid phase on the column material, the operating conditions, the test substances, and the analytical results; further, the w_b/w_h ratio for each column. It should be mentioned that usual carrier gas inlet pressures were used and no attempt was made to optimize the gas velocity; this is the reason for the slightly worse HETP values in some cases. The investigations with the 1/4 in. O.D. columns were carried out with a Perkin-Elmer 154-D Vapor Fractometer with thermistor detector; the BDS column was run on the Model 800 Gas Chromatograph of the same company, equipped with a flame ionization detector.

Results

It is interesting to investigate the w_b/w_h values. Since the correct measurement of the peak width values is difficult (we used a magnifying glass with a millimeter-scale), about $\pm$10 % difference in the ratio was considered as normal. As can be seen, seven 1/4 in. O.D. and the 1/8 in. O.D. columns (marked with m) resulted in symmetrical, Gaussian peak. The three "general purpose columns" with silicone oil, phthalate and sebacate liquid phases (marked with n) gave a slightly worse w_b/w_h ratio probably due to the fact that the two last liquid phases are slightly polar and we analyzed a non-polar paraffinic substance. The picric acid/fluorene and the silicone grease column (marked with o) came next in the line. The first usually does not give symmetrical peaks, thus its position in this scale is evident; the reason why the silicone high vacuum grease gave an asymmetrical peak may be traced to the colloidal silica which is mixed with this grease. The three adsorption columns (marked with p) gave uniformly much larger w_b/w_h ratio than the theoretical value. The large difference

between theoretical and found values for the succinate and the UCON-oil columns (marked with q) could be explained by the support material: with these columns, Chromosorb W or similar material is necessary in order to obtain relatively symmetrical peaks. It is, however, not understood why the actual w_b/w_h value for these two columns is smaller than 1.70; in case of tailing, one would rather expect larger values

The most striking difference can be observed in the case of the two polyester columns. The butanediol succinate (BDS) column was made of the relatively inert HMDS-treated Chromosorb W and as a result, the peaks are practically completely symmetrical. On the other hand, the support material for the diethylene glycol succinate column is the standard diatomaceous earth type material without any treatment which has a considerable adsorption effect on such polar samples as the fatty acid methyl esters. Also, the large deviation of the w_b/w_h ratio from the theoretical value for the adsorption columns can easily be related to the fact that the peaks on adsorption columns usually show some tailing. As mentioned above, most of the other values can also be interpreted in this direction. Thus, the results seem to demonstrate the applicability of this calculation for peak symmetry control.

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A new apparatus for thin-layer chromatography

Various chromatographic techniques have been developed in the last few years with the purpose of reducing time-consuming separations and facilitating the reproducibility of the results.

Thin-layer chromatography on silicic acid, alumina or cellulose, was developed by STAHL who stratified these materials on glass plates[1–3]. This method allows the chromatographic separation of mixtures of substances difficult to separate, such as oil esters, polyterpenes, tars, steroids, bile salts, amino acids, etc.

The two main advantages of STAHL's method for chromatographic separation are the shorter run of the solvents and the uniform chromatographic support obtained by using the standardized gel preparation.

To prepare "thin layers", small devices, manually operated, are now available[4]. These stratifiers do not give good results because the layer presents an uneven thickness which disturbs the chromatographic separation.

This difficulty prompted us to develop an apparatus which would improve the deposition of the adsorbing material on the glass plates.

After a number of attempts to solve this problem we devised an apparatus in which the plates progress at a constant speed ensured by supporting rollers. The plate must pass through an adjustable slit under a reservoir containing the chromatographic support. Details of the apparatus are shown in Figs. 1a and b.

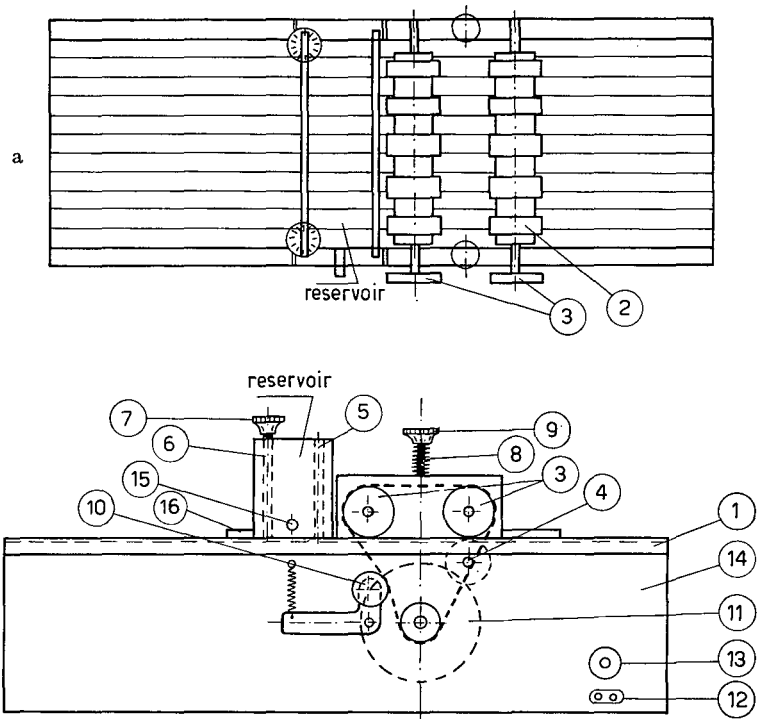

Fig. 1. (a) View of the apparatus from above. (b) Side view.

1. Corrugated board
2. Rubberized rollers
3. Sprocket wheels
4. Supporting sprocket
5. Bulkheads
6. Adjustable bulkheads
7. Adjustable screw
8. Spring
9. Spring screw
10. Pulley
11. Motor
12. Current point
13. Switch
14. Casing of the apparatus
15. Outlet for wash water
16. Glass plate

The apparatus consists of a grooved slab; on this plane are two rollers, belted with rubber rings. These rollers are set in motion by a motor which ensures that the glass plates move along at a constant speed; the motion of the roller is such that it displaces the plate as shown in the layout, from right to left. In this way the plate passes under two bulkheads forming a small room in which the adsorbent is placed. The bulkhead (No. 6) can be regulated by two microscrews and allows stratification at various thicknesses.

With the help of a switch the stratifying process is begun, and a new plate is added every time the preceding one has passed under the reservoir. It is possible in this manner to stratify an indefinite number of plates with the same thickness and a perfect distribution of the adsorbent in all parts of the plate.

With this apparatus* we have obtained good results for the separation of a great variety of substances, in particular amino acids and bile salts.

For special purposes, and in particular for stratifying mixtures of silica gel and silver nitrate[5,6], the apparatus has been constructed with the reservoir in plexiglas.

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* The apparatus used was built by E. Piccolo, Sesto S. Giovanni, Milano.
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A method for the detection of β-phenylethylamines and β-phenylethylamino acids

A convenient method for the detection of β-phenylethylamines on chromatographic paper has not been available. In the course of a study in which it was necessary to treat chromatograms with Ehrlich's reagent after they had been treated with ninhydrin, it was noted that each of a number of substituted β-phenylethylamines appeared as a pink spot. Since these compounds do not react with Ehrlich's reagent alone it was felt that this combination of color reagents might be useful in the detection of β-phenylethylamines. Accordingly, a systematic examination of a series of substituted β-phenylethylamines and related compounds was undertaken.

Procedure and results

The compounds studied were dissolved in methanol–water for application to paper chromatograms. 5 μg of material were routinely chromatographed on Whatman No. 1 paper. The chromatograms were developed in butanol–acetic acid–water (4:1:1). Other solvent systems were also employed, and it was found that if the chromatograms were adequately air dried, the choice of solvent system did not significantly affect the results.

The dried chromatograms were then dipped in ninhydrin–pyridine reagent (0.2 % ninhydrin in acetone–pyridine 9:1), air dried, and heated for 1 min at 105°. After notation was made of the color, the chromatograms were dipped in a modified Ehrlich's reagent (2 % *p*-dimethylaminobenzaldehyde in acetone–conc. HCl 9:1). After the ninhydrin color had completely faded, certain compounds evidenced a pink color which slowly changed to orange. The pink color was maximum approximately 30 min after the strips were dipped in Ehrlich's reagent and the colors were therefore

noted at that time. In the event that 5 μg of material failed to produce a color reaction, the procedure was repeated using 25 μg of the compound. Whether the compounds were applied as free bases, hydrochlorides, or other salts did not alter the results.

From Table I it can be seen that all compounds that produce a purple color with

TABLE I

RESPONSE OF AMINO COMPOUNDS TO THE SPECIFIC TEST FOR β-PHENYLETHYLAMINO COMPOUNDS

Compound (5 μg of chromatographed material)	*Ninhydrin–pyridine reagent (color)**	*Ehrlich's after ninhydrin–pyridine reagent (color)**	*Response to test for β-phenylethyl-amino compounds*
Ethylamine	p	neg.	—
Benzylamine	p	faint y	—
β-Phenylethylamine	p	pk-o	+
Tyramine	p	pk	+
Dopamine	p	pk	+
4-Hydroxy-3-methoxy-β-phenylethylamine	p	pk	+
3,4-Dimethoxy-β-phenylethylamine	p	pk	+
Mescaline	p	pk	+
Amphetamine	faint p	neg**	weak
N-Methyl-β-phenylethylamine	faint p	faint pk	weak
Norepinephrine	p	faint y	—
Epinephrine	faint p	faint y	—
Ephedrine	neg.	neg.**	—
3-Phenyl-1-propylamine	p	neg.	—
Urea	neg.	y	—
p-Methylaminophenol	br	faint tan	—
Pyrrole	neg.	neg.**	—
p-Nitroaniline	y	o	—
N,N-Dimethylaniline	neg.	neg.	—
p-Anisidine	y-o	y	—
Naphthylamine	y-o (vis.)	y	—
Isatin	y (vis.)	faint y-o	—
Marsilid	neg.	neg.	—
Tryptamine	p	p	—
Serotonin	br-p	br-p	—
Bufotenine	faint y	p-b	—
N-Acetyl-5-methoxytryptamine	neg.	b	—
Glycine	p	faint green	—
Glutamic acid	p	faint y	—
Glutamine	p	faint y	—
Lysine	p	faint grey	—
Cysteine	p	faint y	—
Methionine	p	faint y	—
Proline	y	faint p-pk	—
Phenylserine	p	faint y	—
Phenylalanine	p	pk-o	+
3,5-Diiodotyrosine	p	pk	+
3,4-Dihydroxyphenylalanine	p	pk	+
Tryptophan	p	br-p	—
N-Carbobenzoxy-L-tryptophan	neg.	faint p	—

* Color code: p = purple; br = brown; y = yellow; o = orange; pk = pink; b = blue; (vis.) = spots were colored before ninhydrin–pyridine reagent.

** Chromatography of 50 μg of amphetamine revealed a spot faintly p with ninhydrin–pyridine and faintly pk with Ehrlich's after ninhydrin–pyridine. Chromatography of 25 μg of ephedrine revealed a spot p with ninhydrin–pyridine and faintly y with Ehrlich's after ninhydrin–pyridine. Chromatography of 25 μg of pyrrole revealed a spot faintly tan with ninhydrin–pyridine and faintly p-pk with Ehrlich's after ninhydrin–pyridine. All other compounds that failed to produce a color reaction with 5 μg of material did not produce any color with 25 μg.

ninhydrin and a pink color after subsequent treatment with Ehrlich's reagent were β-phenylethylamines and β-phenylethylamino acids. These compounds do not produce a pink color with Ehrlich's reagent alone. Therefore, a compound that:

1. forms a purple color with ninhydrin;
2. forms a pink color 30 min after subsequent treatment with Ehrlich's reagent; and
3. fails to form a pink color with Ehrlich's reagent alone, can be determined, presumptively, to be a β-phenylethylamino compound.

The satisfaction of these three conditions constitutes a positive response to this test. The test is positive for β-phenylethylamine, for β-phenyl-ethylamines substituted on the ring and for β-phenylethylamino acids. Mono substitution on the α-carbon of the amine or mono substitution on the amino group of the amine decreases the sensitivity of the test. The α-amino acids react strongly however. Tertiary amines do not react with ninhydrin[1] and these, therefore, do not give a positive response to this test. Further substitution on the β-carbon of the β-phenylethylamines, as in norepinephrine, produces a final yellow color and, therefore, the response of these compounds is also negative.

In order to determine whether these findings were dependent on the solvents used for the color reagents rather than the ninhydrin and the *p*-dimethylaminobenzaldehyde, ninhydrin reagent was dissolved in (1) acetone alone, (2) acetone–glacial acetic acid (9:1), (3) ethanol alone, (4) water alone. None of these variations affected the results of the test. When *p*-dimethylaminobenzaldehyde was dissolved in ethanol rather than acetone, this also did not alter the results.

Discussion

A study of the mechanism of this test has not been attempted. However, it has been observed that the intensity of the pink color formed after the application of the Ehrlich's reagent is proportional to the intensity of the purple formed after the application of ninhydrin. This suggests that the Ehrlich's reagent reacts with the Ruhemann's purple formed from the reaction of ninhydrin and the β-phenylethylamino compounds. This is also borne out by the fact that N mono substitution and α mono substitution weaken the reaction with ninhydrin and also produce a weaker response to this test. The sensitivity of this reaction has only been studied systematically to 5 μg. However, sensitivity has been found to extend to the anticipated sensitivity with ninhydrin reagent alone.

The finding that many β-phenylethylamino compounds produce a pink color with this test provides a convenient method for the detection of these compounds on paper chromatograms.

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R_M values of some phenols in the system organic non-polar solvent/dimethyl sulphoxide + glycol

In paper chromatography, polar (*e.g.* formamide[1], dimethyl sulphoxide[2], water[3,4]) or non-polar liquids (*e.g.* paraffin oil[5], silicone oil[6]) are frequently used for the impregnation of the paper before development, in order to secure a true partition mechanism of the process. For the sake of simplicity it is preferable to employ a non-volatile liquid, diluted with a volatile liquid (such as acetone, diethyl ether etc.), which would evaporate rapidly in the course of preparation of the strips. The appropriate degree of loading of the paper can then be controlled by the concentration of the non-volatile liquid in the diluted solution, or by repeated impregnations. With moderately volatile liquids used as stationary phases, for instance water[3] or aqueous buffer solutions[4], it is necessary to introduce a weight standard[3,4] or a time standard[7]. The latter methods are time-consuming and may be found difficult in cases when many experiments are to be performed simultaneously; moreover, a time standard depends strongly upon room temperature and humidity. The use, therefore, of volatile liquids as fixed phases, although sometimes advantageous (*e.g.* when paper chromatographic data are utilized for the determination of solvent systems suitable for cascade countercurrent extraction processes, *cf.* refs. [8,9]), should be avoided when possible.

In an earlier paper[10] we have described how, by changing the composition of the polar stationary phase (mixtures of water and dimethyl sulphoxide), the R_F values of certain phenols (and thus the separation efficiency) could be controlled. In order to secure an appropriate degree of impregnation, a weight standard was employed. It seemed that water could be replaced by another, less volatile, liquid of the same solvent class[11] possessing similar solvent properties, and in this way the use of a weight standard could be eliminated. In accordance with previous reports[2] it was found that double impregnation with a 25 % v/v acetone solution of the non-volatile liquid resulted in an appropriate degree of loading (*ca.* 0.5 ml per 1 g of dry paper, which is about optimal for Whatman No. 4 or 41 papers).

As the substitute for water, two polyalcohols were chosen, ethylene glycol and glycerol, which, like water, form a three-dimensional network of strong hydrogen bonds (class I according to EWELL *et al.*[11]).

Experimental

Glycol–dimethyl sulphoxide (DMS) mixtures of varying composition by volume were prepared by mixing weighed aliquots of the two solvents; the mixtures were then diluted with 3 parts by volume of acetone, thus forming a 25 % v/v solution of non-volatile liquid in acetone. The paper strips (Whatman No. 41; 5 × 23.5 cm, cut at right angles to the machine direction), were passed through the impregnating solution at an even rate, blotted between two sheets of filter paper and dried for 15 sec at 60° in a horizontal position. The impregnation was repeated once; this time the strips were passed through the acetone solution in the opposite direction. When the polar fixed phase contained high percentages of DMS ($\geqslant$ 80 %) the strip, after drying in the oven for the second time, was placed between glass plates[2] in order to reduce the absorption of water vapour from the atmosphere.

The phenols were spotted on the starting line from a capillary (*ca.* 0.5 μl, 0.5 % w/v solution of phenols in benzene), and the strips developed with non-polar organic

solvents (downward flow, in glass chambers 6 × 14 × 21 cm, distance of development 16 cm). All these operations were performed as rapidly as possible. The spots were revealed with bis-diazotized benzidine, as in the previous paper.

The results of the experiments are presented in Figs. 1–3, in the form of R_M *vs.*

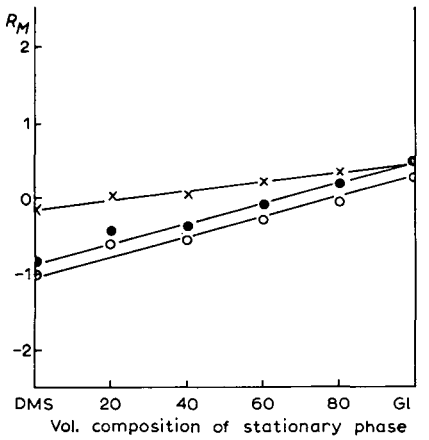

Fig. 1. Diagram of R_M *vs.* volume composition of the stationary phase consisting of mixtures of dimethyl sulphoxide (DMS) and diethylene glycol (Gl). Mobile phase: di-isoamyl ether. ●—● 1-naphthol; O—O 2-naphthol; ×—× 8-hydroxyquinoline.

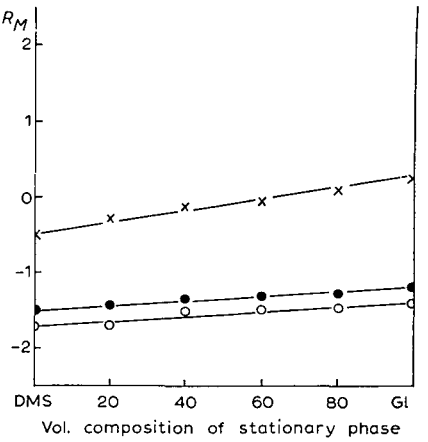

Fig. 2. Diagram of R_M *vs.* volume composition of the stationary phase consisting of mixtures of dimethyl sulphoxide (DMS) and diethylene glycol (Gl). Mobile phase: decalin. ●—● 1-naphthol; O—O 2-naphthol; ×—× 8-hydroxyquinoline.

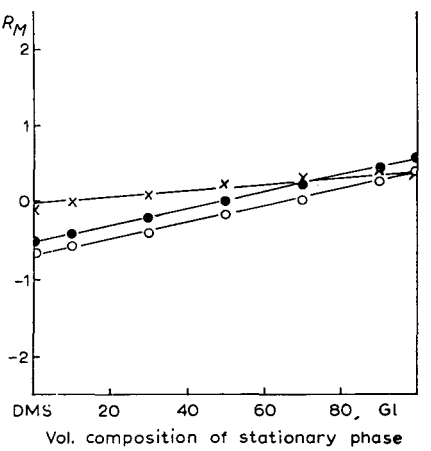

Fig. 3. Diagram of R_M *vs.* volume composition of the stationary phase consisting of mixtures of dimethyl sulphoxide (DMS) and diethylene glycol (Gl). Mobile phase: 20% v/v di-*n*-amyl ether + 80% di-isoamyl ether. ●—● 1-naphthol; O—O 2-naphthol; ×—× 8-hydroxyquinoline.

volume composition plots. As in the previous paper, linear R_M *vs.* volume composition relationships were obtained.

It may be worth noting that the solvent system represented in Fig. 3 is actually a

quaternary one, in which one of the phases is a binary mixture of constant composition. ENGEL *et al.*[12] have pointed out that systems of this type should give linear log K *vs.* molar composition relationships, as in the case of ternary systems (K = partition coefficient of the solute). On the other hand, KEMULA AND BUCHOWSKI[13] stated that volume composition should be used instead of molar composition. Because of the parallelism of the log K *vs.* composition and R_M *vs.* composition relationships (R_M = log Kr; r = ratio of the volumes of the two phases), the experimental data in Fig. 3 confirm KEMULA AND BUCHOWSKI's point of view.

The spots were circular or slightly elongated, as in the case of DMS–water mixtures[10].

Similar results have been obtained with glycerol instead of glycol in the polar phase.

Conclusions

Non-volatile polyalcohols such as diethylene glycol or glycerol may be added to polar liquids employed for the impregnation of paper strips (such as dimethyl sulphoxide, dimethyl-formamide, formamide) in order to modify the solubility of substances in the fixed phase and thus to change their R_F values and separation efficiency. The preparation of the paper before development is not more complicated, the proper degree of impregnation being controlled by the concentration of the non-volatile liquid in the volatile diluting solvent. The results of chromatographic experiments provide further examples of linear R_M *vs.* volume composition relationships.

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L'emploi du réactif de Folin en chromatographie et électrophorèse sur papier

Au cours de nos recherches sur le mécanisme de la détoxification par le formol[1], nous avons été amenées à préparer une base "Mannich"[2], en faisant agir le formol sur un mélange de α-N-acétyl-lysine et de α-N-acétyl-tyrosine. Le produit principal et les produits des réactions accessoires ont été analysés par chromatographie et par électrophorèse sur papier, avant d'être séparés sur une colonne de Chromax*.

La révélation sur papier des composés phénoliques ne pouvait pas être effectuée par la ninhydrine, le groupement α-aminé de la tyrosine étant substitué.

Le réactif de GERNGROSS[3] proposé par ACHER ET CROCKER[4] en 1952 pour révéler la tyrosine et ses dérivés, donne une coloration avec tous les phénols *para*-alkylés, à condition que les positions en *ortho* ne soient pas substituées.

Or dans une base "Mannich" préparée par action du formol sur un couple constitué par un composé aminé et un composé phénolique il y a substitution soit sur une des positions en *ortho* du noyau phénolique, soit sur les deux à la fois.

Pour révéler tous les composés phénoliques sans distinction, nous avons donc eu recours au réactif de Folin, bien connu des biochimistes.

Ce réactif fut proposé pour la première fois en 1912 par FOLIN ET DENIS[5]. Il donne une coloration bleue, en milieu alcalin, avec tous les phénols et leurs dérivés ayant la fonction –OH libre (et avec divers autres composés organiques).

Pour la préparation du réactif de Folin nous utilisons la formule de FOLIN ET CIOCALTEU[6].

Nous révélons les produits phénoliques de la manière suivante: On pulvérise sur le papier le réactif de Folin dilué 1:5. On sèche à $T° \leqslant 70°$, puis on pulvérise une solution de carbonate de soude à 15% (anhydre)[7].

Les produits révélés apparaissent en bleu foncé sur fond bleu pâle. On peut ainsi déceler sur papier, après chromatographie, 0.25 μg de tyrosine et 0.5 μg de α-N-acétyl-tyrosine et après électrophorèse des quantités deux fois plus faibles.

Appliqué à un hydrolysat protéique, le révélateur proposé présente l'inconvénient de donner une coloration bleue avec le tryptophane, en dehors de la tyrosine.

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Avec l'aide technique de
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* Appareil fourni par les Etablissements LKB.
** Professeur M. RAYNAUD.

Book Review

Gas Chromatography 1962, edited by M. VAN SWAAY, Butterworths, London, 1962, price 100 s.

With the increasing bulk of scientific literature, one of the harder tasks facing any research worker is to keep abreast with the developments in a certain field. As far as gas chromatography is concerned, the biennial symposium of the Gas Chromatography Discussion Group of the Hydrocarbon Research Group of the Institute of Petroleum offers to European chemists the possibility of following the highlights of the achievements and developments of this technique and the trend towards further progress.

This volume collects the proceedings of the 4th symposium, held in Hamburg in 1962, in co-operation with the Fachgruppe Analytische Chemie of the Gesellschaft Deutscher Chemiker, and includes the discussions of various contributors and reports on panel discussions.

The book is divided into three sections, theory, apparatus and technique, and applications. Minor contributions are presented in apparatus. Gas chromatography as a technique has made important advances in past years and considering the stage of refinement and perfection attained in commercial apparatus it seems quite difficult to conceive revolutionary improvements. As with any science which grew too fast, however, a considerable amount of work is required to develop the theory and to investigate applications. Large areas of research are still open, the field of micro analysis for instance, as outlined by A. J. P. MARTIN, seems to be one of the more promising. A large section is devoted to physico-chemical studies, such as determination of adsorption characteristics, evaluation of linear gas–solid chromatography, the interaction between various substances and stationary phases, the effect of carrier gas and column pressure on solute retention, etc. The simplicity of the technique renders gas chromatography an ideal tool for the determination of physico-chemical values.

The applications reported concern different fields, the more outstanding contributions are the separation of hydrogen isotopes and their nuclear spin isomers by means of adsorption glass capillary columns, the analysis of permanent gas mixtures and the analysis of flavour volatiles.

This volume maintains the high standard which has been established for the proceedings of these meetings. It can be highly recommended to any laboratory where research in gas chromatography is active.

ARNALDO LIBERTI (Naples)

Announcements

GAS CHROMATOGRAPHY SYMPOSIUM

The Fifth International Symposium on Gas Chromatography to be organized by the Institute of Petroleum Gas Chromatography Discussion Group will be held on September 8, 9 and 10, 1964, at Brighton, England.

Intending delegates, or those wishing to submit papers, should write to:

The Organizing Office,
Fifth International Symposium on Gas Chromatography,
61 New Cavendish Street, London, W. 1.

J. Chromatog., 11 (1963) 280

SYMPOSIUM ÜBER MODERNE METHODEN DER ANALYSE ORGANISCHER VERBINDUNGEN

Das Symposium, organisiert von der Fachgruppe "Analytische Chemie" der Gesellschaft Deutscher Chemiker und von der "Sectie voor Analytische Chemie van de Koninklijke Nederlandse Chemische Vereniging", findet vom 20.–23. Mai 1964 in Eindhoven (Holland) statt. Es stellt die Fortsetzung der Münchener Analytikertagung von 1960 dar und steht unter der Patenschaft der Analytischen Sektion der IUPAC. Als Unterthemen für das Symposium wurden geplant:

1. Elementaranalyse, funktionelle Gruppen
2. Konstitution organischer Verbindungen
   Molekülspektroskopie
3. Trennverfahren
   Wanderungsverfahren, chromatographische Verfahren
4. Analyse von Hochpolymeren
5. Analyse von Naturstoffen
   klinische Analyse.

Das Programm der Tagung wird voraussichtlich Anfang 1964 zur Verfügung stehen. Auskünfte gibt die GDCh-Geschäftsstelle, 6000 Frankfurt (Main), Postfach 9075, Deutschland.

J. Chromatog., 11 (1963) 280

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THE SHAPE OF THE ELUTION PEAKS IN GAS CHROMATOGRAPHY

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It is convenient to describe the shape of elution peaks by the *skew ratio*, R, defined as the ratio of the magnitudes of the slopes of the trailing and leading edges at their points of inflexion; thus when the trailing edge is steeper than the leading edge, the skew ratio is greater than unity.

Various theoretical treatments of gas–liquid chromatography (G.L.C.), using methods first developed for partition chromatography[1,2], start from the concept of the *distribution isotherm* which gives the concentration of the solute in the stationary phase as a function of the concentration of the solute in the moving (gas) phase. In the context of G.L.C. the distribution isotherm relates the *concentration* c of the solute in the liquid phase expressed in moles per unit volume of the phase to the *partial pressure* p of the solute, *cf.* ref. 3, pp. 96 *et seq.* According to both the "plate" treatment[1] and the "rate" treatment[4], the skew ratio should be unity if the distribution isotherm is linear, but should be less than unity if the distribution isotherm is concave towards the pressure axis and greater than unity if the isotherm is concave towards the concentration axis. The skew ratio is thus related to the *sign of the curvature*, $(\mathrm{d}^2c/\mathrm{d}p^2)_{p=0}$, of the graph of c against p near the origin:

$$R \gtrless 1 \text{ according as } \left(\frac{\mathrm{d}^2c}{\mathrm{d}p^2}\right)_{p=0} \gtrless 0 . \qquad (1)$$

Until recently no experimental data have been available to test this prediction, but it has been generally accepted and extended in the following way (*cf.* ref. 3, p. 154). At low concentrations the mole fraction x of the solute in the liquid phase is nearly proportional to the concentration c, so it is assumed that the shape of the distribution isotherm (c *vs.* p) is essentially the same as that of the gas solubility (x *vs.* p) plot, which is linear if the system obeys Henry's law,

$$p = hx , \qquad (2)$$

where h is the Henry's law constant, equal to the vapour pressure of the pure solute only if the solution is ideal over the entire concentration range*. Thus skew ratios greater than unity are said to be attributable to negative deviations from Henry's

* Although in Henry's original formulation[5] of this law the solubility was expressed in terms of amount (presumably mass) per unit volume, the statement of the law in terms of mole fractions has become universal in all thermodynamic work since LEWIS AND RANDALL[6].

law (x *vs.* p plot concave towards x axis) and *vice versa*. Furthermore, since negative deviations from Henry's law are often associated with positive deviations from Raoult's law (which may be stated as eqn. (2) but with h equal to the vapour pressure of the pure solute) and *vice versa*, the skew ratio might be expected to correlate with deviations from Raoult's law.

ASHWORTH AND EVERETT[7] have measured the solubilities of a series of low molecular weight hydrocarbons (C_5 to C_8) in dinonyl phthalate and in squalane, and the same systems have since been studied by G.L.C.[8,9]. Only the solutions of the four normal alkanes and of 2,2-dimethylbutane in dinonyl phthalate show positive deviations from Raoult's law in the low solute concentration region. In the other systems, including all the solutions in squalane, there are *negative* deviations from Raoult's law and positive deviations from Henry's law over the whole of the concentration range ($0 < x < 0.1$) covered by the G.L.C. experiments. According to the foregoing argument, we should therefore expect skew ratios *less* than unity in these latter cases and that the skew ratio should *decrease* with increasing sample size. In fact, both in the work of EVERETT AND STODDART[8] and more recent work in this laboratory[9], while the value of the skew ratio obtained by extrapolating to zero sample size is always just less than unity*, the skew ratio always increases with increasing sample size, being in some cases greater than 3 for 10 μl samples.

This apparent contradiction between theory and experiment has previously been remarked upon by FREEGUARD AND STOCK[10], who have measured the vapour–liquid "isotherms" for these systems and found them all to be concave towards the concentration axis. FREEGUARD AND STOCK state that this is the normal shape of the isotherm for all solutions of vapours in involatile liquids except those showing unusually large negative deviations from Raoult's law. While this is generally true, the situation is complicated by the fact that FREEGUARD AND STOCK have plotted their "isotherms" in terms of the amount of solute per gram of solvent rather than per ml of solution. It is therefore worthwhile to examine again the question of the shape of the distribution isotherm, with the object of establishing rigorously its curvature, since this is the property which through eqn. (1) determines the skew ratio of the elution peaks.

The concentration of the solute in the liquid phase, defined as the number of moles of solute per unit volume of solution, is well approximated by

$$c = \frac{n}{n_s v_s + nv} \tag{3}$$

where n_s and n are the numbers of moles of solvent and solute respectively, and v_s and v are the molar volumes of pure solvent and solute respectively. (The approximation consists in ignoring the volume of mixing, which is always small in dilute solutions.) The relation between the concentration c and the mole fraction x of the solute, defined by

$$x = \frac{n}{n + n_s} \tag{4}$$

* The limiting value of the skew ratio is in every case between 0.80 and 0.85. The fact that it is less than unity is probably due to finite response times of the katharometer and recorder[11].

is then

$$cv_s = \frac{x}{1 - \alpha x}, \tag{5}$$

where

$$\alpha = \frac{v_s - v}{v_s}. \tag{6}$$

Combining eqn. (5) with eqn. (2) gives for the distribution isotherm of a solution obeying Henry's law

$$cv_s = \frac{p}{h - \alpha p}. \tag{7}$$

The curvature of the isotherm is then not zero but

$$\frac{d^2c}{dp^2} = \frac{1}{v_s}\left[\frac{2\alpha h}{(h - \alpha p)^3}\right], \tag{8}$$

and at $p = 0$ has the value

$$\left(\frac{d^2c}{dp^2}\right)_{p=0} = \frac{2\alpha}{v_s h^2} = \frac{2}{v_s h^2}\left(\frac{r - 1}{r}\right) \tag{9}$$

where v_s/v has been set equal to r, the ratio of the sizes of the solvent and solute molecules, so that $\alpha = (r - 1)/r$. The limiting curvature of the distribution isotherm for a system obeying Henry's law is thus always positive unless $v > v_s$. It is extremely unlikely that the molar volume of any relatively involatile liquid suitable for use as the stationary phase in G.L.C. will be smaller than the molar volume of any relatively volatile solute which can be studied by this method. Consequently, *if a system obeys Henry's law we must expect G.L.C. elution peaks to have skew ratios greater than unity even at zero sample size.*

The confusion on this point has arisen because of the assumption that molal concentration $\bar{c}$, molar concentration c, and mole fraction x are linearly related at low concentration, *i.e.*,

$$\bar{c}\frac{M}{1000} \doteq cv_s \doteq \frac{p}{h} \tag{10}$$

instead of

$$\bar{c}\frac{M}{1000} = \frac{p}{h - p}; \qquad cv_s = \frac{p}{h - \alpha p}. \tag{11}$$

While the consequent error in c is trivial, that in the curvature d^2c/dp^2 is important.

The conditions under which a symmetrical peak is to be expected may be examined by expressing d^2c/dp^2 in terms of the activity coefficient, γ, of the volatile solute defined by

$$p = p^\circ \gamma x, \tag{12}$$

where p° is the vapour pressure of pure solute ($x \to 1$).

Combining eqns. (5) and (12) gives, instead of eqn. (11),

$$cv_s = \frac{p}{p^\circ\gamma - \alpha p}, \tag{13}$$

whence

$$\left(\frac{d^2c}{dp^2}\right)_{p=0} = \frac{2}{v_s(p^\circ\gamma^\infty)^2}\left[\alpha - p^\circ\left(\frac{d\gamma}{dp}\right)_{p=0}\right] \tag{14}$$

where γ^∞ is the value towards which γ tends as x tends to zero. Further, since

$$\frac{d\gamma}{dp} = \frac{d\gamma}{dx}\Big/\frac{dp}{dx},$$

while from eqn. (12),

$$\left(\frac{dp}{dx}\right)_{p=0} = p^\circ\gamma^\infty,$$

we find

$$p^\circ\left(\frac{d\gamma}{dp}\right)_{p=0} = \frac{1}{\gamma^\infty}\left(\frac{d\gamma}{dx}\right)_{x=0}$$

Thus $(d^2c/dp^2)_{p=0}$ is zero and $R = 1$ when

$$\left(\frac{d\ln\gamma}{dx}\right)_{x=0} = \alpha = \frac{r-1}{r}. \tag{15}$$

In general, from eqns. (1) and (14)

$$R \gtrless 1 \text{ according as } \left(\frac{d\ln\gamma}{dx}\right)_{x=0} \lessgtr \frac{r-1}{r} \tag{16}$$

For molecules of equal size $R = 1$ when $(d\ln\gamma/dx)_{x=0} = 0$, while for molecules of different sizes the limiting slope of $\ln\gamma$ against x must reach a sufficiently large value in the range 0 to 1. Since nearly all systems relevant to gas chromatography consist of mixtures of molecules of widely different sizes, it is of interest to see what predictions follow from the theories of such solutions.

We consider first athermal solutions. The statistical theory gives[12]

$$\gamma_{ath} = \frac{[r-(r-1)x]^{\frac{1}{2}z-1}}{[q-(q-1)x]^{\frac{1}{2}z}} \tag{17}$$

where z is the average number of nearest neighbours in the quasi-lattice of the liquid, and q is defined by

$$zq = zr - 2r + 2. \tag{18}$$

We shall assume that r in these equations can be identified with that defined earlier by the ratio of the molar volumes of the components (*cf.* ASHWORTH AND EVERETT[7]).

Equation (17) can be written

$$\gamma_{ath} = \frac{r^{\frac{1}{2}z-1}}{q^{\frac{1}{2}z}} \cdot \frac{(1-\alpha x)^{\frac{1}{2}z-1}}{(1-\beta x)^{\frac{1}{2}z}}, \tag{19}$$

where

$$\alpha = \frac{r-1}{r}, \quad \text{as before; and} \quad \beta = \frac{q-1}{q}. \tag{20}$$

Taking logarithms and differentiating we obtain

$$\frac{\mathrm{d} \ln \gamma_{\mathrm{ath}}}{\mathrm{d}x} = \tfrac{1}{2} z \frac{\beta}{1 - \beta x} - (\tfrac{1}{2} z - 1) \frac{\alpha}{1 - \alpha x} \tag{21}$$

so that when $x \to 0$

$$\left(\frac{\mathrm{d} \ln \gamma_{\mathrm{ath}}}{\mathrm{d}x}\right)_{x=0} = \left(\frac{r - 1}{r}\right)\left(\frac{q - 1}{q}\right). \tag{22}$$

Thus for athermal solutions

$$\left(\frac{\mathrm{d} \ln \gamma_{\mathrm{ath}}}{\mathrm{d}x}\right)_{x=0} < \frac{r - 1}{r} \quad \text{and} \quad R > 1 . \tag{23}$$

If $z \to \infty$ and r is large (*i.e.*, the case of a high polymer solution in the Flory-Huggins approximation), $q = r$ and so

$$\left(\frac{\mathrm{d} \ln \gamma_{\mathrm{ath}}}{\mathrm{d}x}\right)_{x=0} = \left(\frac{r - 1}{r}\right)^2_{r \to \infty} \simeq \left(\frac{r - 1}{r}\right) \simeq 1 \tag{24}$$

and symmetrical elution peaks will be observed, at least for very small sample sizes.

For non-athermal solutions, eqn. (17) must be multiplied by

$$\gamma_{\mathrm{th}} = \exp \frac{w}{kT} \left[\frac{q(1 - x)}{q - (q - 1)x}\right]^2 \tag{25}$$

where w is the "interchange energy" defined by

$$w = z \{ \varepsilon^*_{12} - \tfrac{1}{2}(\varepsilon^*_{11} + \varepsilon^*_{22}) \}, \tag{26}$$

and ε_{ij} are the pair interaction potentials[12]. This leads to

$$\left(\frac{\mathrm{d} \ln \gamma_{\mathrm{th}}}{\mathrm{d}x}\right)_{x=0} = -\frac{2w}{kT} \cdot \frac{1}{q} \tag{27}$$

and, adding this term to eqn. (22),

$$\left(\frac{\mathrm{d} \ln \gamma}{\mathrm{d}x}\right)_{x=0} = \left(\frac{r - 1}{r}\right) - \frac{1}{q}\left(\frac{r - 1}{r} + \frac{2w}{kT}\right). \tag{28}$$

Thus when w is positive, as was found for all the solutions studied by ASHWORTH AND EVERETT, the limiting slope of $\ln \gamma$ against x is even less than for the athermal case and the elution peaks will by more unsymmetrical.

Peak symmetry will be observed only when w has a negative value satisfying

$$\frac{2w}{kT} = -\left(\frac{r - 1}{r}\right). \tag{29}$$

The occurrence of large negative values of w is in fact extremely rare and limited to mixtures with a highly polar component. Consequently, since most mixtures of

non-polar liquids of widely different molecular size are described with reasonable accuracy by the statistical theory of solutions, we conclude that the occurrence of skew ratios greater than unity must be regarded as normal in G.L.C. irrespective of the sign of moderate deviations from Henry's law.

ACKNOWLEDGEMENT

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SUMMARY

An analysis is given of the relationship between peak shape and the deviation from ideality of the solution of vapour in the stationary phase. Earlier discussions have obscured the true situation by overlooking the mathematical consequence of assuming that the concentration of the solution is directly proportional to the mole fraction. A more rigorous analysis leads to a relationship between the nature of the peak asymmetry and the behaviour of the activity coefficient of the volatile solute in infinitely dilute solution. Comparison with the theory of mixtures of molecules of different sizes shows that asymmetry of the peak, with the slope of the trailing edge greater than that of the leading edge (skew ratio > 1), must be regarded as normal in gas–liquid chromatography irrespective of the sign of moderate deviations from Henry's law.

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HIGH EFFICIENCY GLASS BEAD COLUMNS FOR GAS CHROMATOGRAPHY

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INTRODUCTION

For a number of years, glass beads have been used as a solid support for gas chromatography. Their main advantage lies in the greater speed of separation compared with that obtainable from a conventional column. However, glass bead packings show a poor fractionating efficiency compared with other types. In this laboratory efficiencies, expressed as height equivalent to a theoretical plate, (HETP), obtained using soda glass beads, 60 to 80 mesh, ranged from HETP = 4.0 to 5.0 mm. Using finer beads (200 mesh), the efficiency can be increased to HETP = 1.5 mm. Columns made from these fine beads, however, have only limited use due to their high impedance to gas flow.

Two factors are thought to be responsible for the comparative inefficiency of these columns:

1. Non-uniformity of stationary phase layer.
2. Adsorption effects.

NON-UNIFORMITY OF STATIONARY PHASE LAYER

Most of the liquid added to the beads is drawn by capillary action to the points of contact between them. This in effect removes all but a very thin layer of liquid from most of the glass surface and forms relatively deep pockets of liquid around the points of contact. Due to this difference in depth of liquid phase the separating efficiency is poor. This has been discussed in some detail by GIDDINGS[1]. The problem therefore is to spread out the stationary phase into a layer of reasonably uniform thickness. In order to do this a rough surface must be produced. Several different methods were tried.

1. Mechanical etching

The beads were ground with different types and grades of abrasive powders. Although scratched and pitted surfaces were produced, no increase in efficiency was obtained.

2. Deposition of metal layers onto the glass surface

(a) A grey layer of silver was deposited on the surface using an ordinary glass silvering formula. The first attempt gave a surprisingly efficient column (HETP = 0.6 mm). However, all subsequent attempts to reproduce this failed.

(b) Nickel and gold deposits were produced on the surface but no improvement over untreated beads was found. In fact, the metal-coated beads usually displayed greater adsorption than the glass ones.

3. Particulate coatings

Particles of diameter 10 μ or less will adhere quite strongly to the surface of the beads in the presence of the liquid stationary phase because of the surface tension of the liquid. It was argued that the addition of particles to the surface of the beads should produce the required roughness to give efficient spreading of the liquid. The types of particle tried fall into two main classes—spherical and irregularly shaped.

(*a*) *Spherically shaped.* Two types were tried, *viz.* micro glass beads and titanium dioxide. Micro glass beads of about 1 μ diameter were added in 1 % w/w quantity to the larger beads together with 0.5% w/w squalane. Titanium dioxide also, with spherical particles of 0.25 μ diameter (Du Pont pyrolytic product) was tried. Quantities used were 1.5 % w/w titanium dioxide, 0.5 % squalane.

In both the above cases no improvement in efficiency over untreated beads was found. This is probably due to the same effect as with untreated beads: the liquid resides mainly at the points of contact, thus giving a very non-uniform layer of liquid.

(*b*) *Irregularly shaped particles.* A variety of powders was tried in this category, including inorganic salts, titanium dioxide and diatomaceous earth.

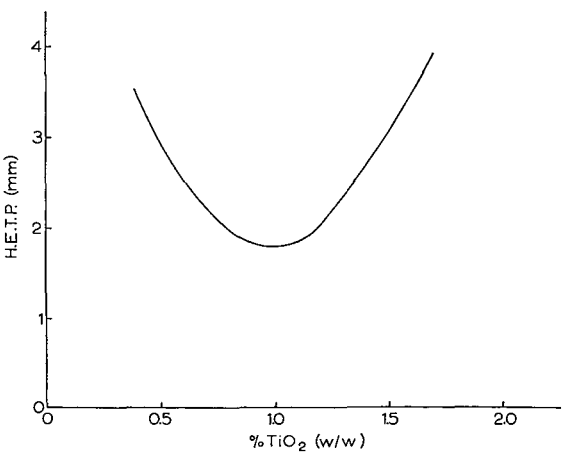

Fig. 1. Effect of additions of ordinary pigment grade titanium dioxide (0.25 % squalane).

The inorganic salts ($BaSO_4$, $BaCO_3$, $CaCO_3$) were prepared in finely divided form and added to the beads in 1.5 % w/w quantity together with 0.5 % w/w squalane. In all cases an improvement in efficiency was noted (HETP = 2.0 mm), however, the peaks tailed badly. This tailing was attributed to the polar nature of the salts.

Various amounts of titanium dioxide of irregular particle shape (conventional rutile pigment grade) were added to the beads containing 0.25 % w/w squalane. The efficiencies obtained show a maximum when plotted against weight of titanium dioxide added (Fig. 1). The best efficiency obtained was HETP = 1.7 mm.

"Celite" 501 and 541 were mixed in 1.5 % quantities with glass beads containing 0.5 % squalane. The efficiencies obtained were HETP = 1.7 mm.

The best results were obtainable with a very fine diatomaceous earth (Johns-Manville "Super-Floss").

The effect of varying both liquid and solid loading on the beads was studied using "Super-Floss" and the results are shown in Fig. 2.

The best efficiency obtained was at a loading of 0.25 % w/w squalane and 1.25 % w/w "Super-Floss" giving HETP = 0.8 mm.

For a given liquid loading the maximum efficiency was found when the ratio of squalane to "Super-Floss" was 1:5.

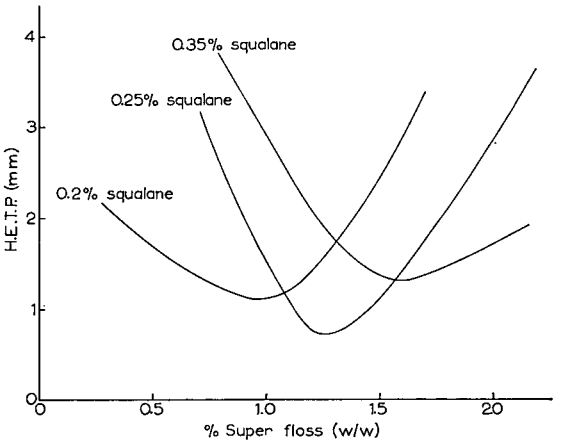

Fig. 2. Effect of "Super-Floss" at three loadings of squalane.

The preparation of the above packing is as follows:

Glass beads (60–80 mesh) are weighed out into an Erlenmeyer flask and the weighed quantity of stationary phase added. The flask is shaken vigorously to spread the liquid. If the stationary phase is too viscous, the minimum quantity of a volatile solvent required to give a solution of usable viscosity is added. The weighed amount of "Super-Floss" is then added to the beads. The mixture is shaken gently with a rotating motion for 5 to 10 min, and any lumps of "Super-Floss" are broken up with a spatula. A few small lumps of "Super-Floss" will remain. These are best left in the packing since any attempt to separate them by means of sieving will damage the particle coating. The beads are then packed into the column in the usual fashion with vibration and dumping to minimum volume.

"Super-Floss" although very fine, retains the typical diatom structures. Grinding coarser grades to similar fineness does not give an equivalent material, the diatom structure being broken down. In mixing "Super-Floss" with the beads, excessive shaking is to be avoided because of the risk of breaking down the particles.

REDUCTION OF ADSORPTION OF SOLID SUPPORT

As a result of the low liquid loading on the support, adsorption of polar compounds on the solid surfaces remains a major problem. There are two methods of at least partially overcoming this.

1. Use of a polar stationary phase

If a sufficiently polar stationary phase is used, adsorption on the solid surfaces will be suppressed. In many cases, however, a polar stationary phase may be undesirable or in fact, no suitable polar phase may be found for the desired separation. In this case a method of deactivating the polar sites on the solid surfaces by means other than the stationary phase is required.

2. Treatment with hexamethyldisilazine (H.M.D.Si.)

A method of deactivation of polar surfaces using this compound has been described[2]. However, this method was found to be long and tedious.

Effective treatment can be obtained by injection of several 2 μl lots of H.M.D.Si. into the column operating at about 120°. As the H.M.D.Si. passes through the column it reacts with the adsorptive sites on the solid support, and most of the adsorptive effects are removed.

This method has several advantages over the published method:

(a) Much simpler operation, *i.e.* deactivation is carried out while the column is in place in the chromatograph, rather than by refluxing column material for several hours, subsequent removal of solvent, etc.

(b) Less time is required. Treatment and subsequent column stabilization requires about 1 h, while other methods take more than a day.

(c) This treatment requires about 20 μl H.M.D.Si. as compared with 10 ml in the published method.

Fig. 3. shows a plot of efficiency *vs.* quantity of H.M.D.Si. added for cyclohexane and benzene. The stationary phase is squalane.

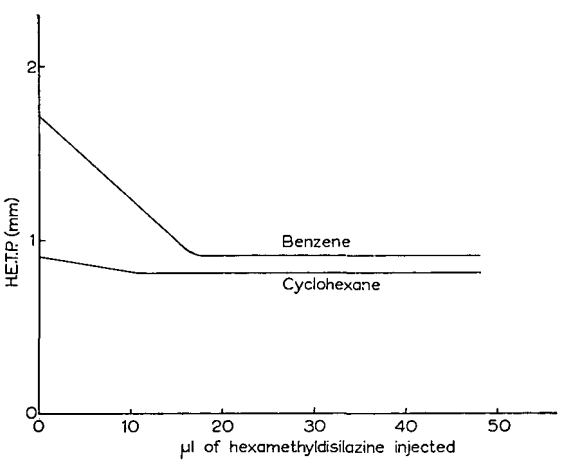

Fig. 3 Progressive effect of hexamethyldisilazine injections (squalane/"Super-Floss").

A certain amount of H.M.D.Si. remains adsorbed loosely on the support and is given off over about 1 h during normal column operation at 120°. The efficiency of the column is somewhat less for polar compounds than for non-polar compounds if a non-polar stationary phase is used. This is shown in Fig. 4, which gives a plot of

efficiency *vs.* time, beginning immediately after treatment of the column with H.M.D.Si.

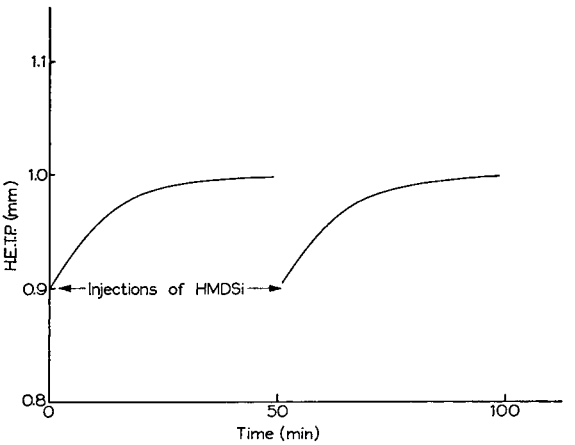

Fig. 4. Effect of loss of physically adsorbed hexamethyldisilazine (benzene on squalane/"Super-Floss").

DISCUSSION

The main source of inefficiency of glass bead columns is the non-uniformity of the depth of the stationary phase layer. This can be effectively overcome by the addition of inert particles of irregular shape onto the surface of the bead. The particles should have a diameter of about 1 to 5 μ. They are held onto the beads by the surface tension of the liquid phase, and effectively spread the liquid over the whole surface.

The maximum efficiency so far obtained reproducibly, HETP = 0.8 mm, compares very favourably with columns using solid supports such as firebrick, diatomaceous earth, etc. The highest efficiency quoted for this type of column (HETP = 0.3 mm) is better than that obtained for the modified glass beads. However, commercially available columns and most of those investigated in this laboratory give a somewhat lower efficiency, HETP = 1.0 to 2.0 mm.

The powder treatment does not, however, affect the speed of separation which makes the glass bead columns so attractive. Alternatively, a column of this type will give the same separation in similar time, but at some 100° lower than the temperature required for a conventional column. This is a considerable advantage when trace analyses are carried out at a high temperature, where bleeding of the stationary phase sets the limit of sensitivity. The effect of the treatment with H.M.D.Si is to remove adsorption caused by hydrogen bonding; it does not apparently improve adsorption effects caused by other polar forces.

SUMMARY

This paper sets out the results of an investigation into the methods of improving the efficiency of glass bead columns for gas chromatography. Efficiencies, expressed as height equivalent to a theoretical plate (HETP) of the order of 0.8 mm, as compared with 0.6 to 2.0 mm for conventional kieselguhr columns and 4.5 mm for normal glass

bead columns have been achieved. The techniques used in obtaining these efficiencies are described.

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A NEW QUANTITATIVE INTEGRAL DETECTOR FOR GAS CHROMATOGRAPHY

THE "BRUNEL" MASS INTEGRAL DETECTOR

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The international adoption of gas chromatographic methods makes the establishing of universally acceptable quantitative standards a matter of special importance. Although quantitative analysis by gas chromatography can be a method of high precision and accuracy, often exceeding those of alternative methods in those cases where alternative methods are available, the attainment of high accuracy may demand time-consuming measurement of zone areas[1,2] as well as calibration of the detector for molecular response[3–10]. These requirements are in addition to those of representative sampling and injection, a stable partition system, a linear detector[7,11,12], and a reliable recorder. Whilst the whole question of quantitative gas chromatographic analysis is complex, the kind of detection system available does much to determine the approach made to any particular problem.

The requirements[13–20] to be met by a useful detector include: high sensitivity, wide applicability, stability, rapidity of response, adaptability to automatic recording, safety, ease of construction, economy, robustness and, a point of special importance in quantitative analysis, a response directly related to some fundamental property such as mass, so that calibration for every sample component and for varying conditions of operation is unnecessary. No single detection system has yet been devised which satisfies all of these demands, and in practice the selection of a detector is determined by the relative importance attached to the various demands listed above.

The progress of gas chromatography during the past ten years has been marked by the invention of a considerable number of different detectors, designed to respond to changes in various physical or chemical properties of the column effluent gas. A limited number of these detectors have been successful in establishing themselves in practice; a successful detector owes its acceptance to its ability to meet some only of the requirements conspicuously well. Thus, the integral titrimetric method of JAMES AND MARTIN[21], and the gas volumetric method of JANAK[22] have the virtues of integral measurement but are of very restricted application. SHAKESPEAR'S katharometer[23], MARTIN'S gas density meter[24,25] and SCOTT'S hydrogen-flame detector[26] have proved their worth in commercial chromatographs. These three detectors are useful at concentrations in the intermediate range, down to about one part in 10^6 of carrier gas, that is, with column loads of about 1 mg. The gas density meter requires only a knowledge of molecular weights for simple calibration. The argon detector of

LOVELOCK[27] and the hydrogen flame ionization detector of MCWILLIAM[28] are conspicuous for extreme sensitivity. Capable of detecting concentrations of solute smaller than one part in 10^{10} of carrier gas, they make possible column loadings of as little as a fraction of a microgram, but they require calibration for molecular response and, like all the other detectors mentioned excepting the first two, they furnish a differential chromatogram.

The advantages of high sensitivity detectors are generally recognized[19]; they are of particular value in the analysis of scarce materials and in the selection of optimum operating conditions. However, with diminishing sample size, errors independent of the detection system may dominate the attainable accuracy of the results[29,30]. The introduction of a predetermined amount of a representative sample becomes more difficult, the spread, purity and reactivity of the stationary phase may complicate or even vitiate the quantitative interpretation of the chromatogram. Further, the effective inertness of the support may no longer be presumed, and the quality of the carrier gas may become more critical.

The recent heavy emphasis on high sensitivity of detection, coupled with corresponding improvements in column efficiency[31,32], has elevated gas chromatography to the status of one of the most sensitive of all methods of analysis. This concentration of attention and effort on microanalytical aspects may well be responsible for some overlooking of the merits of less sensitive detectors, and may have helped to obscure the possibility of other lines of development. It is remarkable that no success appears to have been achieved in applying what must be, in principle at least, the simplest and most direct of all possible methods of detection, namely, the direct continuous weighing of the sample components as they emerge from the column.

We have established the feasibility of a mass integral detector of this kind. By passing the effluent gas from the column through a suitable absorption vessel mounted on a balance, the change in weight brought about by the arrival of absorbable gas or vapour may be observed directly, or may be made to operate a continuous chart recorder. The integrams obtained in this way provide ready quantitation by measurement of the step-heights.

Although so simple in principle, the integral mass detector offers certain important advantages over most of the established detection systems. Since it is the fundamental property of mass which is being measured, molecular response factors are not involved, and no calibration or calculation is necessary in obtaining the gravimetric composition of the sample. Moreover, the need for an integrator[33–36], always an expensive item and not always a reliable one, is eliminated.

In most of our experiments with the mass detector we have used samples in the range 1–20 mg, but we see no reason why this method of detection should not be employed with samples as small as, say, 20 μg, or, at the other extreme, as large as are used in preparative gas chromatography.

EXPERIMENTAL

In our preliminary experiments on mass detection we tried the fairly obvious method of bubbling a fine stream of the column effluent gas through a quantity (about 20 ml) of an involatile solvent, such as di-octyl phthalate, contained in a glass weighing-bottle mounted on the pan of a direct-reading, air-damped analytical balance. The

results of these first tests were highly encouraging, although the success was incomplete. Stepped chromatograms were, indeed, obtained, but they suffered from two serious faults: first, the step-heights did not correspond to the known weights of the sample components, but were about 20 % low; and second, the weight of the absorption system did not remain constant after each absorption, but showed a steady decline following the absorption of each component. The first fault was due to the buoyancy effect of the absorber liquid on the gas inlet tube which, being fixed, was immersed to a depth which varied with the vertical movement of the balance pan during the experiment. The second fault was due to loss, by volatilization, of dissolved components after their initial absorption.

Corrections for these two faults could, however, readily be applied to the recorded weights of the absorption system, and mass integrams could then be plotted. Fig. 1 shows such a corrected mass integram for a mixture of ether, acetone and chloroform separated on a 300 × 6 mm column of 60–80 BSS Celite with 15 % polyethylene glycol adipate at 20°, the absorber liquid being di-octyl phthalate.

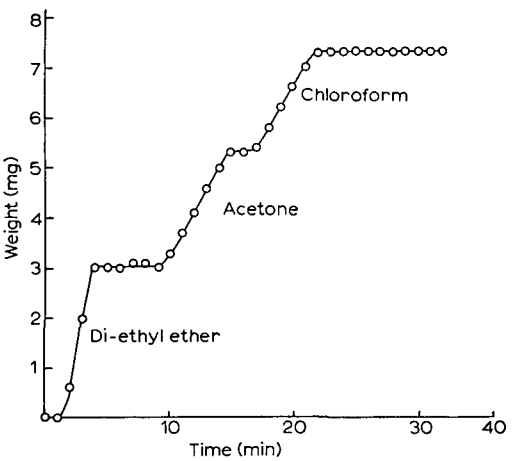

Fig. 1. Mass integram from standard analytical balance (corrected masses).

The feasibility of direct mass detection having thus been established, attention was directed in the next experiments to elimination of the faults of the absorber used in the earlier tests. Furthermore, in all later experiments the changes in weight of the absorber system were followed and recorded automatically by a recording balance (Stanton Automatic Thermo-Recording Balance, Model TR-01).

Buoyancy effects were eliminated by avoiding contact between the gas inlet tube and the absorption liquid in the absorber. The effluent gas from the column, instead of being made to bubble through the liquid, was made to pass close to a layer of liquid lining the interior surface of the absorber. In order to ensure efficient absorption, woven glass cloth, impregnated with absorber liquid, was used to provide a large area of absorbing film.

Errors of the second type, *i.e.*, loss of dissolved component by volatilization from the absorber, were eliminated, as far as many different kinds of samples are concerned, by replacing the simple solvent used in the preliminary tests by a chemi-

cally reactive absorber which, instead of merely dissolving physically the components as they reached the absorber, reacted chemically with them, thus fixing them in a form exerting very low vapour pressure, so that loss by vaporization was negligible. Thus, acidic substances in the column effluent gas were absorbed by solid sodium hydroxide or potassium hydroxide, or by concentrated solutions of these in an involatile solvent such as ethylene glycol. Another chemical absorber of very general usefulness is concentrated sulphuric acid. This effectively absorbs not only amines, but also alcohols, ethers and ketones forming with them, by proton donation, -onium ions of the types: R_3NH^+, ROH_2^+, R_2OH^+ and R_2COH^+. We are currently studying the efficiency of absorption of cooled active charcoal.

A simple form of the mass integral detector, (conveniently referred to as the "Brunel" detector), is shown in Fig. 2. The gas stream from the column is carried into the absorption vessel by a capillary connector of up to 0.5 mm internal diameter and about 12 cm in length. Provided that the diameter of the connector is kept small,

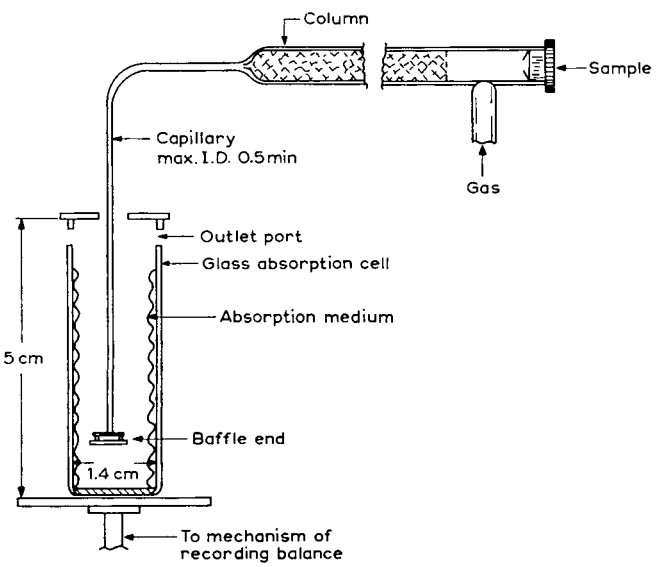

Fig. 2. Mass detector—simplex design.

the length appears to have negligible effect on the resolutions obtainable. The capillary ends in a baffle plate which deflects the stream of gas against the sidewall of the absorber, thus aiding absorption and minimizing the effect of the gas stream on the balance, in the direction of movement of the balance pan. The carrier gas, stripped of its absorbable components, emerges from the absorber through the capillary inlet port, or through side ports. Our experiments indicate that in the cases of amines, alcohols, ethers and ketones, absorption by concentrated sulphuric acid is quantitative, and that these components, once absorbed, are not lost by volatilization. The accuracy obtainable in these cases is determined by the sensitivity of the balance rather than by the absorber. A typical integral chromatogram, obtained using a sulphuric acid absorption cell, is reproduced in Fig. 3. Sample: 3.8 mg; column: 100 × 0.3 cm; support: alkali-treated Celite 80-100 BSS; stationary phase: 10% polyethylene

glycol 400; temperature: 20°; gas-flow: 25 ml nitrogen per minute. Such integrams represent the attainment at the milligram level of an ideal expressed by Dr. A. J. P. MARTIN[20,37] at the 4th International Gas Chromatography Symposium in Hamburg, June 1962, at which a preliminary announcement of our work was made.

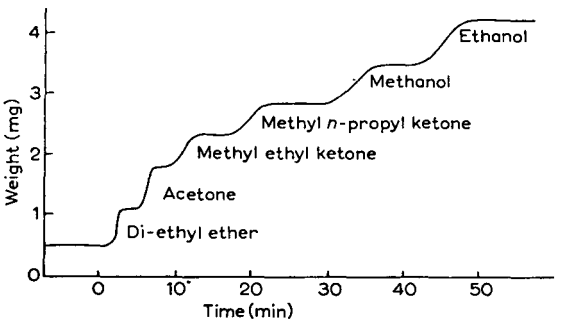

Fig. 3. Mass integram from recording balance.

Development work in progress includes detector design, extension of mass detection to the microgram range, including the use of electrobalances of the Cahn or Sartorius types, and the possibilities of selective absorption.

Patent applications have been made in respect of this invention[38] and rights have been assigned to the National Research Development Corporation.

SUMMARY

The ideal detector for quantitative analysis by gas chromatography would need neither calibration nor the application of integration procedures to a differential chromatogram. This paper describes the experimental realization of a detector possessing these two desirable characteristics. The "Brunel" mass integral detector yields an integral chromatogram in which the absolute masses of the sample components are directly recorded as step-heights.

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GAS CHROMATOGRAPHIC INFORMATION STORAGE AND RETRIEVAL BY THE UNITERM SYSTEM

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Within the last decade gas chromatography has become one of the most popular techniques of organic chemical analysis. This is clearly shown by the number of papers which have appeared on this subject. *Gas Chromatography Abstracts* have published close to 4000 abstracts for the period 1952–1961 and even this figure probably does not represent a complete coverage. The number of publications in this field is growing at such a rate that it is becoming increasingly difficult to keep up with the literature. It is clear from the size of the literature on gas chromatography that collecting it will have little utility without a suitable technique of information retrieval. However, little has been published concerning the suitability of the various information systems for gas chromatography. SPENCER AND JOHNSON[1] have reported details of a punched card system for the storage of gas chromatographic data for hydrocarbons. LEWIS *et al.*[2] expanded this method by using key punched IBM cards and including more data. However, both these systems, as described, have only been applied to the storage of a limited number of concepts.

Of the various systems of information storage and retrieval, we chose the Uniterm system[3,4] for our card file on gas chromatography which at present consists of close to 2500 literature items. Information is being stored under more than three hundred Uniterms. The Uniterm system involves no codes, is highly flexible and simple to operate and both storage capacity and depth of indexing are almost unlimited. However, the direct retrieval of retention data, as described by both SPENCER AND JOHNSON and LEWIS *et al.*, has not been attempted. Although this is undoubtedly possible with the Uniterm system, the punched card system would appear to be the more suitable method for this particular application.

The Uniterm system is essentially a system of co-ordinate indexing consisting of an information store and a concept (Uniterm) store. In the information store the information cards (abstract cards, reprints etc.) are given consecutive integral numbers (non-significant accession numbers) in the order in which they are received, so that each information card is characterised by an arbritary storage number which serves as its address. The information that these cards contain is broken down into a number of simple concepts or bits of information called Uniterms.

The Uniterms, which are subject headings for unit concepts, all of equal hierarchical stature, are stored independently in the form of Uniterm cards, each of which represents a single concept (Uniterm). This store of Uniterm cards makes up the information retrieval system.

Examples of Uniterms are the names of stationary phases, the names of materials

analysed, operating conditions such as temperature, flow rate etc., application of technique such as surface area determination, relative volatility determination etc. As there is no limit to the number of terms which can be chosen there is no limit to the depth of indexing. For example, a generic term such as HYDROCARBONS can be broken down into subgroups such as PARAFFINS, NAPHTHENES, AROMATICS, OLEFINS etc. and the subgroups can be broken down further into specific molecular weight ranges or even individual compounds. Parametric concepts such as COLUMN PROPERTIES can be more rigorously specified by qualifying this Uniterm with the addition of nouns, prepositions, adjectives etc. For example: COLUMNS–CAPILLARY; COLUMNS–PACKED; COLUMN–EFFICIENCY, EFFECT ON; COLUMN–EFFICIENCY, NUMERICAL VALUES; COLUMN–GEOMETRY, EFFECTS OF, etc. The efficiency of the retrieval system depends directly on the specificity of the Uniterms used. The more qualified the concept involved, and consequently the larger the number of Uniterms, the more rapid and efficient will be the information retrieval. However, such systems would require a greater amount of indexing so that the depth of indexing, which is a matter of choice, depends on the individual requirements. Translation of the information into Uniterms is of necessity performed by personnel experienced in the field.

The freedom of choice of terms and the fact that all terms are independent and of equal hierarchical stature makes this system highly flexible, permitting the introduction of new terms as necessary. This is one of the great advantages of the Uniterm system.

Indexing consists of entering the accession number of the information card on the relevant Uniterm card, the column being determined by the terminal digit of the accession number. The accession number 351 is entered in column 1, the number 659, in column 9 etc. The numbers are arranged in ascending order in the columns to facilitate comparison and searching (see Fig. 1).

OLEFINS

0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
100	121	62	93	44	85	406	27	78	99
140	351	182	373	84	555	436	167	98	129
160	371	302	703	174	635	476	187	208	369
200	561	332	783	234	645	636	227	218	439
350	631	372	863	634	665	646	297	278	539
390	691	432	923	644	815	686	347	408	589
410	701	592	973	734	825	736	387	428	599
430	731	602	1053	794	895	766	397	558	679
440	851	642	1133	824		806	407	628	699
540	911	682	1413	1004		816	437	638	729
660	1281	702	1483	1134		976	477	728	779

Fig. 1. A typical Uniterm card.

If card 740 discusses the relative merits of dinonyl phthalate and squalane for the separation of a mixture of paraffins and aromatic hydrocarbons in terms of π bond–polar substrate interaction and vapour pressure effect, the number 740 is written in the 0 column of the following Uniterm cards: DINONYL PHTHALATE–STATI-

ONARY PHASE, SQUALANE–STATIONARY PHASE, PARAFFIN HYDROCARBONS, AROMATIC HYDROCARBONS, SOLUTE–SOLVENT INTERACTION and VAPOUR PRESSURE EFFECTS. Depending on the requirements, the depth of indexing can be increased and Uniterm cards can also be made out for the individual paraffins and aromatics mentioned as well as for the Uniterms, POLAR STATIONARY PHASES and NON-POLAR STATIONARY PHASES.

As the information retrieval consists of a collection of independent concepts it is possible to retrieve information in the form of combinations of specific qualified concepts, which when co-ordinated, represent the sought for idea. This is done by withdrawing the relevant Uniterm cards (representing the simple concepts) and scanning them vertically, columnwise, for common numbers. The common numbers will then represent information cards which contain the information sought, that is, the complex concept represented by the combination of simple concepts. For example, if information is required on the relative merits of polar and non-polar stationary phases for the separation of paraffin–aromatic hydrocarbon systems then the four Uniterm cards POLAR STATIONARY PHASES, NON-POLAR STATIONARY PHASES, PARAFFIN HYDROCARBONS and AROMATIC HYDROCARBONS are scanned for common numbers. A card whose number is found on all four Uniterm cards will contain information on all four concepts. However, it is not always the case that the concepts involved are related in the desired manner. A card may contain information on all four concepts without actually discussing the relative merits of the two types of stationary phases for the separation of paraffin–aromatic systems. The information might concern the separation of paraffins on non-polar stationary phases and the separation of aromatic–olefin systems on polar stationary phases. This does not invalidate the indexing but indicates a lack of depth of indexing or qualification. It can be avoided by increasing the number of Uniterm cards by the addition of the Uniterm PARAFFIN–AROMATIC HYDROCARBON SEPARATION. The desired information would then be sought on the three Uniterm cards (instead of four): POLAR STATIONARY PHASES, NON-POLAR STATIONARY PHASES and PARAFFIN–AROMATIC HYDROCARBON SEPARATION.

Certain Uniterm cards tend to become overloaded. Examples are the Uniterms PARAFFIN HYDROCARBONS and SILICONE STATIONARY PHASES. The greatest amount of work on any single molecular type in gas chromatography has been the separation of the enormous number of paraffin isomers present in natural hydrocarbon fractions. Several hundred articles have been published on this subject. It is clear that a single Uniterm covering this concept will be inadequate. However, making Uniterm cards for each individual isomer will be advisable only for laboratories specifically interested in this subject. A good way to solve this problem is to make Uniterm cards for specific molecular weight ranges, the number of Uniterm cards can thus be limited according to requirements.

Silicones have always been one of the most popular stationary phases and in this case too it is also desirable to increase the depth of indexing. This is readily done by making Uniterm cards for each type of silicone.

Indiscriminate information storage can also result in overloading. For example, an article discussing the separation of ketones and esters might also mention benzene. The storage of this information, which has little informational value relative to the large amount of work published in far greater detail on the separation of aromatics,

will cause overloading which is not compensated for by the informational value involved. This can be avoided by screening (that is, not storing this information) or by the use of the proposed colour coding system mentioned below. The great majority of published papers supply operating conditions and, unless unusual conditions are involved, the storage of information on numerical values of such operating conditions as flow rate and temperature will result in gross overloading and seriously reduce the efficiency of the method.

Some minor refinements to reduce noise and increase retrieval efficiency are planned in these laboratories. As all terminal digits in any column of the Uniterm card are identical there is actually no necessity for their presence. If these terminal digits were eliminated it should make the search for common numbers on several Uniterm cards simpler. For example the numbers 12, 562 and 2532 could be written in column 2 as 1, 56 and 253 respectively with the understanding that the number 2 be added as a terminal digit to any number in column 2 when the abstract card number is sought. Numbers represented by units only could be entered into the corresponding column as zeros to avoid their elimination.

Noise could be reduced by indexing address numbers of cards containing information of particular interest in a different colour. Colour coding could also be used to reduce the number of Uniterm cards by combining two similar Uniterms on one card, for example RETENTION TIME DATA and RETENTION TIME, EFFECT OF VARIOUS PARAMETERS ON.

The compilation of a thesaurus of the Uniterms in use is advisable to avoid duplication. Uniterms seldom consist of single words and multiword Uniterms can frequently be written in several ways, for example TEMPERATURE PROGRAMMING and PROGRAMMED TEMPERATURE. Synonyms also occur such as injection block, flash heater and flash evaporator. A very useful list of Uniterms for gas chromatography and a ready made thesaurus is the index of Gas Chromatography Abstracts. In fact, it should be highly profitable to combine the Uniterm system with Gas Chromatography Abstracts by writing the Gas Chromatography Abstract numbers on the appropiate Uniterm cards (colour coded).

In these laboratories the Uniterm cards have been classified into related groups. For example all Uniterm cards concerning stationary phases are stored together in alphabetic order. Uniterm cards of stationary phase functions such as STATIONARY PHASE COMPARISON, TEMPERATURE LIMIT, CONDITIONING, PRETREATMENT, etc. are similarly stored adjacent to the stationary phase cards. This system greatly facilitates the search for these cards and simplifies Uniterm combinations to build more complex concepts. The subdivision of the Gas Chromatography Abstracts index is a good example of such a classification.

As many of the abstract cards in these laboratories were prepared from references and abstracts, an author index has been found necessary to avoid duplication. This system has been in use three years and has been found to work very satisfactorily.

ACKNOWLEDGEMENT

The authors are indebted to the Israel Mining Industries for permission to publish this work.

SUMMARY

The organization of gas chromatography literature for storage and retrieval by the Uniterm system of coordinate indexing is discussed. In this system the contents of the separate literature items are broken down into simple concepts which are stored independently. By combining these simple concepts into a more complex one retrieval of the sought idea is achieved.

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THE APPLICATION OF GAS CHROMATOGRAPHY TO THE DETERMINATION OF RETENTION DATA OF SOME TRIMETHYL-SILYL ETHERS AND TRIMETHYL-SILYL THIOETHERS

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INTRODUCTION

It has been realised for some time that gas chromatography can be applied to the determination of some physical constants of some compounds, and its use in the analytical field for the analysis of complex mixtures of organo-metallic compounds is ever widening. Following the preliminary recommendations of the International Union of Pure and Applied Chemistry[1] as to the systematised nomenclature for retention data, standard apparatus, control and experimental procedures, specific retention volumes are measured for a homologous series of trimethyl-silyl ethers, and trimethyl-silyl thioethers.

The particular problems encountered in the gas chromatography of these compounds include the tendency to hydrolyse and breakdown on some stationary phases and under some conditions. To overcome these difficulties, high column temperatures are needed to produce reasonable retention volumes for the thioethers. The accuracy and reproducibility of retention volumes is estimated.

EXPERIMENTAL

Preparation of trimethyl-silyl ethers and thioethers

With the exception of the ethyl ether which was available commercially, the methyl, *n*-propyl, isopropyl, *n*-butyl, and *tert.*-butyl ethers were prepared from redistilled trimethylchlorosilane, and the appropriate alcohol[2]. Ammonia was passed into the reaction mixture to remove hydrogen chloride—as ammonium chloride. Samples obtained during fractional distillation of the products were analysed by gas chromatography to estimate their purity.

In contrast the thioethers were prepared by the interaction of trimethylchlorosilane and the corresponding lead mercaptide[3] since no reaction occurs between the chlorosilane and the thiol. Ethyl, *n*-propyl, isopropyl, *n*-butyl and *tert.*-butyl thioethers were prepared in this way. Hexamethyldisilthiane was prepared[3] by the reaction between trimethylchlorosilane and anhydrous sodium sulphide in pressure vessels at 250°.

Apparatus

The gas–liquid chromatographic apparatus used was a Burrell "Kromo-Tog" Mk. 1

instrument. The standard column used was a 2.5 m U-column of 0.5 cm internal diameter glass tubing, the exterior being wound with a heating coil giving a continuous variation of column temperature up to 300°. The temperature was measured by a thermocouple inserted into the top of one leg of the column. The carrier gas used was B.O.C. oxygen-free nitrogen, whose flow-rate was measured at the end of the flow system by a soap-bubble flowmeter. The pressure of the gas after passing through silica gel and Linde "Molecular Sieve" Type 5A drying columns was measured to within 1 mm of mercury using a mercury manometer. All connecting tubes in the gas-system were carefully checked to ensure no possible entry for moisture. Liquid samples were introduced with an error of less than 2 %, using Hamilton 10 or 100 μl syringes through a puncture-type silicone-rubber seal directly on to the top of the column. The detector was a 4-filament type, situated directly at the end of the column. The detector temperature was kept constant at 150° (this was sufficient to prevent any condensation of samples in the detector), and the filament current was 120 mA. The output from the detector was recorded directly by a Honeywell-Brown 1 mV F.S.D. potentiometric recorder.

Stationary phases

The stationary phases used were present to 20 % w/w extent on acid-washed Silocel C-22 firebrick 60–85 mesh; the liquids used were (i) polyethylene glycol 400, (ii) dinonyl phthalate, (iii) tritolyl phosphate, (iv) squalane and (v) Apiezon L grease. Great care was taken to ensure that no moisture was present on the liquid phase or support.

Retention volume

The specific retention volume V_g, is defined as V_R^0, the retention volume fully corrected for dead volume, pressure drop across the column and measured at the column temperature, divided by the weight of the solvent; it is a precise physical property for any substance, a minimum set of conditions for its measurement have been proposed[1]. These recommendations were followed as closely as possible with the apparatus available, and the specifications concerning measurements at different inlet/outlet pressure ratios and column temperatures were observed.

RESULTS

The two stationary phases chosen for systematic measurements of the oxygen ethers were Apiezon L, and tritolyl phosphate, neither of which caused any appreciable hydrolysis of the samples injected on to the column. Investigations showed an increasing amount of hydrolysis of the oxygen ethers to hexamethyldisiloxane when using squalane (2,6,10,15,19,23-hexamethyltetracosane) as a stationary phase as the column temperature was raised from 75° to 110°. Breakdown being almost complete above 120°. Similar results were obtained with polyethylene glycol 400 except the hydrolysis was greater at low temperatures; this must be due to the presence of hydroxyl groups present on the phase which become active at these temperatures. Dionyl phthalate was investigated but rejected because its great selectivity caused retention volumes and volumes which were too large to measure accurately when the recommended specifications were followed.

TABLE I

SPECIFIC RETENTION VOLUMES ON APIEZON L

Solvent	*Apiezon L*				
Temperature °C	*58°*	*80°*	*100°*	*120°*	*140°*
$(CH_3)_3Si$–O–R					
R = CH_3	16.3 ± 0.5	6.73 ± 0.03	4.28 ± 0.08	2.57 ± 0.06	1.50 ± 0.01
C_2H_5	26.2 ± 1.1	10.07 ± 0.26	6.22 ± 0.07	3.72 ± 0.07	2.03 ± 0.06
iso-C_3H_7	33.9 ± 0.4	12.8 ± 0.54	7.93 ± 0.16	4.71 ± 0.09	2.69 ± 0.03
n-C_3H_7	53.7 ± 1.3	19.1 ± 0.7	11.56 ± 0.13	6.96 ± 0.06	3.83 ± 0.08
n-C_4H_9	116.0 ± 0.8	38.8 ± 1.0	22.1 ± 0.7	12.42 ± 0.12	6.64 ± 0.14
$Si(CH_3)_3$	33.7 ± 1.5	12.7 ± 0.5	7.74 ± 0.12	4.65 ± 0.06	2.59 ± 0.04

TABLE II

SPECIFIC RETENTION VOLUMES ON TRITOLYL PHOSPHATE

Solvent	*Tritolyl phosphate*		
Temperature °C	*80°*	*90°*	*100°*
$(CH_3)_3Si$–O–R			
R = CH_3	5.65 ± 0.03	3.70 ± 0.03	2.69 ± 0.04
C_2H_5	7.88 ± 0.11	5.14 ± 0.08	3.64 ± 0.04
iso-C_3H_7	8.76 ± 0.06	6.18 ± 0.04	4.30 ± 0.05
n-C_3H_7	13.70 ± 0.27	9.10 ± 0.13	6.40 ± 0.09
tert.-C_4H_9	12.02 ± 0.07	7.99 ± 0.04	5.65 ± 0.01
n-C_4H_9	26.52 ± 0.53	16.75 ± 0.20	11.19 ± 0.23
$Si(CH_3)_3$	6.57 ± 0.80	4.36 ± 0.08	3.03 ± 0.04

TABLE III

SPECIFIC RETENTION ON VOLUMES ON APIEZON L

Solvent	*Apiezon L*		
Temperature °C	*160°*	*180°*	*200°*
$(CH_3)_3Si$–S–R			
R = C_2H_5	4.33 ± 0.08	2.89 ± 0.03	2.10 ± 0.04
iso-C_3H_7	5.31 ± 0.08	3.43 ± 0.11	2.48 ± 0.07
n-C_3H_7	6.53 ± 0.09	4.25 ± 0.07	3.07 ± 0.05
tert.-C_3H_7	7.65 ± 0.03	5.18 ± 0.14	3.70 ± 0.02
n-C_4H_9	9.95 ± 0.08	6.52 ± 0.08	4.49 ± 0.03
$Si(CH_3)_3$	7.16 ± 0.09	4.80 ± 0.11	3.31 ± 0.04

The only phase found suitable for the gas chromatography of the silyl thioethers was Apiezon L. Complete breakdown of the ethers was noted on phases, *e.g.* tritolyl phosphate even at 80°.

The values of V_g given in Tables I–III are the mean values at each temperature of determinations at inlet/outlet pressure ratios of 1.2, 1.4 and 1.6.

Errors given are on the mean of several determinations and are in almost every case less than 2 %.

DISCUSSION

Chromatography of the trimethyl-silyl ethers

Fig. 1 shows the graphical relationship between log V_g and column temperature for the trimethyl-silyl ethers on Apiezon L and tritolyl phosphate. The relationship is approximately linear for both phases, tritolyl phosphate showing a slight curvature,

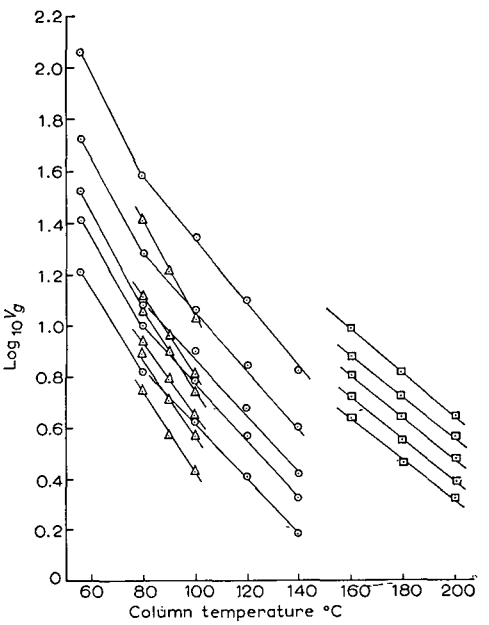

Fig. 1. Mean values for $\log_{10} V_g$ (at inlet/outlet pressure ratios 1.2, 1.4, 1.6) against column temperature, for the series. ⊙ Me_3Si–O–R on Apiezon L; △ Me_3Si–O–R on tritolyl phosphate; ⊡ Me_3Si–S–R on Apiezon L.

with the exception of log V_g values measured at 58° on Apiezon L. The discrepancy is attributed to Apiezon L not being fully liquid at this temperature, thus giving rise to distinct retention characteristics.

Figs. 2 and 3 relate log V_g to the number of carbon atoms in the alkoxy group, for different column temperatures on Apiezon L and tritolyl phosphate respectively. As may be predicted from theoretical considerations, approximately linear plots for the straight chain members were obtained. The exception was the methyl ether (having a greater V_g value than expected) which may be due to some interaction with the stationary phase since other considerations such as "dead time errors" can be

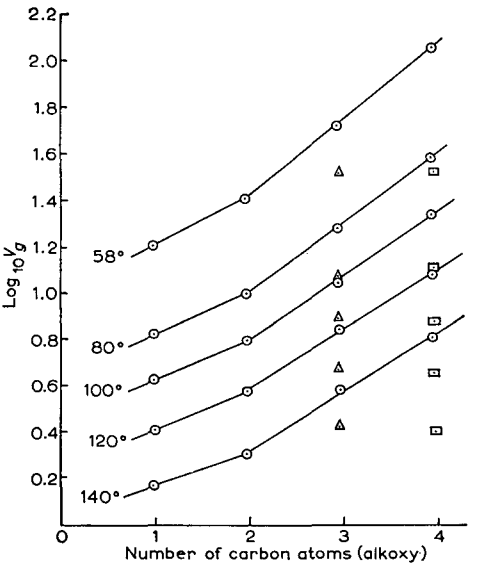

Fig. 2. Mean values for $\log_{10}V_g$ (pressure ratios 1.2, 1.4, 1.6) against number of alkoxy carbon atoms [counting one silicon as carbon in $(CH_3)_3SiOSi(CH_3)_3$], for different column temperatures. 20% Apiezon L stationary phase. ⊙ Normal alkoxy series; △ R = $\cdot CH(CH_3)_2$; ⊡ = $(CH_3)_3SiOSi(CH_3)_3$.

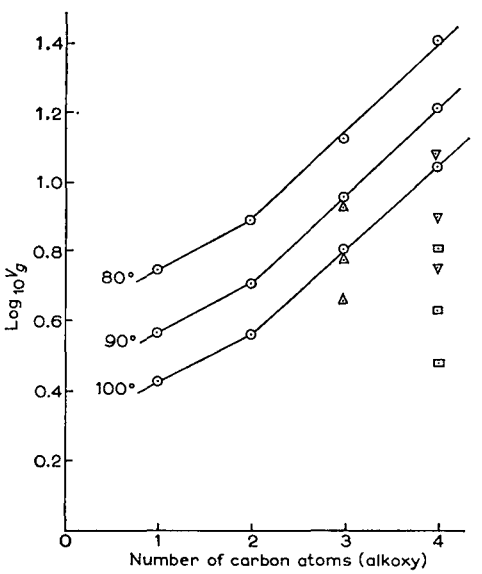

Fig. 3. As Fig. 2 except for 20% tritolyl phosphate stationary phase. ⊙ Normal alkoxy series; △ R = $-CH(CH_3)_2$; ▽ R = $-C(CH_3)_3$; ⊡ = $(CH_3)_3SiOSi(CH_3)_3$.

ignored because of the standard procedures adopted. The branched chain isomers show correspondingly smaller retention volumes, although these are not exactly paralleled by their boiling points (see Fig. 4) which shows the mean values of log V_g against boiling point on 20 % Apiezon L at 100°. Here, straight and branched chain isomers are seen to be linear, with the exception of the *tert.*-butyl ether and hexamethyldisilox-

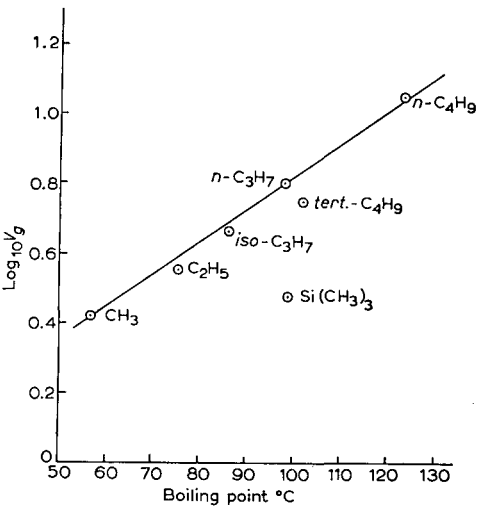

Fig. 4. Mean values of $\log_{10} V_g$ against boiling point for trimethyl-silyl alkyl ethers on 20 % tritolyl phosphate at column temperature 100°.

ane. These two, especially the disiloxane, have much smaller retention volumes than anticipated from their boiling points. Thus there is some indication of selective retention of straight chain compounds over the more bulky branched isomers. Also the compound having an ≡Si–O–Si≡ linkage is eluted much more rapidly than its ≡Si–O–C≡ analogues.

On Apiezon L, hexamethyldisiloxane is seen to have a retention volume very close to that of the isopropyl ether, whilst on tritolyl phosphate, it has a smaller retention volume than the ethyl ether. This indicates selective retention of the disiloxane on Apiezon L as compared with tritolyl phosphate.

Chromatography of trimethyl-silyl thioethers

At the temperatures necessary to elute them, some breakdown into lower boiling components occurred, the proportion of breakdown for the majority of the thioethers increasing from 5 % to 25 % between 160° and 200°. An exception was the *tert.*-butyl thioether which showed a greater tendency to breakdown than the straight chain compounds, and could not be eluted even at 180° at low pressure ratios. Hexamethyldisilthiane showed less degradation than the carbon-containing compounds.

Since the preliminary breakdown products are consistent in form for each thioether under different conditions, but differ between the thioethers, there is scope for a more complete systematic study and characterisation of their decomposition, and this is under active investigation.

As for the oxygen analogues, graphs are drawn of (i) log V_g against column

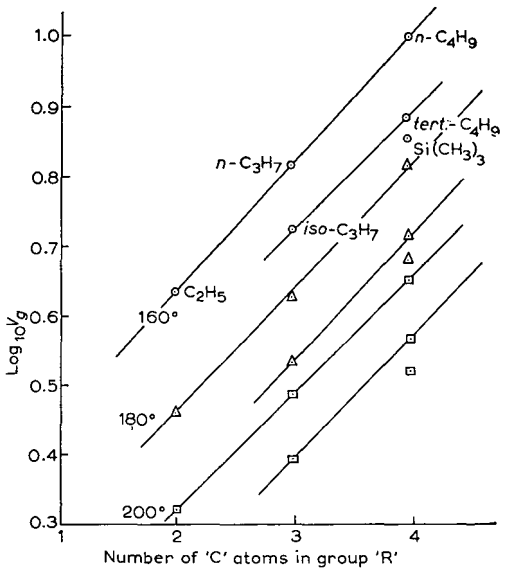

Fig. 5. Mean values for $\log_{10} V_g$ (pressure ratios 1.2, 1.4, 1.6) against number of carbon atoms in group R in thioethers for different column temperatures. 20% Apiezon L stationary phase. ⊙ = 160°; △ = 180°; ⊡ = 200°.

temperature for each thioether (Fig. 1), (ii) log V_g against the number of carbon atoms in the group R (Fig. 5). The former graph has a similar form to that for the oxygen series with some slight curvature. The latter graph also shows a similar form to that obtained for the oxygen series. The normal members of the series are again almost linear while the branched chain isomer have lower value of V_g, consistent with their boiling points. In contrast to the oxygen series on Apiezon L, where the V_g values of

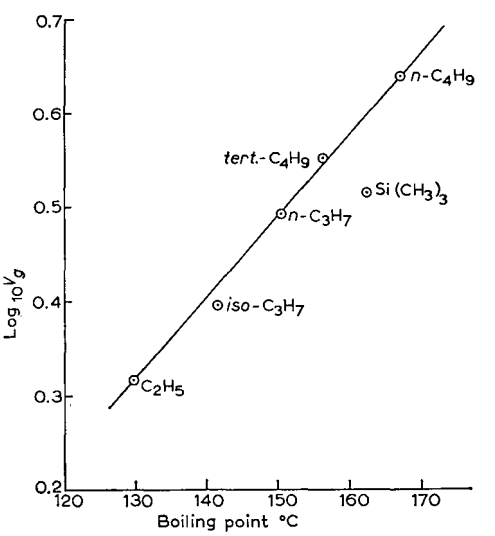

Fig. 6. Mean values of $\log_{10} V_g$ against boiling point for trimethyl-silyl alkyl thioethers on 20% Apiezon L at column temperature 200°.

hexamethylsiloxane and the isopropyl ether are almost identical. In the sulphur series, the retention volume of hexamethyldisilthiane was greater than either of the propyl thioether isomers, and only slightly smaller than that of the *tert.*-butyl thioether.

Since the respective boiling points of these compounds are:

$(CH_3)_3Si–S–Si(CH_3)_3$	163°
$(CH_3)_3Si–S–CH(CH_3)_2$	142°
$(CH_3)_3Si–S–(CH_2)_2CH_3$	151°
$(CH_3)_3Si–S–C(CH_3)_3$	157°

it can be seen that the thioethers are still retarded by the stationary phase in comparison with hexamethyldisilthiane, though not to as great an extent as in the oxygen series. These relative differences are shown in Fig. 6 which shows the graph of log V_g against boiling point (at column temperature 200°).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. E. W. ABEL for samples of the ethers and thioethers. The authors also gratefully acknowledge the award of a D.S.I.R. Maintenance Grant to one of them (P.C.U.).

SUMMARY

The conditions for the gas chromatography of the trimethyl-silyl ethers and the trimethyl-silyl thioethers and similar compounds have been investigated. Efficiencies of various stationary phases have been evaluated, and specific retention data obtained under standard conditions.

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A GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TRACE AMOUNTS OF CARBON

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The high sensitivity of the gas chromatographic technique makes it an ideal tool for application in the field of micro or trace analysis. Even using a conventional thermoconductivity detector, which by standards of gas chromatography is considered to be of low sensitivity, measurements of matter in micro- and submicro-gram region can be executed. The incorporation of gas chromatographic techniques into microanalytical procedures can greatly enlarge the field of microanalysis and provide logical extention to the field of ultramicroanalysis.

Unfortunately, microanalysts of today do not utilize this powerful tool to its greatest advantage. Only a few publications[1–5] dealing with the use of gas chromatography in the field of classical microanalysis have appeared in recent years.

In the field of metallurgy, where trace analysis, especially of hydrogen, oxygen, carbon and nitrogen, plays an important role, the potentials of gas chromatography are finally being recognized. A number of papers dealing with this analytical technique for the analysis of trace impurities in metals were presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy in the spring of 1962.

MOONEY AND GARBINI[6] were able to determine carbon in metals by means of combustion of a sample in an induction furnace, collection of the products of combustion plus oxygen in a liquid nitrogen trap, followed by the gas chromatographic determination of intrapped material using a conventional Perkin-Elmer instrument. FASSEL AND MONTE[7] determined oxygen and nitrogen content of metals using a high-current D.C. carbon-arc discharge in a static atmosphere of helium. A portion of the resulting gas mixture was analyzed using a commercially available gas chromatograph.

SCHULTZ[8] determined nitrogen in metals by fusing the sample in a graphite crucible and transferring the resulting gases into a chromatograph cell with an inert gas for separation and determination.

The purpose of this study was to design and build a unitized analytical instrument for the determination of impurities in metals. The instrumental system consists of a specially designed gas chromatograph integrated with a commercial model induction furnace to form a relatively compact unit, capable of rapid accurate analyses.

The operating model of such an instrument was tested for analysis of carbon in metals. The studies on future use of this instrument for determination of oxygen, nitrogen and hydrogen in metals are in progress at the present time in this laboratory.

EXPERIMENTAL

Chemicals

1. Copper ring accelerator—Leco 550-184.
2. Tin metal accelerator—Leco 501-76.
3. Iron chip accelerator—Leco 501-77.
4. Carbon dioxide gas.
5. Liquid argon.
6. Helium and oxygen.

Apparatus and equipment

1. Induction furnace, Leco Model No. 537

A Leco induction furnace is used for combustion of the sample. The gas stream outlet of the furnace is connected to the Beckman sampling valve and trap. The commercial grade of oxygen gas was found to contain a sufficient amount of impurities to produce a significant blank. A conventional purification train using a copper oxide tube heated to 500°, followed by an ascarite tube, reduced the blank considerably but failed to eliminate it completely. For this reason, a tube containing decomposed silver permanganate prepared by a procedure of LYSYJ AND ZAREMBO[9] heated at 500° will be used instead of copper oxide for future work. The flow system of the combustion train is shown in Fig. 1.

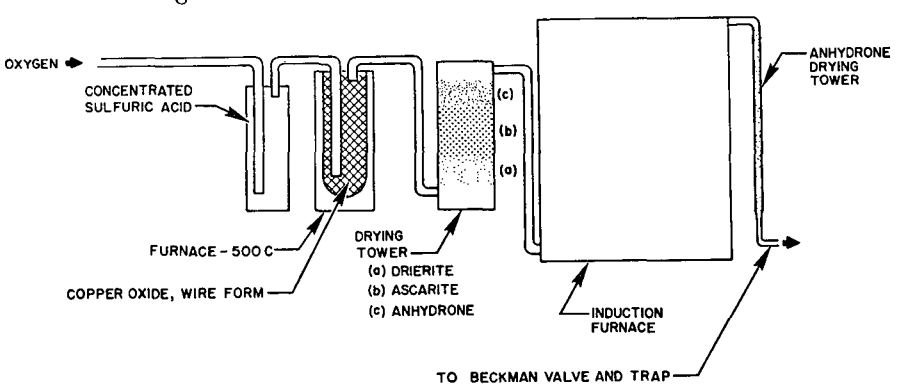

Fig. 1. Induction furnace and purification train.

2. Special gas chromatograph

The instrument is housed in a 20 × 15 × 15 in. aluminum box and incorporates a thermistor Gow-Mac thermoconductivity cell and a Beckman sample valve for collection and introduction of the sample into the helium stream. The thermoconductivity cell Gow-Mac 9677 (AEL) and gas chromatographic column are housed in a Dewar flask, equipped with heating tape and a precision temperature regulator. For the analysis of carbon in metals, a 26 in. long, 3/16 in. diameter copper tube filled with silica gel, 20–200 mesh size, is used. The design of the separating and detecting portion of the instrument is shown in Fig. 2.

To permit collection and introduction of the sample into the helium stream, a Beckman two-way sampling valve was modified in such a way as to permit the

application of a cold trap over the collecting loop. Two stainless steel U-tubes, 1/4 in. diameter and 3 in. long, were incorporated in the valve. By manipulating the position of the valve, one tube is placed in the helium stream and the second in the oxygen stream coming from the exit side of the induction furnace where combustion of the

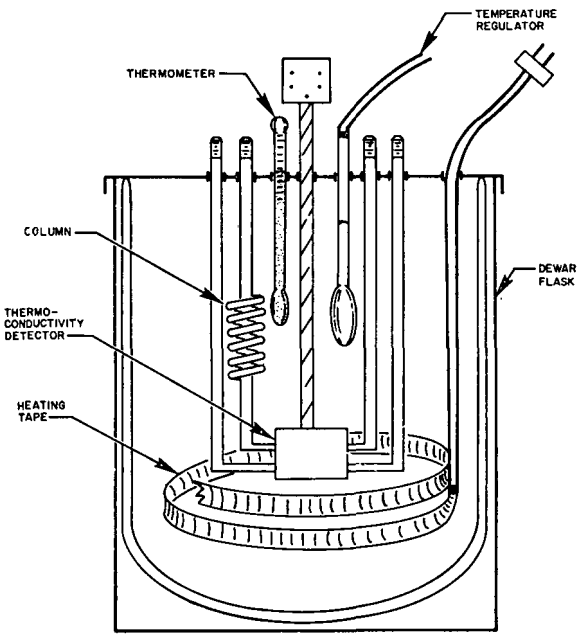

Fig. 2. Detector housing.

sample is conducted. When the liquid argon trap is placed over the collecting tube, carbon dioxide and small amounts of oxygen are condensed. After removal of oxygen by pumping, the carbon dioxide is vaporized and introduced in the helium stream by changing the position of the valve.

A single stream of helium is used in the design of the gas chromatograph. The carrier gas passes first through the reference side of the detecting cell, then through the collecting valve, column, and finally through the detector side of the thermoconductivity cell. The flow system was constructed from 3/16 in. copper tubing and is shown in Fig. 3. The electrical circuit is shown in Fig. 4.

3. *Leeds & Northrup recorder, Speedomax Model G*
4. *Printing integrator, Perkin-Elmer Model 194*
5. *Thermocouple vacuum gauge and control (NRC Equipment Corp.)*
6. *Ceramic crucibles, Leco S28–35*
7. *Vacuum pump, Kinny*
8. *Rotometer flowmeters, Fisher & Porter No. 9143 and 9144*
9. *Oxygen purifying train.*

PROCEDURE

The steel sample, containing 1 mg or less of carbon, is accurately weighed and placed into the ceramic crucible. Then 1.150 g ($\pm$ 10 mg) each of the tin metal and iron chip

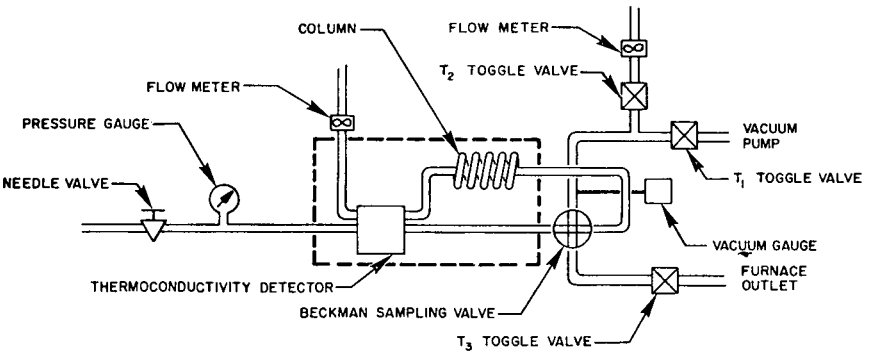

Fig. 3. Flow system of gas chromatograph.

accelerators are added to the sample, followed by one copper ring. It is essential to weigh the catalysts accurately, since they contain sufficient carbon to register an appreciable blank count. The sample is introduced into the furnace, and the apparatus is purged with pre-purified oxygen for 5 min at a rate of flow of 950 ml/min. The carbon dioxide trap is then immersed in liquid argon. The furnace is turned on and left at the maximum setting approximately 1700° C for 3 min and turned off. The entire apparatus is finally purged for an additional 2 min at the same rate of oxygen flow. The carbon dioxide trap is then isolated from the induction furnace by closing

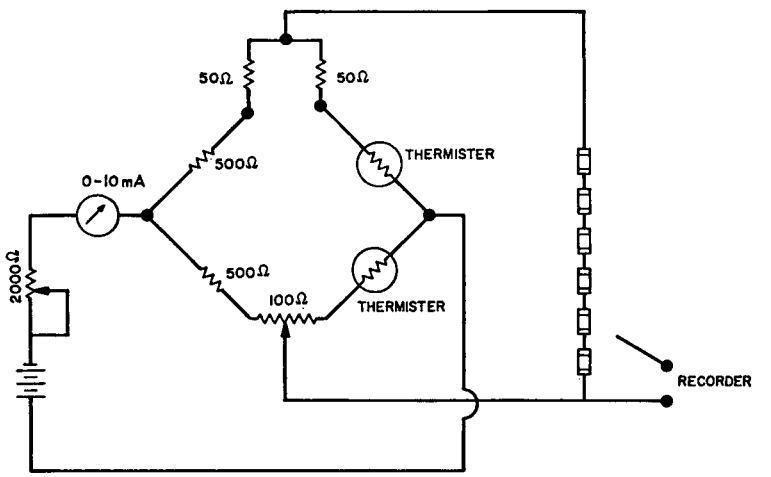

Fig. 4. Detection system electrical circuit.

a toggle valve and evacuated to a pressure of 1–2 mm of mercury to remove most of the oxygen. A small amount of oxygen actually condenses in the trap during the run, although its boiling point is only 2° higher than that of argon; therefore, it was found essential to include this evacuation step to avoid flooding the column. The vapor pressure of carbon dioxide at liquid argon temperature is $4 \cdot 10^{-6}$ mm of Hg, so there exists little danger, except for mechanical loss, of losing any carbon dioxide by vaporization. The Beckman valve is then rotated to place the carbon dioxide trap in

the helium stream, and the liquid argon trap is replaced by a room temperature water bath to rapidly vaporize the carbon dioxide. The following operating parameters were used for every analysis:

Helium inlet pressure—8 lb./in^2.
Helium flow (exit)—54 ml/min
Column and detector block temperature—150°F
Current—6 mA.

The carbon dioxide peak appeared 1.5 min after warming the trap, and was preceded by a small peak due to residual oxygen left after the pumping operation.

The apparatus was initially tested for leaks by following the procedure outlined above, except that the furnace was not heated. The absence of any carbon dioxide peak indicated no carbon dioxide leakage from the atmosphere. However, it was noted that if the furnace was heated using a platinum crucible as a receptor for 3 min during the oxygen flow, a carbon dioxide peak appeared. This was attributed to small amounts of hydrocarbons in the oxygen which were not removed by the purifying train, but which apparently underwent oxidation at the high temperature existing in the induction furnace (approximately 1700° C) in contact with platinum.

RESULTS AND DISCUSSION

The experimental data presented in Table I were obtained using a sample of Bureau of Standards Steel No. 161, which contains 64 % nickel, 17 % chromium, 15 % iron, and 0.342 % carbon.

The precision of the data is considered quite good in view of the fact that inhomogeneities can exist in the carbon content of the small steel samples and the catalysts.

TABLE I

DETERMINATION OF CARBON IN NBS STEEL NO. 161

Blank study							
Oxygen blank (heated furnace) Catalyst + oxygen blank	*Counts*						
	61			*54*			
	360	*352*	*348*	*358*	*354*	*Average*	*356*
Sample wt. (g)	*Sample study*						
	μg of carbon		*Total counts (corr. for blank)*			*Counts/μg of carbon*	
0.2232	763		1604			2.10	
0.2096	717		1413			1.97	
0.2086	713		1566			2.20	
0.2210	756		1654			2.18	
0.1119	383		796			2.08	
0.1095	374		684			1.83	
0.1174	402		830			2.06	
0.0612	209		453			2.17	
0.0610	209		443			2.12	
0.0647	221		473			2.14	
						Average	2.09

Average deviation: $S = 0.079$

The sensitivity of the method can be increased to over 60 times that shown by adjusting the electronic attenuators in the system and by using the detector at a lower temperature. However, in view of the large blank values obtained, greater sensitivities would be less valuable.

The standardization of the instrument was done, also using carbon dioxide as a standard. Measured amounts of carbon dioxide were introduced in a helium stream and determined in the usual manner. The number of counts per microgram of carbon were calculated and presented in Table II. The instrument can also be used for ultramicro determination of carbon in organic matter.

TABLE II

DETERMINATION OF CARBON IN CARBON DIOXIDE GAS*

	mm pressure CO_2	*μg of carbon*	*Number of counts*	*Counts/μg of carbon*
1.	126	177.8	444	2.49
2.	182	256.9	652	2.43
3.	198	279.5	730	2.61
4.	214	302.0	765	2.53
5.	246	347.2	895	2.57
6.	284	400.8	1001	2.49
7.	387	546.2	1397	2.55
8.	404	570.2	1424	2.49
9.	424	598.4	1590	2.57
10.	433	611.2	1598	2.61
11.	438	618.2	1580	2.55
12.	466	657.7	1662	2.52
13.	539	760.7	1994	2.62
14.	551	777.7	1978	2.54
				Average 2.54

Average deviation = 0.045

* Volume of injection loop 19.9 ml. Analysis was done under slightly different operating conditions than standardization using NBS sample 161.

A study is being conducted on the use of the instrument essentially unchanged for the determination of oxygen in metals. The samples are ignited in a graphite crucible in an argon atmosphere in the usual manner, and the carbon dioxide collected and determined as before. The trap-evacuation step would be eliminated, since argon should not condense during the purging and sweeping operations.

An investigation is currently in progress on the determination of nitrogen in metals using a slightly modified apparatus. The carbon dioxide trap under the Beckman valve would be replaced by a trap containing Linde sieves or charcoal cooled with liquid nitrogen. The nitrogen evolved from the metal nitrides heated to high temperatures in a graphite crucible would be swept out with helium or hydrogen into the cooled packed column, and later eluted by warming the trap to room temperature.

SUMMARY

The design of a unitized analytical instrument for the determination of impurities in metals is described. The instrumental system consists of a specially designed gas

chromatograph integrated with the induction furnace to form a relatively compact unit, capable of rapid and accurate analyses.

The operating model of such an instrument was built and tested for analysis of trace amounts of carbon in metals.

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GAS CHROMATOGRAPHY OF THE METHYL ESTERS OF THE AMINO ACIDS AS THE FREE BASE AND BY DISSOCIATION OF THEIR ACID SALTS

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The dissociation of ammonium salts into free acid and base is well known[1]. Early attempts to apply the principle of dissociation of the ammonium salts of esters of amino acids to gas phase analysis resulted either in failure or very poor results[2,3]. Recent experiments in our laboratory have demonstrated that acid salts of fifteen of the twenty common amino acid esters are amenable to gas phase analysis by virtue of their ease of dissociation under the influence of temperature.

Comparative studies were carried out on the amino acid esters in the form of the free base and the salts of hydrochloric and acetic acids. The starting derivative for all of the studies was the methyl ester hydrochloride of the amino acid, usually in the crystalline form[4].

Free base

In this study amino acid esters in the form of the free base were prepared as a reference with which to compare the gas chromatographic behaviour of the acid salts. Gas chromatography of amino acid esters as the free base has been reported[3,5]. Disadvantages of these methods resulting from the use of sodium hydroxide[5] and ammonia[3] were eliminated by the following method of preparation. The free bases were prepared by shaking a methanolic solution of the chloride salt (0.25 M) with about 20 % of its volume of anhydrous Dowex-1 in the hydroxide form. Careful preparation of the resin, to exclude most of the free hydroxyl ions, was carried out by washing the resin in the hydroxide form with deionized water until the pH of the effluent was lower than 8, then dehydrating the resin by continuous washing with anhydrous (0.04 % water) methanol.

Anhydrous solutions of the free bases of amino acid esters prepared under these conditions were found to be stable for periods varying from hours to days. For example, the methyl esters of glycine and leucine showed less than 5 % loss of yield after standing for 18 to 24 hours at room temperature. After 72 hours, the yield from the methyl ester of proline was decreased by 45 %.

The free bases, as well as the salts, were chromatographed either in a laboratory-

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constructed apparatus using nitrogen as a carrier gas and a hydrogen flame ionization detector, or in a commercial apparatus (American Instrument Co., Silver Spring, Md.) with helium as carrier gas and a thermistor detector. The most suitable column packing was found to be 2 % neopentyl glycol succinate on Fluoropak 80 (The Fluorocarbon Co., Anaheim, Calif.). When Chromosorb W was used as inert support, considerable tailing of the peaks occurred, whereas Fluoropak gave sharp peaks as illustrated in Fig. 1. In addition to stability in solution, the methyl esters of the amino acids exhibited equivalent stability during the course of a gas phase analysis. Estimates of yields in excess of 90 % for glycine, leucine and proline were made using the

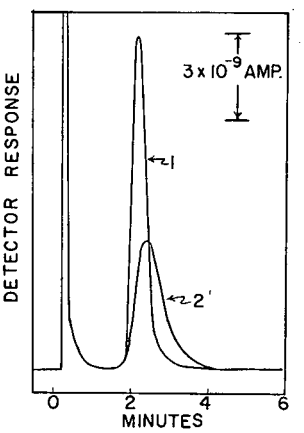

Fig. 1. Gas chromatograms of aspartic acid dimethyl ester. *Curve 1*, free base; *Curve 2*, chloride salt. The curve for the acetate salt was almost the same as that for the free base. Conditions for chromatography were: column and preheat temperature 175°; 6 ft., $^3/_{16}$ in. I.D. glass column packed with 2% neopentyl glycol succinate on Fluoropak 80; carrier gas: nitrogen at 60 c.c./min; detector: hydrogen flame ionization.

hydrogen flame detector, the effective carbon data of STERNBERG *et al.*[6] and methyl decanoate[7] as a primary standard. Diketopiperazine formation in the column was ruled out by a study comparing the behavior of the methyl ester of glycine and glycine anhydride.

The data presented in Table I show that of the twenty common amino acids cysteine, histidine, tyrosine and tryptophan could not be chromatographed satisfactorily in the form of the free base of the methyl ester. Mixtures of the amino acid derivatives chromatographed under the conditions of Table I presented no difficulties except that the pairs glycine–alanine, leucine–isoleucine and phenylalanine–hydroxyproline were unresolved. (Serine and cysteine were also unresolved when the acetate salts were employed. See below.)

Chloride salts

The gas chromatographic behavior of the amino acid methyl ester hydrochlorides as compared with their free bases are summarised in Table I, while a typical peak is shown in Fig. 1. It can be seen that considerable differences exist between the behavior of the various amino acids. Whereas no amino acid ester gave a satisfactory peak at a temperature below about 170°, above this temperature, the esters of alanine, glycine,

TABLE I

GAS CHROMATOGRAPHIC BEHAVIOR OF AMINO ACID METHYL ESTERS AS THE FREE BASE, CHLORIDE AND ACETATE SALTS

Column: 6 ft., $^3/_{16}$ in. I.D., packed with 2% neopentyl glycol succinate on Fluoropak 80. Gas flow 60 c.c./min. Sample size 0.25μmole. The recorded temperature was that of the preheater and column.

	Free base[a]		*Acetate salt*[a]		*Hydrochloride salt*		
	Temp. (°C)	*Retention time (min)*	*Temp. (°C)*	*Retention time (min)*	*Temp. (°C)*	*Retention time (min)*	*Peak area*
Leucine[b]	120	4.3	120	4 3	120	4.7	10 % of free base
	175				175	0.1	80 % of free base
Proline	115	5.2	115	5.2	115	5.5	10 % of free base
	175	0.6	175	0.6	175	0.7	40 % of free base
Serine[c]	150	3.6	150	3.6	150	—	no peak
	175	2.4	175	2.4	175	2.9	small broad peak
Aspartic acid[d]	150	4.8	150	4.8	150	6.7	broad peak
	175	2.0	175	2.0	175	2.3	30 % of free base
							40 % of free base
Phenylalanine[e]	155	9.0	155	9.0	155	11.0	40 % of free base
	180	6.9	180	6.9	180	7.1	80 % of free base
Hydroxyproline	175	5.7	175	5.7	175	6.5	broad peak
	195	2.9	195	2.9			
Cysteine	175	negative result	175	1.9	175	2.0	equal to acetate
Cystine	180	6.7	180	6.7	180	6.7	80 % of free base
Lysine[f]	180	3.5	180	negative result	180	negative result	

[a] Except for cysteine, lysine and arginine, all the above amino acid esters gave similar (to within 10 %) peak areas for both the free base and the acetate salt.
[b] Alanine, glycine, valine and isoleucine gave results similar to those of leucine.
[c] Threonine gave results similar to those of serine.
[d] Glutamic acid gave results similar to those of aspartic acid.
[e] Methionine gave results similar to those of phenylalanine.
[f] Arginine gave results similar to those of lysine.

valine, leucine, isoleucine, methionine, phenylalanine, cysteine and cystine gave peaks of good yields although the retention times were slightly longer than the corresponding free bases. Even at these elevated temperatures, proline, hydroxyproline, serine, threonine, aspartic and glutamic acids gave poor yields while lysine, arginine, tyrosine, histidine and tryptophan gave negative results.

The nature of the inert support for the stationary phase has an important influence on the behavior of the amino acid derivatives. Under conditions which produced peaks of good yield for leucine and phenylalanine on columns packed with Fluoropak 80, negative results were obtained with Chromosorb W as the inert support.

Acetate salts

Acetate salts were prepared from the corresponding chlorides by shaking a solution of the ester hydrochloride in methanol (0.25M) with approximately 20 % of its volume of anhydrous Dowex-1 in the acetate form. The results summarized in Table I indicate that when the free base of an amino acid ester and its corresponding acetate salt both gave peaks, the areas and retention times were similar. This demonstrates that even at temperatures as low as 120°, the phenomenon occurring in the column was dis-

sociation of the salt and chromatography of the free base. With the esters of lysine and arginine no such dissociation was apparent. It is of interest to note that whereas cysteine methyl ester gave negative results in the form of the free base, peaks of good yield were obtained with the corresponding acetate salt.

The consistent behavior of the acetate salts of the amino acid esters as compared with the corresponding chlorides suggests that the acetate salts dissociate more readily at the temperatures employed and are therefore more amenable to gas phase analysis. Combined use of the free base and acetate salt of the methyl esters of the amino acids makes facile the gas phase analysis of all of the common amino acids except histidine, tyrosine and tryptophan.

SUMMARY

When anionic exchange resins were employed to generate the free basic form of the methyl esters of the amino acids, these derivatives were stable for periods of time long enough for gas chromatography. Dissociation of the acetate salts of the amino acid esters into the free base and acetic acid occurred in gas chromatographic columns under the influence of temperatures varying from 120° to 200°. A combination of the use of the amino acid esters as the free base and by dissociation of their acid salts made possible the gas chromatography of the derivatives of all of the common amino acids except histidine, tyrosine and tryptophan.

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PAPER CHROMATOGRAPHIC CHARACTERISTICS OF SUBSTITUTED DIPHENYLETHENES

I. *p,p'*-SUBSTITUTION IN THE PHENYL RINGS

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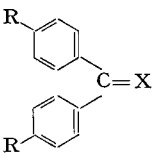
In the investigation of biological effects of diphenylethenes with the general formula



where X is alkylidene or cycloalkylidene, it was found desirable to compare the distribution characteristics of the compounds between a polar and a lipid phase. Owing to the large number of substances, paper chromatography was considered the most suitable method for the purpose in view.

In chromatography of homologous series of substances it is convenient to use R_M, instead of R_F; according to theory the former is a linear function of the number of similar substituents in a molecule. The R_M function was introduced by BATE-SMITH AND WESTALL[1], who in their investigation of phenols and flavones defined R_M as $\log (1/R_F - 1)$. They found a largely linear correlation between R_M and the number of hydroxyl and carboxyl groups. REICHL[2] modified the formula to $R_M = \log R_F/(1 - R_F)$. This modified formula is easier to handle. Any increase in the value of R_F is accompanied by a corresponding increase of R_M (Fig. 1). REICHL determined a fundamental constant and a group constant for all his solvents and found that the R_M value for a substance can be calculated by addition of these constants, this sum giving the R_M value. Among recent investigations of the influence of substituents on the chromatographic characteristics of the compounds studied, that of BARK AND GRAHAM[3], who studied phenoxyacetic acids, deserves mention as does that of KABASAKALIAN AND BASCH[4], who studied the steroids of the pregnane series with the aid of so-called Zaffaroni systems. For the dimethyl sulphoxide–water/isopropyl ether system, SOCZEWINSKI[5] found a linear correlation between R_M and per cent by volume of water in the stationary phase. He assumed that this correlation was generally valid and that thus similar relationships can also be expected for reversed phase chromatography, an assumption confirmed in the present investigation.

An advantage of the R_M value is that it is a simple function of the temperature and the relative volumes of the solvent phases. Using a standard substance for the

chromatograms, any deviations from normal can be readily detected and corrected for in the formula.

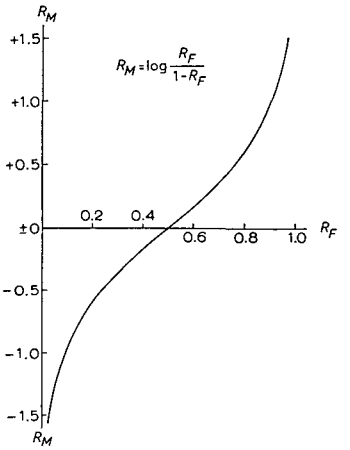


Fig. 1. The R_M function according to REICHL.

MATERIAL AND METHODS

The compounds studied were the substituted diphenylethenes described by MIQUEL *et al.*[6]. Substances that gave more than one spot on a chromatogram or showed other signs of contamination were recrystallized in some suitable solvent.

As reference substance, which was applied to every sheet of chromatographic paper, we used 1,1-bis-(*p*-acetoxyphenyl)-2-ethylbutene-1, recrystallized from 70 % ethanol and diisobutyl ketone to constant melting point. The values for the other spots were corrected with reference to the R_M value of the standard substance. The mean value for the R_F of the standard was calculated on the basis of 125 chromatograms. Six chromatograms in which the R_F of the standard substance differed by more than $\pm$ 0.03 from the mean value were excluded.

The chromatographic paper, Whatman No. 1, 33.5 by 31.5 cm, was impregnated by dipping it into a solution of 5 % by volume of paraffin oil in cyclohexane. Excess solution was removed by blotting the sheet between Whatman No. 3 MM papers, after which the sheet was allowed to dry in the air. Storage of the impregnated paper for a long period up to 6 months appeared to have no effect on the R_F values. The starting line was marked 4 cm from the short edge of the paper, and 10 μl of a solution of each substance (0.5 mg/ml ethanol) was deposited on the paper with the aid of a micropipette. The spots were placed 4 cm apart. The chromatogram was suspended vertically and developed by the ascending technique in "Shandon 13 inch Universal Sheet Chromatanks", containing 400 ml of the various mixtures of methanol and water. Five hours at 22–24° proved to be a suitable running time.

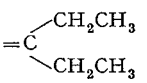
With the amount used, 5 γ substance per spot, well defined spots were obtained; larger amounts gave a certain degree of tailing, especially in the system with the highest water content.

After 5 hours the paper was removed from the tank, air dried, and examined in ultraviolet light (Hanovia Chromatolite, 253.7 mμ).

Most of the compounds studied have an absorption maximum between 240 and 260 mμ, so that the detection method is fairly sensitive, it being possible to recognize substances in amounts of 0.5 γ/cm^2 without difficulty. Substances with one or two phenolic hydroxyls could be demonstrated with about the same sensitivity with Folin–Ciocalteu's reagent[7]. This reagent (diluted with 4 times its volume of water) was sprayed on to the dried paper, which was then placed a few minutes in an atmosphere saturated with ammonia, when blue spots appeared against a practically white background. The R_F values were determined and used for the calculation of $R_M = \log R_F/(1 - R_F)$.

RESULTS AND DISCUSSION

The results are summarized in Tables I–III. Table I shows R_F and R_M values of compounds with various substituents in the *p,p'*-position but with the alkylidene moiety unchanged:

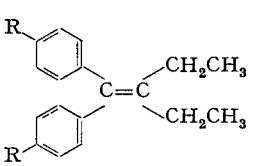


In Tables II and III, on the other hand, the alkylidene moiety is varied but the *p,p'*-diacetoxy-phenyl groups are kept constant. Both the alkylidene and the cycloalkylidene compounds give a linear correlation between R_M and the number of carbon atoms in the chain and ring, respectively (Figs. 2 and 3). The slopes of the different lines representing different mixtures of methanol and water decrease with increasing metha-

TABLE I

R_F AND R_M VALUES OF *p,p'*-SUBSTITUTED 1,1-DIPHENYL-2-ETHYLBUTENE-1 IN METHANOL–WATER SYSTEMS

Paper Whatman No. 1, impregnated with paraffin oil. 5 hours ascending technique. Every R_F value is a mean of 3–6 different chromatograms, excepting the values for the standard substance for which every R_F is a mean of about 30 determinations.



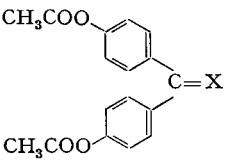
Compound No.	*R*	*Solvent systems, CH_3OH–H_2O*							
		1:1		*1.5:1*		*2:1*		*3:1*	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
10	H	0.00	$-\infty$	0.04	−1.37	0.14	−0.79	0.49	−0.02
10B	OH	0.72	+0.41	0.86	+0.79	0.90	+0.95	0.95	+1.28
10C	CH_3COO*	0.22	−0.55	0.55	+0.09	0.75	+0.48	0.88	+0.87
10D	CH_3CH_2COO	0.06	−1.20	0.24	−0.50	0.52	+0.04	0.81	+0.63
10F	$CH_3(CH_2)_5COO$	0.00	$-\infty$	0.00	$-\infty$	0.00	$-\infty$	0.05	−1.28
10E	$(CH_3)_3CCOO$	0.00	$-\infty$	0.03	−1.51	0.11	−0.91	0.46	−0.07
10A	CH_3O	0.02	−1.69	0.10	−0.95	0.29	−0.39	0.64	+0.25

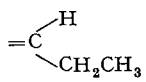
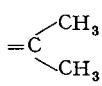
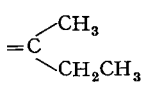
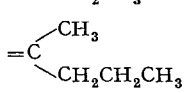
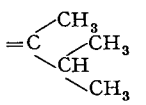
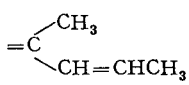
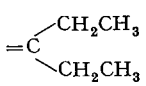
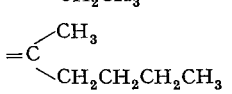
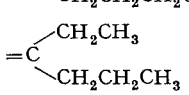
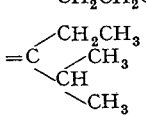
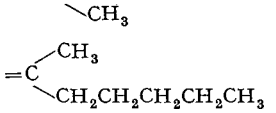
* Standard. For the influence on R_F of varying the alkylidene moiety in this substance, see Tables II and III.

TABLE II

THE INFLUENCE ON R_F AND R_M VALUES OF DIFFERENT ALKYLIDENE CHAINS

(For further explanation, see Table I)



Compound No.	Alkylidene chain X	No. of C atoms in X	Solvent systems, CH_3OH–H_2O 1:1 R_F	1:1 R_M	1.5:1 R_F	1.5:1 R_M	2:1 R_F	2:1 R_M	3:1 R_F	3:1 R_M
2C		2	0.62	+0.21	0.79	+0.58	0.89	+0.91	0.94	+1.20
3C		3	0.44	−0.11	0.67	+0.31	0.83	+0.69	0.91	+1.01
4C		3	0.51	+0.02	0.76	+0.50	0.85	+0.75	0.93	+1.12
5C		4	0.36	−0.25	0.63	+0.23	0.80	+0.60	0.90	+0.95
6C		5	0.23	−0.53	0.52	+0.04	0.72	+0.41	0.89	+0.91
7C		5	0.18	−0.66	0.52	+0.04	0.73	+0.43	0.90	+0.95
8C		5	0.22	−0.55	0.57	+0.12	0.76	+0.50	0.89	+0.91
10C*		5	0.22	−0.55	0.55	+0.09	0.75	+0.48	0.88	+0.87
9C		6	0.14	−0.79	0.42	−0.14	0.64	+0.25	0.86	+0.79
11C		6	0.15	−0.75	0.46	−0.07	0.66	+0.29	0.86	+0.79
111C		6	0.13	−0.83	0.46	−0.07	0.70	+0.37	0.87	+0.83
122C		7	0.08	−1.06	0.30	−0.37	0.56	+0.11	0.82	+0.66

(continued on p. 335)

TABLE II (*continued*)

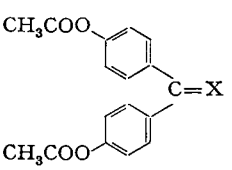
Compound No.	Alkylidene chain X	No. of C atoms in X	Solvent systems, CH_3OH–H_2O							
			1:1		1.5:1		2:1		3:1	
			R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
121C	$=C(CH_2CH_3)(CH_2CH_2CH_2CH_3)$	7	0.09	−1.01	0.37	−0.23	0.57	+0.12	0.79	+0.58
12C	$=C(CH_2CH_2CH_3)(CH_2CH_2CH_3)$	7	0.11	−0.91	0.38	−0.21	0.62	+0.21	0.84	+0.72
131C	$=C(CH_2CH_3)(CH_2CH_2CH(CH_3)CH_3)$	8	0.06	−1.20	0.28	−0.41	0.52	+0.04	0.80	+0.60
13C	$=C(CH_2CH_2CH_2CH_3)(CH_2CH_2CH_2CH_3)$	9	0.03	−1.51	0.17	−0.69	0.39	−0.20	0.74	+0.45

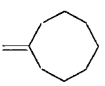
* Standard. For variation of the phenyl substituents, see Table I.

TABLE III

THE INFLUENCE OF DIFFERENT CYCLOALKYLIDENE RINGS ON R_F AND R_M VALUES

(For further explanation, see Table I)



Compound No.	Cycloalkylidene ring X	No. of C atoms in X	Solvent system, CH_3OH–H_2O							
			1:1		1.5:1		2:1		3:1	
			R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
14C		4	0.35	−0.27	0.60	+0.18	0.76	+0.50	0.88	+0.87
15C		5	0.26	−0.45	0.50	±0	0.70	+0.37	0.85	+0.75
16C		6	0.18	−0.66	0.44	−0.11	0.63	+0.23	0.82	+0.66
17C		7	0.12	−0.87	0.36	−0.25	0.58	+0.14	0.79	+0.58
18C		8	0.08	−1.06	0.26	−0.45	0.48	−0.04	0.74	+0.45

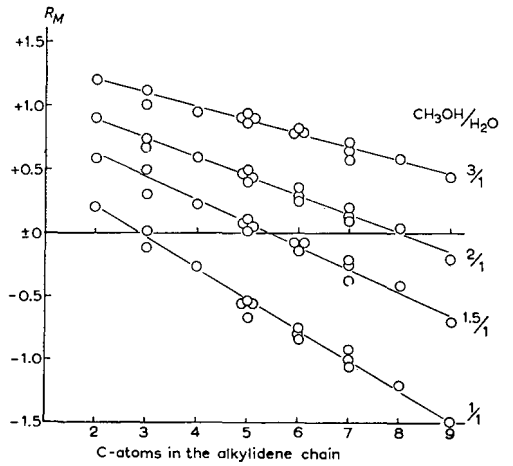


Fig. 2. Relation between the R_M values and the number of carbon atoms in the alkylidene chain of 1,1-bis-(*p*-acetoxyphenyl)-alkenes.

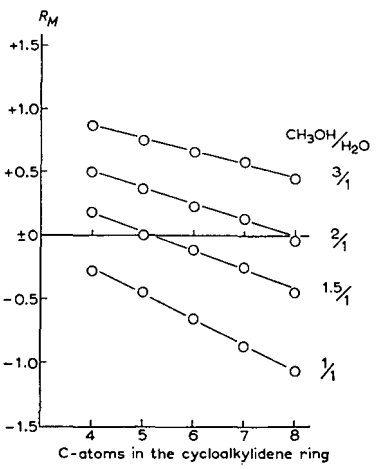


Fig. 3. Relation between the R_M values and the number of carbon atoms in the cycloalkylidene ring of 1,1-bis-(*p*-acetoxyphenyl)-cycloalkylidenemethanes.

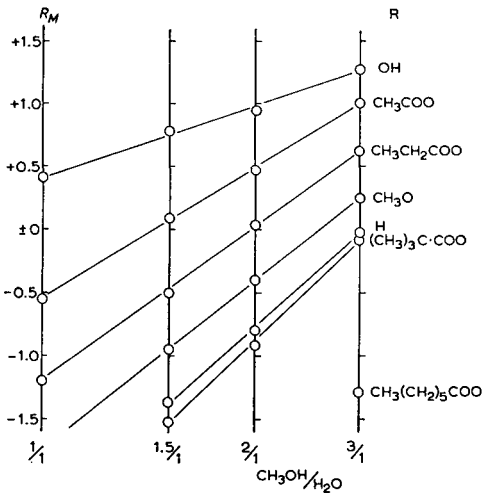
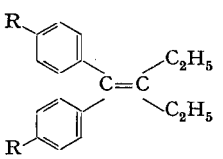


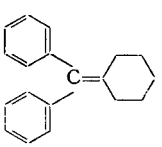
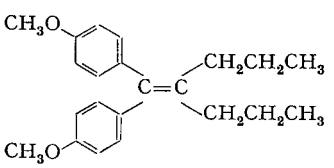
Fig. 4. Relation between the R_M values and the composition of the solvent system methanol–water for the *p,p'*-substituted compounds:



nol content of the system. This inverse relation is discussed by THOMA[8] in his paper on a graphical method for evaluating chromatographic partition parameters.

Fig. 4 shows the relation between R_M and the concentration of methanol in the mobile phase. Corresponding diagrams can also be constructed for all the other compounds studied. A linear relationship was invariably found between R_M and the concentration of methanol in the methanol–water system. The R_F value for similar substances can be easily predicted from the diagrams. A few examples are given in Table IV.

TABLE IV

Compound No.	*Formula*		*CH_3OH-H_2O* 1:1	1.5:1	2:1	3:1
16		expected R_F	0.00	0.03	0.08	0.30
		observed	0.00	0.03	0.09	0.31
12A		expected R_F	0.00	0.05	0.18	0.48
		observed	0.00	0.05	0.17	0.50

The system proved very useful for the purpose in view, but it is of only limited value for chromatography of contaminated solutions, such as urine extracts. For such impure mixtures it is preferable to use a system with a larger capacity such as formamide/toluene or propylene glycol/cyclohexane. It is remarkable that the paraffin oil/methanol–water system gives such a high ΔR_M for small changes in the size of the substituent, an effect not occurring to the same extent with more lipophilic paper, *e.g.* silicone treated or acetylated paper. No significant change in the R_M values was found on branching of (substances No. 7C, 111C, 131C), or introduction of a double bond into the alkylidene moiety (substance No. 8C).

SUMMARY

A series of *p,p'*-substituted 1,1-bis-(phenyl)-alkenes and -cyclo-alkylidenemethanes were studied chromatographically on slightly lipophilic paper and with methanol–water in varying proportions as the mobile phase. A linear correlation was found between the R_M value and the number of carbon atoms in the alkylidene and cyclo-alkylidene moieties. The relation between R_M and the concentration of methanol in the mobile phase was also found to be linear. With the aid of the R_M values determined, it was possible to predict the chromatographic characteristics of new substances in the series, which proved useful for identifying these substances and assessing their degree of purity.

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PAPER CHROMATOGRAPHY OF POLYPHENYLS

QUALITATIVE SEPARATION OF SULPHONIC ACID DERIVATIVES OF DIPHENYL AND *o*-, *m*- AND *p*-TERPHENYLS

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INTRODUCTION

Polyphenyls are a class of compounds the use of which is very promising as moderators and coolants in nuclear reactors.

Some experimental work has appeared in various recent papers on the chromatographic separation of polyphenyls. The separation of isomeric terphenyls on alumina, fuller's earth, silica gel and charcoal has been studied by HELLMANN, ALEXANDER AND COYLE[1] and by WEST[2].

BARRERA[3] has carried out some separations of terphenyls by chromatography on acetylated paper using the technique described by SPOTSWOOD[4]. Good separations of mixtures of diphenyl and *m*-terphenyl are, however, not possible using the procedure described by BARRERA since for diphenyl $R_F = 0.64$ and for *m*-terphenyl $R_F = 0.66$.

The present paper describes a chromatographic method for separating diphenyl and isomeric terphenyls on untreated paper.

EXPERIMENTAL

Sulphonation of polyphenyl mixtures

0.25 g of polyphenyls are mixed with 1.2 ml of concentrated H_2SO_4 ($d = 1.84$, Merck analytical reagent grade) and heated in a thermostat at 50° for 4 h. The mixture of sulphonic acids and excess sulphuric acid is diluted with distilled water, poured into a volumetric flask and made up to 25 ml.

Preparation of the solvent

The solvent is prepared by mixing *n*-butanol, methanol and 3 *N* aqueous solution of $(NH_4)_2CO_3$, in the volume ratio 4:3:2. Some solid ammonium carbonate crystallizes out on mixing the liquids, and is eliminated by centrifugation.

Chromatographic technique

The chromatographic separations were carried out by the ascending technique on 23 × 53 cm strips of Whatman paper No. 20.

The solution for the development was put in a glass chromatographic chamber 31 × 21 × 38 cm 20 h before the introduction of the strips. The strips were then

developed with the solvent for 40 h at room temperature (20°). After development, the strips were dried at 70° in an air oven for 30 min, and sprayed with 0.1 % alcoholic solution of methyl red, diluted in the volume ratio 1:5 with phosphate buffer at pH 7.

RESULTS AND DISCUSSION

The present method of separation takes advantage of the different sulphonation velocities of diphenyl and the isomeric terphenyls under the above-mentioned experimental conditions.

On sulphonation for 4 hours at 50° a mixture of mono- and disulphonic acids is obtained (*viz.* *p*-diphenyl-disulphonic, *o*-terphenyl-monosulphonic and -disulphonic, *m*-terphenyl-monosulphonic and -disulphonic, *p*-terphenyl-monosulphonic acids). With different sulphonation times and different temperatures the composition of the

TABLE I

RESULTS OF SULPHONATION EXPERIMENTS

Compound	*Temp. (°C)*	*Time (h)*	*Disulphonic acid*	*Monosulphonic acid*
Diphenyl	50	4	Present (R_F 0.37)	Not found
	50	5	,, ,,	,, ,,
	100	1–5	,, ,,	,, ,,
	150	5	,, ,,	,, ,,
o-Terphenyl	50	4	,, (R_F 0.23)	Present (R_F 0.54)
	50	5	,, ,,	Not found
	100	1–5	,, ,,	,, ,,
	150	5	,, ,,	,, ,,
m-Terphenyl	50	4	,, (R_F 0.23)	Present (R_F 0.50)
	50	5	,, ,,	,, ,,
	100	1–5	,, ,,	,, ,,
	150	5	,, ,,	Not found
p-Terphenyl	50	4	Not found	Present (R_F 0.44)
	50	5	,, ,,	,, ,,
	100	1–5	,, ,,	,, ,,
	150	5	Present (R_F 0.23)	Not found

mixture of sulphonic acids is also different. At 150° all the disulphonic acids are formed. In this case the chromatographic separation of *o*-, *m*-, and *p*-terphenyl is not possible. Table I shows the results obtained under various sulphonation conditions. In Table II the composition of the different commercial and synthetic mixtures of polyphenyls, used in the present work, are given.

Figs. 1 and 2 show some paper-chromatographic separations. Good separations and readily discernible spots are obtained from mixtures containing, as lower limits, the quantities of each component given in Table III.

The sensitivity of detection for every sulphonic acid useful for the identification, is shown in Table IV.

When higher polyphenyls (quaterphenyls, quinquephenyls) are present, one or more spots with $R_F \leqslant 0.23$ are obtained, and thus do not interfere with the components of the mixture sought.

TABLE II

COMPOSITION OF THE POLYPHENYL MIXTURES USED FOR THE PAPER CHROMATOGRAPHIC SEPARATIONS

Compound	*Commercial mixtures (composition given by the manufacturers)*		*Synthetic mixtures from purified compounds*		
	Santowax R (Monsanto) %	*Santowax OMP (Monsanto)* %	%	%	%
Diphenyl	1	1	32.95	30.77	31.76
o-Terphenyl	9	12	5.78	30.87	32.54
m-Terphenyl	55	64	30.58	32.69	3.97
p-Terphenyl	15	23	30.67	5.65	31.71
High-boiling fraction (quaterphenyl, quinquephenyl, triphenylene, etc.)	10–20	1	—	—	—

TABLE III

PRACTICAL DETECTION LIMITS OF THE COMPONENTS

The limits for separation (readily discernible spots) are given in weight percent of the mixture.

Compound	*wt.* %
Diphenyl	0.5
o-Terphenyl	6
m-Terphenyl	4
p-Terphenyl	6

TABLE IV

SENSITIVITY OF DETECTION

Compound	*Sensitivity* γ
Diphenyl (as disulphonic acid)	10
o-Terphenyl (as monosulphonic acid)	60
m-Terphenyl (as monosulphonic acid)	25
p-Terphenyl (as monosulphonic acid)	60

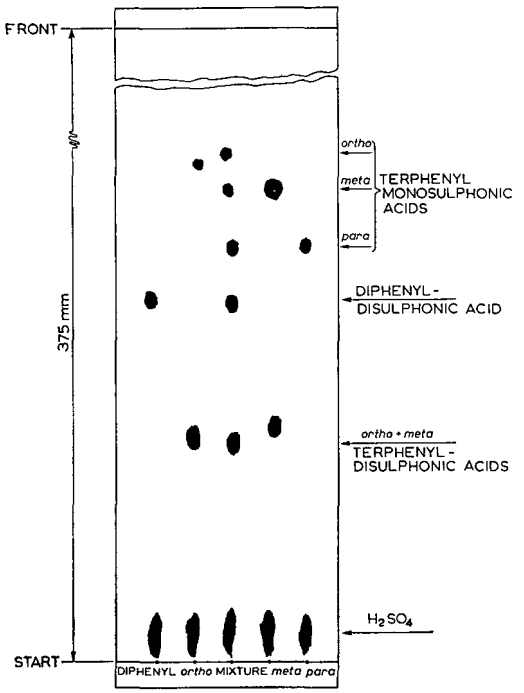

Fig. 1. Paper chromatography of diphenyl, *o*-, *m*-, and *p*-terphenyls and of a synthetic mixture (via the sulphonic acid derivatives).

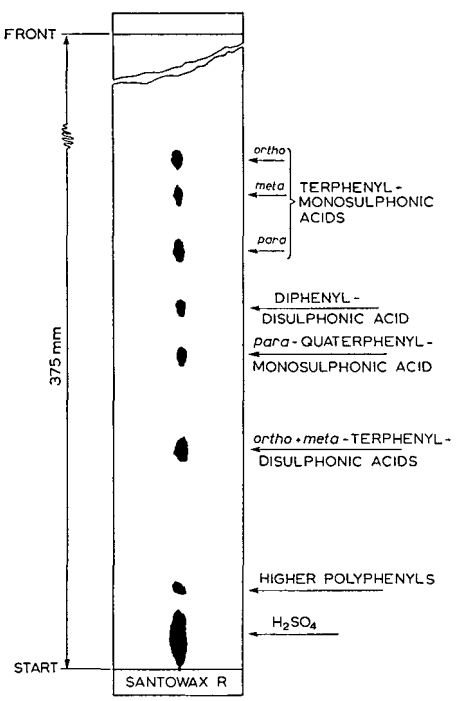

Fig. 2. Paper chromatography of Santowax R (via sulphonic acid derivatives).

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The authors wish to express their appreciation to S.O.R.I.N. for permission to publish this paper.

SUMMARY

A new paper chromatographic method for separating diphenyl and *o*-, *m*-, and *p*-terphenyls is described, in which the mixture of polyphenyls is first sulphonated under controlled conditions.

The migration velocities of the various sulphonic acids are different, and good separations are obtained by paper chromatography. The acids are located on the chromatographic strips by spraying with an indicator.

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CHROMATOGRAPHIC STUDIES ON ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC DERIVATIVES

III. NEW SOLVENTS AND NEW TECHNIQUES

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INTRODUCTION

On previous occasions[1,2] we have studied the paper chromatography and paper electrophoresis of isonicotinic acid hydrazide (INH) and its metabolic derivatives, isolated from blood samples by means of centrifugal ultrafiltration[3,4]. Though efficient, this technique of purification has drawbacks, such as the occasional rupturing of the dialysis bags and the need of heat to concentrate the filtered material.

The recent paper by DAVIS, DUBBS AND ADAMS[5], on an elution–concentration method, led to the development of a technique for the chromatographic isolation of INH-derivatives from wet samples of blood serum. Furthermore, new solvents have been used for the separation of these compounds and papers impregnated with ethylenediaminetetraacetic acid (EDTA) have been tried with good results.

MATERIALS AND METHODS

Chromatographic isolation of INH and derivatives

Blood was drawn from the INH-treated animal and serum was separated as usual, only a few microliters being needed[6]. The serum was then streaked along a line parallel to and at 10 cm from the top of a strip of Macherey-Nagel No. 261 filter paper measuring 40 × 3 cm. No more than 10 μl of serum should be applied to the paper, and the streak must run from edge to edge of the strip, in order to avoid constriction of the spots during the chromatographic run and to force the solvent through the sample. Greater amounts of serum tend to block the passage of the solvent, owing to the precipitation of the proteins by the organic phase.

Paper chromatography was then carried out by the descending method, through the wet sample of serum (drying of the sample prevents the displacement of the solvent). Attention must be paid to the fact that after the paper strip is placed in the chromatographic chamber the initial distance between the solvent and the sample must be about 2 cm, in order to avoid massive precipitation of proteins at the beginning of the run, which would be caused by a solvent front too rich in organic phase.

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Any one of the solvents already studied for the paper chromatography of INH-derivatives[1,2] can be used in the present case. Some of the best results, however, were obtained with a mixture of pyridine and water (65:35), especially when the procedure described above was combined with the elution–concentration method of DAVIS, DUBBS AND ADAMS[5].

In this last case, instead of pressing the filter paper between glass plates, as described by those authors, the following procedure was adopted: a strip of Macherey-Nagel No. 261 filter paper 6 cm wide was cut in the necessary number of segments and the samples were applied to the paper (one on each segment), which was then covered with a bent sheet of aluminium foil, as shown in Fig. 1.

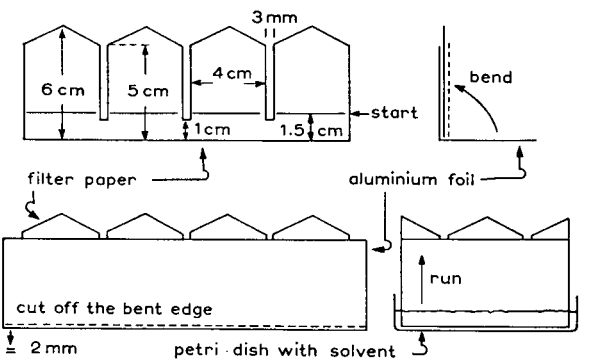

Fig. 1. Setup for the direct chromatographic isolation of INH-derivatives from wet samples of blood serum.

This setup was then run as an ascending chromatogram, after clipping it into a cylinder and cutting off the bent edge of the foil. INH and its derivatives are concentrated at the top of each segment by the evaporation of the solvent, and are easily eluted with a few microliters of pyridine.

Paper chromatography

As before, Macherey-Nagel No. 261 filter paper was used in all the experiments described. In the case of EDTA-impregnated papers the sheets of filter paper were soaked in a 0.2*M* solution of ethylenediaminetetraacetic acid (the excess of which was blotted out with filter paper) and dried afterwards in the oven at 120°. In the case of impregnated papers the solvents were saturated with 0.2*M* EDTA.

The spots were localized as before[2], using the sequential procedure U.V.–$BrCN/NH_3$–GREULACH-HAESLOOP reagent[7].

RESULTS AND DISCUSSION

The method described for the chromatographic isolation of INH-derivatives from wet samples of blood serum was found to be useful in all cases where the amount of the drug administered to the animal was large enough to be detected in the final paper chromatogram, since the volume of the serum sample applied to the paper had to be limited to 10 μl.

Among the solvents commonly used in paper chromatography, pyridine was found to be the only one capable of dissolving all the INH-derivatives with which we were concerned. This led us to study some of the pyridine-containing solvent mixtures already in use, as shown in Table I.

TABLE I

R_F VALUES FOR SOME INH-DERIVATIVES IN SOLVENT MIXTURES CONTAINING PYRIDINE

Compound	*R_F values*			
	1	*2*	*3*	*4*
INH	0.60	0.89	0.74	0.73
Acetyl-INH	0.66	0.92	0.71	0.54
Acetaldehyde INHzone	0.69	0.92	0.76	0.58
NH_4 pyruvate INHzone	0.42/0.59	0.89	0.44/0.59	0.68
NH_4 isonicotinate	0.37	0.87	0.42	0.55
Isonicotinamide	0.67	0.88	0.69	0.72
Diisonicotinyl hydrazide	0.71	0.92	0.74	0.55

Solvent	*Composition*	*Ref.*
1	Pyridine–amyl alcohol–water (40:35:30)	8
2	Pyridine–water (65:35)	9
3	Isoamyl alcohol–pyridine–water (20:25:20)	10
4	Pyridine–isoamyl alcohol–1.6*N* NH_4OH (20:14:20)	11

As can be seen from the results shown in the table, the pyridine–amyl alcohol–water (40:35:30) mixture is especially suitable for the separation of the pyruvic hydrazone. Very good results were obtained by combining it with *n*-butanol saturated with 1% ammonium hydroxide[1] in two-dimensional separations, as shown in Fig. 2. In this case the localization of the spots by means of the GREULACH-HAESLOOP

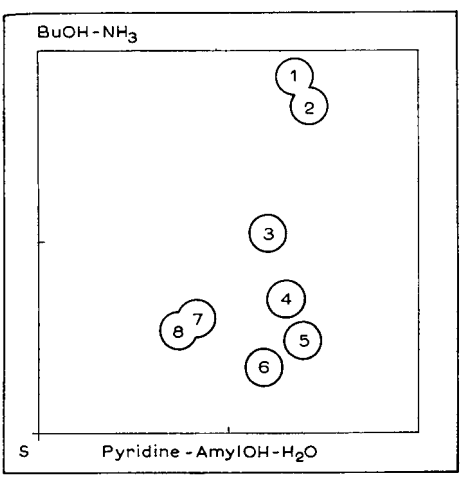

Fig. 2. Map of a two-dimensional paper chromatogram of INH-derivatives: 1 = isonicotinamide; 2 = di-INH; 3 = INH; 4 = acetyl-INH; 5 = acetaldehyde INHzone; 6 and 7 = NH_4 pyruvate INHzone; 8 = NH_4 isonicotinate.

reagent[7] omits the spots of isonicotinic acid and isonicotinamide, making it easier to identify the remaining derivatives.

One of the main problems encountered in the paper chromatography of INH is the frequent formation of "tails", which we tried to avoid by impregnating the paper with a chelating agent, such as EDTA. The results obtained with some EDTA-saturated solvents are shown in Table II. As can be seen, the R_F values found for the INH spots were lower than those obtained with the corresponding water-saturated mixtures[1,2], and higher for the other derivatives. INH-tailing, on the other hand, was either very much reduced or completely eliminated.

TABLE II

R_F VALUES FOR SOME INH-DERIVATIVES IN EDTA-IMPREGNATED FILTER PAPER

Compound	*R_F values*				
	1	*2*	*3*	*4*	*5*
INH	0.66	0.79	0.84	0.76	0.71
Acetyl-INH	0.79	0.87	0.81	0.77	0.58
Acetaldehyde INHzone	0.83	0.84	0.85	0.82	0.58
NH_4 pyruvate INHzone	0.09	0.06	0.56	0.57	0.05
	0.42	0.37	0.76	—	—
NH_4 isonicotinate	0.48	0.34	0.57	0.50	0.23
Isonicotinamide	0.71	0.83	0.80	0.76	0.62
Diisonicotinyl hydrazide	0.80	0.85	0.82	0.85	0.58

Solvent	*Composition*
1	Pyridine–amyl alcohol–0.2 *M* EDTA (40:35:satd.)
2	*n*-Butanol satd. with 0.2 *M* EDTA
3	*n*-Butanol–ethanol–0.2 *M* EDTA (40:10:satd.)
4	Isoamyl alcohol–pyridine–0.2 *M* EDTA (20:25:satd.)
5	Isoamyl alcohol satd. with 0.2 *M* EDTA

ACKNOWLEDGEMENTS

The present work was carried out at the Department of Biochemistry of the Central Laboratory of Tuberculosis, in collaboration with the Institute of Phthisiology and Pneumology of the University of Brazil and with the support of the National Research Council of Brazil.

SUMMARY

The authors devised a method for the direct chromatographic isolation of derivatives of isonicotinic acid hydrazide from wet samples of blood serum. This method is based on forcing the solvent through the sample, applied as a streak, and on the concentration of the isolated material at the top of the filter paper, caused by the evaporation of the solvent.

In addition pyridine-containing solvents were studied and papers impregnated with ethylenediaminetetraacetic acid were tested, in order to obtain better separations of the derivatives and to avoid tailing of the isonicotinic acid hydrazide spots.

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CHROMATOGRAPHY OF COMPOUNDS OF BIOLOGICAL INTEREST ON GLASS FIBER, PARAFFIN-COATED, AND UNTREATED CELLULOSE PAPER*

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The development of a rapid, sensitive, chromatographic method employing glass fiber paper by DIECKERT and coworkers[1] has made possible the analysis of a variety of compounds, including glycerides[2], phospholipids[3–5], sugars[6], polybromostearates[7], saponins[8], tung oil[9], bile acids[10], steroids[11,12], sterols[2,13], triterpenoids[14], and phosphatides[15]. The separation of cholesterol and its esters on paraffin-dipped paper has been described by SMITH[16]. ASHLEY AND WESTPHAL[17] have used paraffin oil-treated paper in the chromatography of aliphatic acids. Cellulose paper, untreated, has been employed for the separation of numerous compounds.

Because reports in the literature of studies employing glass fiber, paraffin-coated, and standard filter paper did not extend adequately to the biological compounds of interest in our studies with the rat, we have explored the use of several new solvent systems for the development of chromatograms prepared on these papers. In the present work, the migration characteristics of selected fatty acids, methyl esters of fatty acids, and other naturally occurring compounds have been studied on glass fiber and paraffin-coated paper. In addition to customary heating of the sulfuric acid-sprayed paper, iodine vapor has been used to detect compounds on glass fiber paper. The migration characteristics of fifty-five selected compounds, including amino acids, vitamins, purines and other biologically important substances, have been determined on untreated cellulose paper in one to six solvent systems.

PROCEDURES

Glass fiber paper***, 9 × 25 cm, was heated in a muffle furnace at 600° for 30 min. The paper was impregnated with a 0.4 % solution of sodium silicate according to the procedure of SWARTWOUT *et al.*[13].

Large filter paper sheets† especially selected for chromatography were used to obtain values listed for compounds in Table III. Paraffin-coated paper was prepared

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** Undergraduate Research Participants, National Science Foundation.

*** No. 934 AH, Reeve Angel and Company, Clifton, New Jersey.

† No. 5-714, Whatman No. 1, 18 × 22 in., from Fisher Scientific Company, Silver Spring, Maryland.

by dipping sheets of this filter paper in a 5 % solution of paraffin in petroleum ether and allowing them to dry.

The compounds used as reference standards were secured from commercial sources. They were dissolved in suitable solvents and made up to contain 500 μg/ml. A line 5 cm from the bottom of the sheets was made with a lead pencil, and the positions of compounds were marked approximately 3 cm apart. Samples were applied to these reference positions with micropipets and allowed to dry. The paper was suspended in a covered rectangular glass jar with the upper 3 cm being held in the solvent by means of glass rods. With the treated glass paper, approximately 7 min were required for the solvent front to migrate to within 2 cm of the bottom edge of the paper. The chromatogram was removed and allowed to dry on a rack in a fume hood until the solvent odor could no longer be detected.

To locate the compounds after chromatography, the glass fiber papers were sprayed with concentrated sulfuric acid from an atomizer, placed on a glass rod support and heated in an oven at 110° for 4 min to produce charred spots. Some of the compounds were detected by reaction with iodine vapor to produce brown spots.

Approximately 15 to 24 h were required for descent of the solvents on the plain filter paper or paraffin-coated paper. The papers were supported on glass rods by stainless steel clamps and allowed to dry in a fume hood. Compounds on untreated or paraffin-coated cellulose paper were detected by reaction with iodine vapor, spraying with a 0.5 % solution of ninhydrin in 95 % ethanol, fluorescence under ultra-violet light, or spraying with bromocresol green or antimony trichloride (24 % solution in chloroform).

RESULTS AND DISCUSSION

The migration characteristics on glass fiber paper of a selected group of fat-soluble compounds in various solvents are shown in Table I.

Two spots were obtained for some of the compounds in the solvents studied. As these compounds were commercial samples which were not further purified, the appearance of two spots represents the presence of impurities therein. 7-Dehydrocholesterol may be separated from cholesterol by use of either isooctane or ethanol–ether–water (1:1:1 or 2:1:2). Ergosterol can be separated from these two compounds in isooctane. Greater separation between linolenic and linoleic acid, or cholesterol and related substances can be obtained by employing longer strips of paper. Limited success was achieved in separating the methyl esters of the fatty acids, hexane–isooctane–chloroform (2:4:1) giving greatest separation.

The R_F values of several compounds on paraffin-coated paper, with butanol–propionic acid–water* and chloroform–acetic acid–liquid paraffin (50:130:10) as solvents are presented in Table II. Slightly greater separation of cholesterol from 7-dehydrocholesterol and ergosterol is achieved in these solvents on paraffin-coated paper than on glass fiber paper.

Cholesterol may be detected on these chromatograms by spraying with Liebermann-Burchard reagent or a solution of ferric chloride in 87 % phosphoric acid (Zlatkis reagent).

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* Fresh solvent was prepared from equal volumes of two solutions: (A) 1246 ml of 1-butanol and 84 ml water, and (B) 620 ml of propionic acid and 790 ml of water.

TABLE I

R_F VALUES OF SELECTED COMPOUNDS ON GLASS FIBER PAPER

Compound	Quantity		R_F values[a]												
	μg	μl	1	2	3	4	5	6	7	8	9	10	11	12	13
Cholesterol	20	—	0.34	1.00	0.97	0.98	0.82	0.58	0.00	0.79	0.00	—	0.93	—	—
Choline	25	—	—	—	—	—	—	0.00	0.96	0.92	0.95	—	0.95	—	0.00
7-Dehydrocholesterol	50	—	0.03	—	0.98	0.96	0.24	0.13	0.96	0.78	0.38	0.79	—	0.74	0.15
	—	—	0.22[b]	—	—	—	0.80[b]	0.63[b]	—	0.82[b]	—	—	—	—	0.62[b]
Dihydrocholesterol	50	—	0.26	1.00	—	—	—	—	—	—	—	0.90	—	0.87	0.63
Ergosterol	50	—	0.01	—	0.99	0.95	—	—	—	0.86	—	0.15	0.93	0.85	—
	—	—	0.15[b]	—	—	—	—	—	—	—	—	0.71[b]	—	—	—
Fumaric acid	100	—	—	—	—	—	—	—	—	0.88	—	—	—	—	—
Linoleic acid	—	2	0.16	0.18	0.83	0.91	0.15	0.12	0.04	0.33	0.12	—	—	—	—
	—	—	—	—	—	—	—	—	0.80[b]	—	0.82[b]	—	—	—	—
Linolenic acid	—	2	0.12	0.20	0.91	0.92	0.23	0.17	0.06	0.39	0.84	—	—	—	—
	—	—	—	—	—	—	—	—	0.84[b]	—	—	—	—	—	—
Oleic acid	—	0.5	0.11	0.14	—	—	—	—	—	—	—	—	—	—	
Methyl arachidonate	—	—	1.00	—	—	—	—	—	—	0.91	—	0.96	0.90	0.96	0.16
Methyl laurate	—	—	—	—	—	—	—	—	—	0.05	—	0.00	—	—	—
Methyl linoleate	—	—	1.00	—	—	—	—	—	—	—	—	0.91	0.96	0.97	—
Methyl linolenate	—	—	1.00	—	—	—	—	—	—	0.87	—	0.94	0.96	0.96	0.63
Methyl myristate	—	—	—	—	—	—	—	—	—	0.89	—	0.92	0.96	—	—
Methyl oleate	—	—	1.00	—	—	—	—	—	—	0.90	—	—	0.97	—	0.97
Methyl stearate	—	—	0.94	—	—	—	—	—	—	—	—	0.90	—	—	—
Methyl palmitate	—	—	—	—	—	—	—	—	—	0.87	—	—	—	—	—

[a] Solvents: 1 = Isooctane
2 = Benzene–ethanol (100:9)
3 = Benzene
4 = Chloroform
5 = Carbon tetrachloride
6 = *n*-Hexane
7 = Ethanol–ether–water (2:1:2)
8 = Isooctane–chloroform (1:1)
9 = Ethanol–ether–water (1:1:1)
10 = Hexane–isooctane–chloroform (2:1:1)
11 = Hexane–chloroform–ethanol–ether (1:1:2:1)
12 = Hexane–isooctane–chloroform (4:1:1)
13 = Hexane–isooctane–chloroform (2:4:1)

[b] Two spots were obtained.

TABLE II

R_F VALUES OF SELECTED COMPOUNDS ON PARAFFIN-COATED PAPER

Compound	*Quantity*		*Detection*[a]	R_F *value*[b]	
	μg	*μl*		*1*	*2*
Adrenaline	50		Brown with I_2	0.29	—
Acetic acid		10	Yellow with BCG	0.20	—
Acetylcholine	50		Brown with I_2	0.40	—
Betaine	50		Brown with I_2	0.98	—
Cholesterol	100		Blue with $SbCl_3$	0.73	0.71
Choline	50		Brown with I_2	0.23, 0.46[c]	—
Cystathionine	20		Purple with Nin	0.02	—
7-Dehydrocholesterol	100		Fluorescent with U.V.	0.84, 0.94[c]	0.86
Ergosterol	100		Fluorescent with U.V.	0.84, 0.92[c]	0.80, 0.89[c]
Ethanolamine		4	Purple with Nin	0.55	—
Fumaric acid		10	Absorbs U.V.	0.54	—
α-Keto-γ-methiol-butyric acid	50		Purple with Nin	0.56	—
Lactic acid		2	Fluorescent with U.V.	0.75	—
Lecithin	100		Brown with I_2	0.85	0.88
Linoleic acid		5	Brown with I_2	0.84	0.76, 0.91[c]
Linolenic acid		5	Brown with I_2	0.84	0.73, 0.89[c]
N-Methylnicotinamide	50		Brown with I_2	0.87	—
Oleic acid		2	Fluorescent with U.V.	0.81	—
S-Adenosylmethionine	50		Purple with Nin	0.09	—
Serine	20		Purple with Nin	0.13	—

[a] I_2 = iodine vapor; BCG = bromocresol green; $SbCl_3$ = antimony trichloride; Nin = ninhydrin; U.V. = ultra-violet light.

[b] Solvents: 1 = butanol–propionic acid–water; 2 = chloroform–acetic acid–liquid paraffin (50:130:10).

[c] Separated into two spots.

In Table III are presented the R_F values of fifty-three compounds of biological interest in six solvent systems. Combinations of lutidine and methanol offer promise as solvents for the two-dimensional chromatography of compounds of biological interest, as excellent separation is achieved in them for a variety of compounds.

TABLE III

R_F VALUES OF SELECTED COMPOUNDS ON UNTREATED CELLULOSE PAPER

Compound	R_F *values* × 100[a]					
	Lutidine	*Methanol*	*Butanol–acetic acid*	*Ethanol–acetic acid*	*Phenol*	*Butanol–propionic acid–water*
Acetylcholine chloride	—	—	—	—	92, 91[b]	75, 64[b]
Adenine	70	—	—	—	—	—
Adenosine triphosphate	—	—	—	—	44, 17, 6[c]	18, 8, 4[c]
Adenylic acid	—	—	—	—	56	21
DL-α-Alanine	—	—	30	—	—	—
L-Alanine	22	70	31	—	—	—
Allantoin	—	50	—	—	—	—
p-Aminobenzoic acid	58	—	81	—	—	—
γ-Aminobutyric acid	—	—	—	44	—	—
DL-α-Amino-*n*-butyric acid	36	55	—	—	—	—

(continued on p. 353)

TABLE III (*continued*)

Compound	R_F values × 100[a]					
	Lutidine	*Methanol*	*Butanol–acetic acid*	*Ethanol–acetic acid*	*Phenol*	*Butanol–propionic acid–water*
4-Amino-5-imidazole-carboxamide	—	48	—	25	—	—
α-Aminoisobutyric acid	—	66	—	—	—	—
α-Aminopimelic acid	54, 15[b]	80, 73[b]	57, 31[b]	46, 26[b]	—	—
L-Aspartic acid	13	67	—	—	—	—
Adrenaline	—	70	—	—	—	—
S-Adenosylmethionine	—	4	—	—	—	—
Choline	36	73	38	39	—	—
Creatine	—	58	—	—	—	—
Creatinine	50	56	42	—	—	—
Cytidilic acid	—	—	—	—	41	14
Cytidine	—	43	—	—	76	42
Ethionine	51, 23[b]	74	52	—	—	—
L-Glutamic acid	13	—	—	—	—	—
Glycine	17	64	—	—	—	—
Guanylic acid	—	31	—	—	73, 38[b]	46, 16[b]
DL-Homocysteine	45, 17[b]	72, 55[b]	—	27	—	—
DL-Homocystine	16	55	—	—	—	—
Homocysteic acid	—	—	—	—	11	9
Homoserine	23	68	22	—	—	—
Isoleucine	47	63	59	—	—	—
α-Keto-γ-methiolbutyric acid	—	59	—	—	—	—
Leucine	50	—	61	—	—	—
Lysine	—	21	—	—	—	—
L-Methionine	45, 24[b]	53, 31[b]	—	—	—	—
Methionine sulfoxide	—	—	—	11	—	—
Methoxinine	—	—	—	—	85	50
N^2-Methylnicotinamide	82	—	81	—	100	77
Methylserine	—	51	—	—	—	—
Nicotinic acid amide	77	—	70	—	—	—
Pantothenic acid	—	71	—	—	—	—
Phenylalanine	47	51	—	—	—	—
Riboflavin	—	31	—	—	—	—
Serine	19	65	—	—	—	—
Taurine	29	—	—	—	—	—
Thiamine	—	—	37	—	—	—
Threonine	23	69	24	—	—	—
Tryptophan	—	35	—	—	—	—
Tyrosine	47	69	—	—	—	—
Urea	49	—	—	—	—	—
Uridylic acid	—	—	—	—	32	15
Valine	38	60	—	—	—	—
Vitamin B_{12}	—	30	—	—	—	—
Xanthine	—	28	—	—	—	—

[a] Solvents: Lutidine = 2,6-lutidine–water (65:35 v/v).
Methanol = absolute methanol–water (95:5 v/v).
Butanol–acetic acid = *n*-butanol–glacial acetic acid–water (80:20:20 v/v/v).
Ethanol–acetic acid = 95% ethanol–glacial acetic acid (95:5 v/v).
Phenol = Mallincrodt, USP "Gilt Label" phenol saturated with a solution of 6.3% sodium citrate and 3.7% potassium dihydrogen phosphate.
Butanol–propionic acid–water = equal volumes of two solutions: (A) 1246 ml *n*-butanol and 84 ml water, and (B) 620 ml propionic acid and 790 ml water.

[b] Two spots were obtained.

[c] Three spots were obtained.

SUMMARY

Selected organic compounds commonly occurring in plant and animal tissues have been chromatographed on glass fiber, paraffin-coated and untreated cellulose paper in a number of different solvent systems. The R_F values of these compounds represent new additions to the literature in the solvent systems studied.

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THE APPLICATION OF PARTITION CHROMATOGRAPHY TO THE SEPARATION AND CHARACTERISATION OF OESTROGENS BY ISOTOPE DILUTION

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INTRODUCTION

The criteria of classical chemistry can seldom be applied to the identification of metabolites particularly of steroid hormones, isolated from tissues or body fluids after experiments with precursors at physiological levels. However, isotope dilution methods, pursued with sufficient rigour, yield information which leaves little doubt of the identity of the material isolated. Chromatography, in conjunction with methods for the measurement of radioactive and unlabelled compounds, plays an important role in the separation and identification of metabolites isolated in experiments of this kind.

We report here chromatographic procedures developed for the separation of mixtures of oestrogens of widely differing polarity and of closely related epimers. Techniques for recording permanently the position of oestrogens on paper chromatograms by autopositive photography and for the determination of ^{14}C on paper by liquid scintillation counting are also described.

MATERIALS AND APPARATUS

Materials

(*a*) *Solvents*

Ethanol was absolute alcohol B.P.C. grade (J. Burrough Ltd., London, S.E. 11). Methanol, ethanol, benzene and light petroleum (b.p. 80–100°) were of A.R. grade. Ligroin, hexane (free from aromatic hydrocarbons) and ethylene dichloride were laboratory reagent grade (B.D.H. Ltd., Poole, Dorset). Solvents used for column chromatography and fluorimetry were redistilled before use, but solvents for paper chromatography were used without further purification.

(*b*) *Reagents*

Potassium ferricyanide and phosphomolybdic acid were of A.R. grade. Sulphuric acid for fluorimetry was either A.R. grade (for dilution to 65 %) or M.A.R. grade (B.D.H., for dilution to 90 %). Ferric chloride was laboratory reagent grade (Hopkin & Williams Ltd., Chadwell Heath, Essex). *m*-Dinitrobenzene was B.D.H. laboratory reagent purified according to CALLOW, CALLOW AND EMMENS[1]. The solution for liquid scintillation counting of ^{14}C was prepared as described by STITCH[2].

(c) Steroids

Oestrone (3-hydroxyoestra-1,3,5(10)-trien-17-one) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Oestradiol-17α (3,17α-dihydroxyoestra-1,3,5(10)-triene) and oestradiol-17β (3,17β-dihydroxyoestra-1,3,5(10)-triene) were supplied by Schering A.G., Berlin. Oestriol-16α,17β (3,16α,17β-trihydroxyoestra-1,3,5(10)-triene) was supplied by Sigma Chemical Corporation, St. Louis, Miss., U.S.A. Dr. T. F. GALLAGHER (Sloan-Kettering Institute, New York, U.S.A.) supplied samples of oestriol-16β,17β (3,16β,17β-trihydroxyoestra-1,3,5(10)-triene) and oestriol-3,16α,17α (3,16α,17α-trihydroxyoestra-1,3,5(10)-triene). Oestradiol-17β 3-methyl ether (3-methoxy-17β-hydroxyoestra-1,3,5(10)-triene) was prepared as described by BROWN[3]. Oestrone 3-methyl ether (3-methoxyoestra-1,3,5(10)-trien-17-one) and oestriol-16α,17β 3-methyl ether (3-methoxy-16α,17β-dihydroxyoestra-1,3,5(10)-triene) were prepared as described by LEVITZ[4]. The infra-red spectra and chromatographic behaviour of the oestrogen methyl ethers were identical with those of authentic specimens supplied by Professor W. KLYNE (M.R.C. Steroid Reference Collection, Westfield College, London, N.W. 3).

Testosterone (17β-hydroxyandrost-4-en-3-one) was supplied by Paines and Byrne Ltd., Greenford, Middlesex. Androst-4-ene-3,17-dione was supplied by Dr. R. I. DORFMAN (Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.).

(d) Supporting phases

Paper chromatography was carried out on Whatman No. 1 filter paper selected for chromatography. Sheets (20 cm × 50 cm) with the grain of the paper parallel to the longer side, were used without purification. If the mobile phase was required to overrun the paper, the end of the paper was cut to form ten equally spaced points.

Celite 535 (Johns Mansville & Co., 20, Albert Embankment, London, S.E. 11), purified according to BAULD[5], was used for column chromatography.

(e) Photographic materials

Photography was carried out on autopositive paper (Extra light weight 42, Kodak Ltd., Kodak House, Kingsway, London, W.C. 2). Photographic developer and fixer were Kodak Duostat brand.

Apparatus

(a) Tanks for paper chromatography

Glass tanks (19 cm × 30 cm × 57 cm, Aimer Products Ltd., 56–58 Rochester Place, Camden Road, London, N.W. 1), lagged all round with expanded polystyrene (sheets, 3.5 cm thick), were used for descending chromatography. The walls were covered inside with sheets of filter paper moistened with mobile and stationary phases. The temperature in the tanks was maintained at 19–21°.

(b) Tubes for column chromatography

Glass tubes (22 cm long, 10–11 mm internal diameter) fitted at one end with a perforated glass disc, were used for column chromatography.

(*c*) *Printing box for photographic recording of paper chromatograms*

After location of carrier steroids by chemical reactions on strips cut from paper chromatograms, each strip was photographed in a printing box (20 cm × 30 cm × 60 cm). Eight 60 W bulbs on the floor of the box in two lines parallel to the longer sides provided the light source. The inside of the box was aluminium painted to aid reflection. Flashed opal glass (25 cm × 45 cm) was mounted above the bulbs as a light diffuser. Above this a sheet of plate glass (27 cm × 50 cm × 5 mm) was mounted. The box was fitted with a lid hinged on one longer edge. On the lower side of this lid a second sheet of plate glass (27 cm × 50 cm × 5 mm) was mounted.

(*d*) *Fluorimeter*

Fluorimetric assay of oestrogens was carried out with an E.I.L. Direct Reading Fluorimeter Model 27A (Electronic Instruments Ltd., Lower Mortlake Road, Richmond, Surrey). An Ilford 601 filter was used as primary filter. In this position it was found to be unstable and was replaced when necessary. The secondary filter was a combination of Chance OY13, Ilford 108 and Chance OB2 filters.

(*e*) *Liquid scintillation counter*

^{14}C was assayed with an Ekco N612 liquid scintillation counter (Ekco Electronics Ltd., Southend-on-Sea, Essex).

METHODS

Methods for paper chromatography

(*a*) *Solvent systems*

Solvents were mixed in the following volumes and allowed to stand for at least 3 h at laboratory temperature (19–22°) before use:

(i) to separate oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β: benzene 200 ml, methanol 140 ml, water 60 ml;

(ii) to separate oestradiol-17α and oestradiol-17β: light petroleum (b.p. 80–100°) 200 ml, methanol 160 ml, water 40 ml, (BUSH system A[6]);

(iii) to separate oestrogen 3-methyl ethers: ligroin 200 ml, methanol 192 ml, water 8 ml[7].

The upper layer was used as mobile phase, the lower layer was the stationary phase.

(*b*) *Loading and running*

Material was dissolved in methanol–chloroform (1:1 v/v) and applied along a line 10 cm from, and parallel to, the shorter edge of the paper. 5–25 μg of each oestrogen per cm gave the best separations. After equilibration of the paper in the tank for at least 3 h, descending chromatography was carried out.

(*c*) *Detection of steroids on paper chromatograms*

The steroids were located by passing a strip (0.5 cm × 45 cm), cut from the centre of the chromatogram, through the reagents.

(i) Free oestrogens were detected at a level of 5 μg/sq. cm, with a mixture of equal volumes of 1 % aq. $FeCl_3$ and 1 % aq. $K_3Fe(CN)_6$[8].

(ii) Oestrogen 3-methyl ethers were detected at a level of 5 μg/sq. cm, with a 10 % solution of phosphomolybdic acid in ethanol[9].

(iii) C_{19} steroids were located with Zimmermann reagent, as described by BUSH[10]. Testosterone gave a blue band whereas 17-oxo-steroids gave a purple band.

(d) Photographic recording of paper chromatograms

The dried coloured strip with the position of the origin and solvent front (if present) marked clearly, was covered with a sheet of autopositive photographic paper and placed between the sheets of plate glass in the printing box. After exposure to the light source for 15–75 sec the photographic paper was developed, washed, fixed, washed and dried.

(e) Determination of ^{14}C after paper chromatography

After removal of a strip for detection of carrier steroids, an area of the paper, from 0.6 cm behind the origin to at least 0.6 cm beyond the solvent front (or to the edge of the paper) and 2.5 cm on either side of the original loading area, was cut into segments (1.2 cm) with this dimension parallel to the solvent flow. Each segment was immersed in liquid scintillator (3 ml) and ^{14}C was assayed with the Ekco N612 counter[11].

Methods for column chromatography

(a) Solvent system

Hexane (110 ml), benzene (90 ml), methanol (140 ml) and water (60 ml) were shaken together and maintained at laboratory temperature (19–22°) for at least 3 h. Celite for the column was treated with the lower layer. The upper layer was used as the first mobile phase. The second mobile phase was prepared by mixing hexane (20 ml) with benzene (80 ml). Ethylene dichloride was the third mobile phase.

(b) Column preparation

Stationary phase (5 ml, see above) was stirred slowly into purified Celite (5 g). The glass tube was filled with mobile phase. Celite-stationary phase was added in small portions and packed to form a column 15 cm high with a MARTIN packer[12]. The flow rate was 24–33 ml/h, with the solvent head at the top of the tube.

(c) Application of oestrogens to the column

The steroid mixture, dissolved in chloroform–methanol (1:1 v/v), acetone or methylene dichloride was transferred to four circular paper discs (8 mm diameter, Whatman No. 1 filter paper). After evaporation of the solvent, the discs were placed on top of the Celite column.

(d) Elution of oestrogens from the column

The mobile phase was run out from the column until the Celite was covered with approximately 0.2 mm. The loaded paper discs were placed on top of the Celite and mobile phase (0.5 ml) was added and allowed to run into the column. A further two volumes (0.5 ml each) were added and allowed to soak in. When the level of solvent was approximately 0.2 mm above the discs the tube was filled with mobile phase No. 1.

After twenty fractions (3 ml each) had been collected the eluting solvent was

changed to mobile phase No. 2. After a further 25 fractions had been collected mobile phase No. 2 was replaced by mobile phase No. 3. In all 60 fractions were collected.

(e) Determination of oestrogens after column chromatography

The eluted carrier oestrogens were detected and quantitatively estimated by fluorimetry with sulphuric acid. The method of BATES AND COHEN[13] (modified in our laboratory by using 45 min heating at 80° with 90 % H_2SO_4, addition of 65 % H_2SO_4 and fluorescence stabilisation for 1 h at 4°) was used.

(f) Determination of ^{14}C after column chromatography

^{14}C was assayed in each fraction by liquid scintillation counting by the procedure described by STITCH[2].

RESULTS

Paper chromatography

(a) Separation of oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β

Oestrone (R_F 0.88 ± 0.05; 19 observations), oestradiol-17β (R_F 0.72 ± 0.05; 19 observations), oestriol-16β,17β (R_F 0.40 ± 0.03; 3 observations) and oestriol-16α,17β (R_F 0.11 ± 0.02; 19 observations) were separated by chromatography in benzene–methanol–water (10:7:3 by vol.), for 3 h. Oestradiol-17α and oestradiol-17β were not separated in this system.

(b) Separation of oestradiol-17α and oestradiol-17β

These epimeric oestrogens were separated by chromatography in light petroleum (b.p. 80–100°)–methanol–water (10:8:2 by vol.), BUSH system A[6], for 30–50 h. The mobility of oestradiol-17α (0.21–0.35 cm/h) relative to that of oestradiol-17β(0.15–0.24 cm/h) was found to be 1.4. Thus in practice clear separations were achieved (Fig. 1) although the absolute mobilities were variable.

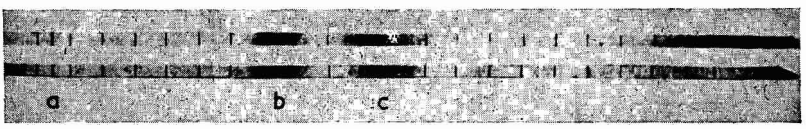

Fig. 1. Paper chromatography of oestradiol-17β (b) and oestradiol-17α (c) in light petroleum (b.p. 80–100°)–methanol–water (10:8:2 by vol.). Photographic record of two different chromatograms. The dark line (a) is the origin.

(c) Separation of oestrogen 3-methyl ethers

We have examined the behaviour of some oestrogen 3-methyl ethers in ligroin–96 % methanol[7], and report the following R_F values: oestrone 3-methyl ether 0.75 ± 0.05 (6 observations); oestradiol-17β 3-methyl ether 0.57 ± 0.05 (9 observations); oestriol-16α,17β 3-methyl ether 0.10 (3 observations). In addition the R_F values of the free oestrogens were oestrone 0.12 (4 observations), oestradiol-17β 0.05 (4 observations), oestriol-16α,17β 0 (2 observations). The R_F values of testosterone (0.13) and androst-4-ene-3,17-dione (0.26) were also recorded.

Column chromatography

Separation of oestrone, oestradiol-17α, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β

These compounds were readily separated, but oestriol-16β,17β and oestriol-16α,17α were not fully resolved. Fig. 2 shows a typical elution pattern.

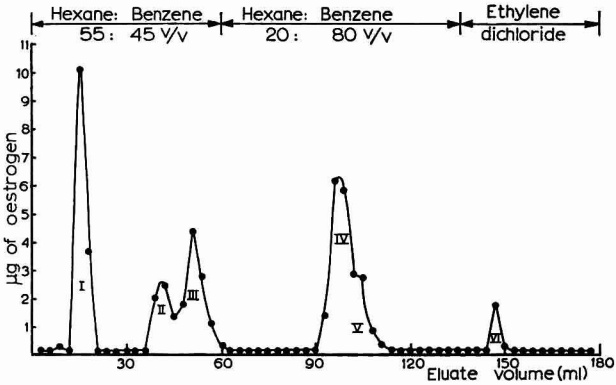

Fig. 2. Elution pattern after partition column chromatography of oestrone (I), oestradiol-17α (II), oestradiol-17β (III), oestriol-16α,17α (IV), oestriol-16β,17β (V) and oestriol-16α,17β (VI). The stationary phase was 70% aq. methanol. The composition of the mobile phase is indicated on the diagram.

Although 2 mg oestradiol-17β chromatographed on this column gave a single recognisable peak, best results were achieved when mixtures containing 50 μg or less of each oestrogen were chromatographed. 71–88% of oestrone, oestradiol-17α, oestradiol-17β and oestriol-16β,17β and 48% oestriol-16α,17β were recovered in the eluate.

Photographic recording of paper chromatograms

The photographic technique provided a permanent record of the chromatogram. Inspection of the distribution of ^{14}C on the paper aligned with the photograph of the position of the carrier enabled any separation of radioactivity from carrier to be observed. Fig. 3 shows the distribution of ^{14}C and location of carrier oestrone, oestradiol-17β and oestriol-16α,17β after incubation of ^{14}C testosterone with rat ovarian tissue[14]. The distribution of 0.3 mμC ^{14}C after chromatography of a mixture of radioactive oestrogens could be determined by this method.

DISCUSSION

To improve the reliability of identification of oestrogens by reverse isotope dilution with chromatographic techniques, we have developed methods for separating oestrogens on paper and on partition columns, for recording permanently the position of oestrogens on paper, and for measuring ^{14}C on paper chromatograms.

The separation of oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β on one paper chromatogram in 3 h reported here, obviates the need for two separate

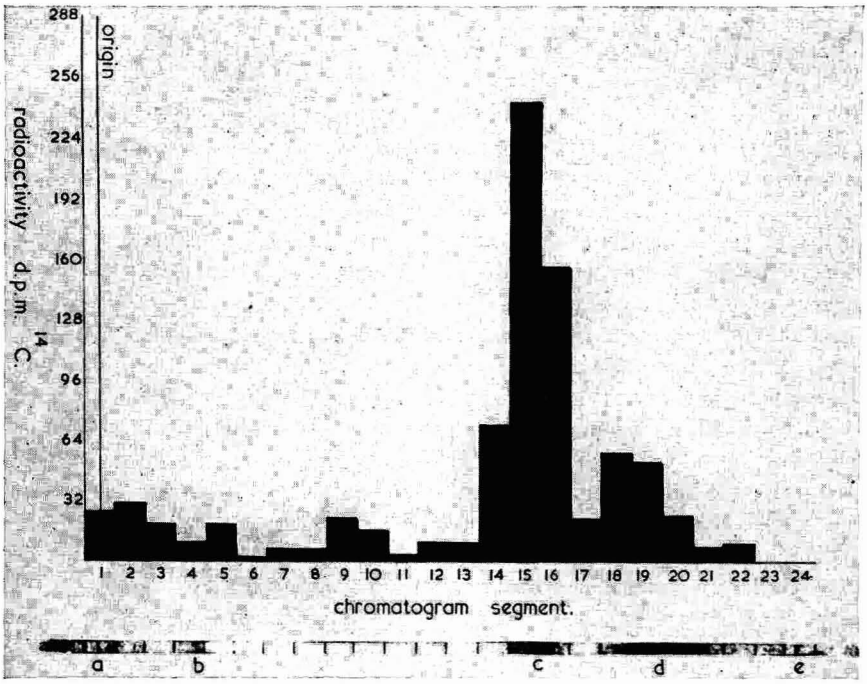

Fig. 3. Paper chromatography of an extract from an incubation of ovarian tissue with ^{14}C-testosterone[14]. Distribution of ^{14}C aligned with the photographic record of the position of carrier oestriol-16α,17β (b), oestradiol-17β (c) and oestrone (d). (a) marks the origin and (e) the solvent front.

chromatograms[15]. The application of BUSH system A to the separation of the oestradiol epimers should be of value as an additional method, although solvent systems have been described for this separation[16–19]. SMITH[7] observed that the 3-methyl ethers of oestrone, oestradiol-17β and oestriol-16α,17β were separated by paper chromatography in ligroin: 96 % aqueous methanol but did not report R_F values. We confirmed that these methyl ethers were separated from each other and found, furthermore, that they were separated from the corresponding parent oestrogens in this system. Oestriol-16α,17β 3-methyl ether and oestrone however, were not separated. The R_F values obtained were recorded.

A major difficulty in the partition column chromatography of oestrogens has been to devise methods capable of handling compounds of widely differing polarity (*e.g.* 2-methoxyoestrone and oestriol-16α,17β). This problem was overcome by loading the column from paper discs and by changing the eluting solvent in two discrete stages.

Several methods utilising partition column chromatography for the resolution of oestrogen mixtures extracted from urine[5,20,21] and blood[22] have been described. The separation of the epimeric oestradiols, however, was not reported in these papers.

Eluates from the column after chromatography of extracts of blood and ovarian tissue appeared sufficiently clean for the determination of carrier oestrogen by H_2SO_4 fluorimetry and of ^{14}C by liquid scintillation counting. However, before strictly quantitative measurements are carried out, it is essential to investigate the possibility of quenching of fluorescence[23] and of phosphorescence[2] by material from the tissue extract.

Comparison of the elution patterns of carrier oestrogens and ^{14}C, together with the determination of specific activity, provided the basis for isotope dilution assay and for the identification of metabolites.

The autopositive photographic process gave a permanent record of paper chromatograms free from subjective error in drawing the position of steroid zones. A major advantage of this technique is that the photographic processing can be carried out in daylight.

A basic requirement for the identification of unknown compounds by isotope dilution techniques is the determination of specific activity at each stage of a serial purification process[24]. Inspection of the distribution of radioactivity and carrier material in eluates from chromatography columns readily allows any separation to be observed. Moreover, direct determinations of specific activity can be made. Measurement of specific activity after paper chromatography, however, is more difficult and usually involves elution of material from the paper. The combination of photography and ^{14}C assay on paper, reported here, although not yielding a direct measurement of specific activity, enables separation of radioactivity and the carrier (equivalent to a change in specific activity) to be detected.

This photographic technique should therefore be of value whenever isotope dilution studies are combined with paper chromatography.

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SUMMARY

Methods are described for separating oestrone, oestradiol-17α, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β by partition column chromatography and for separating oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β on paper.

A separation of oestradiol-17α and oestradiol-17β was achieved on paper in a system originally described[6] for the chromatography of androgens.

Mixtures of oestrone, oestradiol-17β, oestriol-16α,17β and their 3-methyl ethers were resolved by paper chromatography in the system described by SMITH[7].

Autopositive photography was used to record permanently the position of steroids on paper chromatograms.

The application of this technique, combined with liquid scintillation counting of ^{14}C on paper chromatograms, to the identification of radioactive oestrogens is discussed.

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EIN NEUER ABER FARBIGER NACHWEIS VON ALKALOIDEN UND ORGANISCHEN BASEN MIT TETRAPHENYLBORNATRIUM*

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Das von WITTIG[1] synthetisierte TPBNa** hat als Reagenz eine weitgehende Bedeutung erlangt[2]. Der Vorteil liegt in der Erfassung von Kalium-, Ammonium-, Rubidium-, Cäsium- und Thallium-Ionen. Ferner können einwertige Metallkomplexe, Onium-Verbindungen und organische Basen, wie Alkaloide, biogene Amine, basische Aminosäuren und bestimmte synthetische Arzneimittel, nicht nur quantitativ, sondern auch in geringen Mengen genau ermittelt werden[3].

Der Anwendungsbereich von TPBNa wurde von NEU erweitert. Dazu gehört die getrennte Bestimmung von zwei aber gemeinsam mit TPBNa fällbaren Verbindungen, wie Kalium und Triäthanolamin[4]. Ferner der Nachweis und die quantitative Bestimmung der α,ω-Dihydroxypolyäthylenoxyde (PAeO) und der sich davon ableitenden Polyoxyaethylen(POAe)-Sorbitanfettsäureester[5]***, unter Verwendung von TPBNa und Barium-Ionen.

Obwohl das TPBNa als ausgezeichnetes Fällungsmittel für Alkaloide brauchbar ist, sind die damit erhaltenen Niederschläge weiss. Für den papierchromatographischen Nachweis oder den Tüpfel-Test von Alkaloiden konnte das TPBNa deshalb bisher nicht herangezogen werden, weil im Gegensatz zu anderen Alkaloid-Reagentien, z.B. dem DRAGENDORFF-Reagenz, keine Farbreaktion eintritt, die eine Lokalisation der Substanz anzeigt.

In vorliegender Arbeit wird beschrieben, dass TPBNa als farbiger Nachweis für die Feststellung und Lokalisierung von Alkaloiden nach papierchromatographischer Trennung und im Tüpfel-Test verwendet werden kann.

Das Auffinden des Nachweises hat sich aus folgenden Arbeiten entwickelt. In einer vorangegangenen Arbeit hat NEU[6] gezeigt, dass mit dem von demselben Autor zuerst synthetisierten Diphenylborinsäureanhydrid[7] der Nachweis von quartären Ammonium-Verbindungen, wie z.B. Cholin, Acetylcholin, aber auch langkettigen oberflächenaktiven Verbindungen, wie z.B. Pendiomid®, Quartamon®, Bradosol®, Zephirol®, Trypaflavin®, Eulan NK®, Antrenyl® und Triphenyl-dodecylphosphoniumbromid durch die gleichzeitige Verwendung von Flavon-3-olen als charakteristisch gefärbte Niederschläge möglich ist. In dem Zusammenhang hat NEU[9] ferner gezeigt, dass eine Unterscheidung der mit dem Anhydrid der Diphenylborinsäure und quartären Ammonium-Verbindungen bzw. Aminsalzen entstehenden

* Herrn Prof. Dr. FRITZ FEIGL zum 70. Geburtstag in Verehrung gewidmet.
** Tetraphenylbornatrium, als Kalignost® im Handel, Heyl u.Co. Berlin-Steglitz.
*** Tweene®.

Fällungen auf einfache Weise durchführbar ist. Die Differenzierung der mit Diphenylborinsäureanhydrid, Flavon-3-olen und niederen bzw. höhermolekularen quartären Ammonium-Verbindungen entstehenden roten bis rotorangen Niederschläge ist durch ihr Verhalten gegenüber Ammoniak gegeben. Die Farbe bleibt bei den Niederschlägen mit vierbindigem Stickstoff erhalten, während die mit Aminen und ampholytaktiven capillarwirksamen Substanzen erhaltenen Färbungen gegenüber Ammoniak instabil sind.

Das Verhalten von Diarylborinsäureanhydriden als Reagenz in der analytischen Chemie hat NEU[10] zusammenfassend beschrieben.

Vor einiger Zeit hat Verfasser die Frage zu klären unternommen, ob das bei der Umsetzung von POAe und TPBNa verwendete Barium-Ion auch durch andere Substanzen ersetzt werden kann. So wurde beispielsweise geprüft, ob sich Flavonoide dafür eignen und damit zunächst ein qualitativer Nachweis möglich ist. Das Ergebnis zeigte bereits, dass mit der Modifikation der Methode gleichzeitig ein Weg gegeben ist, um Flavonoide im Niederschlag abzuscheiden. Das weitere überraschende Ergebnis war aber die Feststellung, dass für die entstehenden Niederschläge im U.V.-Licht eine aussergewöhnlich starke Fluoreszenz charakteristisch ist. Durch die Verwendung verschieden substituierter Flavonoide traten verschiedene Fluoreszenzfarben auf. Der Farbton liess eine Abhängigkeit von der Konstitution erkennen. Eine Auswertung der Fluoreszenzfarben im Zusammenhang mit der Konstitution wurde schon durchgeführt, über die am angeführten Orte berichtet wird.

Die Übertragung der Reaktion für eine papierchromatographische Prüfung der POAe und Tweene® ergab einen neuen Nachweis, der beschrieben wurde[11].

Auf Grund des Verhaltens der POAe kann angenommen werden, dass sie durch die verwendeten Reagentien ein Verhalten wie Onium-Verbindungen aufweisen.

Bei der Prüfung des Verhaltens von Polyhydroxy-Verbindungen gegenüber den gleichen Reagentien im Tüpfel-Test und durch Chromatographie zeigte sich, dass die Reaktion darauf ebenfalls anspricht. Die Beschreibung erfolgt in dieser Zeitschrift[12].

Für die Papierchromatographie von Alkaloiden und organischen Basen sind eine Anzahl von Fliessmitteln bekannt[13]. Da der Zweck vorliegender Arbeit nicht eine Trennung, sondern einen Nachweis zum Gegenstand hat, ist für die Versuche nur ein Lösungsmittel verwendet worden (siehe Tabelle I).

Das Chromatogramm wurde nach dem Trocknen mit einer 1 %igen Lösung von TPBNa in mit Wasser gesättigtem Butanon gesprüht, das Lösungsmittel durch Trocknen entfernt und dann mit einer 0.015 %igen Lösung eines Flavon-3-ols oder seiner Glykoside in Methanol gesprüht, bzw. durchgezogen und an der Luft getrocknet.

Von den zahlreichen Flavonolen konnte für die vorliegende Beschreibung nur eine Auswahl getroffen werden. Sie beschränkt sich auf ein Flavon-3-ol mit einer Hydroxyl-Gruppe in 5-Stellung, Quercetin (3,5,7,3′,4′-Pentahydroxyflavon), seine beiden Glykoside Quercetin-3-galaktosid (Hyperin) und Quercetin-3-rhamnoglucosid (Rutin) sowie auf ein Flavon-3-ol mit einer in 5-Stellung fehlenden Hydroxyl-Gruppe das Fisetin (3,7,3′,4′-Tetrahydroxyflavon).

Der Vergleich der Eignung der genannten Flavon-3-ole ergibt die absteigende Reihenfolge: Fisetin, Quercetin, Hyperin, Rutin.

Die in 3-Stellung befindliche Hydroxyl-Gruppe hat für den Nachweis anscheinend eine wesentliche Bedeutung, wobei angenommen wird, dass die –C(=O)–C(OH) =C–O– Gruppierung des γ-Pyronringes an dem Ausfall der Reaktion beteiligt ist. Die

Verwendung des nicht substituierten 3-Hydroxy-phenyl-benzo-γ-pyrons wird im Zusammenhang mit einer folgenden Arbeit beschrieben.

In Anbetracht der grossen Anzahl von Alkaloiden wurde zur Feststellung der generellen Brauchbarkeit der Methode eine Auswahl getroffen. Die aufgetragene Menge betrug 20 μg pro Alkaloid. Die Anzeige, ob Stickstoff in einer Substanz in primärer, sekundärer, tertiärer oder in quartärer Bindung vorliegt, ist durch das Auftreten einer charakteristischen Farbreaktion gekennzeichnet. Der Nachweis ist zunächst schnell im Tüpfel-Test zu führen. Damit zeigt sich wieder die Bedeutung des Tüpfel-Testes als allgemeine Voraussetzung für qualitative Nachweise und besonders für papierchromatographische. Die schnelle Durchführung des Tüpfel-Testes, der vor der Papierchromatographie vorgenommen wird, sagt zweierlei aus:

(1) ob die Substanz durch ein Reagenz angezeigt wird und,

(2) kann bereits die für das Papierchromatogramm geeignete Menge vorher festgestellt werden.

Die Farbtöne sind orange, orangerot und rot. Wegen der genauen Charakterisierung des Farbtones, wie diese objektiv anzugeben ist, wird auf die Arbeit von NEU[14] hingewiesen. Das Reagenz entspricht vergleichsweise dem nach DRAGENDORFF.

Von allgemeinem Interesse ist eine Entscheidung in welcher Bindungsform das Stickstoff-Atom vorliegt. Eine Differenzierung, ob ein tertiäres oder ein quartäres Stickstoff-Atom vorliegt, ist unter Verwendung des hier beschriebenen Nachweises möglich. Das Verfahren zur Unterscheidung der Stickstoff-Bindung wird in dieser Zeitschrift beschrieben[15].

VERSUCHSTEIL

Papier: Schleicher u. Schüll 2043 b.

Fliessmittel: *n*-Butanol–Salzsäure–Wasser = 100 ml + 26 ml (25 %ig) + 39 ml.

Reagentien: (a) 1 %ige Lösung von Tetraphenylbornatrium in mit Wasser gesättigtem Butanon.

(b) Das gebrauchsfertige Reagenz wird aus 1 ml 0.1 %iger Fisetin Lösung in Methanol nach Zugabe von 6 ml Methanol bereitet.

TABELLE I

Alkaloid	R_F-*Wert*	*Alkaloid*	R_F-*Wert*
Yohimbin	0.73	Harman	0.81; 0.75
Papaverin	0.72	Histamin	—
Arecolin	0.59	Noscapin	0.74
Novocain	0.26	Eserin	0.70
Atropin*	0.74	Codein	0.58
Chinin	0.59; 0.67	Coniin	0.82
Preocarpin	0.43	Colchicin	0.96
Scopolamin	0.53	Brucin	0.57
Strychnin	0.61	Spartein	0.38
Tetrahydroharman	0.81; 0.77	Cholin	0.20**
Harmalin	0.79; 0.73	Acetylcholin	0.36**

* In gleicher Weise ist das Alkaloid Atropin in einer Tinktur aus *Atropa belladonna* nachweisbar.
** Flecken werden erst nach einigen Stunden sichtbar.

Bei allen Substanzen handelt es sich um Präparate des Handels.

ZUSAMMENFASSUNG

Das zum Nachweis von Alkaloiden vorgeschlagene Tetraphenylbornatrium ist wegen der Bildung weisser Niederschläge nicht im Tüpfel-Test oder im Papierchromatogramm geeignet.

In vorliegender Arbeit wird gezeigt, dass aus Alkaloiden und anderen organischen Basen mit Tetraphenylbornatrium entstehende Niederschläge durch charakteristische kräftige Farbtöne im Tageslicht sichtbar gemacht werden können.

Die Bildung der Farbtöne wird durch zusätzliche Verwendung von 3-Hydroxyphenyl-benzo-γ-pyronen bewirkt. Besonders geeignet sind, unabhängig von weiterer Substitution durch Hydroxyl-Gruppen, solche Phenyl-benzo-γ-pyrone, die in 5-Stellung keine Hydroxyl-Gruppe als Substituenten enthalten.

Das Verhalten der Reagentien wird an einer Anzahl von Modell-Substanzen untersucht und die Brauchbarkeit der Methode festgestellt.

Die auftretenden Farbtöne liegen im orangen, orangeroten und roten Bereich. Die Farben sind im Tageslicht und im U.V.-Licht zu erkennen.

Das Verfahren ist sowohl in der Papierchromatographie als auch im Tüpfel-Test anwendbar.

Für das Tetraphenylbornatrium wird mit vorliegender Arbeit ein weiterer neuer Verwendungszweck beschrieben: Die farbige Kennzeichnung der bisher nur weissen Tetraphenylborate von Alkaloiden.

SUMMARY

Tetraphenylborosodium, which has been recommended as a reagent for the detection of alkaloids, is not suitable for spot tests or for use in paper chromatography, because white precipitates are formed.

In this paper it is shown that the precipitates formed when alkaloids and other organic bases react with tetraphenylborosodium can be made visible in daylight, characteristic strong colours being obtained.

These colours are obtained by the subsequent addition of 3-hydroxy-phenyl-benzo-γ-pyrones. Especially suitable are such phenyl-benzo-γ-pyrones that have no hydroxyl group at position 5, irrespective of further substitution by hydroxyl groups.

The behaviour of the reagents was investigated with a number of model substances and the applicability of the method determined.

The colours obtained fall within the range orange, orange-red and red, and can be observed in daylight and U.V. light.

The method can be used in paper chromatography as well as for spot tests.

In this paper a new application of tetraphenylborosodium is described: the characterization by means of colours of tetraphenylborates of alkaloids, which hitherto have only been obtained as white precipitates.

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DÜNNSCHICHTCHROMATOGRAPHISCHE UNTERSUCHUNGEN AN ANTHOCHLOR-PIGMENTEN

VERGLEICH DES NATÜRLICHEN BRACTEIN-AGLYKONS MIT SYNTHETISCHEM 4,6,3′,4′,5′-PENTAOXY-AURON

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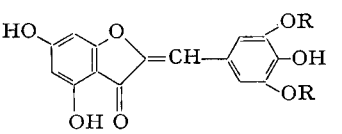
EINLEITUNG

Wir berichteten früher über die Isolierung eines glykosidischen Aurons aus *Helichrysum bracteatum*[1,2], dessen analytische Eigenschaften[2] für das Vorliegen eines bisher unbekannten Naturstoffes der Anthochlorreihe, des 4,6,3′,4′,5′-Pentaoxy-auron-4-glukosids sprechen. Zur endgültigen Konstitutionsaufklärung fehlt die Synthese. Aber weder das Glykosid (= Bractein) selbst noch sein Aglykon sind ohne weiteres zugänglich im Gegensatz zum 4,6,4′-Trioxy-3′,5′-dimethoxy-auron, das sich vom natürlichen Bractein-Aglykon nur durch Methylierung zweier Hydroxygruppen im Seitenphenyl unterscheidet. Eine Entmethylierung des Dimethylderivates in präparativem Masstabe ist wegen der Instabilität der Verbindung wenig aussichtsreich. Wir hielten es aber für denkbar, dass sich die gesuchte synthetische Vergleichssubstanz unter den Bedingungen der Entmethylierung in geringen Mengen bildet, wenn auch vielleicht nur als Zwischenprodukt. Somit musste es darauf ankommen, Aurone in komplexen Reaktionsgemischen nachzuweisen und zu identifizieren. Im folgenden wird zunächst allgemein über die Dünnschichtchromatographie der Aurone und der verwandten Chalkone berichtet. Die neue Methode dient sodann zum Vergleich des natürlichen Bractein-Aglykons mit synthetischem 4,6,3′,4′,5′-Pentaoxy-auron (I).



(I)

R = H: Bractein-Aglykon; R = CH_3: 4,6,4′-Trioxy-3′,5′-dimethoxy-auron

DÜNNSCHICHTCHROMATOGRAPHIE VON AURONEN UND CHALKONEN[9]

Als phenolische Substanzen lassen sich Aurone und Chalkone grundsätzlich mit der papierchromatographischen Technik trennen[3–5]. Werden die im Versuchsteil näher beschriebenen Bedingungen (Versuchsanordnungen von STAHL[6] für phenolische Naturstoffe wurden modifiziert) eingehalten, so erweist sich aber die Dünnschichtchromato-

graphie der Papierchromatographie bezüglich der Trennschärfe als überlegen — von den bekannten sonstigen Vorteilen ganz abgesehen. Zur näheren Untersuchung des Verhaltens der Anthochlore auf dem Dünnschichtchromatogramm verglichen wir zunächst eine Reihe synthetisierter Aurone und Chalkone. Geeignete Laufmittel für hydroxylierte und methoxylierte Aurone sind Gemische von aliphatischen Estern mit einem lipophilen Lösungsmittel unter Zusatz von Ameisensäure. Bei den entsprechenden Chalkonen eignete sich besser ein Laufmittel ohne Säurezusatz, das dagegen mit einem Formamid–Wassergemisch gesättigt ist.

Die beobachteten Gesetzmässigkeiten waren bei beiden Stoffgruppen die gleichen: Mit zunehmenden hydrophilen Eigenschaften einer Substanz sinkt deren R_F-Wert. Die stark hydrophilen Glykoside der Anthochlore bleiben in den verwendeten Laufmitteln (siehe Methodischer Teil) am Startpunkt zurück.

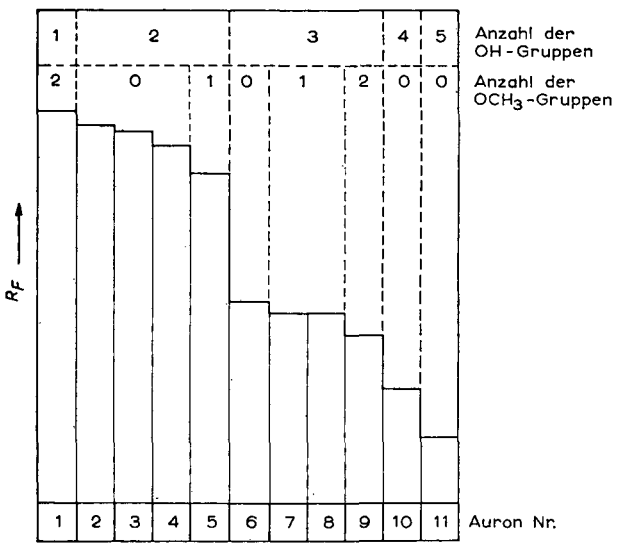


Fig. 1. Graphische Darstellung der R_F-Werte der Aurone (Nr. 1–11) in Abhängigkeit von der Anzahl und der Stellung der Hydroxyl- und Methoxylgruppen. Laufmittel: Benzol–Essigester–Ameisensäure, 4.5:3.5:2.

Wie aus Fig. 1 und Tabelle I ersichtlich, haben Anzahl und Stellung der Substituenten im Auron-Molekül einen deutlichen Einfluss auf den R_F-Wert und zwar:

1. Eine zusätzliche Hydroxylgruppe im Molekül erniedrigt den R_F-Wert beträchtlich,

2. Eine zusätzliche Methoxylgruppe übt dagegen nur einen geringen Einfluss auf den R_F-Wert aus. Es gilt:

$$R_{F(\text{Auron–OH})} \ll R_{F(\text{Auron–OCH}_3)} < R_{F(\text{Auron–H})}$$

Diese Gesetzmässigkeiten gestatten es, aus der Höhe des R_F-Wertes Rückschlüsse auf die Konstitution eines unbekannten Auron-derivates zu ziehen. Da die absoluten Zahlenwerte hier jedoch noch grösseren Schwankungen als bei der Papierchromatographie unterliegen, sollte stets der relative R_F-Wert (Vergleich mit einer bekannten Substanz) bestimmt werden.

Aurone und Chalkone unterscheiden sich durch ihr Farbverhalten im U.V.-Licht: Sämtliche von uns untersuchten Aurone fluoreszieren im U.V.-Licht auf dem Dünnschichtchromatogram gelb bis leuchtend grün, die Chalkone hingegen geben sich

TABELLE I

ÜBERSICHT ÜBER DIE GEPRÜFTEN AURONE

Auron Nr.	*Anzahl und Stellung der Substituenten*	
	-OH	*-OCH_3*
1	6-	3′,4′-
2	6,2′-	—
3	6,3′-	—
4	6,4′-	—
5	6,4′-	3′-
6	6,3′,4′-	—
7	4,6,3′-	4′-
8	4,6,4′-	3′-
9	4,6,4′-	3′,5′-
10	4,6,3′,4′-	—
11	4,6,3′,4′,5′-	—

durch dunkelbraune Farbe zu erkennen. Unter dem Einfluss von Ammoniakdämpfen färben sich die Chalkone tiefrot, die Aurone orange. Das Verhalten der geprüften Chalkone auf dem Dünnschichtchromatogramm zeigt Tabelle II.

Es soll noch darauf hingewiesen werden, dass farbliche Unterschiede nach Behandeln mit Sprühreagenzien auf dem Papierchromatogramm deutlicher zum Ausdruck kommen als auf dem Dünnschichtchromatogramm. Für diesen Nachweis empfiehlt es sich daher, ein Stoffgemisch zunächst dünnschichtchromatographisch mit Hilfe eines Streifenchromatogramms zu trennen, die gewünschte Zone mit Methanol zu eluieren und dann erst das Papierchromatogramm dieses Eluates mit den in Frage kommenden Reagenzien zu besprühen.

TABELLE II

ÜBERSICHT ÜBER DIE GEPRÜFTEN CHALKONE

Anzahl und Stellung der Substituenten		R_F
-OH	*-OCH_3*	
2′-	—	0.77
2′-	4′,6′-	0.46
2′-	4,4′,6′-	0.27
2,2′-	—	0.24
2′-	3,4,-	0.22
2′,4-	—	0.18
2′,4′-	—	0.18
2′,3-	4-	0.13
2′,4′-	4-	0.13
2′,3,4′-	—	0.03

VERGLEICH DES NATÜRLICHEN BRACTEIN-AGLYKONS MIT SYNTHETISCHEM 4,6,3′,4′,5′-PENTAOXY-AURON

Entmethyliert man 4,6,4′-Trioxy-3′,5′-dimethoxy-auron mit Jodwasserstoffsäure nach ZEISEL und chromatographiert das Reaktionsgemisch (siehe Methodischer Teil) nach der o.a. Methode, so findet man neben einer Reihe von nicht näher definierten Zersetzungs- und Reaktionsprodukten eine Substanz, die sich wie ein Auron verhält. Sie hat einen auffallend niedrigen R_F-Wert; das spricht nach den vorher erörterten Gesetzmässigkeiten für die grosse Zahl von OH-Gruppen im Molekül eines Pentaoxyaurons (Fig. 1). Wie wir schon in einer früheren Mitteilung berichteten, verhielt sich die Substanz sowohl auf dem Dünnschichtchromatogramm als auch papierchromatographisch in mehreren Laufmitteln völlig identisch mit dem Aglykon des von uns isolierten Bracteins, sowohl in Bezug auf die Höhe des R_F-Wertes als auch in Bezug auf die Farbe im Tageslicht, U.V.-Licht und nach Bedampfen mit Ammoniak (Tabelle III).

TABELLE III

CHROMATOGRAPHISCHER VERGLEICH DES HYDROLYSEPRODUKTES DES BRACTEINS MIT EINEM REAKTIONSPRODUKT NACH BEHANDELN VON 4,6,4′-TRIOXY-3′,5′-DIMETHOXY-AURON MIT HJ NACH ZEISEL

Laufmittel für Dünnschichtchromatographie:
1 = Benzol–Essigester–Ameisensäure (4.5:3.5:2.0).
2 = Chloroform–Essigester–Ameisensäure (5:4:1).
3 = Toluol–Ameisensäureäthylester–Ameisensäure (5:4:1).
Laufmittel für Papierchromatographie:
a = 60%ige Essigsäure.
b = 50%iges Isopropanol.
c = *n*-Butanol–Eisessig–Wasser (4:1:5).
d = *m*-Kresol–Eisessig–Wasser (48:2:50).
T.L. = Tageslicht; U.V. = U.V.-Licht; NH_3 = Behandeln des Chromatogramms mit Ammoniak.

	R_F-Wert	
Laufmittel Nr.	*Hydrolyseprodukt des Bracteins (Bractein-Aglykon)*	*Reaktionsprodukt nach Behandeln von 4,6,4′-Trioxy-3′,5′-dimethoxy-auron mit HJ*
1	0.25	0.25
2	identische Fleckenhöhen im "Durchlaufverfahren"	
3	identische Fleckenhöhen im "Durchlaufverfahren"	
a	0.15	0.15
b	0.37	0.37
c	0.25	0.25
d	0.03	0.03
	Farbverhalten	
T.L.	gelb	gelb
U.V.	zitronengelb	zitronengelb
T.L.-NH_3	orange-rot	orange-rot
U.V.-NH_3	orange-rot	orange-rot

METHODISCHER TEIL

Herstellung der Vergleichssubstanzen

Die Synthese der Aurone erfolgte in Anlehnung an die Synthese des Aureusidins nach FARKAS *et al.*[7] aus dem entsprechenden Cumaranon und den jeweiligen Aldehyden über das Acetat. Die Chalkone wurden nach der Methode von NADKARNI UND WHEELER[8] durch Kondensation des entsprechenden Acetophenons mit dem geeigneten Aldehyd bei 0° in stark alkalischem Milieu synthetisiert.

Die nachstehend aufgeführten Aurone (1)–(7) sind in der Literatur noch nicht beschrieben und lassen sich folgendermassen charakterisieren:

(1) *6,2'-Dioxy-auron.* Gef.*: C, 70.98, 70.74; H, 3.95, 4.28 %. Ber. für $C_{15}H_{10}O_4$ (254.23): C, 70.86; H, 3.96 %.

Schmp. = ab 310° Zers. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 265, 375 (log ε = 3.93, 4.25). I.R.-Spektrum (in KBr) [cm^{-1}]: 1900 (w), 1820 (w), 1650 (s), 1630 (s), 1600 (s), 1560 (s), 1490 (s), 1450 (s), 1330 (s), 1300 (s), 1260 (s), 1230 (s), 1190 (s), 1160 (s), 1140 (s), 1105 (s), 1040 (m), 960 (w), 945 (m), 910 (m), 860 (m), 850 (m), 830 (s), 780 (m), 760 (s), 695 (m), 665 (m), 660 (m).

(2) *6,3'-Dioxy-auron.* Gef.: C, 70.57, 70.45; H, 4.11, 4.09 %. Ber. für $C_{10}H_{15}O_4$ (254.23): C, 70.86; H, 3.96 %.

Schmp. = 278°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 265, 340 (log ε = 4.03, 4.35). I.R.-Spektrum (in KBr) [cm^{-1}]: 1800 (w), 1660 (s), 1630 (s), 1610 (s), 1600 (s), 1570 (s), 1520 (m), 1480 (s), 1455 (s), 1450 (s), 1360 (s), 1340 (s), 1320 (s), 1290 (s), 1240 (s), 1215 (s), 1195 (s), 1165 (s), 1150 (s), 1135 (s), 1105 (s), 985 (s), 960 (m), 910 (m), 860 (m), 840 (m), 810 (m), 770 (s), 720 (m), 700 (m), 675 (m), 555 (m).

(3) *6-Oxy-3',4'-dimethoxy-auron.* Gef.: C, 68.28, 68.24; H, 4.74, 4.89 %. Ber. für $C_{17}H_{14}O_5$ (298.28): C, 68.45; H, 4.73 %.

Schmp. = 220°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 256, 386 (log ε = 3.82, 4.24). I.R.-Spektrum (in KBr) [cm^{-1}]: 1880 (w), 1670 (s), 1595 (s), 1510 (s), 1480 (s), 1455 (s), 1435 (m), 1410 (m), 1335 (m), 1310 (s), 1285 (s), 1250 (s), 1330 (s), 1190 (m), 1160 (m), 1140 (s), 1120 (s), 1090 (s), 1035 (m), 1015 (m), 970 (m), 950 (m), 920 (w), 870 (m), 840 (m), 820 (w), 800 (m), 760 (m), 730 (m), 695 (m), 660 (s).

(4) *6,4'-Dioxy-3'-methoxy-auron.* Gef.: C, 67.55, 67.55; H, 4.29, 4.46 %. Ber. für $C_{16}H_{12}O_5$ (284.26): C, 67.60; H, 4.26 %.

Schmp. = 269–270°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 255, 393 (log ε = 4.03, 4.50). I.R.-Spektrum (in KBr) [cm^{-1}]: 1680 (s), 1640 (s), 1590 (s), 1510 (s), 1490 (s), 1460 (s), 1425 (s), 1330 (s), 1290 (s), 1260 (s), 1230 (s), 1200 (s), 1170 (s), 1130 (s), 1100 (s), 1035 (s), 970 (m), 960 (s), 880 (m), 840 (s), 810 (m), 790 (m), 780 (m), 760 (m), 730 (m), 695 (m), 655 (m).

(5) *4,6,4'-Trioxy-3'-methoxy-auron.* Gef.: C, 64.10, 64.22; H, 4.09, 4.15 %. Ber. für $C_{16}H_{12}O_6$ (300.26): C, 64.00; H, 4.03 %.

Schmp. = 271–273°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 401 (log ε = 4.47). I.R.-Spektrum (in KBr) [cm^{-1}]: 1840 (w), 1750 (w), 1690 (s), 1640 (s), 1605 (s), 1590 (s), 1520 (s), 1475 (s), 1460 (s), 1420 (s), 1415 (s), 1350 (s), 1310 (s), 1270 (s), 1230 (s), 1190 (s), 1150 (s), 1120 (s), 1060 (s), 1030 (s), 990 (m), 970 (m), 905 (m), 875 (m), 835 (m), 810 (m), 800 (m), 775 (w), 740 (m), 700 (m), 670 (m).

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* Wir danken der Fa. Schering für die Ausführung sämtlicher Verbrennungsanalysen.

(6) *4,6,3'-Trioxy-4'-methoxy-auron.* Gef.: C, 64.08, 64.28; H, 4.20, 4.46 %. Ber. für $C_{16}H_{12}O_6$ (300.26): C, 64.00; H, 4.03 %.

Schmp. = 284°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 265, 395 (log ε = 3.92, 4.49). I.R.-Spektrum (in KBr) [cm^{-1}]: 1670 (s), 1640 (s), 1590 (s), 1520 (s), 1525 (s), 1460 (s), 1435 (s), 1380 (m), 1350 (s), 1310 (s), 1265 (s), 1240 (s), 1205 (s), 1170 (s), 1150 (s), 1120 (s), 1060 (s), 1025 (s), 995 (m), 980 (m), 920 (w), 890 (w), 880 (m), 860 (w), 850 (w), 835 (m), 820 (m), 800 (m), 795 (m), 760 (m), 715 (w), 700 (m), 620 (m).

(7) *4,6,4'-Trioxy-3',5'-dimethoxy-auron.* Gef.: C, 62.00, 62.21; H, 4.58, 4.76 %. Ber. für $C_{17}H_{14}O_7$ (330.28): C, 61.82; H, 4.27 %.

Schmp. = 260°. U.V.-Absorption in Methanol: $\lambda_{Max.}$ [mμ] 260, 399 (log ε = 3.96, 4.43). I.R.-Spektrum (in KBr) [cm^{-1}]: 1670 (s), 1640 (s), 1605 (s), 1580 (s), 1510 (s), 1460 (s), 1445 (s), 1420 (s), 1390 (m), 1355 (s), 1330 (s), 1285 (s), 1245 (s), 1215 (s), 1185 (s), 1150 (s), 1110 (s), 1070 (s), 1000 (m), 920 (w), 910 (m), 880 (m), 830 (m), 820 (m), 770 (m), 720 (w), 710 (m), 690 (m).

Entmethylierungsverfahren

Entmethyliert wurde mit Jodwasserstoffsäure nach ZEISEL. 200 mg Substanz, 2 ml Acetanhydrid, 200 mg roter Phosphor und 5 ml frisch destillierte Jodwasserstoffsäure wurden 1/2 Stunde lang unter Stickstoffatmosphäre auf 140° erwärmt, das Reaktionsgemisch in etwa 10 ml destilliertes Wasser gegossen, mit Natriumbicarbonat bis etwa pH = 4 neutralisiert, vom Phosphor abfiltriert, die Lösung mit Äther ausgeschüttelt, die gewaschene und getrocknete Ätherphase eingeengt und zur chromatographischen Untersuchung benutzt. Eine präparative Isolierung, etwa durch Sammeln des Produktes aus verschiedenen Ansätzen, gelang uns nicht, da die Stabilität der Verbindung so gering ist, dass die Mengen für eine präparative Darstellung nicht ausreichen. Auch mehrfach modifizierte Arbeitsmethoden führten zu keinem besseren Ergebnis; so variierten wir sowohl die Säurekonzentration wie die Reaktionszeit, verwendeten bei der Aufarbeitung als Oxydationsschutz Natriumdithionit oder Natriumthiosulfat, variierten den pH-Wert vor dem Ausschütteln mit Äther oder auch Essigester. Ein Arbeiten im alkalischen Bereich verbot sich mit Rücksicht auf die drei vicinalen OH-Gruppen der Verbindung.

Dünnschichtchromatographie

Als Platten verwendeten wir für die Chromatographie der Aurone gepufferte Kieselgel G-Platten, die nach der Arbeitsvorschrift von STAHL[6] hergestellt wurden. (30 g Kieselgel G in 60 ml Wasser, gepuffert mit 1.5 g Natriumacetat, 1 Stunde getrocknet bei 105°). Als Laufmittel verwendeten wir folgende Gemische:

(1) Benzol–Essigester–Ameisensäure (4.5:3.5:2.0),

(2) Chloroform–Essigester–Ameisensäure (6:3:1) neben (5:4:1),

(3) Toluol–Ameisensäureäthylester–Ameisensäure (5:4:1).

In manchen Fällen bewährte sich, besonders bei Verwendung von Laufmittel (3), das sog. "Durchlaufverfahren": man lässt das Chromatogramm, wenn das Laufmittel das Ende der Glasplatte erreicht hat, noch weiter laufen, so dass die Flecken höher steigen. Man errechnet dann nicht den R_F-Wert, sondern setzt die Höhe der Flecken nur in Relation zu der Höhe des Fleckes einer in ihrer Konstitution bekannten Substanz.

Für die Chromatographie der Chalkone verwendeten wir folgende Platten: 15 g

Kieselgel G und 15 g Kieselgur G in 60 ml Wasser, 1 Stunde bei 105° getrocknet.

Als Laufmittel verwendeten wir:

(4) Cyclohexan–Essigester (7:1, v/v), gesättigt mit 5 ml Wasser und 10 ml Formamid.

Papierchromatographie

Papier: Schleicher & Schüll 2043 bM.

Laufmittel:

(a) Essigsäure (60 %ig),

(b) Isopropanol (50 %ig),

(c) *n*-Butanol–Eisessig–Wasser (4:5:1),

(d) *m*-Kresol–Eisessig–Wasser (48:2:50).

ZUSAMMENFASSUNG

Zur Trennung von Anthochlor-Pigmenten eignet sich die Dünnschichtchromatographie auf gepufferten Platten. Auf Zusammenhänge zwischen Zahl der Hydroxy- und Methoxygruppen im Molekül und dem Verhalten auf dem Dünnschichtchromatogramm wird an Hand von synthetisierten Anthochloren, die teilweise noch nicht beschrieben sind, hingewiesen. Die Methode wird herangezogen zur Identitätsprüfung von natürlichem Bractein-Aglykon, das mit dem durch Entmethylierung von 4,6,4′-Trioxy-3′,5′-dimethoxy-auron erhaltenen Auron verglichen wird.

SUMMARY

Thin-layer chromatography on buffered plates is suitable for the separation of anthochlor pigments. It is shown that a relationship exists between the number of hydroxy and methoxy groups in the molecule and its mobility. Some of the aurones examined were synthesized for the first time. The method is employed to prove the identity of the natural bractein-aglycone, isolated from flowers of *Helichrysum bracteatum*, by comparing it with the product obtained by demethylation of 4,6,4′-trihydroxy-3′,5′-dimethoxy-aurone.

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THE EFFECT OF CALCIUM SULFATE AS THE BINDER IN DEAE-CELLULOSE THIN-LAYER CHROMATOGRAPHY FOR SEPARATING NUCLEIC ACID DEGRADATION PRODUCTS*

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RANDERATH has investigated the separation of purine bases, nucleosides, and ribo nucleotides by thin-layer chromatography[1-3]. His systems for nucleotides appear most appropriate for the separation of a mixture of mono-, di-, and tri-phosphates of a given nucleotide. The separation of the mono-, di-, and tri-phosphates of adenosine also has been accomplished using an ammonium formate solvent system with diethylaminoethyl (DEAE)-Sephadex as the stationary phase by WIELAND AND DETERMANN[4].

In our laboratory it was desirable to find a system which would separate the 2'- and 3'-phosphates of the four principal nucleotides resulting from a basic hydrolysis of ribonucleic acid (RNA), and the 5'-phosphates of the four principal deoxynucleotides resulting from an enzymic hydrolysis of deoxyribonucleic acid (DNA), involving 100 to 1000 μg of sample. Previous work has been concerned primarily with separation of the 5'-phosphates of ribonucleotides[1,3], with the exception of the ammonium sulfate–sodium acetate–isopropanol system to separate 2'- from 3'-phosphates[3]. This system, however, does not separate the phosphates of cytidine from those of uridine, and the high salt content of the solvent requires that the eluant be desalted if further chromatographic separation or enzymic hydrolysis is desired.

MATERIALS AND METHODS

Materials

MN-Cellulose Powder 300 DEAE and 300 G/DEAE were obtained from Macherey, Nagel & Co., Düren, Germany. The latter preparation contains 10% $CaSO_4 \cdot 1/2\ H_2O$ as a binder. All purine and pyrimidine compounds were obtained from Sigma Chemical Corp. except for the following: adenosine-2'- and 3'-phosphates, guanosine-2'- and 3'-phosphates, adenosine, and cytosine from Mann Chemical Company; guanosine from Schwarz Biochemicals; and cytidine and 2-deoxy-adenosine-5'-phosphate from California Corp. for Biochemical Research.

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Preparation of plates

9.5 g of DEAE-Cellulose powder was mixed with 62 ml H_2O and spread onto three 8 in. × 12 in. plates at a thickness of about 375 μ, using a spreader apparatus obtained from Desaga, Brinkmann Corp. The glass plates were dried at 50° for 2 hours. Standards were spotted at 2 cm from the lower edge of the plate and air-dried prior to chromatography. Chromatograms were developed in closed chromatography jars of a diameter of about 21 cm or in open glass trays when a second dimension was desired.

The preparation containing 5% $CaSO_4$ was made by mixing equal volumes of the powder containing 10% $CaSO_4$ and that containing no $CaSO_4$. Thorough washing of the 300 G/DEAE cellulose resulted in removal of $CaSO_4$, leaving a preparation which gave chromatographic results identical to those of the originally $CaSO_4$-free powder (MN-Cellulose 300 DEAE).

Solvent systems

Solvents were: (1) isobutyric acid–ammonia (s.g. 0.90)–water (66:2:32, v/v); (2) distilled water, pH 5.7; (3) hydrochloric acid, pH 2.3; and (4) saturated ammonium sulfate–1 *M* sodium acetate, pH 7.5–isopropanol (80:18:2, v/v)[3]. Times required for the movement of the solvent a distance of 6 in. were, respectively: 6 h, 35 min, 35 min and 2 h.

The isobutyric acid should be redistilled to remove impurities that migrate with the solvent front and interfere with detection and assay of compounds of R_F greater than 0.95.

Preparation of samples

A solution of nucleotides was dried *in vacuo* in a test tube, dissolved in 0.10 ml of 0.10 *N* NH_4OH, and spotted onto the cellulose prior to chromatography. Nucleosides were used from a neutral solution, and purines and pyrimidines from a solution of 0.1 *N* hydrochloric acid.

In order to separate ribonucleotides from a basic RNA hydrolysate, it was first necessary to desalt the mixture. This was done by absorbing the nucleotides onto a small column of charcoal (20 mg Darco G previously washed with 0.1 *N* HCl and distilled water and then made into a 0.3 × 0.6 cm column) to remove salts, and then they were eluted quantitatively with 5 ml of a solution of 95% ethanol–ammonia (s.g. 0.90)–water (5:2:3)[5].

Elution from chromatogram

The spots were located by ultraviolet light and outlined by use of a thin glass rod. The cellulose surrounding the spot was removed with a razor blade, and then the spot itself was removed by scraping with a razor blade held at a 30° angle to the glass. This results in a small cylinder of cellulose which is then placed in a 3 ml conical glass centrifuge tube. To the tube is added 2.5 ml of 0.01 *N* HCl. This is thoroughly mixed and centrifuged at full speed in a clinical centrifuge for a few minutes, and the supernatant decanted through a micro funnel, using Schleicher & Schuell No. 589 blue ribbon filter paper, which had been previously washed with H_2O. This was adequate to trap the cellulose "fines". An alternative successful method was to use a fine

grade sintered glass filter, omitting the micro funnel and filter paper. The elution was repeated twice and gave 95–100 % recovery of the nucleotides.

RESULTS AND DISCUSSION

In Table I are listed the R_F data for the ammonium isobutyrate and the hydrochloric acid solvents. Since the data for distilled water were so similar to those for HCl, and HCl effected slightly better separations in nearly all cases, this data was omitted. When the percent $CaSO_4$ in the cellulose layer is varied, large differences in the R_F of almost all compounds are found with the HCl solvent. Lesser differences are seen with the ammonium isobutyrate solvent.

The best separation of ribonucleotides is obtained with the ammonium isobutyrate solvent. Addition of $CaSO_4$ to the DEAE cellulose tends to separate the 2′- and 3′-phosphates of guanosine, eventually rendering an overlap with the uridylic acid

TABLE I

SEPARATION OF PURINES, PYRIMIDINES, NUCLEOSIDES, AND NUCLEOTIDES

	Compound	R_F in solvent system					
		Isobutyric acid–NH_3 (s.g. 0.90)–H_2O (66:2:32)			0.005 N HCl		
		% $CaSO_4$			% $CaSO_4$		
		0	5	10	0	5	10
Nucleotides	Adenosine-2′-P	0.63	0.62	0.66	0.00	0.04	0.32
	Adenosine-3′-P	0.63	0.62	0.66	0.00	0.04	0.24
	Guanosine-2′-P	0.30	0.29	0.33	0.00	0.02	0.18
	Guanosine-3′-P	0.30	0.24	0.29	0.00	0.02	0.09
	Cytidine-2′-P	0.55	0.55	0.55	0.00	0.09	0.61
	Cytidine-3′-P	0.51	0.50	0.51	0.00	0.05	0.50
	Uridine-2′-P	0.36	0.35	0.37	0.00	0.02	0.48
	Uridine-3′-P	0.36	0.35	0.37	0.00	0.02	0.42
	2-Deoxy-adenosine-5′-P	0.64	0.62	0.69	0.00	0.04	0.28
	2-Deoxy-guanosine-5′-P	0.35	0.32	0.36	0.00	0.02	0.15
	2-Deoxy-cytidine-5′-P	0.60	0.57	0.59	0.00	0.08	0.48
	Thymidine-5′-P	0.47	0.46	0.47	0.00	0.05	0.48
Nucleosides	Adenosine	0.90	0.92	0.91	0.68	0.58	0.62
	Guanosine	0.63		0.59	0.13	0.31*	0.39*
	Cytidine	0.79	0.73	0.78	0.96	0.79	0.94
	Uridine	0.65		0.63	0.30	0.68	0.87
	2-Deoxy-adenosine	0.98	0.96	0.97	0.66	0.70	0.70
	2-Deoxy-guanosine	0.73	0.69	0.73	0.12	0.40	0.50
	2-Deoxy-cytidine	0.91	0.88	0.90	0.93	0.82	0.90
	Thymidine	0.83	0.79	0.81	0.49	0.81	0.90
Bases	Adenine	0.98	0.95	0.94	0.20	0.33	0.44
	Guanine	0.73		0.73	0.10	0.31	0.40
	Cytosine	0.92	0.90	0.94	0.26	0.80	0.90
	Uracil	0.74	0.79	0.73	0.26	0.66	0.75
	Thymine	0.83	0.80	0.83	0.41	0.74	0.85

* Streaking of the spot over an R_F value of 0.1.

spot. If separation of each 2′- from the 3′-phosphate isomer is desired, 0.005 *N* HCl solvent and 10 % $CaSO_4$-cellulose layer is best, giving at least 0.06 difference in the R_F's of the two isomers of each nucleotide. The uridine-2′-phosphate and cytidine-3′-phosphate spots definitely overlap. It is recommended that a mixture of monoribonucleotides be prepared in 0.10 *N* NH_4OH before spotting, since, below pH 10, guanylic acid tends to streak badly. This streaking is more predominant in the HCl solvent than in the isobutyrate solvent. In the ammonium sulfate–sodium acetate–isopropanol solvent, the length of the streak varies almost directly with pH, as seen from Table II.

TABLE II

EFFECT OF pH OF THE INITIAL NUCLEOTIDE SOLUTION ON THE SEPARATION

The R_F's of the upper and lower limits of the spot are given. Stationary phase: 10 % $CaSO_4$–DEAE-cellulose powder.

Compound	*pH of initial solution*	*R_F in solvent*		
		Saturated ammonium sulfate–1 M sodium acetate–isopropanol (80:18:2, v/v)	*0.005 N HCl*	*0.01 N HCl*
Guanosine-2′-phosphate + guanosine-3′-phosphate	2.8	0.08–0.60	0.01–0.36	0.04–0.21
	5.8	0.08–0.63		
	6.5	0.20–0.63		
	7.5	0.30–0.63		0.04–0.21
	9.9	0.35–0.65		
	10.2	0.43–0.65	0.08–0.16	0.11–0.21
	11.0	0.50–0.65		
Adenosine-2′-phosphate + adenosine-3′-phosphate	2–11	0.26–0.32		
Cytidine-2′-phosphate + cytidine-3′-phosphate	2–11	0.73–0.82		
Uridine-2′-phosphate + uridine-3′-phosphate	2–11	0.80–0.87		

The best separation of deoxyribomononucleotides is obtained using the 10 % $CaSO_4$ preparation with ammonium isobutyrate. Whereas 2-deoxy-cytidine-5′-phosphate and 2-deoxy-adenosine-5′-phosphate tend to overlap when no $CaSO_4$ is present; the addition of this binder results in a separation in which the R_F difference increases from 0.04 to 0.10. It is also advisable to prepare a mixture of deoxyribonucleotides in base, since the compounds are more soluble in base, and the purine-deoxyribotides are quite labile in acid.

Dilute HCl affects the best separation of ribonucleosides as well as deoxyribonucleosides (Table I). The $CaSO_4$-free preparation should be used, as the uridine and thymidine spots move up into the range of the cytidine and 2-deoxy-cytidine spots, respectively, when $CaSO_4$ is present. Guanosine tends to streak badly, in the presence of $CaSO_4$, unless the nucleosides are prepared in either a strongly acidic or a strongly basic solution. When spotted from acidic or basic solutions, the R_F of guanosine is increased from 0.39 to 0.52 in 10 % $CaSO_4$. Although ammonium isobutyrate gives a moderately good separation of deoxyribonucleotides, one should be cautioned against use of this solvent if elution of a compound near the solvent front is desired. Impurities

present in commercially available isobutyric acid move with the solvent front and absorb ultraviolet light. A slight tendency to streak was observed when deoxyribonucleotides are prepared at a low pH. Since they are apparently as soluble in neutral or basic pH as in acid, it is desirable to spot these compounds from a solution of pH 7 or higher.

The free bases resulting from RNA hydrolysis are best separated using the ammonium sulfate–sodium acetate–isopropanol system of RANDERATH[3]. Apparently, the solvent has such a high salt content that little difference in R_F's are observed, depending on the presence or absence of $CaSO_4$ in the layer (See Table III). This solvent does not adequately separate thymine from cytosine, but the HCl solvent will

TABLE III

SEPARATION OF FREE BASES WITH SATURATED AMMONIUM SULFATE–1 *N* SODIUM ACETATE–ISOPROPANOL (80:18:2, v/v)

Stationary phase: DEAE-cellulose powder plus $CaSO_4$ as indicated.

Compound	R_F	
	0% $CaSO_4$	*10% $CaSO_4$*
Adenine	0.14	0.13
Guanine	0.23	0.00
Cytosine	0.50	0.56
Uracil	0.62	0.68
Thymine	0.52	0.54

separate the four bases resulting from DNA hydrolysis if the $CaSO_4$-free DEAE-cellulose is used. Addition of $CaSO_4$ causes a one to four-fold increase in the R_F's of all free bases, but moves the guanine spot into the lower range of the adenine spot. Although the isobutyrate solvent effects a fair separation of the bases of DNA, the adenine spot is too near the solvent front to permit direct ultraviolet analysis of the eluant. This solvent cannot be used to separate the bases of RNA because guanine and uracil spots overlap. Free bases should be prepared in 0.1 *N* HCl, owing to their greater solubility at a low pH, and the lessened tendency to streak at low pH.

Two-dimensional chromatograms are easily developed, allowing 1–2 h for drying between solvents. The ammonium sulfate–sodium acetate–isopropanol solvent was unsatisfactory as a first solvent because the salt solvents create a salt gradient over the chromatogram which interferes with the second solvent, Ammonium formate solvents have proven unsatisfactory for either first or second dimensions. However, both HCl and distilled water function nicely as a first solvent, and water, but not HCl, will work as a second solvent when isobutyrate was used in the first dimension. Since the ammonium isobutyrate solvent changes composition in an open system, the R_F's were observed to change somewhat when it was used as the second solvent in an open system. In fact, it has been found that even in a well-sealed chromatography jar, this solvent changes composition slightly from day to day. The effect of aging this solvent on changes in R_F's can be duplicated by preparing a fresh solvent with a greater percentage of ammonia. The desirability of using a two-dimensional system will depend on the extent of separation desired and the number of compounds

in a mixture to be separated. The 8 in. × 8 in. glass plates obtained especially for thin-layer work would be advantageous for two-dimensional chromatograms.

If one wishes to assay the eluted compounds quantitatively by ultraviolet spectrometry, it is advisable to observe two precautions:

(1) The eluant should be filtered as described to avoid the fines which interfere with ultraviolet spectrometry. Glass wool and filter papers not designed for extremely fine particles have proven inadequate. Alternatively, one could wash the cellulose repeatedly in distilled water before use. This procedure, however, will remove the $CaSO_4$ present as "binder", and if $CaSO_4$ is desired, it must be added back. We have not found the presence of $CaSO_4$ necessary for its "binding" properties; both the cellulose preparations described will adhere well to the glass plates in all solvent systems investigated. (2) If isobutyrate were used, the chromatogram should be well-dried to remove this solvent, which absorbs ultraviolet light. If acid-labile nucleotides are present, 24 h at room temperature is best, since this is adequate for drying and destruction of nucleotides does not occur. Otherwise, 1 h at 50–60° is sufficient.

The limitations of the quantities of material which can be handled by the thin-layer methods appear to be few. As little as 10 μg of material can be detected in a spot of 1 cm diameter, and no smearing has been observed when 1 to 2 mg of material was chromatographed.

In Table IV is given a summary of the best systems for separating the compounds of each group of nucleic acid degradation products. Since thin-layer chromatography is

TABLE IV

BEST SOLVENT SYSTEMS AND DEAE-CELLULOSE PREPARATIONS FOR EACH GROUP

Group	*Solvent*	*% $CaSO_4$*
Ribonucleotides, according to bases	Isobutyrate	0
Ribonucleotides, 2′- from 3′-phosphate	HCl	10
Deoxyribonucleotide-5′-phosphates	Isobutyrate	10
Ribonucleosides	HCl	0
Deoxyribonucleosides	HCl	0
Free bases from RNA basic hydrolysis	Ammonium sulfate	no difference
Free bases from DNA enzymic hydrolysis	HCl	0

similar to paper chromatography in most respects, it should be emphasized that the cleanest separations are obtained only if the mixture is first desalted. For purine and pyrimidine compounds, this step is easily accomplished by use of the charcoal column. Serious streaking has been observed in all solvents attempted in the presence of salts.

DISCUSSION

The addition of $CaSO_4$ to the cellulose resulted in better movement of various nucleic acid degradation products. Although the effect of $CaSO_4$ in these studies is not completely understood, at least two explanations are reasonable: (1) a physical change in the tertiary structure of the DEAE-cellulose in the presence of calcium and sulfate ions; (2) the sulfate ions may bind electrostatically with nitrogen moieties of the

diethylaminoethyl groups. The nitrogen of this compound would be in the ammonium (R_3N^+) form at an acidic pH. Electrostatic binding of this ion by sulfate would tend to prohibit a similar bonding by the hydroxyl groups of nucleotide, and, to a lesser extent, by the hydroxyl and amino groups of the purine and pyrimidine bases. This would permit the developing solvent to carry the nucleotides (or possibly their calcium salts) along the cellulose layer in accordance with less powerful interactions, such as Van der Waals' forces and hydrogen bonding.

Several experimental observations tend to support the second suggestion: (1) With the ammonium sulfate solvent a $CaSO_4$ effect is not observed; (2) the $CaSO_4$ effect is less pronounced with nucleosides and free bases; (3) the effect is negligible for adenosine which does not have an hydroxyl group. The fact that all the bases travel to some extent when $CaSO_4$ is absent may be attributed to the predominance of keto and imino forms over the hydroxyl and amino forms, the latter being more capable of binding to the R_3N^+ ion.

SUMMARY

Thin-layer techniques involving layers of diethylaminoethyl-cellulose, with and without $CaSO_4$, are presented for the complete separation and quantitative elution of the major degradation products of ribonucleic acid and deoxyribonucleic acid. Emphasis is given to the effects of the pH of the material to be separated and to the percent $CaSO_4$ present in the cellulose as "binder".

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART VII. THE HYDROLYSIS OF PHOSPHORUS TRIHALIDES AND PSEUDOHALIDES

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It has been reported[1] that the phosphorus trihalides and pseudohalides may be hydrolysed in alkaline solution, giving rise to a variety of lower oxy-acids of phosphorus. A number of these acids have been reported to contain phosphorus–phosphorus bonds, *e.g.* hypophosphate, and such a hydrolytic reaction is one of the few methods of synthesizing such bonds, starting from monomeric materials. If the mode of formation can be elucidated, it may prove possible to synthesize compounds containing such bonds. Using the combined techniques of paper and anion-exchange chromatography, this paper describes a study of the hydrolysis, in sodium hydroxide solution, of phosphorus trichloride, tribromide and triiodide, and the pseudohalides, phosphorus triisocyanate, triisothiocyanate, and tricyanide.

EXPERIMENTAL

Preparation of the phosphorus trihalides and pseudohalides

1. Phosphorus trichloride, B.D.H. reagent grade was twice redistilled under a stream of dry nitrogen, and the fraction boiling between 75° and 76° collected.

2. Phosphorus tribromide, B.D.H. reagent grade was twice redistilled under reduced pressure, and the fraction boiling at 20–21° at 0.1 mm pressure was collected.

3. Phosphorus triiode was prepared by the direct union of the elements in purified carbon disulphide[2], and was twice recrystallised from this solvent before use.

PI_3: P calc., 7.53 %; P found, 7.51 %.

4. Phosphorus triisocyanate $P(NCO)_3$, was prepared by distillation of a sample of polymerized compound. When freshly prepared, phosphorus triisocyanate is a liquid (d = 1.439), which upon standing polymerizes to a yellow-white solid which is insoluble in common organic solvents[3].

The phosphorus triisocyanate which was in the polymerized form was twice distilled under dry nitrogen, and the fraction boiling at 169° collected. The resulting liquid was hydrolysed immediately.

5. Phosphorus triisothiocyanate $P(NCS)_3$ was synthesized by the addition of freshly distilled phosphorus trichloride to a solution of ammonium thiocyanate in

liquid sulphur dioxide. Ammonium chloride precipitates leaving phosphorus triisothiocyanate in solution[4]. After filtering off the ammonium chloride, the sulphur dioxide was allowed to partially evaporate, whereupon the phosphorus triisothiocyanate separated out as a colourless oil. The oil was separated from the remaining sulphur dioxide, dissolved in a fresh sample of solvent, and the separation process repeated. All attempts to distil the oil resulted in the formation of the deep brown coloured polymer reported by DIXON[5], this polymer also formed on standing. The substance was freshly prepared and treated three times with liquid sulphur dioxide before use.

$P(NCS)_3$: P calc., 15.10 %; P found, 14.98 %.

6. Phosphorus tricyanide $P(CN)_3$ was prepared by the reaction between phosphorus trichloride and silver cyanide in benzene[6]. The phosphorus tricyanide was resublimed (98°, 0.1 mm) before use.

$P(CN)_3$: P calc., 28.42 %; P found, 28.49 %.

During all the work described above, precautions were taken to exclude moisture by handling the materials in a dry-nitrogen filled, dry box.

Hydrolysis of the phosphorus trihalides and pseudohalides

All hydrolysis experiments were carried out under identical conditions using a stock solution of 5.063*N* sodium hydroxide solution and keeping the ratio: moles of $P(X)_3$/ moles of NaOH constant at ratio of 1:7.

The calculated volume of sodium hydroxide solution, equivalent (subject to the above criterion) to 5.0 ml of the halides or pseudohalide to be hydrolysed was contained in a three-necked reaction vessel under a stream of dry nitrogen. The reaction vessel was surrounded by ice-water and its contents stirred with a magnetic stirrer. The sample to be hydrolysed was introduced from a 5 ml syringe pipette fitted with a ground-glass joint, so effectively stoppering the reaction vessel. After passing through the reaction vessel, the nitrogen stream was passed through a series of four traps, each containing 20 ml of 5.0 % w/v mercuric chloride solution. This solution was to precipitate any phosphine that may be formed during the hydrolysis, as the yellow complex $P(HgCl)_3$ which was then estimated by the method reported by BEYER[7]. After completion of the hydrolysis reaction, the resultant solution was analysed by paper and anion-exchange chromatographic techniques.

Analysis

(1) *Anion-exchange chromatography.* 5 ml of the solution obtained after the hydrolysis were diluted to 50 ml in a graduated flask with distilled water, giving Solution A. 5 ml of the solution were then pipetted on to the anion-exchange column, and analysed using the procedure reported in a previous communication[8].

In some cases, the concentration of orthophosphite present in 5.0 ml of Solution A is too large to allow a satisfactory analysis; due to column overloading with respect to this anion. In these cases, it was found necessary to dilute a 5 ml aliquot of Solution A to 25 ml, and to pipette 5 ml of this solution on to the anion-exchange column.

Thus by carrying out two analyses:
(i) for anions with retention volumes greater than orthophosphite, and
(ii) for anions with a lower retention volume than orthophosphite, and including orthophosphite,
a complete and accurate estimation of the products can be obtained.

Anions with a retention volume greater than pyrophosphate, that is after passage of 1.1 l of the potassium chloride eluant, were eluted by passage of 4 *N* hydrochloric acid down the column. All anions containing two atoms of phosphorus were assumed to have retention volumes greater than pyrophosphate.

All chromatographic analyses were performed as soon as possible after hydrolysis thus minimising any errors due to breakdown of unstable anions formed during the hydrolysis.

(2) *Paper chromatography.* One spot, approximately 3 mm diameter, of the solution obtained after hydrolysis was run on to the starting line of a paper chromatogram measuring 3 in. × 15 in. and using Whatman No. 541 chromatographic paper. The spot was allowed to dry, and equilibrated in a glass tank containing the solvent to be used. The chromatograms were developed by a downward elution technique using both acid and basic solvents as described by EBEL[9,10]. After eluting for 13 h, the chromatograms were dried and sprayed with KARL-KROUPA'S reagent[11]. The identity of hydrolysis products was then determined by comparison with a standard separation of a known mixture of phosphorus oxy-acids, developed on the same chromatogram.

RESULTS AND DISCUSSION

The relative percentages of phosphorus oxy-anions formed during the hydrolysis of the phosphorus trihalides and pseudohalides estimated by anion-exchange chromatography are given in Table I (see Figs. 1 and 2), whilst Table II gives the species detected by paper chromatography.

TABLE I

RELATIVE PERCENTAGES OF PHOSPHORUS OXY-ANIONS FORMED DURING THE HYDROLYSIS OF PHOSPHORUS TRIHALIDES AND PSEUDOHALIDES

*Species**	PCl_3	PBr_3	PI_3	$P(NCO)_3$	$P(NCS)_3$	$P(CN)_3$
P^1	–	1.3	0.9	10.6	–	3.7
P^3	84.3	41.9	42.3	84.7	92.5	79.0
P^5	6.1	8.9	18.6	–	–	–
P^2–P^4	4.9	17.1	3.6	3.8	3.3	3.8
P^4–P^4	2.4	5.9	4.6	–	0.1	–
P^3–O–P^5	–	–	3.6	–	–	**
P^5–O–P^5	–	–	1.6	–	–	–
Anions containing more than 2 atoms of phosphorus	2.9	23.8	24.7	1.0	3.2	4.20

* The nomenclature used to describe the oxidation state of oxy-acids of phosphorus is that proposed by BLASER AND WORMS[12].

** Some isohypophosphate[6] was detected in this case (retention volume 890 ml) but was not separated from an unidentified species[9] (retention volume 810 ml) see Fig. 2F. This species was not formed in the hydrolysis of any other halide or pseudohalide and was found to be very stable to alkali at room temperature.

TABLE II

PHOSPHORUS OXY-ACIDS DETECTED BY PAPER CHROMATOGRAPHY

PCl_3	PBr_3	PI_3	$P(NCO)_3$	$P(NCS)_3$	$P(CN)_3$
—	—	—	P^1	P^1	P^1
P^3	P^3	P^3	P^3	P^3	P^3
P^5	P^5	P^5	—	P^5	P^5
P^2–P^4	P^2–P^4	P^2–P^4	P^2–P^4	P^2–P^4	P^2–P^4
P^4–P^4	P^4–P^4	P^4–P^4	—	P^4–P^4	—
P^4–P^3–P^4	P^4–P^3–P^4	P^4–P^3–P^4	P^4–P^3–P^4	P^4–P^3–P^4	P^4–P^3–P^4
		P^3–O–P^5		*	P^3–O–P^5
		P^5–O–P^5			P^5–O–P^5
**	**	**	**	**	**

* In the hydrolysis of $P(NCS)_3$, in addition to the species shown above, two unidentified species were observed on acid solvent chromatograms, having R_x values of 0.9, and 0.15 respectively. R_x = distance travelled by unknown oxy-anion/distance travelled by orthophosphate.

** In all cases, some species were formed which remained at or near the starting line and were unidentified. Presumably these are high-molecular weight phosphorus oxy-anions *e.g.* $-(P^3)_6-$ (see ref. 12).

In no case was sufficient phosphine formed to cause precipitation in the mercuric chloride traps.

In addition to the hydrolyses of the trihalides and pseudohalides in caustic soda solution, hydrolyses were carried out in 2 *N* hydrochloric acid, and the resulting solutions examined qualitatively by using paper chromatography. The three trihalides formed orthophosphite and a trace of orthophosphate while the pseudohalides formed orthophosphite only.

It appears from the results that the reaction products become more complicated as the atomic number of the halide increases. This is accompanied by an increased rate

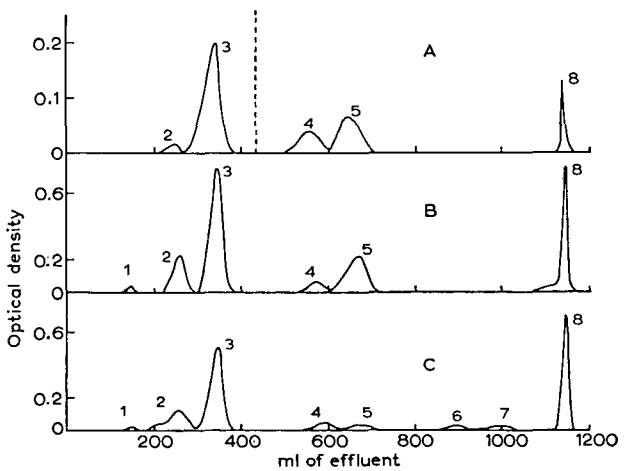

Fig. 1. Typical ion-exchange elution patterns of the phosphorus trihalides. A = PCl_3; B = PBr_3; C = PI_3. Nomenclature: (1) hypophosphite; (2) orthophosphate; (3) orthophosphite; (4) hypophosphate; (5) diphosphite; (6) isohypophosphate; (7) pyrophosphate; (8) anions containing two or more phosphorus atoms eluted with 4*N* HCl; (9) unidentified species. The dotted line signifies that the pattern was compounded from two separate runs as described in the experimental section.

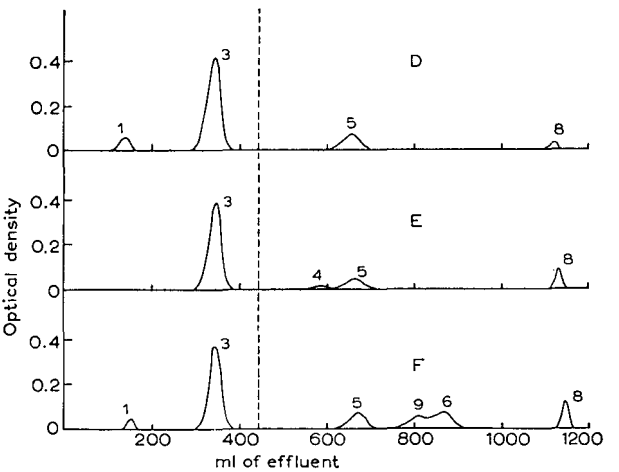

Fig. 2. Typical ion-exchange elution patterns of the phosphorus pseudohalides. D = $P(NCO)_3$; E = $P(NCS)_3$; F = $P(CN)_3$. For nomenclature see Fig. 1.

of hydrolysis and a decrease in the Pauling electronegativity value[13] of the halide (Table III). The values for the pseudohalides were calculated from infra-red data using the method proposed by BELL[14].

The analytical figures for the hydrolysis of phosphorus triiodide and phosphorus tricyanide cannot be strictly compared with those obtained for the other trihalides and pseudohalides, as they are solids and were hydrolysed in that form. This procedure increases the rate of hydrolysis, which becomes almost explosive for phosphorus tricyanide. Solutions of phosphorus triiodide in carbon disulphide, and phosphorus tricyanide in diethyl ether, were hydrolysed by dropping the solutions into caustic soda solution and violently agitating the two immiscible liquids. Phosphorus tricyanide formed only orthophosphite, while phosphorus triiodide produced orthophosphite and a trace of orthophosphate.

The formation of oxy-anions having an oxidation state different from that of the original halide is probably due to disproportionation, but the interesting facet of the reaction is the formation of phosphorus-bonds. The reason for this is by no means clear as yet and further work is being carried out, with a detailed study of the hydrolysis of phosphorus tribromide under a wide variety of differing experimental conditions in order to clarify this situation.

TABLE III

PAULING ELECTRONEGATIVITY VALUES OF THE SUBSTITUENT HALIDES AND PSEUDOHALIDES

Halide or pseudohalide	*Electronegativity*
Cl	3.0
Br	2.8
I	2.5
CNO	3.7
CN	3.4
CNS	3.2

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SUMMARY

Paper and anion-exchange chromatographic techniques have been employed to study the formation of a variety of phosphorus oxy-anions in the hydrolysis of phosphorus trihalides and pseudohalides in caustic soda solution.

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PAPIERCHROMATOGRAPHISCHE TRENNUNG VON ZIRKON UND NIOB

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Die analytische Chemie von Zirkon und Niob hat durch die Herstellung neuer Legierungen an Bedeutung gewonnen. Die Trennung dieser Elemente ist auch deshalb wichtig, weil ^{95}Zr und dessen Folgeprodukt ^{95}Nb häufig angewendete Indikatoren sind. Die Reinheitsprüfung der beiden Radionuklide ist schliesslich ebenfalls von Interesse.

Die gravimetrische Trennung dieser Metalle ist zeitraubend und oft nicht befriedigend. Dagegen gibt es einige Extraktionsverfahren, die sich hauptsächlich mit den Radionukliden ^{95}Zr und ^{95}Nb befassen und teilweise zu guten Ergebnissen führen. Die wässrige Phase besteht hierbei meist aus Fluorwasserstoffsäure[1,2] oder Salpetersäure[3–6], während sich die organische Phase aus Benzol bzw. Xylol mit Zusatz von Thenoyltrifluoroaceton (TTA)[3–6], Methyläthylketon[1,7] oder Tetrachlorkohlenstoff mit Zusatz von Tri-*n*-butylphosphinoxyd[2] zusammensetzt.

Auch säulenchromatographische Versuche zur Trennung dieser Metalle sind beschrieben. Als Säulenmaterial kommen Zellulose[8–10], Aluminiumoxyd[11], Anionenaustauscher[12–16] und Kationenaustauscher[17] sowie mit Tri-*n*-butylphosphat präpariertes Silikagel[18] in Frage. MOORE[19] nutzt eine oft gemachte Beobachtung, dass trägerfreies ^{95}Nb zu einem erheblichen Teil an Glas adsorbiert wird, für die quantitative Trennung der beiden Metallionen aus.

Es sind wenige Arbeiten bekannt geworden, die sich mit der papierchromatographischen Trennung von Zirkon und Niob befassen[20–21]. Bei der Untersuchung der Kernspaltprodukte haben CROUTHAMEL UND FUDGE[20] diese Trennung unter Verwendung von Fluorwasserstoffsäure durchgeführt, die dann von HARDY UND SCARGILL[5] modifiziert wurde. Auch GRAND-CLEMENT *et al.*[21] geben eine Vorschrift dafür an. Im folgenden wird eine papierchromatographische Methode beschrieben, die ohne Fluorwasserstoffsäure auskommt und somit in normalen Laboratoriumsgefässen ausgeführt werden kann. Aus den Extraktionsversuchen war die Selektivität von Methyläthylketon für Niob bekannt[1,7]. Die unterschiedlichen Eigenschaften der Oxalatokomplexe der beiden Metalle sind in der Literatur beschrieben und zur säulenchromatographischen Trennung an Anionenaustauschern ausgenutzt worden[12,16].

Es wurden die optimalen Bedingungen für die Trennung von Zirkon und Niob unter Verwendung von Methyläthylketon und Oxalsäure untersucht.

EXPERIMENTELLES

Die Versuche wurden in einem Glaszylinder bei Zimmertemperatur und ohne Stabilisierung mit Papierstreifen von Whatman No. 1 bzw. Schleicher & Schüll No. 2043b

durchgeführt. Die Metallionen wurden als Oxalatokomplexe aufgetragen. Die Lösungen enthielten etwa 5 mg/ml inaktives Metall bzw. 1 μC ^{95}Zr/ml.

Entwicklung

Die Färbung der Chromatogramme erfolgte mit Morin. Die Flecke sind bei normalem Licht für Zirkon gelb und für Niob braun. Unter U.V.-Licht ist der Nachweis der Farben besonders leicht. Zirkon fluoresziert gelb, während Niob eine wenig fluoreszierende dunkle Farbe zeigt. Auch Quercitin ergibt bei normalem Licht für Zirkon einen gelben und für Niob einen braunen Fleck, wenn das Chromatogramm mit Ammoniak-Dämpfen behandelt wird. Die Auswertung der Papierchromatogramme der Radionuklide ^{95}Zr und ^{95}Nb wurde mit Strahlungsmessgeräten vorgenommen.

Fliessmittel

In Vorversuchen wurde eine Grundlösung, bestehend aus Methyläthylketon, Wasser und Dioxan im Verhältnis 5:1:1, hergestellt. Dieser Lösung wurden Oxalsäure und/oder Salzsäure verschiedener Konzentrationen zugesetzt. Die Konzentration der Oxalsäure wurde von 0.05 bis 1 Mol/l verändert, wobei die Molarität der Salzsäure versuchsweise 0.1 betrug. Es zeigte sich, dass ab 0.1 Mol/l eine Trennung erfolgt; die günstigste Konzentration der Oxalsäure lag jedoch bei 0.25 Mol/l. Die Laufzeit betrug

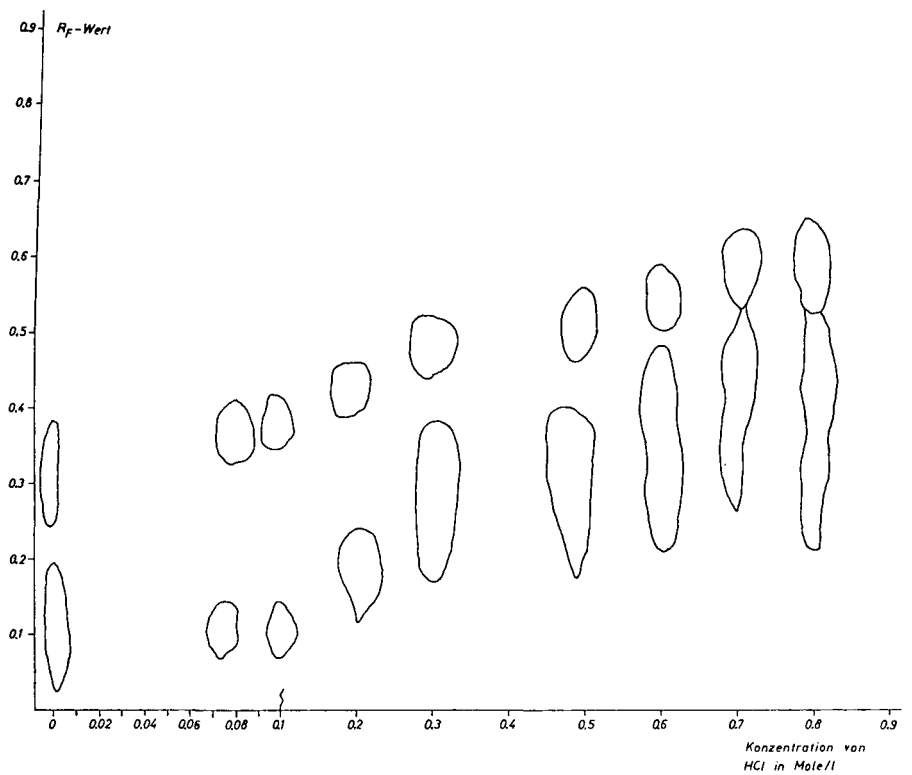

Fig. 1. Abhängigkeit der Trennung von Zirkon und Niob von der Konzentration der Salzsäure in einem Fliessmittel bestehend aus: Methyläthylketon–Dioxan–Wasser (5:1:1), bei einer konstanten Konzentration der Oxalsäure von 1 Mol/l.

bei einer Laufhöhe von 30 cm etwa 4 Stunden. Werden die Metallionen nicht als Oxalatokomplex aufgetragen, so ist eine Mindestkonzentration von 1 Mol/l notwendig.

Unter denselben Bedingungen wurde die Oxalsäure durch andere komplexbildende organische Säuren ersetzt. Während Mandelsäure, Zitronensäure und Phthalsäure keine befriedigenden Ergebnisse lieferten, konnte mit Weinsäure (1 *M*) eine Trennung erzielt werden. Bei einer Laufzeit von 8 Stunden wurden R_F-Werte von 0.6 für Niob und 0.05 für Zirkon gefunden. Niob zeigte allerdings eine Schwanzbildung.

In einer weiteren Versuchsreihe wurde bei konstantem Oxalsäuregehalt von 1 Mol/l die Konzentration der Salzsäure von 0 bis 1 Mol/l verändert. Aus Fig. 1 ist zu ersehen, dass eine Konzentration von 0.07 bis 0.15 Mol/l die günstigste ist. Eine Steigerung der Konzentration der Salzsäure bewirkt eine Verschlechterung der Trennung. Ein oft vorgenommener Zusatz von Wasserstoffperoxyd[16] zum Fliessmittel brachte hier keinen Vorteil.

Ergebnisse

Aus den Versuchen ist zu entnehmen, dass das günstigste Fliessmittel eine 0.25 *M* oxalsaure und 0.1 *M* salzsaure Lösung von Methyläthylketon–Dioxan–Wasser im Verhältnis 5:1:1 ist. Die R_F-Werte betragen hierbei etwa 0.1 für Zirkon und etwa 0.4 für Niob (Fig. 1).

Die Fig. 2 zeigt das Radiopapierchromatogramm einer Trennung von ^{95}Zr und ^{95}Nb. Gezählt wurde mit einem NaJ-Kristall. Daraus ersieht man, dass ein Teil des Zirkons am Startpunkt bleibt. Diese Tatsache, die auch sonst beobachtet worden ist[5], beruht wahrscheinlich darauf, dass Zirkon in der Lösung in verschiedenen Formen vorliegt[18].

Dass es sich hierbei um Zirkon und nicht um Niob handelt, kann durch die Betrachtung der γ-Linien in einem Spektrometer geklärt werden. Während der Niobfleck die 0.23-MeV-Linie des ^{95m}Nb enthielt, konnte diese Linie bei den anderen Flecken nicht nachgewiesen werden. Auch die Ansprechwahrscheinlichkeit in den Strahlendetektoren kann zur Entscheidung dieser Frage herangezogen werden. Bedingt durch die Ähnlichkeit der γ- und die Verschiedenheit der β-Energien der beiden Nuklide ist das Verhältnis der Zählausbeute von ^{95}Zr in einem β- bzw. γ-Detektor verschieden von der des Niobs. Misst man das Papierchromatogramm in einem β- und anschliessend in einem γ-Zählrohr, so kann durch den Vergleich der Ergebnisse geschlossen werden, dass der Startfleck dem Zirkon zuzuordnen ist. Die Messungen hierzu müssen der Nachbildung des Niobs wegen unmittelbar nach der Trennung vorgenommen werden.

Grenzen der Trennung

Um die Möglichkeit der Trennung sehr geringer Mengen eines Elementes in Gegenwart eines grossen Überschusses des anderen zu untersuchen, wurde etwa 1 ml einer 1 μC/ml aktiven trägerfreien ^{95}Zr–^{95}Nb-Oxalatlösung jeweils mit 1 ml einer 5 mg/ml Metallionen enthaltenden Zirkon- bzw. Nioboxalatlösung versetzt. Die anschliessend erfolgte Trennung zeigte eine geringfügige Erniedrigung des R_F-Wertes von Niob bei Zusatz von Zirkonträger bzw. eine geringfügige Erhöhung des R_F-Wertes von Zirkon in Gegenwart von Niobträger. Die Trennung erfolgte aber in beiden Fällen befriedigend.

Trennung des Molybdäns von Zirkon und Niob

In einem Nachversuch wurde die an uns herangetragene Frage untersucht, ob eine

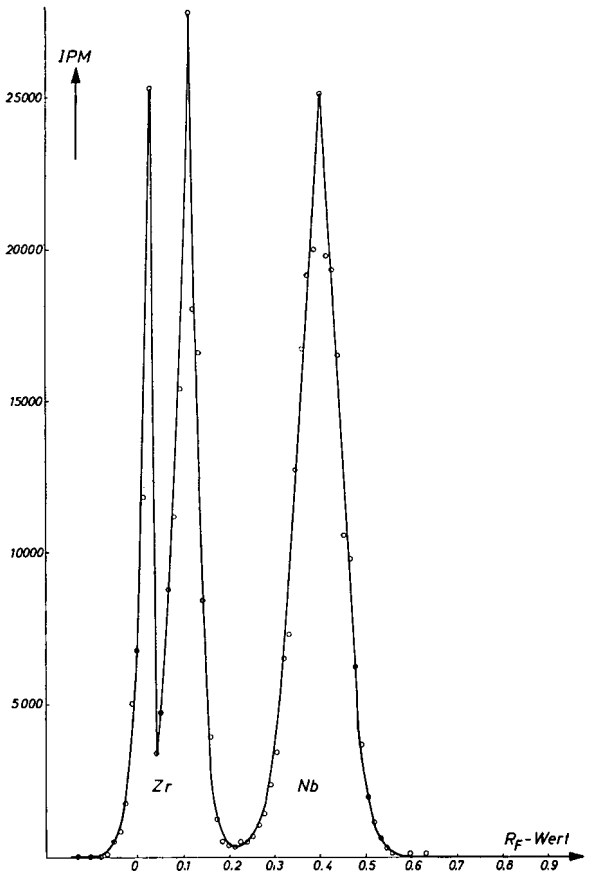

Fig. 2. Radiopapierchromatogram der Trennung von Zirkon und Niob mit dem Fliessmittel Methyläthylketon–Dioxan–Wasser (5:1:1), wobei die Molarität der Oxalsäure 0.25 und die der Salzsäure 0.1 beträgt.

Trennung von Molybdän von den beiden anderen Elementen möglich ist. Auch hier wurde der Oxalatokomplex des Metalles aufgetragen. Mit demselben Fliessmittel, das für die Trennung von Zirkon und Niob verwendet wurde, konnte eine Trennung der 3 Metallionen erzielt werden. Hierbei betrug der R_F-Wert für Molybdän 0.7. Im übrigen gilt auch hier für die Konzentration der Oxalsäure dasselbe wie für Zirkon und Niob geschildert wurde.

DANK

Herrn Doktor K. HOGREBE danken wir herzlich für die wohlwollende Unterstützung dieser Arbeit. Fräulein E. BARSIECK führte die Laboratoriumsversuche in dankenswerter Weise durch.

ZUSAMMENFASSUNG

Die papierchromatographische Trennung von Zirkon und Niob wurde mit einem Fliessmittel, bestehend aus einer 0.25 M oxalsauren und 0.1 M salzsauren Lösung von

Methyläthylketon, Dioxan und Wasser (5:1:1), durchgeführt. Die R_F-Werte betragen hierbei für Zirkon 0.1 und für Niob 0.4. Molybdän ergibt unter denselben Bedingungen einen R_F-Wert von 0.7.

SUMMARY

Zirconium and niobium were separated by paper chromatography with the solvent system methyl ethyl ketone–dioxan–water (5:1:1), 0.25 *M* in oxalic acid and 0.1 *M* in hydrochloric acid. The R_F values in this system are 0.1 for zirconium and 0.4 for niobium. Under the same conditions the R_F of molybdenum is 0.7.

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CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME INDIVIDUAL RARE EARTHS ON PAPER BY MEANS OF FLUORESCENCE

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The applications of fluorescence in inorganic analysis have hitherto been seriously limited by the presence of numerous interfering substances (activators, quenching substances and other substances that form fluorescent compounds with the reagent employed) and also by the poor selectivity of the organic reagents normally employed for the formation of fluorescent compounds.

Recent progress in separation techniques, however, and the extremely small quantities of an element that can be revealed by means of analyses based on fluorescence, encouraged us to work out simple and rapid methods for the separation of some elements from all interfering substances, and to initiate a study of the fluorescent properties of a number of inorganic compounds with a view to finding more sensitive and more selective reagents than those at present employed.

We have elsewhere[1] discussed the conditions governing the formation and the fluorescent properties of inorganic precipitates containing traces of uranium.

Treatment with zinc acetate followed by trisodium phosphate yields highly sensitive fluorescent spots with uranium (identification limit 10^{-3} γ). This test has also been used for rapid field determination of uranium in natural waters[2].

In the work described here, a series of fluorescent rare earth compounds was examined, some of which are already known from the literature (rare earth tungstates). A special study was made of the conditions governing the formation on paper of compounds that exhibit a characteristic, stable, fluorescence. Attention was also paid to the quenching of a large number of inorganic ions, and a description is given of a paper chromatographic technique for the separation of rare earths from interfering ions. Separation on paper was decided upon because of the rapidity and simplicity of this operation, and also because the lanthanide elements can be revealed directly, after separating the interfering substances, by spraying the appropriate reagents on the paper.

EXPERIMENTAL

Reagents

Rare earths. The solutions were prepared from rare earth oxides (Johnson, Matthey & Co, London). In the case of europium 99.995 % pure europium oxide (Johnson, Matthey Specpure) was used.

Tributyl phosphate. TBP (Fluka) 100 %.
Sodium tungstate (Merck). 15 % solution in water.
Ammonium oxalate (Merck). Saturated solution.

All other chemicals used were Merck analytically pure reagents.

Formation of fluorescent spots on filter paper

On a Whatman No. 1 paper was micropipetted 0.005 ml of a solution of known concentration of the ion under examination. After quick drying in hot air, the paper was sprayed with the appropriate reagents in order to form the fluorescent compound and then examined under U.V. light (280 mμ). The concentration of the solution was then reduced to the point of the maximum dilution at which the ion in question could still be revealed. The smallest quantity (by weight) of the ion that can be detected on paper is related to the volume taken for the test. The values given in Tables I and II are for volumes of 10^{-3} ml of solution, a volume which is still easy to measure and yields spots of about 0.3 cm diameter.

Procedure for the study of interference

The interference of various ions was studied by determining the quantity of a given ion that reduces the fluorescence by 50 %. The quantity in question may be found without difficulty by comparing the fluorescent spots obtained with 0.005 ml of solution containing, respectively,

(a) 50 γ/ml of the ion under examination,

(b) 50 γ/ml of the ion under examination plus a known amount of the ion being studied for interference,

(c) 25 γ/ml of the ion under examination.

The quantity of the interfering ion is varied up to the point where the intensity of the fluorescent light of spot (b) is equal to that exhibited by spot (c).

RESULTS

Studies were carried out on a series of reagents capable of producing fluorescent compounds on paper with the elements of the lanthanide series.

In Table I are listed the reagents giving positive results. For each reagent the colour of the fluorescent light and the identification limit on paper are given.

Of all the reagents examined, sodium tungstate gave the best results for traces of dysprosium, samarium and europium, while ammonium oxalate exhibited a certain selectivity for terbium and europium. Cerium forms weakly fluorescent compounds with sodium phosphate, sodium pyrophosphate and borax only. As regards the individual lanthanide elements, terbium can be readily identified with ammonium oxalate by means of spot tests on paper. In fact, with the reagent in question, as may be seen from Table I, only terbium and europium give appreciably fluorescent compounds. The two fluorescent spots are nevertheless clearly distinguishable, that of terbium being yellow-green and that of europium red. Dysprosium is likewise readily identifiable with sodium tungstate, in that it is the only lanthanide element giving off a whitish-pink fluorescent light with that reagent. Europium gives a highly sensitive reaction on paper with sodium tungstate (red fluorescence; identification limit 10^{-3} γ) but its identification is difficult in the presence of appreciable

TABLE I

SPOT TESTS OF RARE EARTHS BY MEANS OF FLUORESCENCE ON PAPER

Reagent employed	*Spot test sensitivity and fluorescence colour**					
	Ce	*Sm*	*Eu*	*Gd*	*Tb*	*Dy*
Sodium tungstate	d.s.	0.01 γ (10 γ/ml) red	0.001 γ (1 γ/ml) red	i.s.	d.s.	0.002 γ (2 γ/ml) whitish pink
Ammonium oxalate	d.s.	2 γ (2000 γ/ml) red	0.025 γ (25 γ/ml) red	i.s.	0.025 γ (25 γ/ml) yellow-green	0.1 γ (100 γ/ml) whitish pink
Sodium ortho-phosphate	5 γ (5000 γ/ml) blue	i.s.	0.1 γ (100 γ/ml) red	i.s.	d.s.	i.s.
Sodium pyro-phosphate	5 γ (5000 γ/ml) blue	i.s.	0.5 γ (500 γ/ml) red	i.s.	d.s.	i.s.
Sodium molybdate	d.s.	i.s.	0.4 γ (400 γ/ml) red	i.s.	d.s.	i.s.
Borax	5 γ (5000 γ/ml) blue	i.s.	0.2 γ (200 γ/ml) red	i.s.	d.s.	i.s.
Ammonium vanadate	i.s.	i.s.	2 γ (2000 γ/ml) red	i.s.	i.s.	i.s.

* The values in parentheses are dilution limits; i.s. = invisible spot; d.s. = dark spot.

amounts of samarium, the latter, in fact, producing fluorescent spots of a similar colour (identification limit with tungstate $10^{-2}\gamma$). Thermo-quenching tests, however, showed that when the fluorescent spots of europium and samarium are warmed at the same time by means of hot air, as the temperature rises (from 30° to 80°) the colour of the fluorescence veers from red to yellowish orange in the case of samarium, while the red fluorescence of europium only weakens slightly.

With thermo-quenching, therefore, it is possible to identify europium and still obtain useful indications for identification of samarium.

According to POLLARD *et al.*[3] and STEVENS[4] lanthanum, lutetium and gadolinium may be identified with morin in that they are the only lanthanide elements that form fluorescent (yellow-green) compounds with that reagent, so that there are now seven lanthanide elements that can be revealed and identified by means of fluorescent techniques on paper. In addition, cerium (III) can be revealed and identified by fluorescence, though with a lesser degree of sensitivity (blue fluorescence with borax; identification limit 5 γ).

Separation of rare earths from interfering ions

A large number of ions interfere with the reactions described. Table II shows the quantities of the interfering ions, which quench the fluorescence of the spots obtained with sodium tungstate by 50 %.

In order to bring about a rapid separation of rare earths from the interfering ions, we applied simple paper chromatographic separation methods relying on the high degree of selectivity with which certain organic solvents extract lanthanide elements.

The procedure is as follows: 10^{-2} ml of the solution under examination is spotted on to a small strip of Whatman No. 1 paper at about 2–3 cm from one end. After drying, the end is dipped into the organic solvent and left there till the latter rises to a height of a few centimetres (approx. 2 or 3 minutes). The strip is then dried with

TABLE II

THE AMOUNTS OF VARIOUS ELEMENTS THAT QUENCH THE FLUORESCENCE OF 0.25 γ OF EUROPIUM BY 50%

Element	*Amount* γ	*Element*	*Amount* γ	*Element*	*Amount* γ
Sr(II)	20	Ba(II)	5	Th(IV)	3
Cd(II)	10	Be(II)	5	Fe(III)	2
Zr(IV)	8	Bi(III)	5	Pb(II)	2
La(III)	5	U(VI)	5	Fe(II)	1
Ce(III)	5	Ce(IV)	4	Cr(III)	0.7
Hg(I)	5	Hg(II)	4	Cu(II)	0.2
Tl(I)	5	Y(III)	4	Ni(II)	0.2

hot air and the desired reagents sprayed on it in order to form the fluorescent compound. Of the different solvents employed, the best results were obtained with 100 % TBP saturated with concentrated HNO_3. This solvent extracts quantitatively on paper all the lanthanides provided one has taken the precaution to add a drop of aluminium nitrate as salting-out agent at the starting point. Some sixty inorganic cations were studied (among them all the interfering ions listed in Table II) under the conditions described, and only U(VI), Th(IV), Zr(IV) and Ce(IV) accompanied the rare earths to the solvent front.

From Table II it can be seen that thorium and uranium do not interfere with the fluorescence test provided they are present in only small amounts, yet they may be easily separated from the rare earths by means of ascending chromatography with mesityl oxide. The latter solvent, in presence of HNO_3, extracts uranium, thorium and other tetravalent elements, leaving the rare earths at the starting point. Therefore, in order to separate on paper the group of rare earths from the other elements the following procedure may be adopted: first elute with mesityl oxide–HNO_3 to a height of about 20 cm. Dry and elute again with TBP–HNO_3 up to 15 cm. The rare earths will be well separated (Fig. 1). Their presence may be revealed with sodium tungstate or ammonium oxalate.

Separation of individual lanthanide elements on ion-exchange paper and identification by means of fluorescence

With TBP as solvent the rare earth group can be separated from most inorganic elements, but the individual lanthanide elements cannot be separated.

In earlier work[5] we separated mixtures of La, Ce, Nd and Sm by ascending chromatography on paper impregnated with zirconium phosphate. The eluent used was a strong acid such as $HClO_4$, which was employed to demonstrate the complexing

power of zirconium phosphate. Separation of lanthanides with a higher atomic number was, however, not possible by such means. To separate mixtures of La, Ce, Sm, Eu, Tb, Dy and Lu (the only lanthanide elements at present detectable by means of fluorescence on paper) we utilised the well-known complexing power of

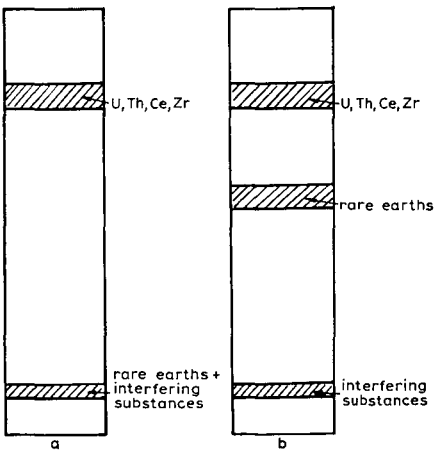

Fig. 1. (a) Elution with mesityl oxide–HNO_3 (first step). (b) Elution with TBP–HNO_3 (second step).

lactic acid, the complexes formed with the lanthanide elements being separated on ion-exchange paper. We employed strips of Amberlite SA-2 ion-exchange papers (manufactured by Rohm & Haas). The separations were effected by descending chromatography with 10 % lactic acid as eluent.

The findings are given in Fig. 2*. In all probability better results might be obtained with other eluents such as ethylenediaminetetraacetic acid solution. The separations obtained, however, are sufficient to permit the identification of the individual lanthanide elements in a rare earth mixture, not only by their position relative to one another but also by the colour of the fluorescence produced with the specific reagents employed, and, again, by thermo-quenching.

If there are other interfering ions present in the rare earth mixture these should be separated with TBP, as described earlier, before attempting separation of the individual rare earths by ion exchange. The separation may be carried out by first extracting uranium, thorium, cerium and zirconium with mesityl oxide–HNO_3, and then extracting the rare earths with TBP–HNO_3.

The rare earths are then put in water and the individual elements are separated by ion-exchange chromatography.

Extractions with mesityl oxide or TBP may also be performed directly on ion-exchange paper, as already described for non-impregnated paper, in which case the problem remains of eliminating TBP from the paper before proceeding with the next ion-exchange separation by means of elution with complexing acids. This is possible with carbon tetrachloride once the strips have been made alkaline with sodium carbonate in order to reduce losses of rare earths into the CCl_4. This procedure may

* It should be noted that on ion-exchange paper the sensitivity of fluorescence tests is somewhat reduced.

find useful application in the rapid qualitative isolation of rare earths from such minerals as monazite.

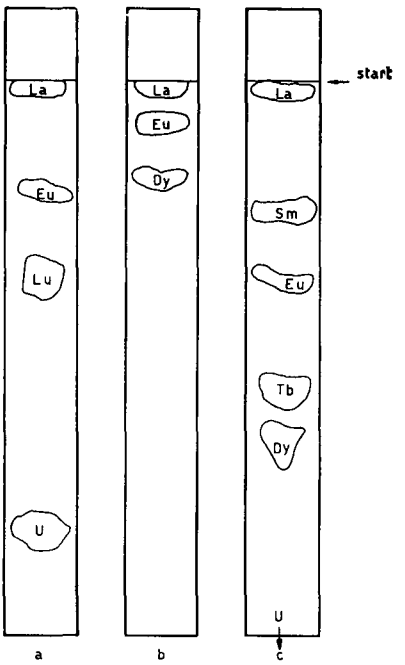

Fig. 2. (a) Separation of La, Eu, Lu and U(VI) by descending chromatography. Elution with aqueous lactic acid; pH = 3; time 2 h. (b) Separation of La, Eu, Dy; pH = 2.4; time = 3 h. (c) Separation of La, Sm, Eu, Tb, Dy and U(VI); pH = 1.5; time 28 h.

CONCLUSION

Identification test by means of fluorescence on paper showed a high degree of sensitivity and were to some extent specific for individual rare earths, such as Sm, Eu, Dy and Tb. With simple separations on paper the tests may be conducted even in presence of interfering ions.

In addition to the application in detecting individual earths in various compounds, minerals included, the test described may be usefully adopted in chromatography and electrophoresis in order to show the position of these elements on filter paper. Reagents normally employed to reveal rare earths on paper (Thoron, 8-hydroxyquinoline and morin) are less sensitive and not very specific.

SUMMARY

The conditions for the formation of some U.V. fluorescent rare earth compounds were studied. It is possible to detect by fluorescence some rare earth ions on paper with high sensitivity. The spot tests employed are moreover very specific for Eu, Sm, (sodium tungstate as reagent; red fluorescence; identification limit: Eu 10^{-3} γ; Sm $10^{-2}\gamma$), for Dy (sodium tungstate as reagent; whitish pink fluorescence; identification limit: $2 \cdot 10^{-3}\gamma$) and for Tb (sodium oxalate as reagent; yellow-green fluo-

rescence; identification limit $2.5 \cdot 10^{-2}\gamma$). Separation of the rare earth group from interfering substances was carried out by extracting with mesityl oxide (which removes tetravalent cations) followed by extraction with tributyl phosphate–HNO_3. The individual rare earths were separated on Amberlite SA-2 ion-exchange paper employing aqueous lactic acid as eluent.

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Notes

A rapid method for evaluating gas–liquid chromatography stationary phases

Introduction

The liquid used as stationary phase in the G.L.C. column should be chemically stable and involatile at the operating temperature and should preferably be thoroughly evaluated before use. Since the analyst requires reproducible retention data, evaluation of the stationary phase with respect to the constancy of retention data is more significant than investigations of chemical changes[1] or redistribution of the liquid phase in the column[2,3] or measurement of the loss of material from the column[4,5].

Previous attempts to detect changes in retention[3,6] have employed specific retention volumes which are difficult and time consuming to measure reliably and are irrelevant in the context of relative retention data. As the evaluation of stationary phases is of prime importance to gas chromatography the author proposes to describe a simple and rapid method which can be run in conjunction with the routine analytical work of a laboratory.

Procedure

The method involves the following steps:

1. Preparation and conditioning of the column.
2. Determination of the maximum operating temperature.
3. Preparation of suitable calibration solutions.
4. Chromatography of the calibration solutions whilst the column temperature is alternated between a convenient temperature in the range 60° to 100° and the maximum operating temperature.
5. Calculation of relative retentions in retention index units and processing of the data.

1. The column packing should be coated by the usual technique and the column conditioned at moderate temperatures (not exceeding 100°) thus ensuring minimum thermal degradation of the stationary phase.

2. The most probable maximum operating temperature should be obtained by measurement of the detector signal due to stationary phase bleed preferably using one of the ionisation detectors. The retention data subsequently obtained will serve to confirm whether or not this was the highest temperature at which constant retentions can be obtained.

3. The calibration solution should be a mixture containing at least four *n*-alkanes, preferably successive homologues, and at least five solutes which have retentions between those of the *n*-alkanes. The solutes should be selected so as to

have as wide a range of solvent interactions as possible. Therefore molecules with either strong dipoles, or hydrogen bond acceptor or donator properties or readily polarisable groups should be included. Only compounds which give symmetrical peaks on the chromatogram should be used. The mixtures should be made up as ~ 5 % w/v solutions in a volatile solvent, 30–40 petrol or *n*-heptane.

4. The column temperature should be alternated daily between a convenient temperature in the range 60–100° and the maximum operating temperature. The column temperature should remain constant within the period the chromatograms are obtained (drift not to exceed 0.2°) and be reproducible from day to day (within ± 0.2°). The carrier gas flow rate should also remain constant within a run and be reproducible.

5. Retention distances should be measured from the point of injection to peak maxima on the recorder chart and the retention indices calculated according to KOVATS[7] using the expression:

$$I = 100\,N + 100\,n\left(\frac{\log R_S - \log R_N}{\log R_{N+n} - \log R_N}\right)$$

where N = the carbon number of the first *n*-alkane used and $N + n$ that of the second,
R_S = the retention of the solute,
R_N = the retention of *n*-alkane carbon number N,
R_{N+n} = the retention of *n*-alkane carbon number $N + n$.

The constancy of the retention data can be tested by plotting against the column life. Since no column dead volume correction (which will vary from column to column) is applied, the retention data is not suitable for documentation.

Experimental and results

In order to illustrate the method of evaluation one of the new nitrile silicone stationary phases was examined.

Chromatograms were obtained using an apparatus consisting of glass columns (5 ft. long and 4 mm I.D.) with a modified flame ionisation detector[8]. The carrier gas was a 3:1 (by volume) mixture of hydrogen and nitrogen. The flow rate was controlled solely by a standard BHR 12 regulator (British Oxygen Gases). The column was packed with a 20 % w/w mixture of nitrile silicone fluid XF-1150 (General Electric Company of U.S.A.) and acid washed 60–70 mesh celite. The column was heated by means of a vapour jacket, methanol 65° and *n*-pentanol 138°. The carrier gas which was dried over linde molecular sieve was allowed to flow continuously, the column being maintained at elevated temperature during the day (~ 8 h) and left at room temperature overnight. The calibration solutions were introduced by means of stainless steel capillary pipettes.

The results which are summarised in Table I reveal that nitrile silicone XF-1150 stationary phase is capable of giving constant retention data at temperatures up to 138° over a period of at least 120 h (approximately 15 working days).

Conclusions

The method described is capable of giving constant retention data with stationary phases which do not undergo chemical changes with column usage. The particular

TABLE I

THE CONSTANCY OF RETENTION DATA WITH COLUMN USAGE

Column: 20% nitrile silicone XF 1150, celite; carrier gas flow rate: 60 ml/min at 65° and 138°.

Substance	*Temp.(°C)*	$I_{initial}$	I_{final}	δI	I_{mean}	σ
n-Hexane thiol	65	1141	1142	+ 1	1143	1.2
Pentan-1-ol	65	1181	1181	Nil	1182	1.2
n-Butyl cyanide	65	1252	1255	+ 3	1255	1.8
Di-*tert.*-butyl disulphide	65	1329	1330	+ 1	1330	0.7
1-Iodoheptane	65	1358	1360	+ 2	1360	1.0
Methyl fumarate	138	1718	1719	+ 1	1719	0.9
Octadec-1-ene	138	1850	1850	Nil	1850	0.4
Dodecan-1-ol	138	1955	1955	Nil	1955	0.8
Di-*n*-heptyl ketone	138	2076	2076	Nil	2076	1.1
1-Chlorohexadecane	138	2137	2137	Nil	2137	0.6

Where $I_{initial}$ and I_{final} are the initial and final retention indices and I_{mean} the mean of 7 determinations carried out over a period of 3 weeks.

δI is the change of retention index between initial and final observations.

σ is the standard deviation in retention index units.

attractions of the method are its simplicity and rapidity. Chromatograms need not take longer than 3 h for duplicate determinations, and the calculations less than 30 min as no dead volume correction need be computed[9]. Thus it should be possible to evaluate a number of stationary phases at one time, the columns being available for routine analysis purposes after a day's calibrations have been completed. Once stationary phases have been evaluated by this technique methods of column characterisation may then be investigated. Such an investigation is now in progress at N.R.P.R.A.

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A modified design of a high temperature argon beta-ray detector for gas chromatography

In 1958, LOVELOCK[1,2] described an ultra sensitive detector for gas chromatographic work, whose operation is based on the unique ionisation properties of argon gas. Using this detector, which is relatively insensitive to changes in temperature, pressure and gas flow, as little as 10^{-12} moles of most organic substances can be detected.

A high temperature is desirable for optimum sensitivity of the argon detector and is also essential to prevent condensation in gas chromatography of high boiling-point compounds. Although argon β-ray detectors are now used extensively, very little work has so far been done using this highly sensitive detector at temperatures above 300°.

There are two main difficulties in the use of these detectors at high temperatures: (i) the insulation of very high voltages at high temperatures, and (ii) the choice of suitable connecting tubes between the column and the detector body.

TERANISHI *et al.*[3] used silicone gaskets and glass tubing to connect the column to the detector and silicone rubber as an insulator. This limits the range to below 240°. UPHAM *et al.*[4] connected the column and detector directly with teflon sleeves. GUDZINOWICZ[5] and his coworkers used sapphire as an insulator at high temperatures and in a recent paper[6] they have replaced the usual silicone rubber or teflon connecting tubes between column and detector with glass to metal ball and socket connections. But even then the detector does not function properly at very high temperatures, *e.g.*

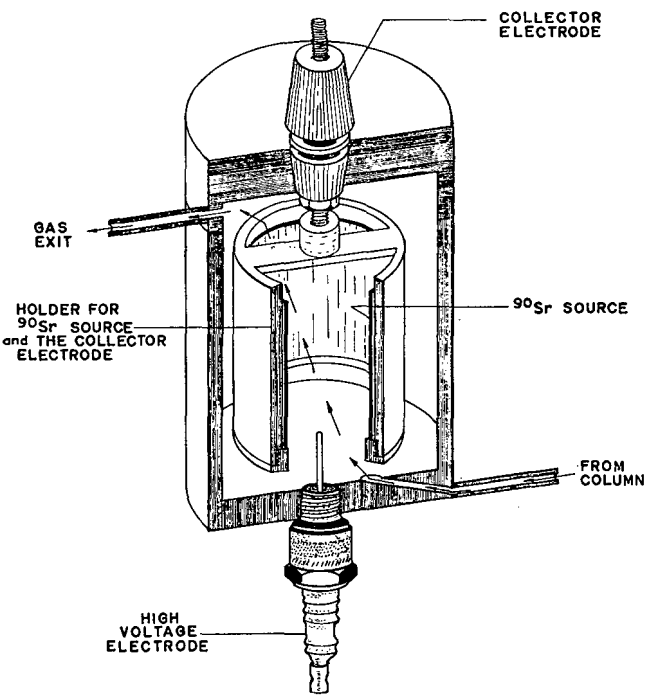

Fig. 1. β-Ray ionisation detector.

500°, when expansion of the metal to glass connection and electrical leakage through glass become serious problems.

To avoid these difficulties we have modified the design so that neither the high voltage nor the low voltage points are connected to the body of the detector. In the modified design the metal column is directly connected to the metal detector body using a swagelok coupler* without any insulating material in between. The high voltage is applied through an extended spark plug probe and the collector electrode is isolated from the body of the detector by a porcelain insulated "Anticorona" plug. A gold coated ^{90}Sr source** (10 mC) is used in order to prevent any diffusion of active ^{90}Sr at high temperatures. The details of the design are given in Fig. 1.

The detector has been working quite satisfactorily for about a year. It has been used at temperatures up to 500° in conjunction with our high temperature gas chromatographic unit[7] and it is felt that it can be worked at still higher temperatures.

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* A swagelok metal-metal union NO (400-6) supplied by Crawford Fitting Co., 884 East 140th Street, Cleveland, 10, Ohio, U.S.A.
** Supplied by Radiochemical Centre, Amersham, U.K.

Chromatographie préparative sur plaques.

Confection d'un mélange (gel de silice/plâtre) permettant un étalement homogène de couches épaisses et leur séchage sans craquelures. Application au moyen d'un nouvel appareil

La confection des couches épaisses de gel de silice pour la chromatographie préparative pose quelques problèmes. En effet, les mélanges de gel de silice et de plâtre commercialisés à l'heure actuelle sous le nom de Gel de silice G (selon STAHL) renferment en général 10 ou 13 % de sulfate de calcium (plâtre minute). Si l'on effectue la suspension de ces mélanges dans un volume d'eau double du poids d'adsorbant, la pâte ainsi obtenue durcit trop rapidement pour être étalée de façon homogène.

Si l'on augmente le volume d'eau par rapport à l'adsorbant, l'étalement est plus satisfaisant, mais les couches ne sont plus assez épaisses. Dans tous les cas, d'ailleurs, le séchage des couches épaisses est défectueux. Il est difficile d'obtenir des plaques sans craquelures même si l'on a la précaution de les laisser sécher à la température ambiante pendant plusieurs heures avant de les placer à l'étuve à 110°.

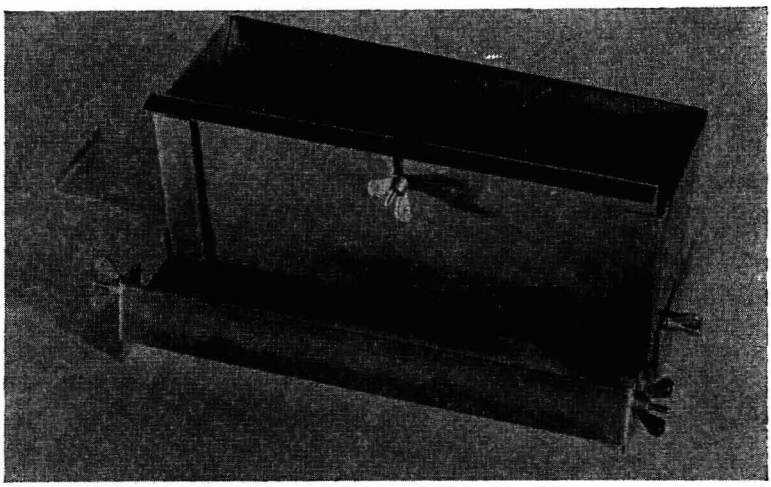

Fig. 1. Applicateur pour chromatographie préparative sur plaques.

On peut remédier à tous ces inconvénients si l'on prend le soin de procéder de la manière suivante:

(1) Ralentir la vitesse de prise du plâtre en portant le Gel de silice G (selon STAHL) à une température de 500° environ pendant 4 heures. Tamiser sur tamis 110 (norme française) soit 80 (norme TYLER) pour éliminer les grumeaux qui auraient pu se former.

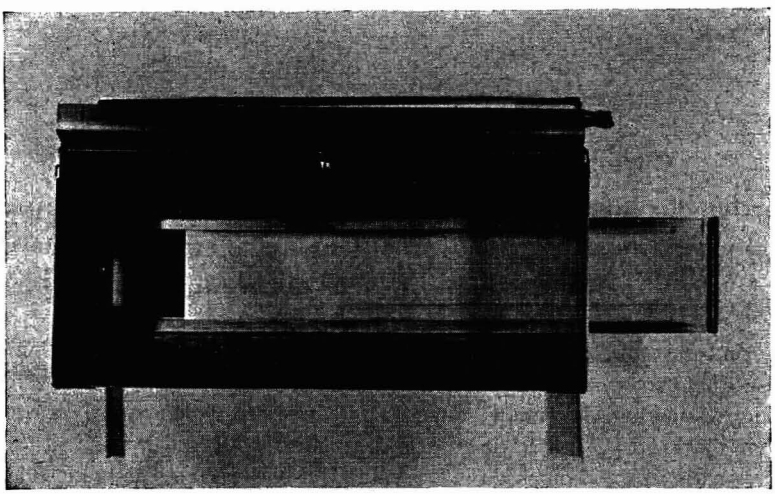

Fig. 2. Applicateur pour chromatographie préparative sur plaques.

(2) Diminuer le taux de sulfate de calcium du Gel de silice G et l'amener à 5 %. Pour ce faire, ajouter au mélange gel de silice/plâtre vendu dans le commerce un certain pourcentage de gel de silice obtenu soit en lavant abondamment le mélange pour en éliminer le plâtre, soit en se procurant du gel de silice pur de même granulométrie. Utiliser un agitateur mécanique pour homogénéiser la poudre.

(3) Ajouter 275 cm^3 d'eau à 150 g de la poudre ainsi obtenue.

(4) Étaler la suspension sur des plaques de verre (20 × 20 cm) soit avec un applicateur de type courant soit avec l'applicateur* dont nous donnons la reproduction (Figs. 1 et 2) qui permet de réaliser 5 plaques de 1 mm d'épaisseur à la fois ou 2 à 3 plaques de 2 mm d'épaisseur.

(5) Faire sécher les plaques de la manière suivante:

– surmonter un réchaud électrique d'une cheminée – confectionnée au moyen d'une boîte métallique ouverte aux deux extrémités – sur laquelle on place une feuille d'amiante;

– poser les plaques une à une sur cette cheminée et chauffer modérément de façon à obtenir l'évaporation de la majeure partie de l'eau; le degré de séchage souhaitable s'estime d'après la couleur de la plaque qui passe du gris au blanc;

– transférer les plaques dans une étuve à 110° pour parfaire le séchage et pour les activer.

Les couches épaisses réalisées dans ces conditions sont toujours homogènes même sur 2 mm d'épaisseur et permettent une meilleure résolution des différents constituants des lipides. On obtient ainsi, pour un mélange de 50 à 60 mg de lipides solubles dans l'acétone (origine biologique) des bandes qui correspondent aux R_F suivants:

Esters du cholestérol	0.87
Esters méthyliques	0.65
Triglycérides	0.40

en utilisant le mélange d'élution: éther de pétrole–éther sulfurique–acide acétique (90:10:1).

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R_M values in adsorption chromatography

The concept of R_M values developed by BATE-SMITH AND WESTALL[1] has been successfully applied to the analysis of steroids chromatographed in liquid–liquid[2] and gas–liquid[3] partition systems. LEDERER[4] has suggested that this method of analysis might usefully be extended to adsorption systems, using data easily obtained from thin-layer chromatography. This note is intended to show that, although general rules can be formulated for adsorption systems, the precise determination of ΔR_{Mg} and ΔR_{Mr} values (as defined by BUSH[2]) is not as simple as in partition systems. Irregularities in relative elution properties have been noted and it is suggested[5] that these may be attributed to the fact that substances adsorbed onto rigid surfaces can possess different conformations from those which they have in solution. The following results also illustrate these points.

Steroids were chromatographed on Stahl chromatoplates[6] using as adsorbents Silica Gel G and Alumina G (E. Merck & Co., Darmstadt). For the purposes of comparison, the same steroids were chromatographed in a gas–liquid system using as stationary phase QF-1, a fluorinated alkyl silicone polymer[7]. Steroids on chromatoplates were detected as coloured spots after spraying with concentrated sulphuric acid in ethanol and heating at 110° for 10 minutes.

Table I shows that, in general, saturated ketones are eluted ahead of α,β-unsaturated ketones which are eluted ahead of alcohols on Silica Gel G when chloroform is the eluant.

Table II shows the ΔR_{Mr} values for the conversion of a 5α-3-ketone to a 5β-3-

TABLE I

R_F VALUES ON SILICA GEL G WITH CHLOROFORM AS ELUANT

Steroid	R_F*
5α-Pregnane-3,20-dione	0.86
5β-Pregnane-3,20-dione	0.85
5α-Androstane-3,17-dione	0.79
5β-Androstane-3,17-dione	0.65
Pregn-4-ene-3,20-dione	0.76
19-Norpregn-4-ene-3,20-dione	0.60
Androst-4-ene-3,17-dione	0.52
3α-Hydroxy-5β-pregnan-20-one	0.35
3β-Hydroxypregn-5-en-20-one	0.37
17β-Hydroxy-5β-androstan-3-one	0.36
3α-Hydroxy-5α-androstan-17-one	0.33
3β-Hydroxyandrost-5-en-17-one	0.35
20β-Hydroxypregn-4-en-3-one	0.25
17β-Hydroxyandrost-4-en-3-one	0.21
17β-Hydroxy-19-norandrost-4-en-3-one	0.21
5β-Pregnane-3α,20α-diol	0.10
Pregn-5-ene-3β,20β-diol	0.18
5β-Pregnane-3α,20β-diol	0.13
5α-Androstane-3β,17β-diol	0.14

* All steroids were chromatographed on a single plate.

TABLE II

COMPARISON OF ΔR_{Mr} (3-KETONE (5α) → 3-KETONE (5β)) VALUES IN ADSORPTION AND GAS–LIQUID PARTITION SYSTEMS

Compound	ΔR_{Mr} (3-ketone (5α) → 3-ketone (5β))		
	Adsorption systems		Gas-liquid partition system
	Alumina G	Silica Gel G***	6% QF-1 (250°)
Cholestan-3-one	— 0.26*	— 0.07	— 0.04
Pregnane-3,20-dione	+ 0.03*	+ 0.05	— 0.04
Androstane-3,17-dione	+ 0.40**	+ 0.14	— 0.04
17β-Hydroxyandrostan-3-one	+ 0.12**	+ 0.04	— 0.04

* Eluant: chloroform–toluene(1:3).
** Eluant: chloroform–benzene(1:1).
*** Eluant: benzene–chloroform (1:3).

ketone for two adsorption systems and the gas–liquid partition system. It can be seen that in the partition system the 5β-3-ketone is always eluted ahead of its 5α-isomer with a consistent ΔR_{Mr} value of — 0.04. No such relationship is found in the adsorption systems.

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Some pitfalls in studies related to gas chromatography

This report discusses certain pitfalls in studies on natural products involving gas chromatography.

Qualitative and semiquantitative investigations on aroma-bearing constituents frequently involve procedures in which the constituents are ultimately obtained in an ether solution at very low concentrations, *e.g.*, 1 l of solution containing 0.01% solute. The ether solution is then evaporated to a small volume (*e.g.*, 1 ml or less)

for subsequent gas chromatographic analysis. In contrast to strongly concentrated solutions[1,2], the percentage losses of solutes during evaporation of such weakly concentrated solutions may be quite large, as shown in Table I.

TABLE I

LOSSES OF METHYL ESTERS ON EVAPORATION OF SOLVENT FROM ETHER SOLUTIONS BY VARIOUS METHODS

Method	*Method of solvent removal*[a,b]	*Over-all loss (%) of methyl esters of indicated n-acids*				
		C2	*C3*	*C4*	*C6*	*C8*
1	SB → 30 ml → SBC → 1 ml	95	85	96	78	43
2	SB → 10 ml → SN → 1 ml	96	91	97	76	36
3	STC → 30 ml → SBC → 1 ml	93	71	62	37	29
4	SBC → 1 ml	84	75	61	47	0

[a] Each solution contained the following amounts dissolved in either 1 l (methods 1–3) or 30 ml (method 4) of ether: methyl propionate (b.p. 80°), 36 mg; methyl acetate (b.p. 57°), methyl *n*-butyrate (b.p. 103°), methyl caproate (b.p. 151°), and methyl caprylate (b.p. 192°), 20 mg each.
[b] SB = steam bath. STC = 80 cm Stedman column (reflux ratio, 10:1). SBC = 25 cm micro spinning band column (reflux ratio, 7:1). SN = stream of nitrogen at or slightly below room temperature.

The solutes were a homologous series of esters present in very low concentrations in ether*; the concentrations were intended to simulate what may be encountered in a study of aromatic substances. The evaporation procedures included two methods sometimes encountered in the literature: simple boiling off on a steam bath and fractional distillation. The latter was conducted on two columns, one of high throughput and the other of low holdup. Since data on theoretical plate numbers were not available, the columns were conventionally operated under arbitrary conditions which represented a compromise between estimated efficiency and time of distillation. The over-all percentage loss for each procedure was determined by gas chromatography of the concentrates using a standard area–concentration curve for each component. Although minor quantitative discrepancies are observed in Table I, the magnitude of the losses can be approximated. Losses with all methods were high; considering time *vs.* efficiency, it is questionable whether the relatively slow (8 h) fractional distillation (method 3) would represent a significant advantage over the more rapid method 2 for routine use in the type of investigation under discussion. Except for methyl caprylate, the losses on the micro spinning column alone (method 4) were high considering that only 29 ml of solvent were removed. Although this may be indirect evidence that the larger column is more efficient, the exclusive use of this column is not feasible due to its relatively large holdup. Of course, lower losses might be obtained by using higher efficiency columns or reflux ratios or by some other procedure; however, the high ratios of solvent to solute make effective quantitative separation exceedingly difficult. In general, these data emphasize the need for caution in reporting semi-quantitative estimations or the absence of components in such studies without a knowledge of losses due to solvent removal.

A second pitfall concerns traces of impurities in solvents or reagents. Reports occasionally appear in which gas chromatograms from natural products are later

* This mixture would not be expected to form azeotropes[3].

shown to have contained extraneous peaks from solvents or reagents[4]. The traces (*e.g.*, 0.005–0.03 %) of carbonyls, ethanol, etc., found in commercial anhydrous ether of the highest purity are concentrated to some extent on evaporation of the ether and may yield significant peaks on chromatograms. Distillation of such ether before use will reduce but not eliminate these peaks. Blank runs should be made to assure that the extraneous peaks are known or, if necessary, the ether may be further purified by an efficient chemical method, such as that of FIESER[5].

Another pitfall is illustrated by a recent experience in this laboratory. In determining the phenols of tobacco smoke, a method was initially examined which yielded a low (about 10 %) recovery of added authentic phenol. Although a solvent (ether) evaporation step was included in the method, the over-all loss of phenol could not be accounted for entirely by the loss on solvent removal. Interest was centered on a preliminary step in the procedure in which an aqueous 0.5 % sodium hydroxide solution containing the phenol (17 mg per 200 ml solution) was continuously extracted for 24 hours with ether (750 ml) to remove extraneous, water- and ether-soluble, nonacidic substances. Gas chromatography of this ether extraction after concentration showed a significant amount (about 50 %) of the missing phenol therein. Further investigation showed that synthetic sodium phenolate gives a peak for free phenol on gas chromatographic analysis. The most plausible explanation was that continuous extraction with (wet) ether removed small but quantitatively significant amounts of sodium phenolate in a cumulative manner, thus contributing to the over-all loss.

Many other examples of pitfalls could be cited, *e.g.* the inadvertent removal of water-soluble components from ether solutions during washing to remove extraneous acid or base, and the failure to recognize extraneous peaks (usually from laboratory air) during headspace vapor analysis with flame ionization detectors. The continuing development of sensitive analytical instruments makes adequate recognition of methodological shortcomings increasingly necessary.

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Bestimmung von O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-thiophosphat in technischen Produkten nach vorhergehender Abtrennung der Begleitstoffe mittels Dünnschichtchromatographie

Das O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-thiophosphat (I) gehört zu den neuzeitlichen Insektiziden mit niedriger Toxizität gegen Warmblüter[1] und ist unter der Bezeichnung "Verbindung 009"[2], Sumithion[3] und Bayer 41831 bekannt.

Je nach der Technologie der Herstellung kann das technische Produkt ausser (I) noch Begleitverbindungen enthalten, wie: O-Methyl-O,O-di-(3-methyl-4-nitrophenyl)-thiophosphat (II), O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-phosphat (III), 3-Methyl-4-nitrophenol (IV), O-Methyl-O,O-di-(3-methyl-4-nitrophenyl)-phosphat (V), gegebenenfalls S-Alkyl-isomere des Hauptbestandteils (I).

Ähnlich dem Parathion[4-6] sind die Stoffe (I–V) polarographisch aktiv. In der für die Polarographie bestimmten Lösung (enthaltend 40 % Methylalkohol und 60 % Sörensensche Boratpufferlösung mit pH 9.2, zusätzlich 0.02 % Gelatine) ergeben sie gut entwickelte Stufen. Die Halbstufenpotentiale weisen gegen die gesättigte Kalomelelektrode in diesem Medium folgende Werte auf: (I) — 0.69 V; (II) — 0.65 V; (III) — 0.68 V; (IV) — 0.98 V; (V) — 0.64 V. Aus diesen Werten für die Halbstufenpotentiale geht hervor, dass eine direkte polarographische Bestimmung von (I) durch die Koinzidenz der Stoffe (II), (III) und (V) verzerrt wird. Die Bestimmung von (I) wird durch (IV) nicht gestört. Dies ermöglicht die polarographische Verfolgung der Hydrolyse von (I) in einem gepufferten alkalischen Medium gemäss dem Sinken des Grenzstroms von (I). Wir haben festgestellt, dass eine Hydrolyse von (I) im Grundelelektrolyten praktisch nicht in Betracht kommt.

Da, wie erwähnt, eine direkte polarographische Bestimmung von (I) durch die Anwesenheit der Stoffe (II), (III) und (V) verzerrt wird, haben wir den Gehalt an (I) indirekt polarographisch in der Weise bestimmt, dass wir die Begleitstoffe vorher chromatographisch auf einer Schicht, bestehend aus SiO_2 ("Siloxyd"[7]), unter Verwendung eines Gemisches aus Petroläther (Kp. 60–80°) mit einem Gehalt an 1.4 Vol.-% Aceton abgetrennt haben. Die Sichtbarmachung der abgetrennten Verbindungen wurde im U.V.-Licht einer Quecksilberdampfentladungsröhre (Philips Philora HPW 125 W) vorgenommen. Die abgegrenzten Flecke der Begleitstoffe haben wir mittels Vakuum in ein vorbereitetes Saugröhrchen, das nachher als Kolonne dient, abgesaugt[8], mit 4 ml Methanol eluiert und mit dem Grundelektrolyten auf 10 ml ergänzt, worauf wir nach Entfernung des Sauerstoffs die Kurve der Begleitverbindungen im technischen Produkt registriert haben. Unter denselben Bedingungen haben wir die Kurve des technischen Produkts direkt, ohne einer vorhergehenden chromatographischen Abtrennung der Begleitstoffe, registriert. Nach dem Abrechnen der Stufenhöhen des direkt registrierten technischen Produkts haben wir mit Hilfe eines Standards von (I) den prozentuellen Gehalt an (I) ausgewertet. Diese Methode wurde unter Verwendung künstlicher Gemische dieser Stoffe experimentell nachgeprüft. Der relative Fehler bei 5 Bestimmungen überschritt nicht $\pm$ 2 %. Das in der angeführten Weise analysierte technische Produkt wurde gleichzeitig mittels der Methode der Isotopenverdünnung ausgewertet. Für die Isolierung des reinen verdünnten radioaktiven [^{35}S]-O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-thiophosphats haben wir die rasche präparative Dünnschichtchromatographie herangezogen, wie vorher erwähnt, nur in 10-fach

grösseren Mengen. Das Standard-^{35}S-(I) haben wir durch Mikrosynthese hergestellt, und zwar unter Reaktionsbedingungen gemäss[2], und dieses mittels präparativer Papierchromatographie im System Olivenöl–Essigsäure[9] gereinigt. Der Hexanextrakt von ^{35}S-(I) wurde mit dem gleichen Volumen Acetonitril[10] durchgeschüttelt. Das ^{35}S-(I) verblieb im Acetonitril, das Öl in der Hexanschicht. Der radiometrisch ausgewertete Verteilungskoeffizient von (I) im System Acetonitril–Hexan weist den Wert von 38 auf. Die Ergebnisse der Methode der Isotopenverdünnung unterscheiden sich von dem Durchschnittswert der polarographischen Bestimmung desselben Produkts im Rahmen der Fehler der polarographischen Methode.

Die Quantitativität des Absaugens des Adsorbens von der Oberfläche der Glasplatte haben wir mit Hilfe des ^{35}S-(I) untersucht. Dabei haben wir festgestellt, dass auf der abgesaugten umgrenzten Stelle des Fleckens nur 0.2 % der ursprünglichen Aktivität zurückbleibt. Den Verlauf des Eluierens aus der SiO_2-Säule haben wir radiometrisch untersucht. Es stellte sich dabei heraus, dass sich der Hauptanteil der Aktivität in dem ersten ml Methanol vorfindet, dagegen konnte im vierten ml keinerlei Aktivität mehr gemessen werden. Unter Zuhilfenahme der durch präparative Dünnschichtchromatographie gereinigten Standardstoffe (I, II, III, IV) vermochten wir die Anwesenheit dieser Stoffe im technischen Produkt zu identifizieren. Beim Chromatographieren des technischen Produkts beobachteten wir einen wenig intensiven Flecken (R_F 0.86), der nicht identifiziert wurde. Er kommt wahrscheinlich irgendeinem der S-Alkyl-isomeren zu.

Die R_F-Werte von (I, II, III, IV) im angeführten System sind die folgenden: (I) 0.71; (II) 0.36; (III) 0.06; (IV) 0.16.

Die Dünnschichtchromatographie als eine rasche Separationsmethode weist im Vergleich mit der Papierchromatographie eine Reihe von Vorteilen auf[11]. Im gegebenen Fall ist es möglich, diese Methode in geeigneter Weise beim Studium der Stabilität von (I) im U.V.-Licht und der thermischen Zersetzung zu benutzen.

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The separation of the nucleotides of an alkaline hydrolysate of ribonucleic acid by thin-layer chromatography

A number of purines, pyrimidines, nucleosides and nucleotides can be separated by thin-layer chromatography[1-3]. Using the procedure described below, guanylic, uridylic, adenylic and cytidylic acids released by alkaline hydrolysis of ribonucleic acid may be separated by this technique. Ribonucleotides from both yeast ribonucleic acid (L. Light & Co.) and from samples of nucleic acid extracted from rye seedlings by a modification of the method of TRIM[4] have been separated. Ribonucleic acid samples were hydrolysed in 0.3 *N* KOH at 37° for 18 h. Potassium was removed either by precipitation as the perchlorate or by passing the hydrolysate through a small column of cation exchange resin in the hydrogen form. The separated components of hydrolysates were identified by their ultraviolet absorption spectra and by the fact that each corresponded in position on the chromatograms to the appropriate pure nucleotide (British Drug Houses Ltd.) chromatographed by itself adjacent to the mixture.

The thin layers are prepared by suspending 0.5 g of a DEAE-cellulose powder suitable for thin-layer chromatography (Serva-Entwicklungslabor, Heidelberg, Germany) in 4 ml of water. The resulting slurry is poured onto an 18 × 6 cm glass plate and spread in a uniform layer along the plate using a glass rod. The rod is kept at a set distance above the plate by two pieces of wire (310 μ diam.) wrapped around the rod 5.5 cm apart, the diameter of the wire determining how thick a layer of the cellulose is spread. The layer is dried at room temperature and solutions of nucleotides applied about 2 cm from one end.

Two developments of the chromatograms are necessary, the first of which is by propan-1-ol–ammonia (sp. gr. 0.880-ANALAR)–water (60:30:10) at 40° allowing the front to reach at least 7 cm beyond the point of application of the nucleotides (45 min). After drying, the plate is developed again in the same direction by 0.24 *M* acetic acid at 20 to 25° until the front of this solvent just reaches the position reached by the front of the first solvent (15 min). The nucleotides are located by examination of the chromatogram in U.V. light.

The distances travelled by the individual nucleotides relative to the distance from the point of application to the position reached by the solvent fronts are: guanylic acid 0.21; uridylic acid 0.34; cytidylic acid 0.82; (the -2′- and -3′- phosphates of each of these are not resolved) and adenylic acid 0.53, 0.62 (the -2′- and -3′- phosphates separate). The use of a dilute weak acid (0.16 or 0.24 *M* acetic acid) in a single development did not separate guanylic from uridylic acid but did separate the two adenylic acid isomers and the cytidylic acid from one another and from the mixture of guanylic and uridylic acids. In the double development the first solvent separates guanylic acid (nearest the origin) from the other nucleotides and thus makes possible the complete separation on subsequent development with 0.24 *M* acetic acid. Satisfactory separations could not be achieved if the first solvent was freshly prepared using an ordinary reagent grade of 0.880 ammonia. Solvent so prepared had to be left to stand overnight before giving satisfactory results. Using ANALAR ammonia (British Drug Houses Ltd.,) a freshly prepared solvent gave a good separation.

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Separation of sulphidimines of sulphides on paraffin-treated paper

Several methods have been described for the paper chromatographic separation of the sulphidimines prepared from aliphatic sulphides and Chloramine T. LEAVER AND CHALLENGER[1] used both conventional techniques and reversed phase chromatography. PETRANEK AND VECERA[2] also used reversed phase techniques and the sulphidimine prepared by reacting the monochloroamide of *p*-nitrobenzenesulphonic acid with the sulphide instead of Chloramine T.

In studies on the metabolism of marine algae the volatile compounds produced in the cultures were examined by aspirating, into mercuric cyanide, mercuric chloride and mercuric acetate[3,4]. The volatile sulphides were precipitated as co-ordination compounds with mercuric chloride and converted to the sulphidimine by the method of LEAVER AND CHALLENGER[1]. The separation between the sulphidimines from dimethyl sulphide, ethyl methyl sulphide and diethyl sulphide was not sufficient to permit the distinguishing of sulphidimine-like spots, believed to be due to cyclic sulphides, from the former. Tetrahydrothiophene was found to behave in a similar manner when reacted with Chloramine T[5] and recrystallised from benzene.

The purpose of this communication is to suggest that the method of ASATOOR[6] using paraffin-impregnated paper, may with small modifications be used to give improved separation of the short-chained aliphatic sulphides chromatographed as sulphidimines.

Experimental

A strip of Whatman 3 MM paper (23 × 57 cm) was placed in a shallow tray (24 × 60 cm) containing a 5% solution of liquid paraffin (sp. gr. 0.83–0.87) in 80–100° petroleum ether. The solution was allowed to flow over both surfaces of the paper by tilting the tray from side to side and by turning the paper. The paper was dried by

pressing between two sheets of absorbent paper and dried at room temperature for an hour. The solvent was prepared by mixing chloroform, water and liquid paraffin 25:25:1 and allowing it to stand for 12 h. The chromatograms were run by the descending technique for 7 h. The lower organic phase was placed in a dish on the floor of the tank. The sulphidimines were located by spraying with acidified potassium iodide (1 % in 0.2 *N* hydrochloric acid) and heated at 80°.

TABLE I

CHROMATOGRAPHY ON PARAFFIN PAPER

Sulphidimine	R_{methyl}
Dimethyl sulphide	1
Methyl ethyl sulphide	0.72
Diethyl sulphide	0.51
Di-*n*-propyl sulphide	0.20
Ethyl isopropyl sulphide	0.31
Ethyl *n*-propyl sulphide	0.29
Tetrahydrothiophene	0.65

Results

Owing to slight variations in impregnation it was found advisable to use the technique of ASATOOR[6] and express the rate of movement of each sulphidimine spot relative to that of the sulphidimine of dimethyl sulphide. The R_{methyl} values for the average of six experiments are set out in Table I.

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A new detection method for aromatic compounds

In paper chromatography, gaseous compounds are particularly suitable for spot indication as they will not distort the spot. In our institute we have employed iodine vapour as a developer[1].

Owing to its higher vapour pressure and reactivity bromine was also thought to be suitable. A number of organic compounds were exposed to the action of bromine vapour. It was found that, except for a few instances, the spots had only a very slight pale yellow hue, or were colourless, and that as soon as the paper strips were removed

from the bromine atmosphere the spots tended to fade very rapidly. This would indicate that bromine is not as capable of forming adsorption compounds or, when it does form such compounds, they are weaker than those with iodine. This phenomenon may be explained by the atomic weight of bromine which is lower than that of iodine[2]. On the other hand it is likely that bromine, which has a higher normal potential, will have a greater effect upon chromatographed compounds than iodine and will involve them in chemical reactions as well.

It was assumed that when the spots of chromatographed organic aromatic compounds are exposed to the action of bromine vapour, a substitution reaction will probably take place as follows:

$$ArH + Br_2 \rightarrow ArBr + HBr$$

If this reaction actually takes place, hydrogen bromide will be liberated and can be detected with an acid–base indicator.

This assumption has been confirmed by our preliminary experiments. When paper strips with the spots of various aromatic compounds were exposed to bromine vapour and subsequently sprayed with the solution of a suitable acid–base indicator after removal of excess bromine vapour by standing the papers in the air, the spots of a number of compounds showed up in a vivid red colour. Since HBr is a strong acid dimethyl yellow (4-dimethyl amino-azobenzol; colour change red to yellow, pH 2.9–4.0) proved more suitable for our purpose.

Procedure

After development and removal of the acid and basic solvents the papers are placed in a desiccator (apparatus shown in Fig. 1). About 10 to 15 ml of bromine are placed in the glass bottle and bromine vapour is pumped into the desiccator for 5–10 sec using a nitrogen cylinder at a pressure of 0.5 atm. After leaving the bromine for 1–2 min, the papers are aerated for 15–20 min in a fume chamber with a good draught; then they are sprayed with a 0.5% alcoholic solution of dimethyl yellow. The spots of the reacting compounds will show up in red on a yellow background.

In the paper chromatographic separation of basic materials for drugs, the following developers have been employed: *n*-butanol–water–glacial acetic acid (4:5:1) and *n*-butanol saturated with 1.5 *N* ammonium hydroxide. Development of the chromatogram was by the procedure described in an earlier paper[3]; the only difference was a longer period of drying in order to remove completely the acetic acid and the ammonia.

Table I gives the sensitivity of some organic compounds employing the above reaction.

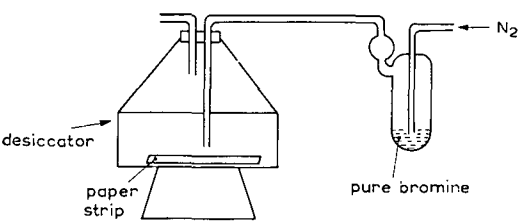

Fig. 1. Apparatus for detection of spots.

It should be noted, however, that some impurities in the paper migrate with the solvent front and are also coloured red in the process of development; consequently, they are apt to impair the reaction of compounds with R_F values close to 1.0. This

TABLE I

Denomination of substance	*Quantity of substance applied µg*	
	1	*2*
Acetanilide	10++	15+
Amidazophen	5+	10+
Atropine sulphate	50+	30 (yellow)
Azophen	5+++	5+++
Thiamine hydrochloride	20+	30+
Ethyl-morphine chloride	15+	15+
Phenacetin	30+++	15++
Phenolphthalein	15+++	10+++
Physostigmine salicylate	30++	30+
Hexamethylenetetramine	30 (yellow)	15 (yellow)
Potassium guaiacol-sulphonate	10+	10++
Codeine chloride	30++	30++
Caffeine	20++	20++
β-Naphthol	10+	10++
Methyl *p*-hydroxybenzoate	15+++	15+++
Ethyl *p*-aminobenzoate	10+	10+
Sodium [1-phenyl-2,3-dimethyl-pyrazolon-(5)-yl-(4)]-methyl-aminomethanesulphonate	10++	10++
Papaverine hydrochloride	30++	60+
Resorcinol	10+++	10+++
Salicylic acid	10++	10++
Strychnine nitrate	30+	30
Sulphanilamide	15+++	15+++
Amethocaine hydrochloride	30++	15+++

Mixtures: 1 = Butanol–glacial acetic acid–water (4:1:5).
2 = Butanol saturated with 1.5 N ammonia.
+ = definitely noticeable; ++ = strong; +++ = very strong.

effect can be eliminated by washing the paper with distilled water prior to chromatography.

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Rapid detection of gallic acid esters by centrifugal chromatography

Gallic acid esters are used in the food industry as antioxidants for oils and fats. Some of them are even among the antioxidants most often used (*e.g.* propyl gallate, being intermediate between the natural antioxidants (α-tocopherol, flavonoids, etc.) and the synthetic ones, not occurring in nature (BHA, BHT*). The amount of antioxidant that is permitted as additive to fats is very low, and usually does not exceed 0.01–0.05 % of fat weight; hence accurate identification and determination of these substances is often difficult, especially where various mixtures of antioxidants have been used for fat stabilisation.

In all the methods so far described, antioxidants are isolated from fats by extraction, usually with alcohol. Their detection is effected either by chromatographic separation on paper[1–4] (acetylated paper, or with a stationary less polar phase), or by separation on chromatoplates[5–8] (silica gel or polyamide powder). The latter method is preferable, mainly on account of its greater rapidity.

Centrifugal chromatography has recently been found increasingly useful in the detection of various groups of substances; it has the advantage of shortening the time taken for chromatographic separation. Centrifugal chromatography can be carried out in the simple apparatus described by DEYL, PAVLÍČEK AND ROSMUS[9]. In the present work the method was used for rapid separation and detection of gallic acid esters.

Experimental

Apparatus. Centrifugal chromatography was carried out in a simplified version of the rotary chromatographic apparatus described by PAVLÍČEK AND DEYL (number of revolutions 700/min). Whatman No. 3 paper was used for the chromatographic separation; the diameter of the chromatographic paper disc was 18 cm.

Solvent systems. Methanol–benzene in various ratios; methanol–carbon tetrachloride in various ratios; water–ethyl acetate (97.5:2.5); ethyl acetate; ethyl acetate saturated with water; methyl acetate; butyl acetate; and the classical mixture: *n*-butanol–acetic acid–water (4:1:5).

Procedure. About 0.05 ml of methanolic solution of the respective gallate is spotted on the start at a distance of about 1.5 cm from the centre of rotation. After drying, the chromatographic paper is stretched on and fixed in the rotary chromatographic apparatus, which after having been perfectly sealed is put into operation. The inflow of the solvent introduced through a special capillary tube, is adjusted in such a way as to prevent any possible irregularity of the flow during the chromatographic process. When the solvent system has advanced about 14 cm, the operation is interrupted, the chromatogram removed, dried and the substances detected with an alcoholic 1 % ammoniacal 1 % silver nitrate solution.

Results

Various mixtures of gallates were separated by centrifugal chromatography, using the above-mentioned solvent systems. The results are given in Table I. The most suitable solvent system proved to be a mixture of carbon tetrachloride and methanol in the

* BHA = 2(and 3)-*tert.*-butyl-4-methoxyphenol; BHT = 2,6-di-*tert.*-butyl-*p*-cresol.

TABLE I

R_F VALUES OF GALLIC ACID AND ITS ESTERS

Solvent system	R_F					
	GA	*MG*	*EG*	*PG*	*OG*	*LG*
Ethyl acetate	0.53	0.81	0.88	0.94	0.97	0.98
Ethyl acetate satd. with water	0.50	0.89	0.92	0.93	0.94	0.95
Methanol–benzene (1:9)	0.0	0.30	0.38	0.58	0.88	0.98
Methanol–benzene (1:4)	0.06	0.35	0.46	0.58	0.95	0.98
n-Butanol–acetic acid–water (4:1:5)	0.78	0.88	0.91	0.93	0.97	0.97
Carbon tetrachloride–methanol (4:1)	0.08	0.33	0.45	0.50	0.86	0.95
Methanol–benzene (1:19)	0.00	0.07	0.12	0.29	0.62	0.78
Methanol–benzene–amyl alcohol–water (2:1:1:1)	0.67	0.92	0.97	0.97	0.98	0.98

GA = gallic acid; MG = methyl gallate; EG = ethyl gallate; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate.

ratio 4:1. The advantage of centrifugal chromatography lies especially in its rapidity; in addition, the separation of some substances with close R_F values is better than with the usual arrangement. Thus, a good separation of gallic acid esters in the solvent system carbon tetrachloride–methanol (4:1) takes 5–6 hours without centrifugal force; in the rotary arrangement the same substances are separated within 30–45 minutes. Whatman No. 3 chromatographic paper proved best for the purpose; the capacity of the method is also fairly high.

We were successful in separating even highly complex mixtures of gallic acid esters. The method was verified in the separation of gallic acid esters isolated from stabilized fats. For this purpose about 100 g of fat was weighed out and mixed with 100 ml petroleum ether. This solution was then extracted 3 times with 50 ml 60% ethanol each time; the combined alcoholic extracts were evaporated on a water bath, and the dry residue dissolved in 1 ml methanol. This solution was then spotted on the chromatographic paper. We have observed that the R_F values of substances isolated from fats are lower than those of the corresponding pure gallates, owing to the presence of residues of fat, fatty acids and other lipoidic substances. Their presence, however, has no substantial influence on the quality and accuracy of the separation.

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Micro-electrophoresis of mucopolysaccharides on agarose gel

The human bloodvessels contain several mucopolysaccharides. In the course of our investigations on anticoagulant activities of extracts from human aorta, we developed a micro-method, which permits the separation and analysis of mucopolysaccharides, using only microgram amounts of material.

The method is based on the micro agar gel electrophoresis according to WIEME[1]. Using commercial agar* in preparing the gels for electrophoresis, we found that the agar gel itself develops a considerable metachromasia on staining with Toluidine Blue or Azure A. From a theoretical point of view, it could be suspected that the sulphate groups in the agar molecules were responsible for the metachromatical staining. Consequently we prepared sulphate-free agar ("agarose") according to the original method of ARAKI[2] and we did in fact observe that the gels prepared from this agarose no longer yield a metachromatic stain.

In this paper the technique of micro-electrophoresis of mucopolysaccharides will be described. Results obtained with this method in studies of heparin and its derivatives will be published elsewhere (JAQUES *et al.*[3]).

Material and methods

Material. The heparin sample used was heparin Leo. Chondroitinsulphuric acid A and hyaluronic acid were gifts from Dr. SZIRMAY and Dr. DOYLE.

Agarose was prepared from Difco Agar Noble, according to ARAKI, with technical modifications in the procedure. A description of a similar procedure is given by HJERTEN[4].

Barbiturate buffer pH = 8.6, containing 10.4 g sodium barbiturate and 1.84 g barbituric acid.

Methods

Electrophoresis. This was performed in a gel of 0.9% agarose in barbiturate buffer, pH 8.6, according to the micro-method of WIEME[1] on microscope slides. A voltage of 20 V/cm was applied. The electrophoresis time was about 7 min. Cooling during electrophoresis was achieved with the aid of petroleum ether, boiling range 28–40°.

Fixation. (a) For mucopolysaccharides: after the electrophoretic run, the slides were immersed in a solution of 0.1 % Cetavlon in distilled water for 1 h, to precipitate the mucopolysaccharides. In later experiments the Cetavlon solution was prepared with physiological saline to obtain maximal precipitation.

(b) Combined fixation for proteins and mucopolysaccharides: the slides were treated for 15 min with a mixture of 1 vol. neutralized formol and 4 vol. methanol. Afterwards they were immersed for 1 h in 0.1 % Cetavlon solution.

Drying. The slides, after fixation of the mucopolysaccharides, were dried at 37° in the usual manner by covering with filter paper.

Staining. (a) For mucopolysaccharides: the slides were stained for 15 min either in a solution of Toluidine Blue or in a solutionof Azure A. The background was rinsed colourless in 1 % acetic acid solution.

* Difco "Agar Noble"; Reinagar, Behringwerke; "Oxo-agar", Oxo Ltd.

Staining solutions

Toluidine Blue	40 mg		Azure A	20 mg
distilled water	20 ml		distilled water	5 ml
dry acetone	80 ml	or	acetone	40 ml
			methanol	180 ml

(b) Combined staining for proteins and mucopolysaccharides: the slides may be stained according to HEREMANS AND VAERMAN[5] with Alcian Blue and Azocarmine.

In our hands better results were obtained by the following procedure:
(1) 15 min in Toluidine Blue solution.
(2) Rinse background colourless in 1 % acetic acid solution.
(3) Stain for 5 min with a solution of 300 mg Lissamine Green in 100 ml 1 % acetic acid.
(4) Rinse in 1 % acetic acid solution.

The mucopolysaccharides will be stained red-purple, while the proteins are stained green.

Results

With the method described it is possible to analyse mucopolysaccharides using very small quantities. Heparin can still be demonstrated in a solution which contains 0.01 mg/ml, chondroitin-sulphuric acid A in a concentration of 0.1 mg/ml and hyaluronic acid in a concentration of 0.1 mg/ml.

The smallest absolute quantity of heparin, still demonstrable is 0.01–0.02 γ. With the pH and the buffer system used in our studies, a good separation of serum proteins and mucopolysaccharides is obtained (Fig. 1). The electrophoretic mobility of heparin is about twice that of human serum albumin. Chondroitin-sulphuric acid A has a somewhat smaller velocity, while hyaluronic acid has the smallest mobility (Fig. 2).

The method described in this paper is also suitable for direct tissue electrophoresis. Analysis of bone marrow can easily be made.

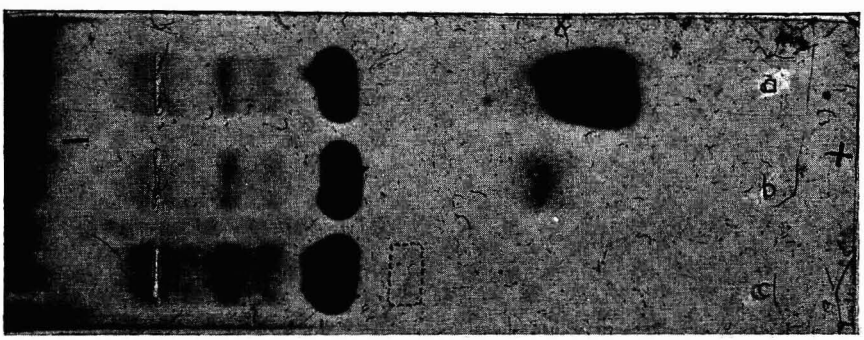

Fig. 1. Microelectrophoresis on agarose gel of a mixture of serum and mucopolysaccharides. Barbiturate buffer pH 8.6; combined staining for proteins and mucopolysaccharides. (a) Human serum containing heparin in a concentration of 0.5 mg/ml. (b) Human serum containing chondroitin-sulphuric acid A in a concentration of 0.5 mg/ml. (c) Human serum containing hyaluronic acid in a concentration of 0.5 mg/ml. The spot of the hyaluronic acid (indicated by dotted lines) is not visible in the reproduction.

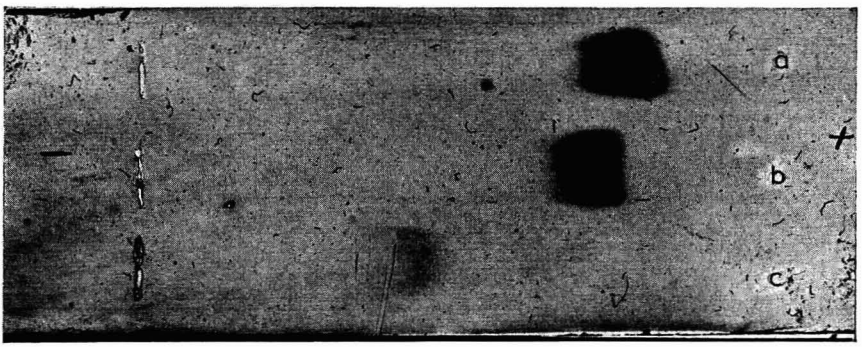

Fig. 2. Microelectrophoresis on agarose gel of mucopolysaccharides. Barbiturate buffer pH 8.6; Toluidine Blue staining. Absolute amount of material applied on the strip: (a) heparin 0.5 γ; (b) chondroitin-sulphuric acid A 0.5 γ; (c) hyaluronic acid 0.5 γ.

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A simple device for maintaining a constant pressure in liquid–solid chromatography

In liquid–solid chromatography work it is generally necessary to exert a pressure on the eluant solution, so that it passes through the column at the required flow rate.

In order to have a constant flow rate during a run (after the solid adsorbent has packed down) it is necessary to maintain a constant air pressure on the eluant. In general, the required pressure is not very high and, according to the case, varies from 50 to 400 mm Hg. A low pressure compressed air line is therefore necessary, and it is important that the pressure does not undergo considerable changes.

In our laboratory an efficient constant pressure device has been set up. It is illustrated in Fig. 1, variations of pressure do not exceed 1–2 mm Hg. The device is easily constructed, and has been used successfully during several months of uninterrupted work.

The air compressor A provides air at a pressure of 8–9 kg/cm² which is reduced to the required value by the pressure reducing valve C and is then collected in the receiver E. The air at low pressure in E then passes solenoid valve F and goes on one side to the

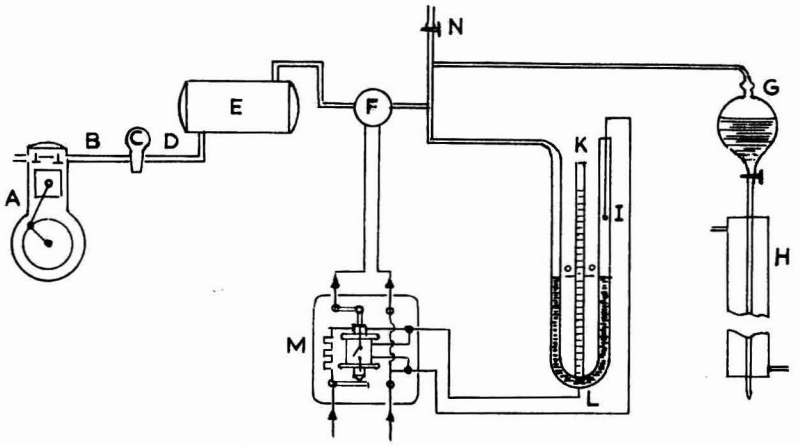

Fig. 1. Schematic diagram of device. A = Air compressor. B = High pressure line. C = Pressure reducing valve. D = Low pressure line. E = Low pressure air reservoir (capacity: 20 l). F = Solenoid valve. G = Eluant reservoir. H = Column. I = Movable electric contact. K = U-shaped tube. L = Fixed electric contact. M = Relay. N = Stopcock.

eluant solution contained in the reservoir G connected by an air-tight joint to the chromatographic column H. On the other side it passes into the U-shaped tube K containing mercury, which is in electrical contact with the relay M through L. The air pressure causes the mercury to rise in the branch of the U-shaped tube in which there is a movable contact I. When the mercury reaches this contact, it closes the circuit between I and L in such a way that it operates the relay M which opens the feeding contact of the solenoid valve F, which closes and stops the compressed air flow. Thus the pressure in the apparatus to the right hand side of the solenoid valve will remain constant at some prefixed value dependent on the level of I unless the pressure is lowered, as a result of a fall in the eluant solution level, or owing to a small leak. The mercury meniscus then breaks contact with I, the relay closes the feeding contact, the solenoid opens to admit air from E which raises the mercury levels and re-establishes the contact between I and L. By adjusting the pressure at a value slightly higher than the pressure required in the column, the variations of the air pressure in this section will not be more than 1–2 mm Hg.

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News

EUROPEAN SOCIETY FOR BIOCHEMICAL PHARMACOLOGY

Biochemical methods are becoming increasingly important for the development of new drugs and thus a new branch of medical sciences, on the borderline between pharmacology and biochemistry, and applying techniques from both disciplines, has come into being, biochemical pharmacology. In view of the great potentialities of this new field of investigation, a new Society, the European Society for Biochemical Pharmacology, has been formed with the aim of promoting collaboration and personal contacts between scientists engaged in this type of research, particularly in European Countries.

The constitutional meeting of the Society was held at the Istituto Superiore di Sanità in Rome, February 6th.

The Organizing Committee has elected Dr. E. B. CHAIN, F. R. S., Professor of Biochemistry, Imperial College of Science and Technology, London and Scientific Director of the International Centre for General Biochemistry and Chemical Microbiology, Istituto Superiore di Sanità, as President. The Organizing Committee consists of the following scientists: Prof. Z. M. BACQ (Liège); Prof. C. HEYMANS (Ghent); Dr. J. M. BARNES (Carshalton, Surrey); Dr. E. C. DALGLIESH (Stoke Poges, Slough, Bucks.); Prof. C. E. DODDS (London); Prof. A. HADDOW (London); Dr. A. SPINKS (Macclesfield, Ches.); Prof. P. DESNUELLE (Marseille); Dr. J. JACOB (Paris); Dr. J. THUILLIER (Paris); Prof. P. HOLTZ (Frankfurt/Main); Prof. H. MASKE (Hoechst, Frankfurt/Main); Prof. R. PFLEGER (Bemberg); Prof. G. B. MARINI-BETTÒLO (Rome); Prof. A. DI MACCO (Milan); Prof. S. GARATTINI (Milan); Prof. G. GIACOMELLO (Rome); Prof. A. SOLDI (Milan); Prof. E. TRABUCCHI (Milan); Prof. B. UVNAS (Stockholm); Dr. H. LANZ (Basel); Dr. A. PLETSCHER (Basel).

Applications for membership and all other enquiries should be addressed to the Secretary, Prof. RODOLFO PAOLETTI, Institute of Pharmacology, University of Milan, Via A. del Sarto 21, Milan, tel. 719 060.

The first general meeting is planned to be held in Milan, in Spring 1964.

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EDITORIAL

Two years ago we were forced, as a result of the number of papers awaiting publication, to increase the yearly page number from 1200 to 1800. We have now arrived at a stage where unless we increase again the number of pages we shall be forced to reject over 25 % of all acceptable papers which are submitted. It was thus decided to publish four volumes of 600 pages each in 1964.

This volume of publication on a topic which is not even a branch of a science but only a technique comes as a surprise both to the editor and the publishers. We can suggest only two possible explanations, either the technique which has very wide applications is being exploited rather quickly, or chromatography is not a technique but a new approach to chemistry. In the first case we would expect a decrease soon in the volume of research, in the latter even a further increase is not unlikely.

While announcing the (temporary?) increase in the annual volume number we would also like to announce two further alterations. Review articles will not appear in the next volumes of the Journal of Chromatography but only in the "Chromatographic Reviews" in order to make more space for original papers. The bibliography section will, however, be expanded to include also thin-layer chromatography and gas chromatography. These new sections will be under the editorship of Dr. K. MACEK.

THE APPLICATION OF A LONG-PATH INFRA-RED CELL IN GAS CHROMATOGRAPHY

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INTRODUCTION

Several recent papers[1-4] have described the use of gas–liquid chromatography to separate the components of mixtures containing unknowns, followed by infra-red spectroscopy for their identification. Other papers have described the combination of gas–liquid chromatography with other spectroscopic techniques[5-7]. Two approaches may be distinguished. The fractions after leaving the column and detector may be condensed out and subsequently transferred to a suitable gas or liquid infra-red cell. This procedure has obvious advantages with relatively involatile samples, provided the quantities are large enough to be manipulated.

Alternatively the gas stream may be led directly from the detector into an infra-red gas cell. This approach avoids the loss of time, and possible loss of sample involved in condensing it before examination. It has, however, the disadvantage that under normal gas–liquid chromatographic conditions, the concentrations, at least of minor components, are too small for sufficiently intense spectra to be obtained in a normal infra-red gas cell of about 10 cm path. One means of overcoming this difficulty is the use of mechanical or electrical scale expansion on the infra-red spectrometer. The degree of expansion is, however, limited to about five-fold, if a wide wavelength range is to be scanned, by imperfections of the background trace obtained with a double-beam spectrometer. Scale expansion also leads to loss of sensitivity, which must be compensated for by increasing the amplifier gain or slit-widths.

A long-path cell therefore offers a better method of obtaining the necessary band strength. The present paper describes the construction of such a cell, and illustrates its use in obtaining the infra-red spectra of chromatographically separated components.

EXPERIMENTAL

(a) *Long-path cell*

Since long-path cells of small volume are not normally available for commercial spectrometers, a simple cell has been constructed to fit the double-beam unit of the Grubb-Parsons G.S. 2A spectrometer. The design uses the conventional 3-mirror arrangement. Fig. 1 shows how the cell is arranged with respect to the spectrometer optics. The path of the central ray through the cell is indicated for sixteen passes. In order to retain a sharp image on the entrance slit, the horizontal axis of the long-path cell is inclined at approximately 20° to the normal beam direction, so that the

final image falls at the correct distance from the slit. The two mirrors M_1 and M_2 (2.2 cm square) and the larger mirror M_3 (3.0 by 1.7 cm) were all cut from a single concave mirror of 7½ cm focal length, and subsequently surface aluminised. These mirrors and the plane mirrors M_4 and M_5 were mounted on a ¼ in. brass plate, which in turn was fixed at the correct height by a support screwed on to the double beam unit. The cell could thus be removed and replaced as required without loss of alignment.

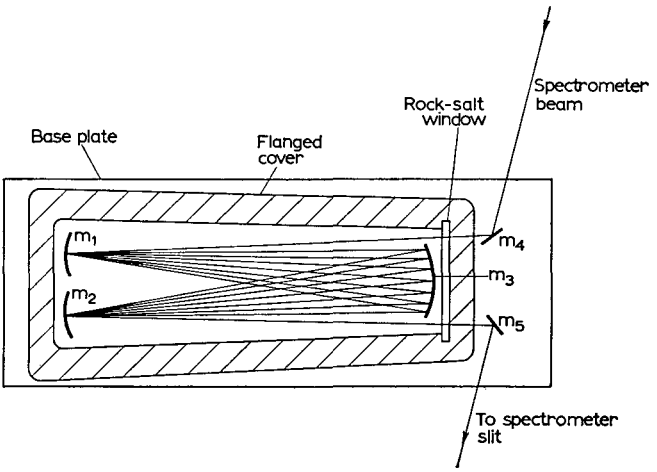

Fig. 1. Plan of long-path cell.

Up to 7 images could be obtained on M_3, corresponding to 16 passes, giving a path-length of 240 cm. After the optical adjustments were made, the cell was completed by covering the mirrors with a flanged brass cover of minimum volume (about 130 c.c.), one end of which was fitted with a rock-salt window. The flange rested on a rubber ring which fitted into a groove in the base-plate. Sufficient pressure could be obtained on the flange by means of clips to provide a gas tight seal. With this arrangement about 40 % transmission was obtained through the cell. An attenuator was placed in the blank beam so that full scale deflections could be obtained.

(b) Gas chromatography

A 4 ft. by 4 mm column packed with 20 % dinonyl phthalate on 60–30 mesh "Celite", was used, and could if necessary be heated by means of an electrically heated jacket. A flame ionisation detector was used, in which about 5 % of the carrier gas stream (nitrogen) was diverted to the flame. This flame was maintained by independent supplies of air and 50/50 hydrogen/nitrogen. Satisfactory signal-to-noise characteristics were obtained when this detector was connected to an amplifier of Type 1E/114 (Gas Chromatography Ltd.) and a Type DSP (2) Sunvic Recorder. Minimum lengths of polythene tubing were used to connect the detector to the column and long-path cell, so as to avoid remixing of fractions. The column was operated with its outlet at atmospheric pressure.

(c) Mode of operation

Because of the relatively large volume of the cell, it was not feasible to flow the

effluent gas continuously through it, since remixing of the separated components would occur. The following procedure, however, proved satisfactory, using two three-way taps connected as shown in Fig. 2.

Gas flow through the system was started, tap T_1 being turned to waste by-passing the long-path cell, until the emergence of a fraction from the column was indicated by the detector. The gas flow was then diverted to the cell and the infra-red spectrum

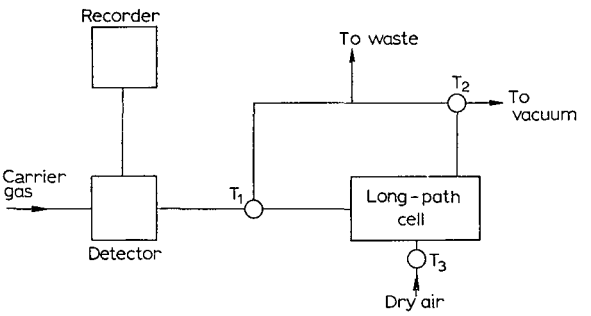

Fig. 2. Arrangement of chromatograph and long-path cell.

scanned rapidly by hand until a band was found. The spectrometer was held at this wavelength until maximum absorption was obtained. About 30 sec normally elapsed between attainment of maximum concentration in the detector and in the gas cell. Taps T_1 and T_2 were then closed, the gas flow stopped and the spectrum of the fraction measured, usually from 5–15 μ. This takes about 4 min. Except with very close sample peaks, diffusion on the column during this period was insignificant. The long-path cell was then evacuated via T_2, and refilled with dried air through T_3, after which gas flow was restarted, by-passing the cell until the next fraction was detected. The procedure was then repeated.

RESULTS

The apparatus was tested by separating several synthetic mixtures, and obtaining the infra-red spectra of the components. Three examples are illustrated below.

(*a*) *Separation of ketones*

One drop (about 3.5 μl) of a mixture of equal amounts of acetone, methyl ethyl ketone, methyl isopropyl ketone and diethyl ketone was placed on the column with a hypodermic syringe, and eluted at room temperature by nitrogen at 20 ml/min. Fig. 3 (a) shows the resulting spectra. They are sufficiently strong for the components to be readily identified by comparison with the spectra of the corresponding liquids given in the Sadtler collection.

(*b*) *Separation of esters*

Fig. 3(b) illustrates an identical experiment involving ethyl formate, ethyl acetate, isopropyl acetate and ethyl propionate. Again a spectrum of good quality was obtained from each component.

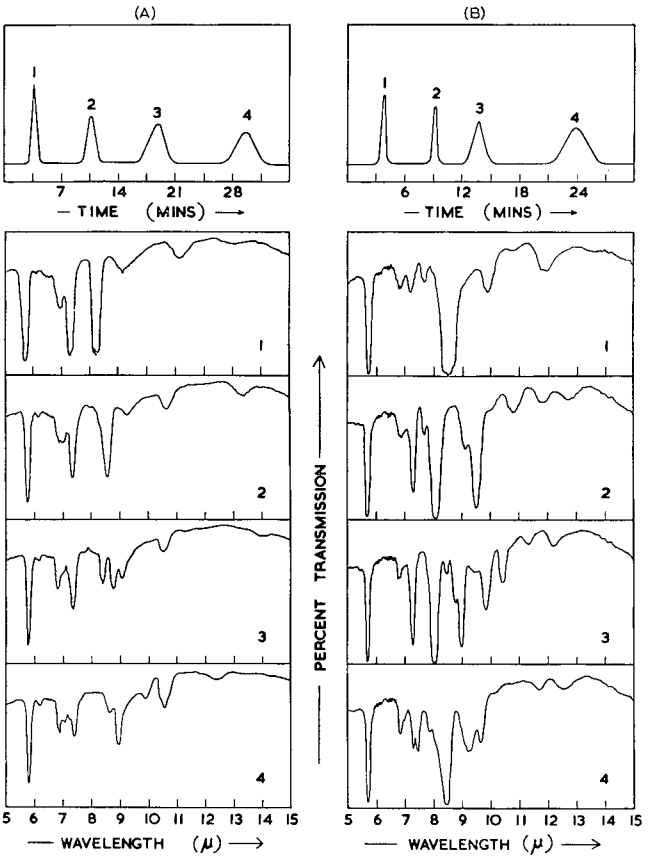

Fig. 3. Gas chromatograms and infra-red spectra. (a) Separation of ketones. (1) acetone; (2) methyl ethyl ketone; (3) methyl isopropyl ketone and (4) diethyl ketone. (b) Separation of esters. (1) ethyl formate; (2) ethyl acetate; (3) isopropyl acetate and (4) ethyl propionate.

(c) *Separation of a ten-component mixture*

A rather more rigorous test was made on a mixture of equal parts of diethyl ether, diisobutyl ether, acetone, methyl isopropyl ketone, isopropyl acetate, ethyl propionate, cyclohexane, toluene, 1-pentanol and cyclohexanol. Two drops (7 μl) of sample were used, and because of the wide variation in the volatility of the components, the column temperature was increased from 37° to 90° during the experiment. Once again, as shown in Fig. 4, an adequate spectrum of each component was obtained. Similar separations involving mixtures of ethers, hydrocarbons and alcohols were also carried out. It must be stressed that in separations of this kind, the limiting factor in the quality of the infra-red spectra is the degree of separation of the components on the column. Satisfactory spectra can be expected only if resolved peaks are obtained in the chromatogram.

In order to assess the value of the method in the identification of small amounts of impurity, one drop of a solution of 1 % of acetone in ethyl acetate was added to the column, and the spectrum of the acetone determined. The three strongest peaks in its

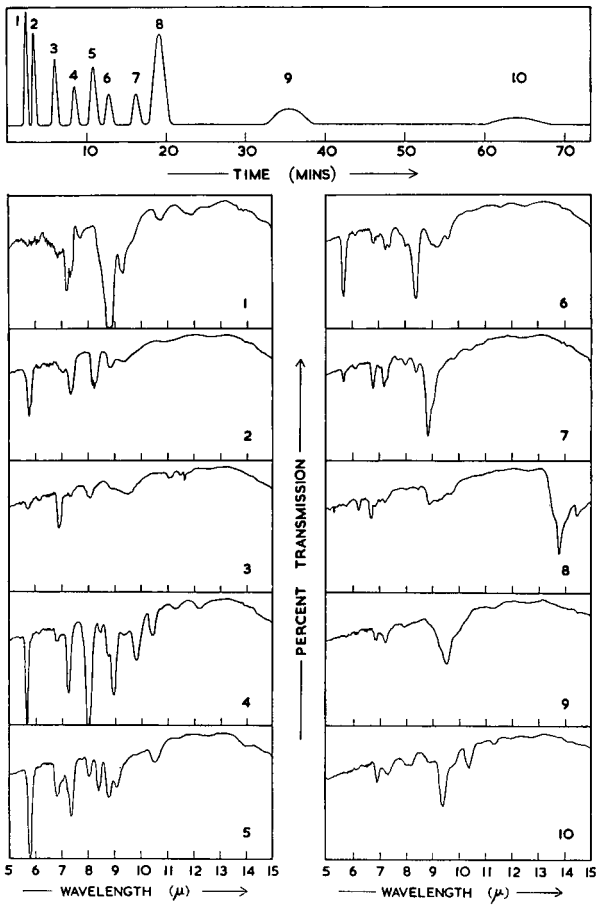

Fig. 4. Gas chromatogram and infra-red spectra of components of a ten-component mixture. (1) diethyl ether; (2) acetone; (3) cyclohexane; (4) isopropyl acetate; (5) methyl isopropyl ketone; (6) ethyl propionate; (7) di-isobutyl ether; (8) toluene; (9) 1-pentanol; (10) cyclohexanol.

spectrum, at 5.75, 7.3 and 8.2 μ showed optical densities of about 0.1. This indicates that in favourable cases as little as 0.00003 g of an impurity could be identified.

ACKNOWLEDGEMENTS

We are grateful to Mr. R. A. SAUNDERS for helpful discussions and to Mr. G. PERRY for experimental assistance.

SUMMARY

A long-path infra-red cell has been used to obtain the spectra of the separated components of mixtures, as they emerge from a gas chromatograph. The advantages and limitations of this method of identifying these components are considered.

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THE APPLICATION OF GOLAY COLUMNS OF LARGER INTERNAL DIAMETER

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INTRODUCTION

The use of capillary columns for the gas chromatographic separation of volatile substances dates from the pioneer research of GOLAY[1] by whose name such columns are also sometimes recognized. The various applications of Golay columns have been described by numerous later authors[2], and, since it appears that no doubt exists as to the reproducibility of their manufacture[3] and characteristic operational features for quantitative and qualitative analysis, the way is now clear for their general application in gas chromatography. The work described here is concerned with the use of Golay colums with internal diameters of 1.0 mm. The geometrical parameters, the separation efficiency, the loading capacity and the necessary initial instrumental requirements by which the first results were obtained are separately discussed[4].

THEORY*

By analogy with the phenomena attending distillation and liquid–liquid partition, it is customary in gas chromatography (GC) to adopt the working hypothesis of the "theoretical plate height" (usually designated HETP) and the concept of the "number of theoretical plates (n)", factors which are interrelated with the column length L by the expression:

$$\mathrm{HETP} = \frac{L}{n} \tag{1}$$

VAN DEEMTER AND GOLAY have furnished a further equation to provide a relation between the geometrical parameters and other variable quantities resulting from the use of the columns:

$$\mathrm{HETP} = \frac{B}{u} + [C_G + C_L]u = \frac{2D_G}{u} + \frac{r^2}{D_G}\left[\frac{1 + 6k + 11k^2}{24\,(1 + k)^2}\right]u + \frac{d_F{}^2}{D_L}\left[\frac{2k}{3\,(1 + k)^2}\right]u \tag{2}$$

The minimum HETP is given by eqn. (3):

$$\mathrm{HETP}_{\min} = 2\sqrt{[B\,(C_G + C_L)]} \tag{3}$$

* For a list of symbols used, see p. 450.

If one considers C_L to be very small compared with C_G (which is permissible to a first approximation), eqn. (4) follows:

$$\mathrm{HETP_{min}} = \frac{d}{2}\sqrt{\left[\frac{1 + 6k + 11k^2}{3\,(1 + k)^2}\right]} \qquad (4)$$

which provides a simplified, direct relation between $\mathrm{HETP_{min}}$ and the column diameter d. This equation provides the reason for the concentration of our past work on the use of the smallest possible column diameter (in the region of 0.25 mm).

The further diminution of column diameter, to attain lower plate heights, is limited by purely practical problems of tube-stretching, the quality of the tube's inner surface, the loading capacity and the pressure gradient along the column length.

Since, in order to attain a small HETP, a small film thickness, d_F, of the stationary phase must also be chosen the loading capacity of the Golay column is perforce restricted. It is already evident that, for sample sizes of 0.5–1 μl, stream-splitting before the column in the ratio 1:100 to 1:200 must be adopted, to avoid "overloading". It is very difficult to separate such small samples of mixtures containing many, equally distributed components, and this at the same time gives rise to the need for a highly sensitive detector, *e.g.* a flame ionization detector (FID).

In three particular instances it is especially desirable to increase substantially the loading capacity of Golay columns:

(1) In the case of mixtures of widely differing component-concentrations (*e.g.* so-called trace analyses), the given sample size must be so chosen that the least abundant component present is estimable by the detector. In order to maintain simultaneously the main components in the linear part of the distribution isotherm, a highly sensitive detector (FID) must be combined with a column of greater capacity.

(2) At present the efficiency of GC in the separation of mixtures by the use of Golay columns is essentially higher than in the identification of single components. It is understandable, therefore, that identification is usually made by another method. Since it is not always possible to make use of a mass spectrometer[5] for this purpose, it is necessary to collect the separated components at the base of the column. Although a higher concentration ratio between substance and carrier gas is obtainable in the case of Golay columns of small diameter, such small absolute sample sizes are not suitable for analysis by other chemical or physico-chemical methods. The combination of the separation efficiency of Golay columns with their ability to "prepare" components therefore primarily requires an increase in their loading capacity.

(3) The wide application of GC in industrial routine analysis demands a careful examination of the balance between the initial outlay on equipment and the issuing analytical results. Moreover, the high number of theoretical plates (from about 100000) of Golay columns, operable only in conjunction with an FID, is by no means essential for all analytical problems. In numerous cases numbers of theoretical plates from 20000–25000 suffice, and in this instance, macro-Golay-columns may be introduced without difficulty and run in conjunction with a simple thermal conductivity detector (TCD)[4].

It is evident from these considerations that under three different sets of problematic conditions it is desirable to increase the loading capacity of Golay columns. This can

be achieved by raising the "working volume V_n" of a plate. For the latter, KEULEMAN'S[6] equation applies:

$$V_n = \frac{V_G + KV_L}{n} \tag{5}$$

It is possible to increase the working volume under constant geometric conditions (constant V_G) by raising the volume V_L of the stationary phase. In the case of Golay columns this in effect means an increase in film thickness d_F. The stability of the film, however, decreases with increasing thickness, so that the manufacture of such "thick film columns" is not recommended. By changing the geometric parameters (*e.g.* by increasing the column diameter) one can profitably alter the gas volume V_G in a simple way. The volume V_L of the liquid phase is also increased, and the desired increase in plate volume is attained.

Equation (4) shows that an increase in $HETP_{min}$ is paralleled by a drop in the number of plates. Table I reports the gas volumes V_G attained in the case of different geometrical parameters.

For the successful application of Golay columns, the magnitude of the film thickness d_F in relation to the column diameter d, is of great importance.

TABLE I

GAS VOLUMES V_G OF GOLAY COLUMNS WITH DIFFERENT DIMENSIONS

Column radius r (mm)	*Column length L (m)*	*Gas volume V_G (ml)*
0.125	25	1.2
0.125	50	2.4
0.250	50	9.7
0.500	100	78.0

Since, in all analytical separations, the time required for analysis is an important factor, the interdependence of film thickness, column diameter, gas volume, and carrier gas flow, is discussed below, taking into consideration several k values for different substances.

From the simple relations:

$$k = \frac{t_r'}{t_M} = K \cdot 4 \frac{d_F}{d} \tag{6}$$

and:

$$t_M = \frac{V_G}{u} \tag{7}$$

the corrected retention time t_r' is obtained:

$$t_r' = K \frac{4 d_F}{d} \cdot \frac{V_G}{u} \tag{8}$$

From eqn. (8) the following conclusions can be drawn:

Columns possessing a relatively high gas volume V_G will show a longer analysis time for a particular separation than columns with smaller gas volumes (if the ratio

d_F/d is initially taken as constant). If the carrier gas flow is not too small, the influence of V_G on the analysis time is reduced. Since, however, the flow cannot be chosen independently of the film thickness, owing to its influence on the plate height and on the resolving power, a definite limit to the film thickness already exists. Normally, this should be as small as possible (eqn. 2) as the plate height is then smaller, the efficiency of the column is only to a small extent dependent on the magnitude of the partition coefficient and of the carrier gas flow, and the latter can be relatively high without disadvantage. The reasons for a flat curve in the HETP/u diagram becomes more apparent since, from eqn. (9):

$$u_{opt} = \frac{8D_G}{d}\sqrt{\left[\frac{3\,(1+k)^2}{1+6k+11k^2}\right]} \qquad (9)$$

the optimum flow rate u_{opt} is proportional to $1/d$. The optimum flow rate, corresponding to $HETP_{min}$, therefore decreases with increasing column diameter. When working with optimum flow rate the analysis times increase with increasing column diameter.

This attempt to obtain the smallest possible d_F/d ratio militates against the desirability (also on practical grounds) of relatively high loading capacity of the columns. On the other hand, of course, in order to guarantee a complete coating of the tube wall, the film thickness must not fall below a certain value.

These considerations, which have been made essentially with regard to columns with the smallest possible HETP (highest possible number of theoretical plates), are completed by taking carefully into account a further feature, the resolving power. This is by no means always proportional to the number of theoretical plates of a column, but is dependent in many cases upon the form and nature of the film on the tube wall.

In conclusion, comparison of columns of diameter $0.25 \leqslant d \leqslant 0.5$ mm with those of diameter $d = 1$ mm, shows that on the grounds discussed above, the ratio d_F/d for the two column types differs only insignificantly. Since the surface area of the tube wall increases only linearly with increasing diameter, while the column volume is proportional to the second power of d, the columns of greater diameter yield a greater gas volume V_G in relation to the volume V_L of the liquid phase. The loading capacity is, for all substances, greater than that in the case of columns of small diameter, so that stream-splitting before the column can be avoided.

Especial difficulties arise in the theoretical interpretation of measurements on loading capacity. Experimentally the sample size is continually raised until a distinct deviation of the peak form from the shape of a Gauss curve is established. When such a "concentration" range is run, the linear part of the distribution isotherm falls off at a particular value of the sample size, so that the calculation of the number of theoretical plates and of the plate height respectively from the peak shape is no longer permissible. Therefore only the reciprocal value of the relative half-band width, *i.e.* the quotient $t_r/w_{1/2}$, was applied to the interpretation.

In the case of relatively high k values the change of t_r with the concentration is negligible, to a first approximation. Furthermore the quotient $t_{r2}/(t_{r2}-t_{r1})$ (t_{r2} and t_{r1} are the retention times of the two base points of a peak) once again indicates the change of the peak form with concentration.

The particular geometric parameters of the Golay columns under discussion yield a very small pressure gradient for the necessary flow rates (see Table II). Hence, for the first time in gas chromatography, columns are available, possessing an approximately uniform flow over the whole column length without special apparatus.

TABLE II

CARRIER GAS FLOW u (COLUMN EXIT AT NORMAL PRESSURE) AS A FUNCTION OF THE INLET PRESSURE FOR A 100 m COPPER COLUMN (1.0 mm I.D.), CARRIER GAS He, 40° COLUMN TEMPERATURE

p (kg/cm²)	u (ml/min)
0.1	7.5
0.2	15.0
0.3	23.0
0.4	31.0
0.5	39.0
0.8	62.5
1.0	78.0
1.5	117.0

The advantages arising from the application of pressure in the column have already been described elsewhere[4]. In the work here described the results correspond to new experimental conditions.

APPARATUS

For the measurements, the Fractometer F6 (Bodenseewerk Perkin-Elmer & Co., GmbH., Germany) was used. Thermistors and hot wires were used as detectors, as also was an FID.

Columns were developed from copper tubing. The dimensions are reproduced in the various cases.

As stationary phases, squalane, apiezon L, apiezon M and polypropylene glycol were used.

RESULTS

The upper half of Fig. 1 shows the plate height HETP as a function of the linear flow u for three different film thicknesses (calculated from the coating process). The smallest film thickness gives the nearest approach to the ideal function in this respect. The loading capacity of the column is, however, like the resolving power (see the lower half of Fig. 1), so small that practical application is out of the question.

This example illustrates a criticism of the separating efficiency of these columns, namely that the HETP is not sufficient. In each case the resolving power must also be measured. There is no essential difference between the resolving power of the other film thicknesses, so that from the functional representation in the upper part of Fig. 1 the middle thickness is to be preferred.

The upper part of Fig. 2 shows the HETP as a function of the linear, pressure-corrected flow u for squalane as stationary phase, working with several absolute

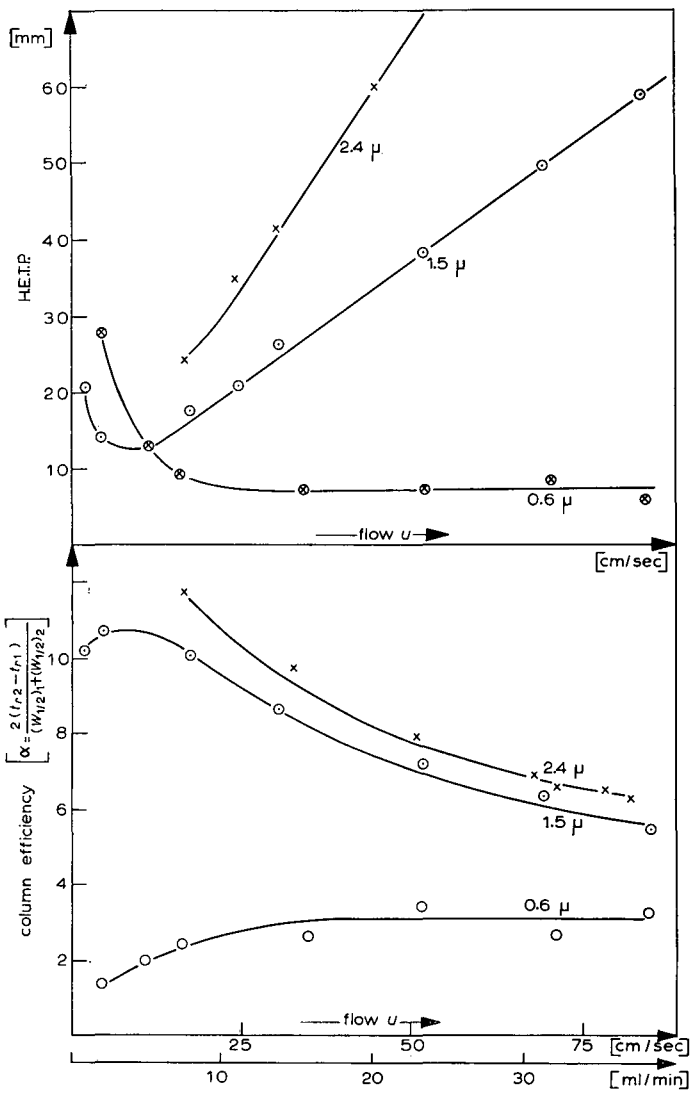

Fig. 1. HETP and column efficiency as a function of carrier gas flow (helium) for three different film thicknesses d_F. 100 m copper column (1.0 mm i.d.), Apiezon M as stationary phase; column temperature 40°; sample 2,4-dimethylpentane, 0.1 μl.

pressures in the column. In the lower part the retention times of the same measurements are represented as a function of the linear, pressure-corrected flow (in the form of $1/u$). These measurements show that results of the analysis are independent of the absolute pressure and that only the linear flow in the column is of significance. (It could be established—see also ref. 4—that the influence of the absolute pressure in a column on the half-band width—and therefore also on the HETP—is dependent on the ratio of component boiling-point to working temperature.)

The lower half of Fig. 2 also verifies eqn. (8), according to which the retention

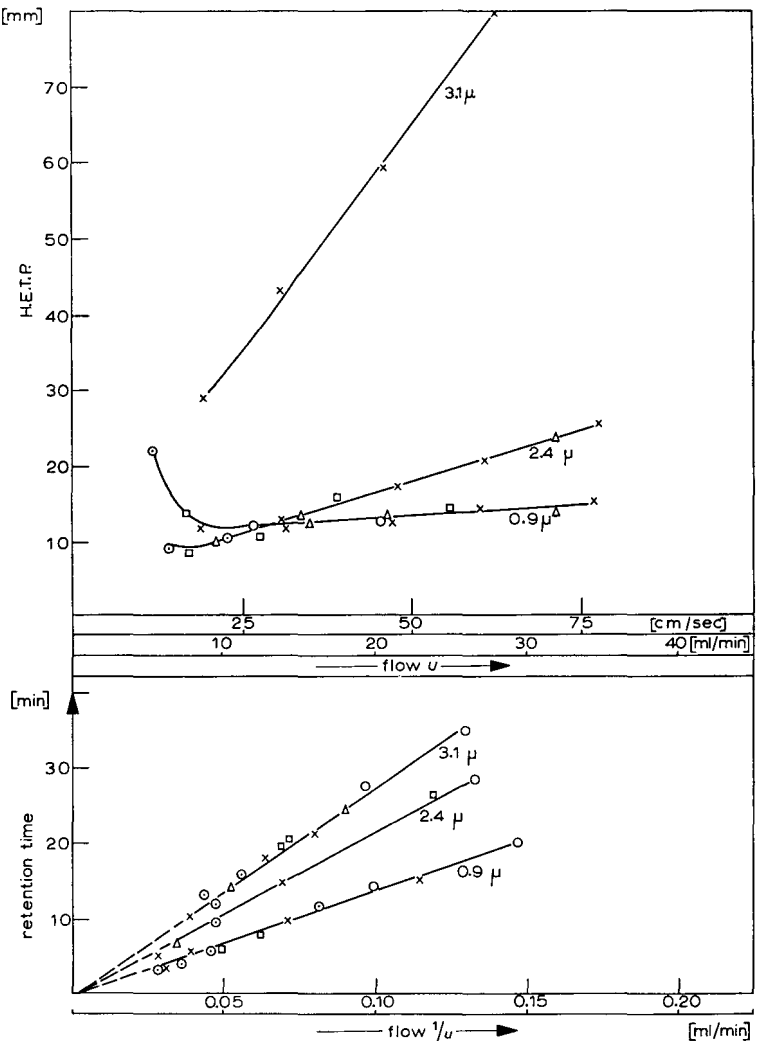

Fig. 2. HETP as a function of flow u, and retention time t_r as a function of flow $1/u$ for different pressures in the column. (×) 0.2 atm; (○) 0.5 atm; (△) 1.0 atm; (⊙) 2.0 atm, excess pressure. Film thicknesses d_F of 0.9; 2.4 and 3.1 μ. 100 m copper column (1.0 mm i.d.); stationary phase squalane; column temperature 40°; sample 2,4-dimethylpentane, 0.1 μl.

time t_r for a particular column diameter d, a definite film thickness d_F, and for a constant gas volume V_G, must, for a particular substance, be independent of absolute pressure in the column, and only dependent on the flow, being proportional to $1/u$.

In Fig. 3 the reciprocal value of the relative peak-base width $t_{r2}/(t_{r2}—t_{r1})$ is represented as a function of the sample size. A distinct difference is evident in the loading capacities of the two columns with internal diameters of 0.5 and 1.0 mm respectively. Only the horizontal part of the curve corresponds to symmetrical peaks of a form similar to that of a Gauss function. With the onset of the fall-off of the curve, the peaks lose their symmetry and assume a near-triangular form[4].

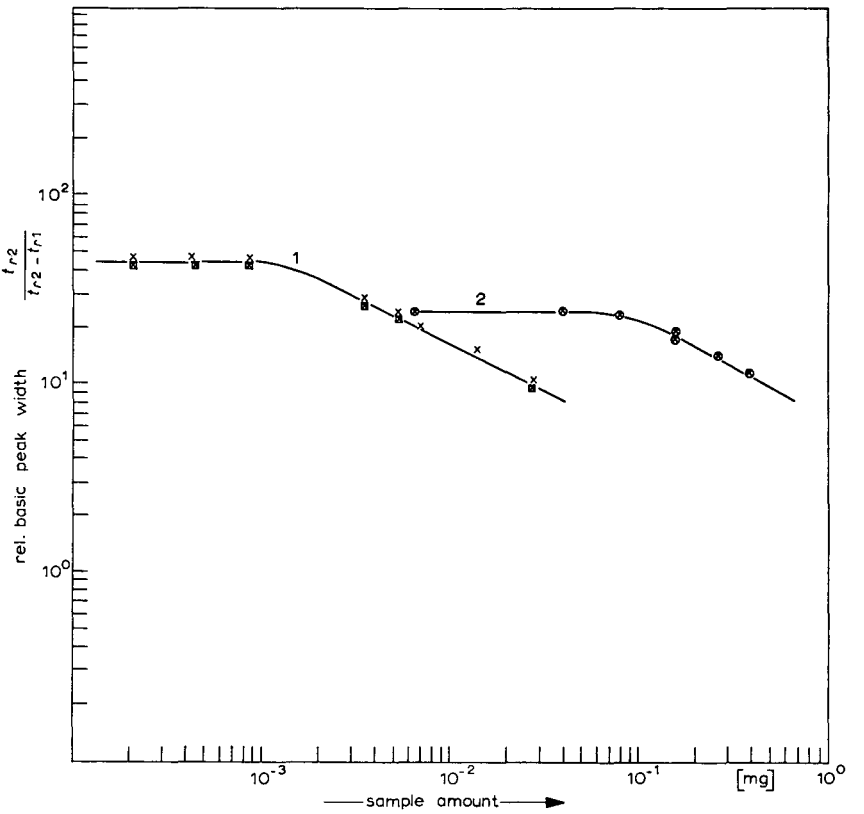

Fig. 3. Admissible sample size of Golay columns. Curve 1: 50 m copper column (0.5 mm i.d.); stationary phase 7:8-benzoquinoline; column temperature 70°. Curve 2: 100 m copper column (1.0 mm i.d.); stationary phase polypropylene glycol; column temperature 80°. (×) *m*-xylene; (⊠) *p*-xylene; (⊗) 3-methylbutanol-1.

Fig. 4 shows the resolving power $t_r/w_{1/2}$ as a function of the sample size (*n*-butanol) for two different layer thicknesses. The thicker films yield a higher resolving power for greater sample sizes. The differences in the case of small sample sizes are in agreement with theory. It should be pointed out at this juncture that these relations are highly dependent on the chosen sample and the working conditions, particularly the working temperature. This is also illustrated in Fig. 5. Here the resolving power for *n*-butanol is again reproduced as a function of the sample size. In the upper part the results at 100° are indicated, and in the lower part the working temperature was 120°. The pressure *in* the column served as a parameter.

The linear flow in the column was the same in each set of experiments. While working at the lower temperature, below the boiling point of *n*-butanol, the course of the function was approximately independent of the two pressures, while at 120° (b.p. of *n*-butanol 117°) the two curves overlap. Thus by the application of pressure one can also in this case obtain a good separating power with large sample sizes.

Fig. 6 shows how greatly the influence of pressure in the column is dependent on the ratio of working temperature to boiling point. The results shown are those obtained with ethanol at a working temperature of 120°.

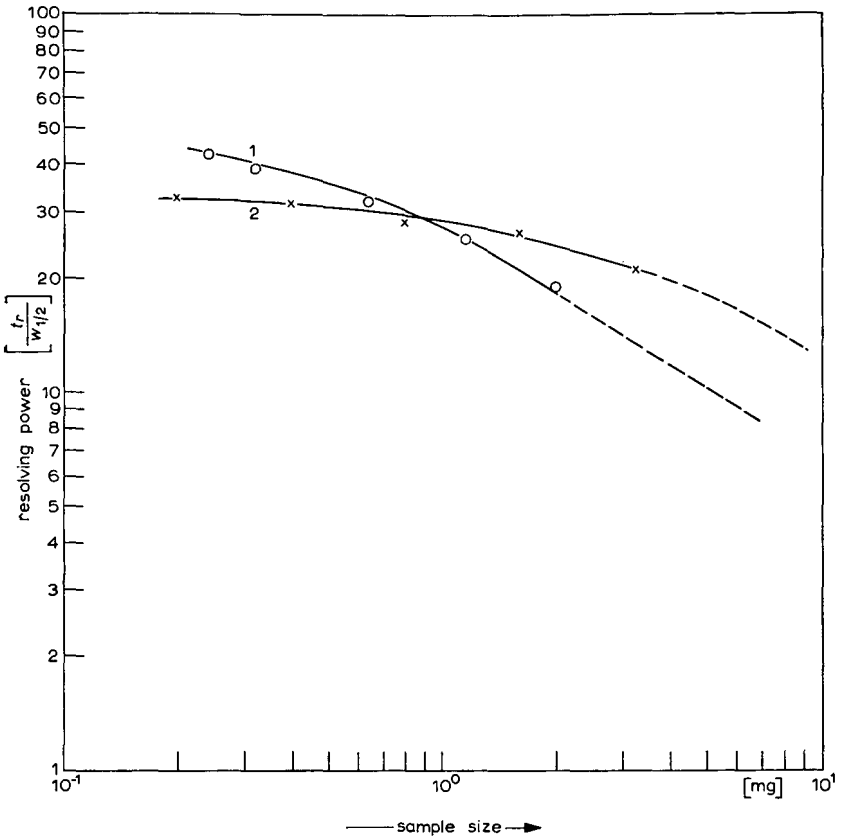

Fig. 4. Resolving power $t_r/w_{1/2}$ as a function of sample size (mg) for two film thicknesses d_F with *n*-butanol as sample. Curve 1: $d_F = 1.5\ \mu$; curve 2: $d_F = 3.1\ \mu$. 100 m copper column (1.0 mm i.d.); stationary phase polypropylene glycol; column temperature 100°.

DISCUSSION

The theoretical considerations and experimental results have shown that it is possible and advisable to use Golay columns of a relatively large diameter. With regard to the analysis time, which must lie within a definite limit, the carrier gas flow velocity must lie between 10–25 ml/min on account of the relatively large gas volume of these columns. Since such velocities greatly exceed the optimum flow velocity, the film thickness must be so chosen that the HETP increases only slightly with increasing flow. From this point of view the ratios d_F/d are taken with $\sim$ 1/1000 maximum.

In order to achieve operative simplicity when using Golay columns of smaller diameter, *e.g.* by avoiding the necessity of stream-splitting and the use of the thermal conductivity detector, particular attention must be paid to the loading capacity of the columns. Since relatively thick films are desired, the d_F/d ratio should lie above 1/1000.

It is therefore obvious that, in order to take both tendencies into account, it is necessary to effect a compromise. Indeed, by the choice, given a certain film thickness, of particular working conditions—*e.g.* the ratio of working temperature to boiling

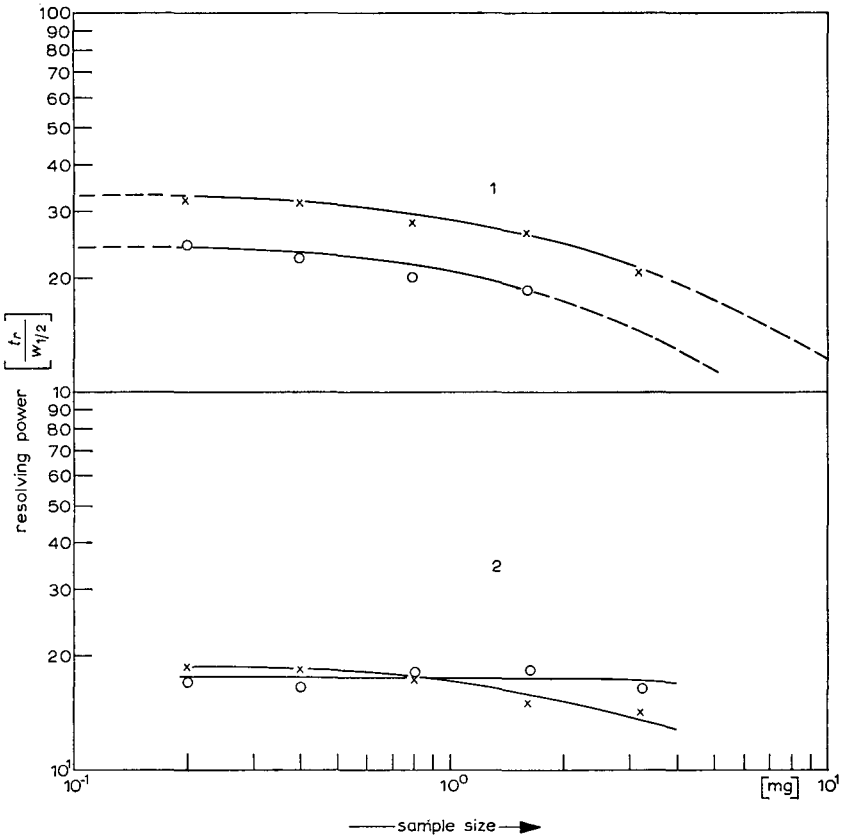

Fig. 5. Admissible sample size of columns (100 m copper; 1.0 mm i.d.; stationary phase polypropylene glycol; film thickness $d_F = 3.1\ \mu$) for *n*-butanol as sample at 100° (curve 1), and 120° (curve 2). Excess pressure in the column (×) 0.5 atm and (O) 2.0 atm; 19.3 ml/min He; 20.0 ml/min He carrier gas flow.

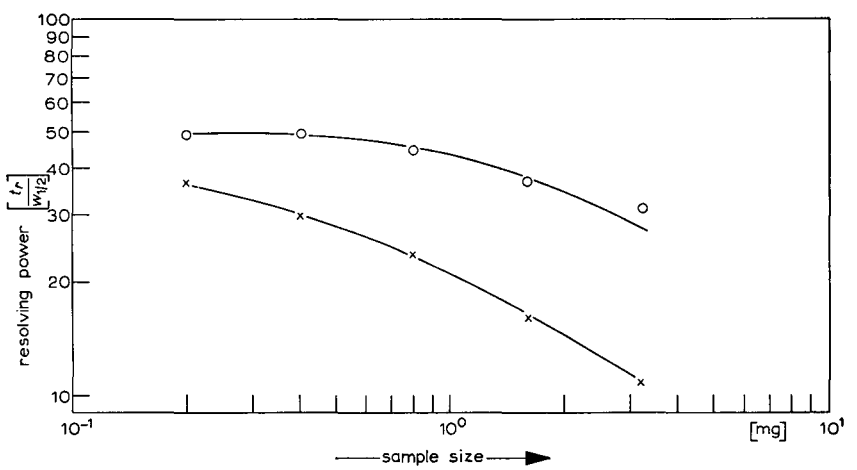

Fig. 6. Admissible sample size of columns (100 m copper; 1.0 mm i.d.; stationary phase polypropylene glycol; film thickness $d_F = 3.1\ \mu$) for ethanol at 100° with 0.5 atm (×) and 2.0 atm (O) excess pressure in the column. Carrier gas flow 19.8 ml/min He in both cases.

point, and the application of pressure in the column—an increase in the loading capacity may be attained. For a theoretical description of the ensuing phenomena, *e.g.* peak form, loss of separating power under different working conditions and pressures, a further series of experiments must be carried out.

When the efficiency of Golay columns of large internal diameter is compared with that of those of small diameter, it is evident that in the former case a much smaller outlay on equipment is required (no FID, no stream-splitting). In the case of the bigger column the resolving power is smaller. Furthermore it is not possible to choose convenient and practicable sample amounts for analysis of component mixtures of wide boiling range ($\Delta T_s > 100°$), for, with too much sample, apparent overloading effects ensue and the peaks lose their symmetry. As regards loading capacity, however, the bigger Golay column is a real improvement on the smaller. For trace analyses (that is, the analyses of mixtures of high concentration differences), the high loading capacity of the column can be combined with the high sensitivity of an FID. Owing to the large gas volume of the column, dead-space problems in the apparatus play only a small part.

Compared with packed columns, Golay columns of large diameter have the advantage of a higher separating efficiency, attainable in the same analysis time. The economic considerations of these columns should be emphasized, when the column is run to give the same resolving power as that of a packed column, namely that the carrier gas consumption is itself between 1/3 and 1/10 of that of a packed column. Resolving power is normally higher.

With increasing resolving power, the consumption decreases to about 1/20. The small flow velocities reduce the noise level and yield warming-up times favourable to the use of thermal conductivity detectors. The small pressure gradient across the big Golay columns leads to an approximately uniform flow throughout the whole column and reduces all leakage problems in the complete apparatus. The big Golay column is also of much broader application than the packed column:

(a) Carrier gas flows of 30–40 ml/min yield in shorter analysis time separations comparable to those of packed columns.

(b) Carrier gas flows of 15–25 ml/min yield in the same analysis time separations which are better than those of a packed column.

(c) Flows of less than 10 ml/min yield the maximum efficiency of the columns, which is essentially higher than that of a packed column.

The advantage of this column type therefore lies in the fact that, *with one column*, the working conditions can be so chosen as to yield the desired performance.

SYMBOLS USED

d	column diameter
d_F	film thickness of the stationary phase
D_G	diffusion coefficient in the gas phase
D_L	diffusion coefficient in the liquid phase
HETP	theoretical plate height
$HETP_{min}$	minimum theoretical plate height
K	partition coefficient
k	retention time ratio t_r'/t_M

L	column length
n	number of theoretical plates
r	column radius
t'_r	corrected retention time $t'_r = t_r - t_M$
t_M	retention time of a substance insoluble in the liquid phase
u	carrier gas flow velocity
v_n	volume of a theoretical plate
V_G	gas volume of a column
V_L	volume of the liquid phase of a column
$w_{1/2}$	half-band width
$t_r/w_{1/2}$	resolving power.

SUMMARY

The application of Golay columns of larger internal diameter is described from a theoretical and practical point of view. The influence of the geometry and of the ratio film thickness to diameter on the admissible sample size is discussed.

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DER EINFLUSS DES TRÄGERMATERIALS AUF DIE QUANTITATIVE, GASCHROMATOGRAPHISCHE ANALYSE HÖHERER FETTSÄUREMETHYLESTER*

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Die rasche Entwicklung der Gaschromatographie in den letzten Jahren hat in vielen Laboratorien zu ihrer Anwendung als quantitativer Bestimmungsmethode höherer Fettsäuren geführt. Ihre Zuverlässigkeit ist jedoch noch fragwürdig. Eine kürzlich von KAUFMANN und Mitarb. beschriebene Ringanalyse verschiedener Institute ergab für das gleiche Fettsäuregemisch eine Fehlerbreite von $\pm$ 25 %; dies zeigt, mit welchen Unsicherheitsfaktoren die quantitative Aussage noch belastet ist[1].

Zur quantitativen Auswertung des Chromatogrammes wird im allgemeinen bei höheren Fettsäuren die Bandenfläche gemessen, da diese bei Stoffen mit grösserer Retentionszeit von Schwankungen der Flussgeschwindigkeit und Temperatur unabhängiger ist als die Gipfelhöhe. Um die Bandenfläche auf die Gewichtsmenge beziehen zu können, muss der Untersucher in den meisten Fällen eine Eichung des Gaschromatographen für die gegebenen Arbeitsbedingungen vornehmen. Die verschiedenen Möglichkeiten dieser Eichung sind folgende: Es wird entweder der Probe ein interner Standard bekannter Gewichtsmenge zugesetzt oder die Probe in einem bekannten Volumen aufgenommen und mit einem Eichchromatogramm ähnlicher Zusammensetzung und bekannter Konzentration verglichen. Eine Eichung wird nicht als erforderlich angesehen, wenn die Gesamtfettsäuremenge vorher gewogen wird und dann, unter der Voraussetzung, dass alle Komponenten flüchtig sind und im Chromatogramm erscheinen, der prozentuale Anteil jeder Komponente aus den unterschiedlichen Flächengrössen ermittelt wird[2,3].

Um die Fehlergrenze bei der quantitativen Fettsäurebestimmung in biologischem Material so niedrig wie möglich zu halten, haben wir vor und nach jedem Testchromatogramm ein Eichchromatogramm bekannter Konzentration an Methyllaurinat, Methylmyristinat, Methylpalmitat, Methylstearat, Methyloleat und Methyllinolat aufgegeben. Dabei lässt sich nach mehrstündigem Chromatographieren eine Zunahme der Bandenfläche für die gleiche Fettsäuremenge in Abhängigkeit von der Kettenlänge und der Durchgasungsdauer zwischen zwei Chromatogrammen beobachten, obwohl die Gipfel symmetrisch und gut getrennt sind. Aus 80 Tagesschreibungen über jeweils 7 Stunden errechnet sich zum Beispiel eine mittlere Zunahme für Methylmyristinat von 7 % und für Methylstearat von 18 % gegenüber der Ausgangsfläche zu Beginn des Tages. Die ungesättigten Fettsäuren zeigen eine Flächenzunahme in der

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gleichen Grössenordnung wie Methylstearat. Diese Erscheinung ist unabhängig vom Detektor, da immer die gleiche absolute Konzentration aufgetragen wird und ebenfalls unabhängig von der Art und Konzentration der flüssigen Phase (Apiezon und verschiedene Polyester, 10 oder 20 %). Lediglich der Wechsel des Trägermaterials von Sterchamol zu Kieselgur führte zu einer deutlich verbesserten Reproduzierbarkeit.

Diese Beobachtungen weisen darauf hin, dass es sich bei der Zunahme der Flächengrösse offenbar um einen entscheidenden Einfluss des Trägermaterials handelt, und dass die äusserlichen Kriterien für ein inertes Verhalten der Trägerstoffe — Symmetrie der Banden und Trennbarkeit von zwei benachbarten Komponenten — nicht ausreichen, um ihre Brauchbarkeit für die quantitative Analyse zu beurteilen. Viele Arbeitskreise haben sich in den letzten Jahren mit den Adsorptionskräften der Trägerstoffe und der Ausschaltung durch Vorbehandlung beschäftigt, aber es wurde fast ausschliesslich auf eine Verbesserung der Eigenschaften des Chromatogrammes nach den oben genannten Kriterien geachtet[4–16]. Es erschien uns daher gerechtfertigt, in einer speziellen Versuchsanordnung die beiden hauptsächlich gebräuchlichen Trägerstoffe Sterchamol und Kieselgur auf ihre Verwendbarkeit bei der quantitativen Analyse höherer Fettsäuremethylester zu untersuchen.

METHODISCHES

Die Trägerstoffe werden entweder unbehandelt, wie sie käuflich zu beziehen sind*, oder nach vorheriger Behandlung, wie in Tabelle I angegeben, untersucht. Als flüssige Phase wird in allen Fällen 10 % Reoplex** aufgetragen. Die fertig gepackte Säule (Länge 240 cm) wird 24 Stunden vor Gebrauch konditioniert. Die beiden Standardsubstanzen, Methyllaurinat und Methylstearat, sind hochgereinigte Präparate

TABELLE I

UNTERSUCHTES TRÄGERMATERIAL

Sterchamol	unbehandelt
Sterchamol	HCl und KOH gewaschen
Sterchamol	mit Hexamethyldisilazan vorbehandelt
Sterchamol	mit 2 % Na-Laurinat vorbehandelt
Kieselgur	unbehandelt
Kieselgur	HCl und KOH gewaschen

der California Corporation for Biochemical Research. Die 1-^{14}C-Palmitinsäure wurde aus dem Radiochemical Center, Amersham, bezogen und zusammen mit Trägerpalmitinsäure nach der Methode von METCALFE UND SCHMITZ in Methylpalmitat überführt[17]. Alle Untersuchungen werden in einem Barber-Colman-Gaschromatographen ausgeführt.

Auf der fertig konditionierten Säule werden 75 μg einer der oben angegebenen Methylester in Abständen von 5 Min. über einen Zeitraum von 20 Min. chromatographiert und die prozentualen Flächenveränderungen gegenüber der ersten Bandenfläche errechnet. Die Säulentemperatur beträgt 186°, die Flussgeschwindigkeit des Argonträgergases 75 ml/Min.

* Sterchamol und Kieselgur 0.2–0.3 mm, Fa. E. Merck, Darmstadt.
** Reoplex (Diäthylenglykoladipat), Fa. E. Merck, Darmstadt.

Die in Heptan gelöste Probe wird mit einer Hamilton-Mikrospritze ohne Unterbrechung des Gasstromes durch eine Siliconkautschukmembran direkt auf die Säule aufgegeben. Der Nachweis erfolgt durch eine Argonionisationskammer, die Bandenflächen werden automatisch mit einem DISC-Integrator registriert. Alle Bedingungen werden über den Zeitraum der Untersuchung sorgfältig konstant gehalten. Vor jeder neuen Serie wird die Säule mindestens 3 Stunden mit Trägergas (75 ml/Min.) gespült, damit retinierte Reste von Fettsäuremethylestern eluiert werden. Bei den Versuchen mit radioaktivem Methylpalmitat wird, entsprechend einer Methode von KARMEN UND TRITCH, das Palmitat nach Verlassen der Ionisationskammer in getrennten Fraktionen an Anthracenkristalle aus dem Trägergasstrom kondensiert und die Impulse im Flüssigkeitsszintillationsspektrometer gemessen[18].

Vorbehandlung der Trägerstoffe

1. Waschen mit konz. HCl und 0.5 *N* methanolischer KOH wie von FARQUHAR und Mitarb. angegeben[12].

2. Vorbehandlung mit Hexamethyldisilazan* nach dem Verfahren von BOHEMEN und Mitarb.[13].

3. Vorbehandlung mit Na-Laurinat: 400 mg Na-Laurinat werden in wenig Wasser und 50 ml heissem Aceton gelöst und zu dieser Lösung 20 g Sterchamol hinzugegeben. Das Gemisch wird auf einem Wasserbad (70°) solange gerührt, bis der Rückstand fast trocken ist und anschliessend 14 Std. im Vakuumtrockenschrank bei 95° vollständig getrocknet.

ERGEBNISSE

Die Fig. 1 und 2 zeigen das Verhalten von unbehandeltem Sterchamol und Kieselgur unter den vorher beschriebenen Versuchsbedingungen. Wenn auf diesen Säulen in Abständen von 5 Min. Fettsäuremethylester chromatographiert werden, nimmt die

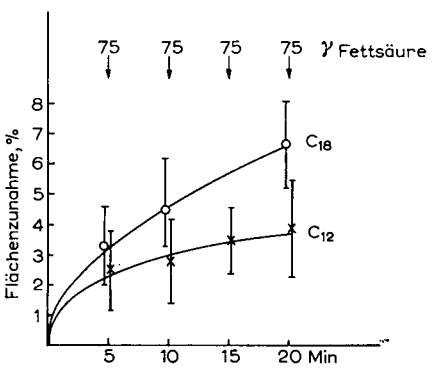

Fig. 1. Sterchamol unbehandelt.

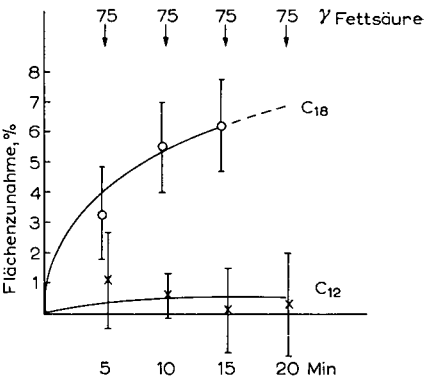

Fig. 2. Kieselgur unbehandelt.

Bandenfläche gegenüber der Ausgangsfläche beim Sterchamol für Methylstearat um nahezu 7 % und für Methyllaurinat um etwa 4 % zu. Beim Kieselgur beträgt die Zunahme für Methylstearat 6 %, während für Methyllaurinat praktisch keine Zu-

* Firma Fluka, Buchs (Schweiz).

nahme zu beobachten ist. Jeder Punkt in diesen und den nachfolgenden Abbildungen repräsentiert den Mittelwert aus 6–10 Einzelmesserien, die getrennt für die beiden Fettsäureester durchgeführt wurden. Die vertikalen Linien veranschaulichen die errechneten Standardabweichungen.

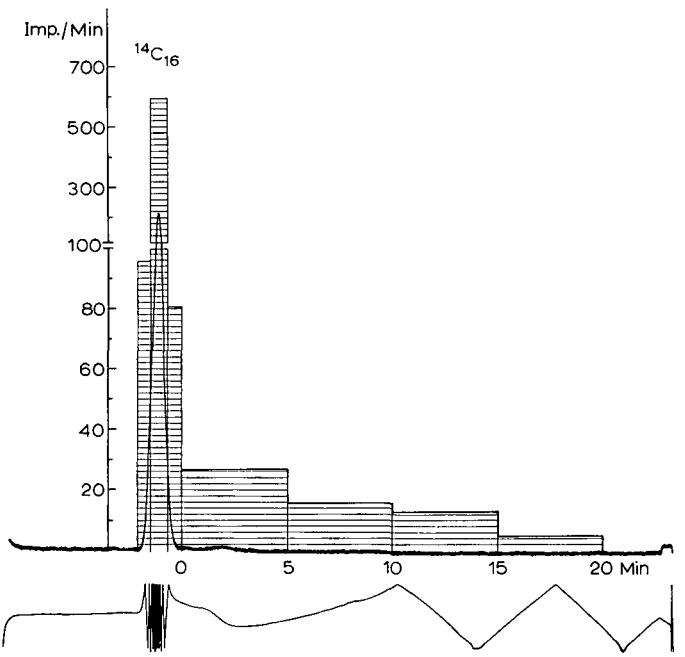

Fig. 3. Sterchamol unbehandelt.

Die Fig. 3 zeigt ein Chromatogramm von 1-^{14}C-Methylpalmitat auf einer Säule mit unbehandeltem Sterchamol. Die schraffierten Kästchen stellen Impulse/Minute in der auf der Abszisse angegebenen Zeit dar. Im Anschluss an den Gipfel lassen sich noch 8 % der Gesamtfettsäuremenge nachweisen, die bei der quantitativen Bestimmung aus der Bandenfläche nicht mit erfasst werden. Dieses Ergebnis lässt darauf

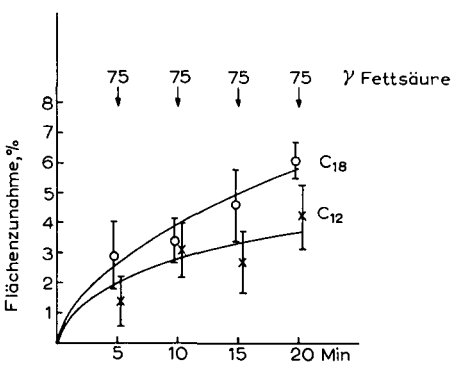

Fig. 4. Sterchamol HCl und KOH behandelt.

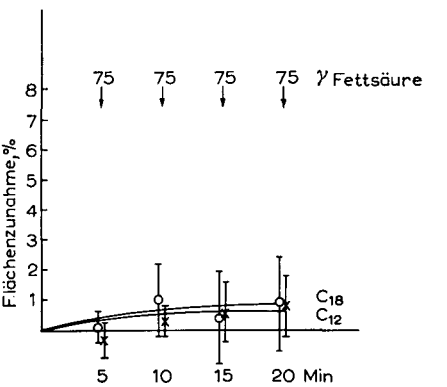

Fig. 5. Kieselgur HCl und KOH behandelt.

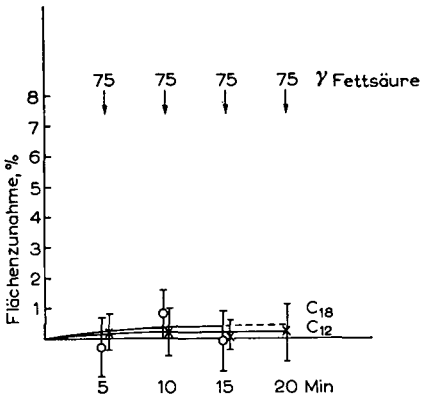

Fig. 6. Sterchamol Hexamethyldisilazan behandelt.

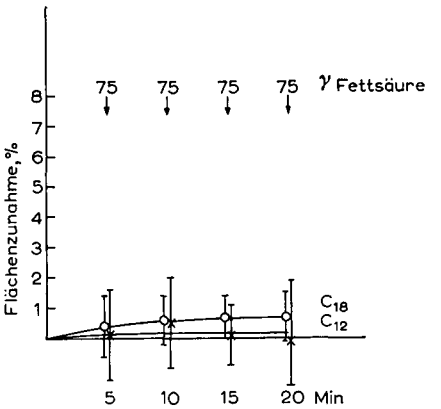

Fig. 7. Sterchamol 2% Na-Laurinat behandelt.

schliessen, dass die zunächst adsorbierte Fettsäuremenge wieder kontinuierlich vom Trägergas eluiert wird.

Eine Vorbehandlung des Trägermaterials mit HCl und methanolischer KOH führt beim Sterchamol nicht zu dem gewünschten Ergebnis. Wie Fig. 4 zeigt, ist keine Änderung gegenüber dem unbehandelten Sterchamol eingetreten (Vergleich mit Fig. 1). Beim Kieselgur ist diese Vorbehandlung ausreichend, um praktisch alle Adsorptionskräfte auszuschalten. Aus Fig. 5 geht hervor, dass keine Flächenzunahme für Methylstearat gegenüber der Ausgangsfläche mehr zu beobachten ist. Erst die Behandlung des Sterchamols mit Hexamethyldisilazan oder mit Na-Laurinat ergibt

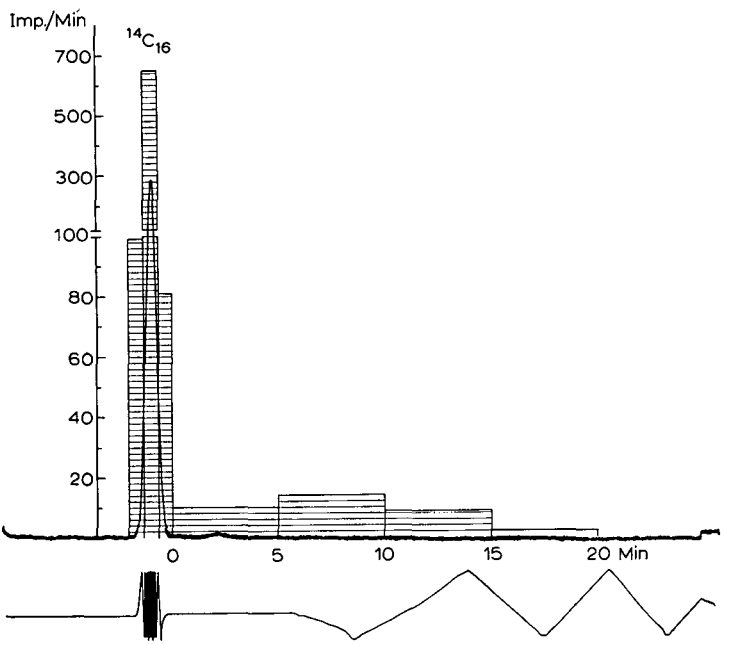

Fig. 8. Sterchamol 2% Na-Laurinat behandelt.

ein gutes Resultat (Fig. 6 und 7) und macht auch diesen Trägerstoff für die quantitative Gaschromatographie höherer Fettsäuremethylester brauchbar. Fig. 8 zeigt ein der Fig. 3 entsprechendes Chromatogramm von 1-^{14}C-markiertem Methylpalmitat auf einer mit Na-Laurinat vorbehandelten Säule. Im Anschluss an den Gipfel lassen sich nur noch 4 % seiner Gesamtaktivität nachweisen.

DISKUSSION

Die Versuchsanordnung gestattet unabhängig vom Detektor das Verhalten der Trägerstoffe im Hinblick auf die Adsorption höherer Fettsäuremethylester zu untersuchen. Die vorgelegten Befunde zeigen, dass beim käuflichen Sterchamol und Kieselgur ein Teil der Fettsäuremethylester adsorbiert wird, obwohl diese Trägerstoffe äusserlich symmetrische Gipfel im Chromatogramm liefern. Die Adsorption ist umso umfangreicher, je grösser die Retentionszeit ist, und bedeutet eine erhebliche Fehlerquelle bei der Eichung mit nur einem internen Standard. Der Verlauf der Kurven in den Fig. 1, 2 und 4 lässt eine allmähliche Absättigung aktiver Zentren am Trägerstoff vermuten und nicht, wie ORR UND CALLEN[19] für ihren Fall annehmen, einen Umesterungsprozess mit der Polyesterphase der Säule. Dagegen spricht auch die Beobachtung der gleichen Phänomene an Apiezon-Säulen mit einem unpräparierten Trägerstoff. Die Radioaktivitätsmessung zeigt, dass der Fettsäuremethylester offenbar reversibel adsorbiert und mit dem Trägergasstrom wieder eluiert wird. Für die Adsorption werden übereinstimmend von vielen Autoren Reste an Kieselgel im Trägermaterial verantwortlich gemacht, deren Ausschaltung durch eine chemische Umsetzung mit methanolischer KOH[3,12], Dimethyldichlorsilan[7,10,16] oder Hexamethyldisilazan[13] mit Erfolg versucht worden ist. Eine zweite Möglichkeit ist die physikalische Abdeckung der aktiven Zentren durch einen Metallüberzug[9] oder durch Zusatz von Stearat[4] oder Capronat[5,6,11] zur flüssigen Phase.

Einige dieser Möglichkeiten sind in der vorliegenden Arbeit erprobt worden. Die Behandlung mit methanolischer KOH reicht aus, um Kieselgur zu einem brauchbaren Trägerstoff für die quantitative Gaschromatographie zu machen. Die gleiche Behandlung führt beim Sterchamol zu keinem Erfolg, wahrscheinlich aufgrund der anderen chemischen Zusammensetzung. In diesem Fall aber ergibt eine Vorbehandlung mit Hexamethyldisilazan oder Na-Laurinat eine nahezu vollständige Ausschaltung der Adsorptionskräfte. Die Chromatographie einer markierten Fettsäure lässt kaum noch ein Austreten von Radioaktivität im Anschluss an den Gipfel erkennen und widerlegt damit den Einwand, dass es sich in Fig. 3 um den Nachweis von Bruchstücken der markierten Fettsäure handeln könnte. Dieses Ergebnis hat darüber hinaus eine Bedeutung für die fraktionierte Radioaktivitätsmessung in biologischem Material, wenn z.B. auf eine hochmarkierte Fettsäure unmittelbar eine Fettsäure mit geringer spezifischer Aktivität folgt, muss der Blindwert möglichst niedrig sein.

Die Forderung nach einem inerten Trägermaterial wird immer wieder gestellt. Wie notwendig eine sorgfältige Vorbehandlung für eine zuverlässige quantitative Aussage und eine fraktionierte Radioaktivitätsmessung ist, haben die vorgelegten Untersuchungen gezeigt; darüberhinaus lassen sich die sonst häufigen Eichungen einschränken und führen damit in der Routineanalyse zu einer erheblichen Zeiteinsparung.

ZUSAMMENFASSUNG

Es wird eine vom Detektor unabhängige Versuchsanordnung beschrieben, die den Einfluss des Trägermaterials bei der quantitativen gaschromatographischen Bestimmung höherer Fettsäuren zu prüfen gestattet. Die Ergebnisse lassen den Schluss zu, dass bei Verwendung von unpräpariertem Trägermaterial eine reversible Adsorption von Fettsäuremethylestern stattfindet, die die quantitative Aussage mit einem erheblichen Fehler belastet. Eine Vorbehandlung des Kieselgurs mit konz. HCl und methanolischer KOH und des Sterchamols mit Hexamethyldisilazan oder Na-Laurinat reduziert diese Adsorption auf ein zu vernachlässigendes Minimum und beseitigt so die Fehlerquellen, die die Brauchbarkeit der beiden Trägerstoffe beeinträchtigen.

SUMMARY

A procedure is described to test the influence of solid supports on quantitative gas-chromatographic analysis of fatty acid methyl esters. The results show that considerable adsorption takes place when untreated kieselguhr or firebrick is used, despite the fact that symmetrical peaks are obtained in the chromatogram. For the preparation of a suitable support with low adsorptive properties treatment with acid and alkali is an adequate procedure for kieselguhr, while firebrick has to be pretreated with hexamethyldisilazane or sodium laurinate to obtain reproducible results in quantitative analysis.

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GAS-CHROMATOGRAPHIC IDENTIFICATION OF PHENYLALKANES AND BICYCLIC HYDROCARBONS

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INTRODUCTION

Gas-chromatographic techniques have led to great progress in the analytical aspects of chemical kinetics, but for most systems the lack of known retention times makes it still necessary to synthesise many of the possible reaction products for their identification. In the present work a comparison has been made of the gas-chromatographic retention volumes of a set of hydrocarbons which are representative of the kind of substances formed by reaction of hydrocarbon radicals with aromatic compounds[1]. Monocyclic and bicyclic compounds with aliphatic, olefinic and aromatic groups have been studied. Empirical rules gained from these series should help in the identification of homologous products. The literature lists a few of these series using different liquid phases. SPENCER AND JOHNSON[2] determined the relative retention volumes for isomeric phenylalkanes (phenyl-C_6 to -C_{20}) separated on an asphalt column. BEAVEN *et al.*[3] have compared the retentions of alkyl-biphenyls using Apiezon M as liquid phase.

Table I gives the retention indices and their temperature gradients as found for Emulphor O and Silicone Oil DC 710 as liquid phases. The representation by the index numbers makes it possible to compare the results of homologous series and to join the data to the large number of indices published[4,5].

The retention index is defined by [5,6]:

$$I_T = 200 \frac{\log r\ (Y/nP_{(z)})}{\log r\ (nP_{(z+2)}/nP_{(z)})}$$

where $r\ (nP_{(z+2)}/nP_{(z)})$ is the relative retention volume of two succeeding even numbered n-alkanes that precede and succeed the substance Y. $r\ (Y/nP_{(z)})$ is the relative retention volume of the substance Y compared with the n-alkane with z carbon atoms. The retention indices of the even numbered n-alkanes are by definition $100 \cdot z$ for each column and temperature (ethane = 200; butane = 400; hexane = 600...).

EXPERIMENTAL

The substances were either of commercial origin or prepared by published methods. Their structures have been checked by infrared spectroscopy, by mass spectrometry

TABLE I

RETENTION INDICES AND THEIR TEMPERATURE GRADIENTS

Compound	*DC-710 column*			*Emulphor O column*		
	Temp.	$I_{130°}$	$10 \times dI/dT$	*Temp.*	$I_{130°}$	$10 \times dI/dT$
Benzene				130–170	862	3.6*
Toluene				130–170	963	3.4*
1-Phenylethane				130–170	1053	3.8*
1-Phenylpropane	130–170	1048	6.4	130–170	1136	4.2
Isopropylbenzene				130–170	1104	3.5*
1-Phenylbutane	110–150	1151	5.0	125–170	1219	4.2
tert.-Amylbenzene	110–150	1188	3.5	125–170	1254	6.6
1-Phenylpentane	110–200	1248	3.5	125–170	1313	4.8
3-Phenylpentane	110–150	1175	3.8	125–170	1232	5.0
1-Phenylhexane	110–200	1348	3.7	125–170	1417	5.3
2-Phenylhexane	110–200	1283	3.5	125–170	1342	4.5
3-Phenylhexane	110–200	1254	3.6	125–170	1310	4.6
2-Phenyl-4-methylpentane	110–150	1130	2.7	125–170	1289	4.1
1-Phenylheptane	110–200	1447	3.3	125–170	1515	5.0
Styrene				150–190	1128	7.0
3-Phenylpent-2-ene	110–200	1275	3.5	125–170	1362	5.2
2-Phenyl-4-methylpent-2-ene	110–200	1328	3.2	125–170	1413	4.3
3-Phenylhex-3-ene	110–200	1350	3.6	125–190	1434	4.8
3-Phenylhex-2-ene						
2-Phenylhex-2-ene	120–200	1399	3.1	125–190	1490	5.2
Bicyclohexane	110–200	1369	7.3	125–170	1390	9.2
Cyclohexylcyclohex-1-ene	110–200	1398	7.2	125–190	1422	8.4
Cyclohexylcyclohex-2-ene	110–200	1398	7.2	125–190	1436	9.4
Cyclohexylcyclohex-3-ene	110–200	1398	7.2	125–190	1447	9.2
Phenylcyclohexane	110–200	1437	7.1	125–190	1521	9.2
Phenylcyclohexa-2,5-diene	110–200	1474	6.9	125–190	1592	9.6
Phenylcyclohex-1-ene	110–200	1535	7.1	125–190	1645	9.3
Biphenyl	110–215	1541	7.6	150–190	1712	9.2
2-Methylbiphenyl	110–215	1548	6.9	150–190	1680	8.5
3-Methylbiphenyl	110–215	1638	7.5	150–190	1805	10.4
4-Methylbiphenyl	110–215	1646	8.0	150–190	1819	9.2
2,2′-Dimethylbiphenyl	110–215	1560	7.0	150–190	1667	7.1
2,3′-Dimethylbiphenyl	110–215	1637	6.9	150–190	1755	10.9
2,4′-Dimethylbiphenyl	110–215	1672	7.0	150–190	1780	9.6
3,3′-Dimethylbiphenyl	110–215	1735	7.3	150–190	1900	10.7
3,4′-Dimethylbiphenyl	110–215	1742	7.3	150–190	1912	9.7
4,4′-Dimethylbiphenyl	110–215	1752	7.3	150–190	1920	10.0
Diphenylmethane	110–215	1595	7.2	150–190	1743	9.2
2-Methyl-diphenylmethane	110–215	1685	7.8	150–190	1826	9.7
3-Methyl-diphenylmethane	110–215	1686	7.8	150–190	1827	9.2
4-Methyl-diphenylmethane	110–215	1691	7.4	150–190	1840	8.9
Bibenzyl	110–215	1671	8.0	150–190	1818	10.5
1,1-Diphenylethylene	110–215	1675	6.7	150–190	1820	10.0

* Values measured by WEHRLI AND KOVATS[5].

with a magnetically focussed mass spectrometer, and using a combination of a gas–liquid chromatograph with a time of flight detector unit[7]. The diene structures were confirmed by NMR spectroscopy. The isomers of the dimethylbiphenyls and the methylbiphenyls were cross checked by decomposing *o*-, *m*- or *p*-bitoluyl peroxides in toluene and in benzene.

Chromatography columns

The following columns were used:

(a) 5 % Silicone Oil DC 710 on Celite, 120 to 150 μ, columns of 1, 3 and 5 m length and 1000 to 4000 theoretical plates, designated subsequently as D.

(b) 5 % Emulphor O (BASF), a polyethylene glycol octadecyl ether, on Celite, 120 to 150 μ, columns of 3 m with 3400 theoretical plates, designated as E.

All columns used gave symmetrical elution curves. Argon was used as carrier gas and the detector was a Pye Argon Ionisation Detector. The columns were heated in vertical Pye heating jackets. The jackets of some of the columns were packed with coarse aluminum powder to ensure equal heat dissipation over the whole column length. The temperatures were checked at different positions. The same columns were used for measurements at different temperatures.

RESULTS

The retention indices plotted *vs.* $1/T$ gave straight lines for temperature intervals of about 100°. The statistical analysis of the results also showed that values obtained from different columns were indistinguishable. The mean values listed in Table I are calculated from measurements at the different temperatures by fitting a linear regression line for $I = f(1/T)$. The values have been tabulated in accordance with standard practice and the temperature gradients have been transformed to $10 \times (dI/dT)$ values at 130°.

The indices determined for the D columns are calculated from measurements made (a) at 3 to 4 temperatures when the index was less than 1550, and (b) at 6 to 7 temperatures when it was greater than 1550. The values for the E columns (c) were determined at 3 to 4 temperatures. The errors of the interpolated 130° point have been calculated from the variation about the regression line. They are (a) $\pm$ 2 ($\pm$ 0.6)*, (b) $\pm$ 3 ($\pm$ 0.9), (c) $\pm$ 3 ($\pm$ 1) for the indices and their temperature gradients for a 10° interval respectively.

DISCUSSION

Some empirical relationships may be deduced from the results, which will aid in the identification of unlisted hydrocarbons with homologous structure.

1. The homologues of phenylalkanes which possess a side chain exceeding three carbon atoms, show an index increase of 100 units for each additional methylene group. In these compounds the phenyl group has a functional retention index of 750 when measured on the D column and 816 when measured on the E column. The isomeric phenylalkanes are eluted according to their boiling points.

A methyl group added to biphenyl or methylbiphenyl increases the index number

* 95 % confidence limit.

on both columns by 95 when in the *meta* position and by 105 in the *para* position. The *o*-methylbiphenyls deviate from the expected values. The functional retention index for this group varies from 0 to 25 for the D columns, and from —32 to —50 for the E column. BEAVEN *et al.*[3] have already noticed the greatly reduced retention volumes of *o*-alkylbiphenyls. He attributed this effect to a reduction of the overall biphenyl-type conjugation in these sterically hindered compounds. The differences of the indices on this logarithmic scale correspond within a series of these compounds directly to the differences of the molar polarisations. The values $R = (n^2 - 1)/(n^2 + 2)$. M/d, calculated from API data are for *o,o'*-dimethylbiphenyl 60.4, for *o,m'*-dimethylbiphenyl 61.2 for *m,p'*-dimethylbiphenyl 62.2 and for *o*-methylbiphenyl 61.0 and for *m*-methylbiphenyl 61.8.

2. The temperature dependence of the indices is mostly 7 to 8 for the D columns and 8–10 units for the E column. The dependence becomes about half this value for monocyclic compounds. These characteristics effectively help to distinguish between monocyclic and bicyclic compounds.

3. Identification may also be made by comparing the differences of the indices between the two columns. For instance, at 130° they are:

20 – 50 for cyclohexylcyclohexenes
60 – 80 for phenylalkanes
80 – 110 for phenylalkene and phenylcyclohexane
110 – 130 for phenylcycloalkene
140 – 150 for diphenylmethane and substituted methyldiphenylmethane
165 – 170 for biphenyl and *m*- and *p*-methyl-substituted biphenyls.

ACKNOWLEDGEMENTS

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SUMMARY

Retention indices and their temperature coefficients have been measured with Silicone Oil DC 710 and Emulphor O as liquid phases. Some experimental rules are put forward.

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A GENERALIZATION OF THE RETENTION INDEX SYSTEM INCLUDING LINEAR TEMPERATURE PROGRAMMED GAS–LIQUID PARTITION CHROMATOGRAPHY

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The determination of retention data serves different purposes and the choice of the type of retention parameter depends on the purpose.

If the investigation is concerned with the study of the physical phenomena underlying the behaviour of compounds in gas chromatographic systems, the specific retention volume will be the parameter of choice. Defined as:

$$V_{g(x)} = RT/M_L\gamma_x P^\circ_x \qquad (1)$$

it describes peak positions in terms with a physical meaning.

However, the majority of the users of the gas–liquid partition chromatography (GLPC) technique are not directly interested in the study of these physical phenomena but in the identification of the components of mixtures. The difficulties encountered in the accurate determination of the specific retention volume (the more as many instrument manufacturers tend to forget the installation of proper gauges for measurement of column inlet pressures) and the reproduction of these determinations, together with the elaborate calculations involved, make this parameter impractical for routine identification work. The necessity of simple and reproducibly determinable retention parameters for this type of work was very clearly expressed by PRIMAVESI[1].

To achieve this goal, it is obvious that the accurate determination of as many operational variables as possible must be eliminated.

The first attempt in this direction was the *relative retention*, defined as:

$$r_{x,s} = V_{g(x)}/V_{g(s)} = t'_x/t'_s \qquad (2)$$

This relative retention eliminated, it is true, many operational variables; it has, however, the drawback that the choice of the reference material is completely at the discretion of the investigator. And apart from the often used *n*-pentane, one may encounter in the literature reference materials such as carbon tetrachloride[2], hexadecanal[3] and coumarone[4].

To obtain a fixed reference point SMITH[5] introduced the *theoretical nonane system*. This system is based on the fact that under identical, isothermal conditions, the higher members of a homologous series show the relationship:

$$\log V_{g(x)} = A + Bn_x \qquad (3)$$

between the retention volume and the number of C-atoms. Using the normal paraffins as the reference series, the constants A and B are calculated and the value $n_x = 9$ is substituted in the equation to find the reference point. The theoretical nonane index is now defined as:

$$r_{x,9} = \frac{V_{g(x)}}{V_{g(9)}} = \frac{t'_x}{t'_9} \tag{4}$$

The principal disadvantage of this system is the determination of the reference point by extrapolation and not by direct observation.

The *retention index system*, introduced by KOVÁTS[6] was also based on the *n*-paraffinic series as the reference materials. However, by using the *n*-paraffins eluting directly before and after the compound under investigation as the reference points the extrapolation was eliminated. Fixed reference points are obtained in this way by attaching to each *n*-paraffin the retention index:

$$I = 100\, n \tag{5}$$

The position of the peak of a compound is now found from:

$$I = 100\, i \frac{\log V_{g(x)} - \log V_{g(n)}}{\log V_{g(n+i)} - \log V_{g(n)}} + 100\, n \tag{6}$$

It should be noted here that KOVÁTS uses the logarithms of the retention volumes and further that he showed that I is linearly dependent on temperature with in most cases a very small temperature coefficient.

It is an advantage of isothermal GLPC that when comparing retention data obtained in one chromatogram it is permissible to replace V_g by t'. In temperature

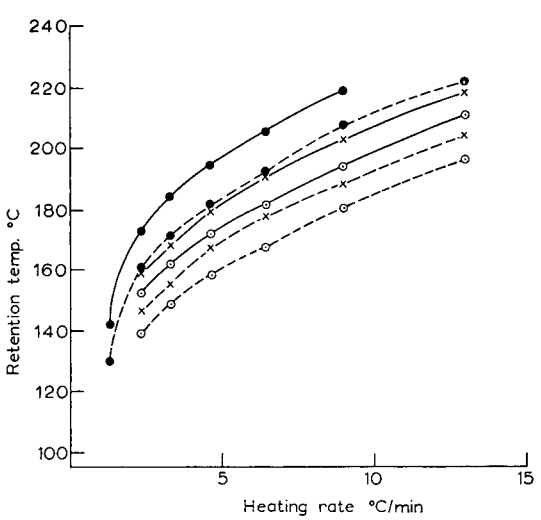

Fig. 1. Retention temperatures. Program start: 80°, program end: 225°. — — — benzyl acetate; ——— benzyl butyrate; ●——● flow rate 25.2 ml/min; ×——× flow rate 51.3 ml/min; ⊙——⊙ flow rate 75.0 ml/min.

programmed GLPC, however, this replacement is not allowed, which makes direct application of the retention parameter systems described impossible.

The retention parameter most frequently encountered in linear temperature programmed GLPC is the *retention temperature*. Unless applied under strictly identical conditions, this parameter will vary depending on heating rate and carrier gas flow-rate. In Fig. 1 this is shown for two benzyl esters. However, as might be seen from Fig. 2, the difference in retention temperatures between two compounds is remarkably constant.

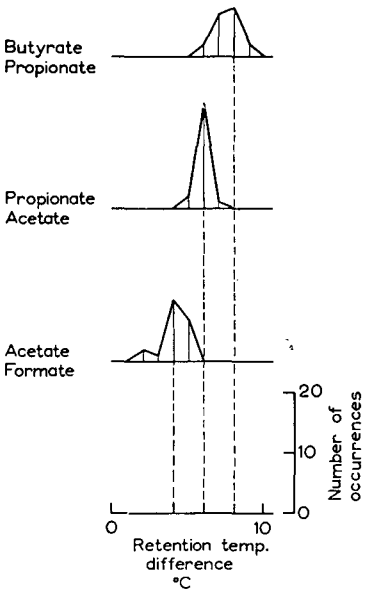

Fig. 2. Benzyl esters. Program start: 80°. Program end: 225°. Heating rates: 1,3; 2.3; 3.3; 4.6; 6.4; 9.0; 13°/min. Flow rates: 25.2; 51.3; 75.0 ml/min.

Remembering that in many cases in linear temperature programmed GLPC for the members of a homologous series the equation:

$$t'_x = C + Dn_x \tag{7}$$

will hold, we found that the retention index may be generalized to include also linear temperature programmed GLPC by rewriting eqn. (6) as:

$$I = 100\, i \frac{X - M_{(n)}}{M_{(n+i)} - M_{(n)}} + 100\, n \tag{8}$$

In isothermal GLPC, the retention index is found now by substituting for X, $M_{(n)}$ and $M_{(n+i)}$ the logarithms of the adjusted retention volumes (adjusted retention times) of respectively the compound and both markers. In linear temperature programmed GLPC for X, $M_{(n)}$ and $M_{(n+i)}$ either the retention temperatures or the adjusted retention times are substituted. Here an advantage of this way of operation over isothermal operation demonstrates itself, as gas holdup-time does not need to be

measured, which is specifically of importance in detectors which are relatively insensitive to air.

Using the same column packing, we expected that in all cases in which the temperature coefficient of the retention index is small the retention index for a compound would be practically the same in isothermal and in linear programmed GLPC, thus extending the usefulness of the retention index. The expectation proved to be true (Table I).

TABLE I

RETENTION INDICES UNDER VARIOUS CONDITIONS OF OPERATION

Instrument: F & M 500; katharometer. Stationary phase: Carbowax 20M, 20% on Celite

Compound	*Isothermal at 125°*		*Programmed 75–228° at 4.6°/min*
	Flow rate (ml/min)		*Flow rate (ml/min)*
	55.6	*80.0*	*80.0*
Ethyl formate	820	822	822
Ethyl butyrate	1032	1032	1032
Ethyl valerate	1130	1136	1130–1128
Ethyl hexanoate	1226	1228	1228–1227

Although the retention index system is based on the *n*-paraffinic series, it is sometimes useful to have at hand a secondary reference set. If the retention indices of the members of the secondary set are known, values obtained in the secondary system may be converted into standard retention indices, using the equation:

$$I = \frac{[S_{(x)} - S_{M(n)}]\,[I_{M(n+i)} - I_{M(n)}]}{100\,i} + I_{M(n)} \qquad (9)$$

In our work we found the ethyl esters of the *n*-fatty acids to be a useful secondary reference set, in which case we attach to each ethyl ester the index $S = 100\,(n-2)$. The standard retention indices of these esters are given in Table II, together with the retention indices of many other compounds. From this table also an impression may

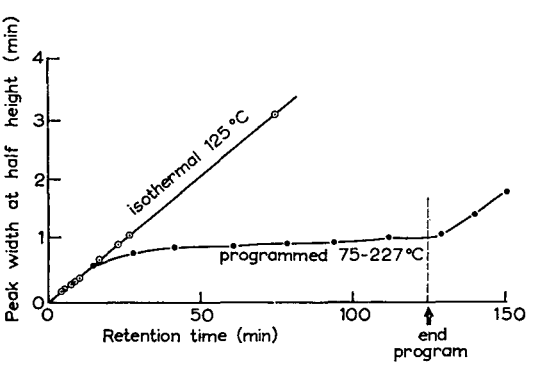

Fig. 3. Peak width.

TABLE II

RETENTION INDICES OF ESTERS AND OTHER COMPOUNDS

Compound	*Column*	
	*SE 30**	*Carbowax 20 M***
Methyl ester of		
Propionic acid		885–885
Butyric acid		970–972–971
Isobutyric acid		903
Valeric acid	808	1081–1081–1085
Isovaleric acid		1013
Hexanoic acid	907	1183–1182–1183
Isohexanoic acid		1094
Heptanoic acid	1008	1282–1281
Octanoic acid	1109	1378–1380
Nonanoic acid	1211	1484–1487
Decanoic acid	1310	1584–1588
Undecanoic acid	1410	1694–1696
Dodecanoic acid	1513	1800–1801
Myristic acid	1714	2002–1998
Hexadecanoic acid	1911	2190
Octadecanoic acid	2098	
Benzoic acid	1080	1631
Phenylacetic acid	1156–1154	1759–1761–1762
Salicylic acid	1181–1181	1794
p-Hydroxybenzoic acid	1435	
o-Hydroxyphenylacetic acid	1260–1269	
p-Hydroxyphenylacetic acid	1460	
Cinnamic acid	1363	2065
o-Hydroxycinnamic acid	1430	
m-Hydroxycinnamic acid	1690	
p-Hydroxycinnamic acid	1498	
Anthranilic acid	1325	2259
β-Hydroxybutyric acid	1320	1464–1457
2-Hydroxyisobutyric acid	1118	
2-Methylbutyric acid	758	980
2-Keto-octanoic acid	1200	
Crotonic acid		1102
Furoic acid	950	
Dimethyl ester of		
Oxalic acid	837	1381–1383
Malonic acid	895	1489–1489–1490
Succinic acid	1000–1004	1576
Glutaric acid	1105	1686–1687–1689
Adipic acid	1213	1804
Pimelic acid	1313	1908–1909
Suberic acid	1416	2010
Azelaic acid	1519	2102–2100
Sebacic acid		2213–2210
Ethyl ester of		
Formic acid		822
Acetic acid		866
Propionic acid	700	940
Butyric acid	787	1032
Isobutyric acid		950
Valeric acid	884	1130–1128

(continued on p. 468)

TABLE II (*continued*)

Compound	*Column*	
	*SE 30**	*Carbowax 20 M***
Isovaleric acid		1064
Hexanoic acid	979–983	1228–1227
Heptanoic acid	1080–1081	1324–1327
Octanoic acid	1181	1422–1427
Decanoic acid	1379–1379	1631
Lauric acid	1579	1840
Myristic acid	1780	2038
Palmitic acid	1979	2238
Stearic acid	2175	
Salicylic acid	1261	1828
Cinnamic acid	1447	2108
Lactic acid	801	
Diethyl ester of		
Oxalic acid	948	
Malonic acid	1035	
Succinic acid	1139	
Propyl ester of		
Acetic acid	704	
Isopropyl ester of		
Acetic acid		866–866
Butyl ester of		
Acetic acid	802	1065
Benzoic acid	1360	1871
Isobutyl ester of		
Acetic acid		1002–1002
Isobutyric acid	901	1090
Cinnamic acid	1598	
Benzoic acid	1318	1799
Amyl ester of		
Acetic acid	896–900	1169
Benzoic acid	1462	
Salicylic acid	1535	
Isoamyl ester of		
Acetic acid	853	1116–1116
Benzoic acid	1425	1921
Hexyl ester of		
Formic acid	913	1216
Acetic acid	993–993	1264
Butyric acid	1177	1406
Isobutyric acid		1337
Benzoic acid	1565	2068–2070
Salicylic acid	1684	2208
Hexanoic acid	1371	1606
Isohexyl ester of		
Acetic acid		1208

(continued on p. 469)

TABLE II (*continued*)

Compound	*Column*	
	*SE 30**	*Carbowax 20 M***
Heptyl ester of		
Acetic acid	1096	
Octyl ester of		
Salicylic acid	1895	
Nonyl ester of		
Acetic acid	1296	1569
Decyl ester of		
Acetic acid	1395	1674
Dodecyl ester of		
Acetic acid	1595	
Benzyl ester of		
Formic acid	1057	1687
Acetic acid	1141	1728
Propionic acid	1237	1791
Butyric acid	1325	1870
Cinnamic acid	1682	
Phenylethyl ester of		
Cinnamic acid	2143	
Anthranilic acid	2088	
Cinnamyl ester of		
Formic acid	1332	
Acetic acid	1422	2125
Propionic acid	1519	2194
Isobutyric acid	1562	2179
Isovaleric acid	1663	2289
Cinnamic acid	2052	
Allyl ester of		
Hexanoic acid	1062–1060	1360
Heptanoic acid	1163	1463
Octanoic acid	1262	1566
Alcohols		
Methanol		866–866
Ethanol		895–899
Isopropanol		866–866
Butanol		1121–1130
Isobutanol		1067
Amyl alcohol		1228–1228
Isoamyl alcohol	723	1184
Hexanol	854	1325–1323
Heptanol	957	1422–1422–1427
Octanol	1057–1058–1059	1533
Benzyl alcohol	1020	1858–1860
Phenylethyl alcohol	1197	1893–1895
Cinnamyl alcohol	1295	2238–2238

(continued on p. 470)

TABLE II (*continued*)

Compound	Column	
	*SE 30**	*Carbowax 20 M***
Aldehydes		
Butanal		866
Hexanal		1080–1080
Heptanal	895	1184–1183
Nonanal	1091	1387–1385
Decanal	1193	1498–1498
Undecanal	1296	1603–1608
Dodecanal	1397	1711–1708
Tridecanal	1501	1815–1817
Hydratropic aldehyde	1080	1631
o-Methoxycinnamaldehyde	1512	
Vanillin	1379	
Ethylvanillin	1446–1442	
Ketones		
Acetone		822
Methyl ethyl ketone		882–882
Methyl isobutyl ketone	719	
Methyl amyl ketone	873	1184–1178
Methyl hexyl ketone	973	1280–1276
Methyl heptyl ketone		1383–1380
Methyl nonyl ketone	1280	1597–1596
Methyl decyl ketone	1384	
Methyl undecyl ketone	1485	1807–1809
Diacetyl		956
Miscellaneous		
Dihydrocoumarin	1361	
Anisole	902	1341

* 25% Silicone rubber SE 30 on Celite; operated under linear temperature programmed conditions.

** 25% Carbowax 20M on Celite; operated under linear temperature programmed conditions.

be obtained of the reproducibility. The difference between two determinations ranged from 0 to 9 with an average difference of 2.

In practice, we run chromatograms of the mixture under investigation without and with a set of reference materials from which we determine the retention indices. By marking the temperature on the chart at 5° intervals and using the chart as a graph we not only easily obtain at the same time retention temperatures and the accurate end point of our program, but also have a control on the regularity of the rise in temperature.

It should be further remarked here that in quantitative work in linear temperature programmed gas–liquid partition chromatography the method for calculation of peak areas by using retention time × peak height is not applicable (see Fig. 3). Hence also the method of SMITH AND LEVI[7] for the estimation of peak width-at-half-height from a graph of known peak widths-at-half-height *versus* retention time is not applicable.

SYMBOLS USED

V_g	specific retention volume
R	gas constant
T	absolute temperature (°K)
M_L	molecular weight of the stationary phase
γ	activity coefficient
P°	saturated vapor pressure
$r_{x,s}$	relative retention of compound X with regard to the reference material S
t'	adjusted retention time
n	number of carbon atoms in the compound
I	retention index
i	difference in numbers of carbon atoms of the reference materials
$M_{(n)}$; $M_{(n+i)}$	reference material with (n); $(n + i)$ carbon atoms
S	retention index in the secondary reference system
A; B; C; D	constants
subscripts	refer to the compounds

SUMMARY

The different ways of describing peak positions on gas chromatograms are reviewed. The retention index is preferred to the theoretical nonane system and the relative retention.

The equation given by KOVÁTS for the calculation of the retention index in case of isothermal operation is transformed to a more general form to include also the case of linear temperature programmed operation. This generalized equation gives the same retention index for both ways of operation.

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SEPARATION OF SULFONES BY GAS CHROMATOGRAPHY

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INTRODUCTION

Sulfones of the general formula R_2SO_2 are used for insecticides, fuel additives, plasticizers, anti-icing additives[1] and many other applications. With increasing use of the sulfones taking place the analysis of materials containing them is becoming more important. This study was made to determine the optimum column materials and conditions for effectively separating the sulfones by gas–liquid chromatography.

EXPERIMENTAL

Sulfones

Dimethyl sulfone was obtained from Crown Zellerback Corporation, *n*-propyl, *n*-butyl and phenyl sulfone from Eastman, ethyl, isopropyl, isobutyl, *sec.*-butyl, *n*-amyl, isoamyl, *n*-hexyl, butadiene, tetramethylene (sulfolane) and *sym.*-trimethylene trisulfone from Wateree Chemical Co., ethyl 2-hydroxyethyl, 2-hydroxyethyl, 4-chlorophenyl, 3-aminophenyl, 4-aminophenyl, 4-hydroxyphenyl, dibenzothiophene, 2,4-dimethylsulfolane, and 2,4-dimethyl-3-sulfolene from Aldrich Chemical Co., 3-methylsulfolane, sulfolene, and 3-methylsulfolene from Phillips Petroleum Co., and methyl sulphonal from British Drug Houses Ltd. The *tert.*-butyl and methyl *p*-nitrophenyl sulfones were prepared in this laboratory.

Since a solid sample vaporizer was not available, the solid sulfones were injected into the gas chromatograph as a solution in an organic solvent. Acetone was found to be a very good solvent for most of the sulfones and was generally used to facilitate injection into the gas chromatograph. Chloroform was used for dibenzothiophene sulfone since it is a better solvent for this compound. Both solvents emerged from the column quickly and did not interfere with further study of the chromatogram.

Equipment

One of the instruments used for this study was constructed in this laboratory, the other was an F and M Model 609. The detector system for the former (Figs. 1 and 2) was similar to that of FELTON AND BUEHLER[2] using direct flow of the gas stream past Veco model airplane glo-plugs, type No. 109 with 7/32 in. reach. The connecting wire was silver soldered to the cap of the glo-plug. The power supply was a 4 V rechargeable lead storage battery which gave 2.4 V at the detector terminals. This voltage gave

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considerable greater sensitivity than the 1.5 V recommended by Veco, however, it was a practice to flush the system with helium before turning on the detector switch in order to prolong the life of the glo-plugs. The D.C. source gave more stable operation than an A.C. power supply.

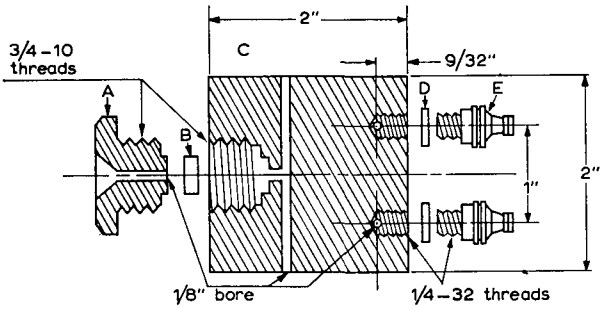


Fig. 1. Injector-detector block cross section. A. Section of a 3/4 in. 10 threads/in. machine bolt. B. Silicone rubber disc septum, 3/8 in. diameter, 1/8 in. thick. C. 2 in. cube piece of stainless steel. D. Copper glo-plug gasket, 1/4 in. I.D. E. Veco No. 109 glo-plug.

The temperature of the isothermal oven constructed by BEUERMAN AND MELOAN[3] was controlled to within 1 degree during a days use by heating tapes and a powerstat variable transformer. The injector-detector block was maintained at a slightly higher temperature than the oven by means of a separate heating tape and powerstat.

Column materials and preparation

The columns were 3 ft. long and made of 1/4 in. O.D. aluminum tubing obtained from the Ben McKalip Co., the support was coated by covering 100 g of solid support with

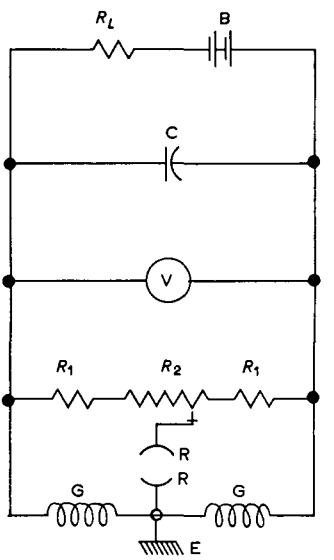


Fig. 2. Electrical circuit for the detector system. B = 2 V Gould National Battery Co. type EP 9 rechargeable lead-acid storage batteries. C = 500 mF 50 V D.C. capacitor. G = Veco No. 109 glo-plug. R = recorder leads. $R_1 = 47\,\Omega$, 1 W resistor. $R_2 = 25\,\Omega$, 10 turn helipot. R_L = resistance of trunk line cables. V = voltmeter, 0 to 3 V.

20 g of the liquid phase. Acetone was used as the solvent for the Carbowaxes and benzene for the silicones and Apiezon greases.

Column conditions

The columns were compared at 200°, as this is the temperature limit for the Carbowax 1500, with 15 p.s.i.g. of helium pressure at the inlet and atmospheric pressure at the outlet. The helium flow rate was measured with a soap bubble flow meter at the exhaust. Temperatures of 225° and 250° were used on those columns with higher temperature limits than 200°.

RESULTS AND DISCUSSION

The identification of the sulfones was verified by plotting the logarithm of the adjusted retention volume, V_R', (the retention volume from air) *vs.* the number of carbon atoms as shown in Figs. 3, 4 and 5, and mentioned by PECSOK[4]. The numbers refer to similar numbers on Table I. The members of homologous structural type formed a straight or slightly curved line on these plots. The branched alkyl sulfones had shorter retention times than the straight chain compounds. The sulfone group is very polar and groups which tended to shield this group produced sulfones which had shorter rentention times.

Thermal stability

No difficulty was observed due to possible thermal decomposition of any of the sulfones within the injection port or the column at operating temperature of 250°. This would be expected because of their great stability and very low reactivity[5]. This is contrary to experience with the sulfoxides and their disproportionation reactions which caused considerable trouble[6].

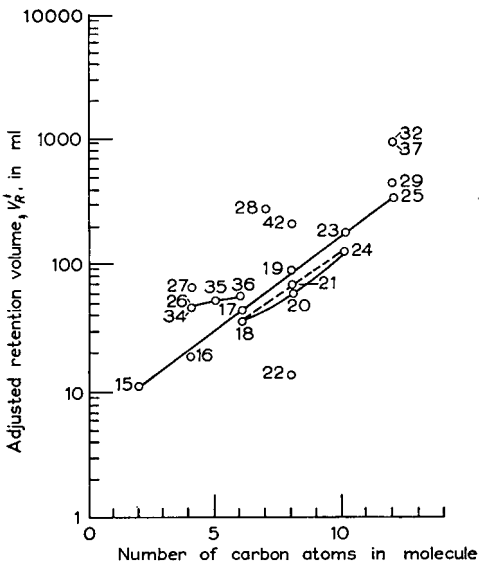
Column characteristics

Liquid phases giving satisfactory results were Carbowax 1500, Carbowax 20-M, Dow Corning silicone oil 550, Silicone gum rubber (Fisher Scientific), and Apiezon L and M greases from James G. Biddle Co. These liquid phases have high temperature limits which are necessary for several of the sulfones, particularly the aryl sulfones.

Solid supports found to be satisfactory were Haloport F, 60-80 mesh Gas-Chrom P (acid and alcoholic base washed), Gas-Chrom Z (acid washed and silanized), and 35-80 mesh Chromosorb (silver plated as described by ORMEROD AND SCOTT)[7], which gave only moderately acceptable results. Untreated Chromosorb and Chromosorb W were found to be unsatisfactory due to tailing of these polar compounds and of the water sometimes present in the sample. The most nearly symmetrical and sharpest peaks with the least amount of tailing for a given partition liquid were obtained when Gas-Chrom Z was used as the solid support.

The shape of the peaks was quite good when using any of the listed liquid phases except when the compounds had large retention volumes, such as phenyl and 4-chlorophenyl sulfones. These had a sloping leading edge and steeper backsides, which were considerably improved by raising the column temperature. Our instrument did not allow us to push this to the limit to see if good symmetrical peaks could eventually be obtained although it seemed possible that it could be done.

Fig. 3. The adjusted retention volume, V_R', plotted against the number of carbon atoms in the molecule for the sulfones. The column was 3 ft. long and contained Apiezon L grease on Gas-Chrom Z. The points represent the sulfones having the corresponding numbers in Table I, and the volumes are those given in the table for the above column.



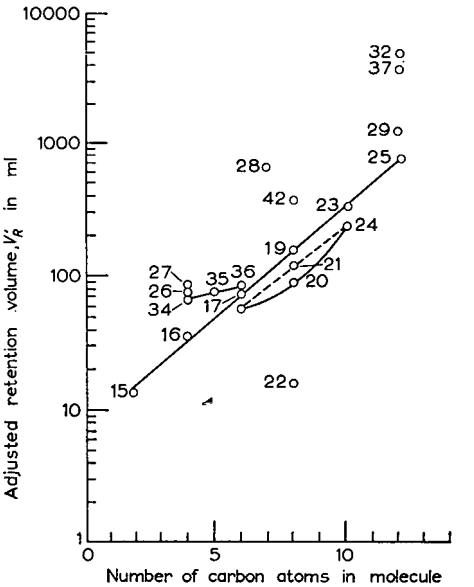


Fig. 4. The adjusted retention volume V_R', plotted against the number of carbon atoms in the molecule for the sulfones. The column was 3 ft. long and contained Silicone gum rubber on Gas-Chrom Z. The points represent the sulfones having the corresponding numbers in Table I, and the volumes are those given in the table for the above column.

Fig. 5. The adjusted retention volume, V_R', plotted against the number of carbon atoms in the molecule for the sulfones. The column was 3 ft. long and contained Carbowax 20-M on Gas-Chrom Z. The points represent the sulfones having the corresponding numbers in Table I, and the volumes are those given in the table for the above column.

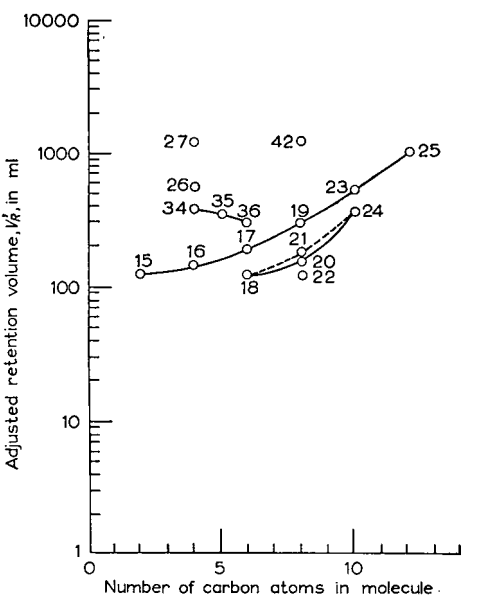


TABLE I

ADJUSTED RETENTION VOLUMES, V_R', OF THE SULFOXIDES IN ml

	Column No.	*1*		*2*		*3*		*4*		*5*		*6*
	°C	*200*	*250*	*200*	*250*	*200*	*250*	*200*	*225*	*200*	*250*	*200*
	He flow ml/min	*165*	*165*	*200*	*200*	*140*	*110*	*143*	*143*	*165*	*165*	*130*
	Sulfone											
15	Methyl	11		14		11		23		122		109
16	Ethyl	25		36		19		49		141		122
17	*n*-Propyl	46		74		43		91		193		162
18	Isopropyl	38		58		36		69		123		105
19	*n*-Butyl	95		156		92		192		298		286
20	Isobutyl	58		94		61		105		153		135
21	*sec.*-Butyl	77		124		72		133		178		157
22	*tert.*-Butyl	13		16		14		26		122		111
23	*n*-Amyl	211		346		189		393		525		495
24	Isoamyl	147		242		134		287		359		325
25	*n*-Hexyl	495	110	790	206	368	89	882	318	1000	195	891
26	2-Hydroxyethyl	61	26	76	34	47	38	109	60	541	122	417
27	Ethyl 2-hydroxyethyl	71	28	86	36	67	41	144	87	1210	198	1270
28	Methyl *p*-nitrophenyl	570	123	680	196	281	87	882	353		905	5400
29	Phenyl	775	187	1250	328	478	114	1780	565		695	4630
30	3-Aminophenyl*											
31	4-Aminophenyl*											
32	4-Chlorophenyl		520	5000	980	1010	197		1370		1630	
33	4-Hydroxyphenyl*											
34	Sulfolane	44		70		46		109		370		300
35	3-Methylsulfolane	54		78		54		128		343		267
36	2,4-Dimethylsulfolane	58		86		58		123		297		215
37	Dibenzothiophene	2300	445	3730	670	1000	167	4300	1330		2040	
38	Sulfolene*											
39	3-Methylsulfolene*											
40	2,4-Dimethyl-3-sulfolene*											
41	Butadiene*											
42	Methyl sulphonal	240	72	386	128	215	70	608	218	1210	215	900
43	*sym.*-Trimethylene trisulfone**											

Columns: 1 = Apiezon M; 2 = Apiezon L; 3 = Silicone gum rubber; 4 = Silicone oil 550; 5 = Carbowax 20-M; 6 = Carbowax 1500.

* Did not emerge in 30 min at 250° on Silicone gum rubber column.

** A suitable solvent in which this compound is soluble could not be found.

No retention volumes are given for the aminophenyl, hydroxyphenyl, and butadiene sulfones or any of the sulfolenes as these were not observed to emerge in 30 min. at 250°, the maximum operating temperature of the chromatograph, on the silicone gum rubber column.

Table I shows that the silicone gum rubber column generally gave the smallest retention volumes and Carbowax 20-M gave the longest. The carbowaxes gave the best separations of the sulfones from other components; however, the silicones and Apiezon greases give better separation and more linear curves of the log of retention volume *vs.* the number of carbon atoms for homologous sulfones.

By using flow rates less than those in Table I and varying the column temperatures to obtain the desired retention times, while using a liquid phase with a higher

temperature limit than the column temperature, quite satisfactory separations can be obtained. It may also be desired to use greater column lengths to increase the resolution of the components. The use of programmed temperature gas chromatography has considerable advantage with these compounds as can be seen by Figs. 6 and 7, the

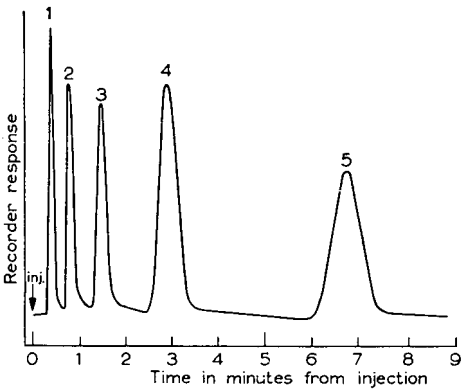


Fig. 6. Isothermal gas chromatogram of a mixture of sulfones. Instrument = F & M 609. Injection port temperature = 230°. Column temperature = 175°. Helium flow = 100 ml/min. Sample size = 0.5 μl. Attenuator setting: peak 1 at 64, others at 32. Column = 3 ft. long, Carbowax 20-M on Gas-Chrom Z. Peak 1 = acetone solvent. Peak 2 = dimethyl sulfone = 13% by weight. Peak 3 = diethyl sulfone = 15% by weight. Peak 4 = *n*-propyl sulfone = 37% by weight. Peak 5 = *n*-butyl sulfone = 35% by weight.

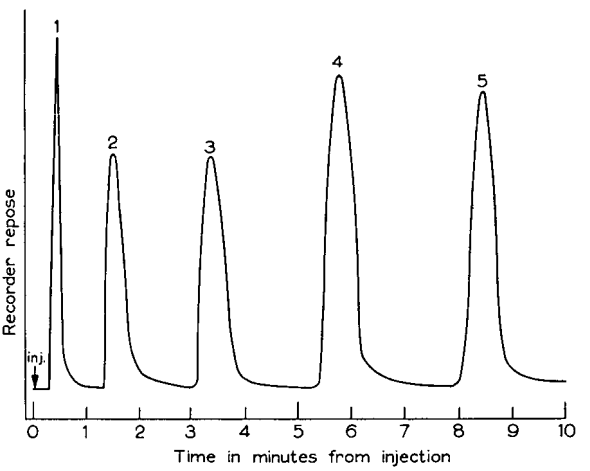


Fig. 7. Programmed temperature gas chromatogram of a mixture of sulfones. Instrument = F & M 609. Injection port temperature = 230°. Column temperature at start = 125°. Temperature programming rate = 13° per min. Helium flow = 100 ml/min. Sample size = 0.5 μl. Attenuator setting = peak 1 at 64, others at 32. Column = 3 ft. long, Carbowax 20-M on Gas-Chrom Z. Peak 1 = acetone solvent. Peak 2 = dimethyl sulfone = 13% by weight. Peak 3 = diethyl sulfone = 15% by weight. Peak 4 = *n*-propyl sulfone = 37% by weight. Peak 5 = *n*-butyl sulfone = 35% by weight.

separation of four homologous sulfones. A weight percent standard should be used for calibration and quantitative analysis.

Fig. 8 shows the separation of a sulfide, a sulfoxide and a sulfone since this combination occurs together quite frequently in commercial products.

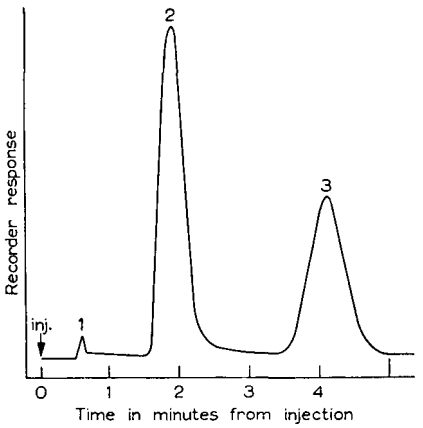


Fig. 8. Gas chromatogram of a mixture containing a sulfide, a sulfoxide and a sulfone having the same R groups. Instrument = F & M 609. Injection port temperature = 160°. Column temperature = 150°. Helium flow = 90 ml/min. Sample size = 0.4 μl. Attenuator setting = 64. Column = 3 ft. long, Carbowax 20-M on Gas-Chrom Z. Peak 1 = *tert.*-butyl sulfide. Peak 2 = *tert.*-butyl sulfoxide. Peak 3 = *tert.*-butyl sulfone.

ACKNOWLEDGEMENT

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SUMMARY

Sulfones were separated by gas–liquid chromatography; the best separations were obtained using Carbowax 1500 and 20-M on Gas-Chrom-Z, a silanized diatomaceous solid support. The results are compared and discussed for other liquid phases and supports. The sulfones studied were: dimethyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec.*-butyl, *tert.*-butyl, butadiene, *n*-amyl, isoamyl, *n*-hexyl, phenyl, methyl *p*-nitrophenyl, tetramethylene, *sym.*-trimethylene trisulfone, ethyl 2-hydroxyethyl, 2-hydroxyethyl, 4-chlorophenyl, 3-aminophenyl, 4-aminophenyl, 4-hydroxyphenyl, dibenzothiophene, 2,4-dimethylsulfolane, 2,4-dimethyl-3-sulfolene, 3-methylsulfolane, sulfolene, 3-methylsulfolene and methyl sulfonal.

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THIN-FILM CHROMATOGRAPHY IN THE STUDY OF CARBOHYDRATES*

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Thin-film silicic acid chromatography[1] has been developed by STAHL and coworkers[2–5] into an important analytical technique of great adaptability[6–8] which has been modified for micropreparative use[9] and quantitative analysis[9,10].

The method has found application in the study of a broad range of compounds including terpenes[1,2,11], organic peroxides[3], protein hydrolyzates[12,13], pyrethrins[14], indole and its derivatives[15], alkaloids[5,16], lipids[17–22], steroids[23,24], 2,4-dinitrophenylhydrazones[25], and in inorganic analysis[7]. Apart from its application in the separation of mixtures of a few simple sugars[26], digitalis and podophyllum glycosides[27], mono-O-methyl, mono- and di-O-isopropylidene[28], O-acyl[29–31], and 2,4-dinitrophenylhydrazone[32] derivatives, the technique has had only limited usage in the carbohydrate field. This paper reports the extension of thin-layer silicic acid chromatography to the qualitative investigation of a variety of partially or completely derivatized, low molecular weight carbohydrates and to the quantitative determination of the composition of a mixture of methylated sugars.

Three solvent systems have been employed. Benzene–ethanol–water–ammonium hydroxide (200:47:15:1)[33] (solvent A) is preferred for the separation of mixtures of O-methyl sugars and their glycosides. 1-Butanol–acetic acid–ethyl ether–water (9:6:3:1) (solvent B) is employed for the separation of carbohydrates with low degrees of substitution, whereas 1-butanol–acetic acid–water (2:1:1)[34] (solvent C) gives satisfactory resolution of sugar acids or acid derivatives.

In general, the R_F values of fully derivatized compounds are high (see Tables IV, V and VI), a decrease in R_F being observed as the number of free hydroxyl groups in the molecule is increased. Thus, the R_F of tetra-O-methyl-D-glucopyranose is 0.38 in solvent A, those of the tri-O-methyl-D-glucoses are of the order of 0.15 whereas the di-O-methyl-D-glucoses exhibit R_F values of approximately 0.05 (see Table III).

The separation of similarly derivatized diastereoisomers is slight. For example, in solvent A, the R_F of methyl tetra-O-acetyl-β-D-glucopyranoside is 0.76 whereas the R_F of methyl tetra-O-acetyl-β-D-mannopyranoside is 0.79 (see Table II). An exception to this generalization is the excellent separation of D-glucosaccharo-1,4-lactone from D-glucosaccharo-3,6-lactone in solvent C, the R_F of the former being 0.43 whereas the R_F of the latter is 0.85 (see Table VII).

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The pentoses and their derivatives have higher R_F values than the hexose analogues. However, this relationship is reversed in the case of the respective glyconic acids. Accordingly, L-arabinose and D-glucose have R_F values of 0.57 and 0.49 respectively in solvent B (see Table I), whereas the R_F value of L-arabonic acid is 0.35 and that of D-gluconic acid is 0.40 in solvent C (see Table VII).

The development of the chromatoplates requires 0.5 to 3.5 hours depending upon the relative volatility of the solvent and the room temperature. Solvent A affords the most rapid and solvent C the slowest development.

It is observed that the migration of a particular compound is decreased by repeated use of the solvent. For this reason it is recommended that fresh solvent be employed each time a chromatoplate is developed.

The compounds are detected by spraying the developed plates with concentrated sulfuric acid or alkaline potassium permanganate[35,36] and heating until suitable color formation occurs.

Sulfuric acid is effective in detecting all carbohydrate compounds. Sugar alcohols, glyconic acids, inositol and their derivatives require stronger heating than reducing sugars and their derivatives, a property useful in differentiating compounds having similar R_F values. Glycerol and glyceryl hexosides give transient pink spots which change to brown on standing at room temperature. Pentoses yield spots with a purple cast whereas sulfur-containing derivatives give yellow-green spots. Hexoses, hexose-containing oligosaccharides and their derivatives give yellow-brown to black spots.

The alkaline permanganate spray readily detects all compounds with free hydroxyl groups or compounds which are derivatized with alkali-labile groups. The spray gives bright yellow spots on a purple background which fade to white spots on a brown background on standing at room temperature. This spray is preferred for the detection of polyols and inositol.

With partially derivatized carbohydrates ammoniacal silver nitrate[37], *p*-anisidine–trichloroacetic acid[38] or aniline hydrogen oxalate[39] may be used. The sensitivity of the ammoniacal silver nitrate is lower on silicic acid than on chromatography paper. Acylated derivatives are readily detected (*cf.* ref. 29) as the ferric hydroxamates[40].

A mixture of 3-O-methyl-D-glucopyranose, 2,3,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucose can be resolved using solvent A, and quantitatively eluted[10] and estimated using the phenol–sulfuric acid colorimetric determination[41]. This procedure permits a rapid determination of the molar ratios of the mono-, di-, tri- and tetra-O-methyl components in the hydrolysate of a methylated polysaccharide.

Thin-layer silicic acid chromatography affords a simple, rapid and sensitive method for the qualitative and quantitative study of low molecular weight carbohydrates. It is of particular value for carbohydrates which are not readily detected by conventional chromatography. By this means reactions in carbohydrate chemistry involving the preparation or transformation of derivatives can be followed with speed and facility, and, as a result of the sensitivity of this technique, very low concentrations of side products or impurities can be detected.

EXPERIMENTAL

(*a*) *Solvents*

The following solvents were used for the separation of partially or completely derivatized carbohydrates:

Solvent A[33]. Benzene–ethanol–water–ammonium hydroxide (sp. gr. 0.8), 200:47:15:1 parts by volume. The upper phase of this two phase system is used. It has been observed that the substitution of glacial acetic acid for the ammonium hydroxide gives slightly sharper bands or spots.

Solvent B. 1-Butanol–acetic acid–ethyl ether–water, 9:6:3:1 parts by volume (single phase system).

Solvent C[34]. 1-Butanol–acetic acid–water, 2:1:1 parts by volume (single phase system).

(*b*) *Spray reagents*

Spray 1: Concentrated sulfuric acid. Spray 2[35,36]:0.5 % potassium permanganate in *N* NaOH.

(*c*) *Preparation of plates*

A slurry of Silica Gel G (Merck and Co.) (30 g) in water (66.5 ml) is applied to 5 × 20 cm or 20 × 20 cm smooth glass plates using the apparatus of STAHL[2,4].

The plates are dried overnight at 135°. The serrated plates, after drying, are scraped with a straight-edge to remove adsorbent from the ridge peaks.

(*d*) *Chromatographic procedure*

The compounds are dissolved in suitable solvents and applied to the chromatoplate with glass capillaries[3]. The plates are developed in pre-equilibrated battery jars or wide-mouth bottles by the ascending technique.

(*e*) *Detection of carbohydrate compounds*

The developed plates are dried in the air and sprayed with either concentrated sulfuric acid or a solution of 0.5 % potassium permanganate in *N* sodium hydroxide. The plates are then heated in a suitable oven at about 100°. With the alkaline permanganate spray heating for 0.5 to 2 min is required whereas if the plates are preheated before the spraying, the color develops almost instantaneously. With the sulfuric acid spray a heating time of 5 to 10 min is usually sufficient. Whereas reducing sugars and their derivatives can be detected at about 100°, sugar alcohols, glyconic acids, inositol and their derivatives require heating to approximately 150°.

(*f*) *The separation of partially or completely derivatized sugars, polyols, glyconic and glycuronic acids and inositol*

The results of this study are summarized in Tables I to VII.

(*g*) *The quantitative determination of a mixture of 3-O-methyl-D-glucopyranose, 2,3,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucose*

3-O-Methyl-D-glucopyranose (0.00404 g, 2.0 μmole), 2,3,6-tri-O-methyl-D-glucopyranose (0.00135 g, 0.61 μmole) and 2,3,4,6-tetra-O-methyl-D-glucose (0.00061 g, 0.25 μmole) are dissolved in methanol (approx. 0.2 ml).

A portion of this solution is applied to a 20 × 20 cm glass plate coated[24] with Silica Gel G, using a capillary tube. The sample is applied at 1 mm intervals along a straight line 16 cm long equidistant from each side and 3.2 cm from the bottom of the plate. The plate is developed in solvent A by the ascending technique.

TABLE I

R_F VALUES FOR FREE SUGARS, POLYOLS AND *myo*-INOSITOL IN SOLVENT B

Compound	R_F	*Compound*	R_F
D-Ribose	0.59	Melibiose	0.15
L-Arabinose	0.57	Lactose	0.09
D-Xylose	0.57	Raffinose	0.13
D-Glucose	0.49	Glycerol	0.58
D-Mannose	0.55	Erythritol	0.52
D-Fructose	0.51	D-Mannitol	0.38
Maltose	0.29	D-Glucitol	0.39
Cellobiose	0.32	Galactitol	0.36
Isomaltose	0.16	Maltitol	0.10
Laminaribiose	0.26	*myo*-Inositol	0.27
Sucrose	0.25		

TABLE II

R_F VALUES FOR GLYCOSIDES AND DERIVATIZED GLYCOSIDES OTHER THAN O-METHYL ETHERS

Compound	R_F *in solvent*	
	A	*B*
Methyl β-L-arabinopyranoside	0.03	0.48
Methyl α-D-glucopyranoside	0.02	0.48
Methyl β-D-glucopyranoside	0.02	0.52
Methyl α-D-mannopyranoside	0.02	0.51
n-Amyl β-D-glucopyranoside	0.04	0.69
Phenyl β-D-glucopyranoside	0.03	0.69
Methyl β-maltoside	0.01	0.31
Methyl β-lactoside	0.00	0.17
Methyl α-isomaltoside	0.00	0.19
Methyl 3,6-anhydro-α-D-glucopyranoside	0.03	0.59
Methyl 6-O-benzyl-α-D-galactopyranoside	0.08	0.69
Methyl tetra-O-acetyl-β-D-glucopyranoside	0.76	0.79
Ethyl tetra-O-acetyl-β-D-glucopyranoside	0.78	0.80
n-Propyl tetra-O-acetyl-β-D-glucopyranoside	0.83	0.83
Phenyl tetra-O-acetyl-β-D-glucopyranoside	0.82	0.81
Methyl tetra-O-acetyl-β-D-mannopyranoside	0.79	0.81
Methyl hepta-O-acetyl-β-maltoside	0.74	0.74
Methyl 4,6-O-benzylidene-α-D-glucoside	0.21	0.81
Methyl 4,6-O-benzylidene-β-D-glucoside	0.21	0.79
Methyl 2,3-anhydro-4,6-O-benzylidene-α-D-alloside	0.83	0.76
Methyl 2,3-di-O-tosyl-4,6-O-benzylidene-α-D-glucoside	0.90	0.91

TABLE III

R_F VALUES OF O-METHYL ETHERS OF SUGARS AND POLYOLS IN SOLVENT A

Compound	R_F
2,3-Di-O-methyl-D-xylopyranose	0.15
2,3,4-Tri-O-methyl-D-xylopyranose	0.28
2,4-Di-O-methyl-D-glucopyranose	0.05
2,6-Di-O-methyl-D-glucopyranose	0.05
2,3,6-Tri-O-methyl-D-glucopyranose	0.18
2,4,6-Tri-O-methyl-D-glucopyranose	0.13
2,3,4,6-Tetra-O-methyl-D-glucopyranose	0.38
2,4,6-Tri-O-methyl-D-galactopyranose	0.17
2,4-Di-O-methyl-L-fucopyranose	0.11
1,2,5,6-Tetra-O-methyl-3,4-O-isopropylidene-D-mannitol	0.60

After removal of solvent by evaporation at room temperature the center of the plate is covered with a band of polyethylene film 12 cm wide situated 4 cm from each side of the plate and covering its entire length, so as to leave a 2-cm strip, containing a portion of the sugar mixture, on each side of the protective covering. The exposed areas of the plate are sprayed with sulfuric acid and the plate heated for 5 min at 100° to develop color. The bands are located and marked. The silicic acid of the appropriate areas containing each component is then sucked off the plate through a drawn out tube into distilled water (3 ml) in a test tube by means of a water aspirator. The glass tubing and rubber connections used to conduct the silicic acid into the water are rinsed with distilled water (1 ml) and the two solutions combined. The silicic acid is separated by centrifugation and aliquots (1 ml) of the clear, colorless supernatant are used in the determination of carbohydrate by the phenol–sulfuric acid method[41]. The absorbancies of triplicate samples are determined at 485 mμ in a Coleman Jr. spectrophotometer[41]. The average absorbance is used to determine the weight of carbohydrate present by reference to standard curves previously constructed for the purpose.

A blank experiment revealed the presence on the developed chromatoplate of two readily visible, narrow yellow bands having R_F values of 1.00 and 0.78. The colored

TABLE IV

R_F VALUES OF O-METHYL ETHERS OF GLYCOSIDES

Compound	R_F *in solvent*	
	A	*B*
Methyl 4-O-methyl-α-D-glucopyranoside	0.05	0.57
Methyl 6-O-methyl-α-D-galactopyranoside	0.06	0.45
Methyl 2,3-di-O-methyl-α-D-glucopyranoside	0.13	0.64
Methyl 2,6-di-O-methyl-α-D-glucopyranoside	0.15	0.62
Methyl 4,6-di-O-methyl-α-D-glucopyranoside	0.23	0.64
Methyl 3,4-di-O-methyl-α-D-mannopyranoside	0.21	0.57
Methyl tetra-O-methyl-α-D-glucopyranoside	0.52	0.75
Methyl tetra-O-methyl-α-D-mannopyranoside	0.54	0.69
Methyl hepta-O-methyl-β-maltoside	0.76	0.65

TABLE V

R_F VALUES OF ESTERIFIED SUGARS, POLYOLS AND *myo*-INOSITOL

Compound	R_F *in solvent*	
	A	*B*
Tetra-O-acetyl-D-xylopyranose	0.84	0.84
Penta-O-acetyl-α-D-glucopyranose	0.82	0.79
Penta-O-acetyl-β-D-glucopyranose	0.81	0.79
Penta-O-acetyl-β-D-galactopyranose	0.81	0.77
Hexa-O-acetyl-D-mannitol	0.83	0.73
Hexa-O-acetyl-*myo*-inositol	0.79	0.82
3,4,5,6-Tetra-O-acetyl-D-glucosazone	0.74	0.88
Tri-O-acetyl-D-glucal	0.83	0.79
Hexa-O-acetylxylobiose	0.72	0.84
Octa-O-acetyl-α-maltose	0.79	0.85
Octa-O-acetyl-β-cellobiose	0.64	0.84
Octa-O-acetyl-β-laminaribiose	0.62	0.81
Octa-O-acetyl-sucrose	0.63	0.77
Octa-O-propionyl-α-maltose	0.92	1.00
6-O-Tosyl-tetra-O-acetyl-β-D-glucopyranose	0.84	0.87
6-Deoxy-6-iodo-tetra-O-acetyl-β-D-glucopyranose	0.88	0.87
Tetra-O-benzoyl-D-fructofuranose	0.87	0.87

TABLE VI

R_F VALUES OF ACETAL AND MERCAPTAL DERIVATIVES OF SUGARS AND POLYOLS

Compound	R_F *in solvent*	
	A	*B*
1,2-O-Isopropylidene-D-glucose	0.10	0.79
1,2,5,6-Di-O-isopropylidene-D-glucose	0.49	0.90
1,2,3,4-Di-O-isopropylidene-D-galactose	0.47	0.84
6-O-Tosyl-1,2,3,4-di-O-isopropylidene-D-galactose	0.90	0.89
6-Deoxy-6-iodo-1,2,3,4-di-O-isopropylidene-D-galactose	0.93	0.89
3,4-O-Isopropylidene-D-mannitol	0.05	0.68
1,2,3,4,5,6-Tri-O-isopropylidene-D-mannitol	0.92	0.88
4,6-O-Ethylidene-D-glucitol	0.02	0.61
1,2-O-Isopropylidene-3,5-O-benzylidene-6-O-acetyl-D-glucofuranose	0.83	0.87
D-Galactose diethyl mercaptal	0.05	0.66
L-Arabinose diethyl mercaptal	0.12	0.77
L-Fucose diethyl mercaptal	0.18	0.76
L-Rhamnose diethyl mercaptal	0.21	0.78

TABLE VII

R_F VALUES OF SUGAR ACIDS AND THEIR DERIVATIVES

Compound	R_F *in solvent C*	*Compound*	R_F *in solvent C*
D-Ribonic acid	0.38	Calcium D-galactonate	0.38
D-Ribono-γ-lactone	0.61	D-Galactono-γ-lactone	0.61
L-Arabonic acid	0.35	D-Galactono-δ-lactone	0.47
Calcium L-arabonate	0.33	D-Galacturonic acid	0.32
L-Arabono-γ-lactone	0.64	D-Mannuronic acid	0.36
L-Arabono-δ-lactone	0.58	D-Mannurono-γ-lactone	0.53
L-Arabonic phenylhydrazide	0.62	L-Rhamnono-γ-lactone	0.64
D-Gluconic acid	0.40	D-Gulonic acid	0.31
D-Glucono-γ-lactone	0.68	D-Gulono-γ-lactone	0.53
D-Gluconic phenylhydrazide	0.60	D-Gulono-δ-lactone	0.43
D-Glucurono-γ-lactone	0.58	α-D-Glucoheptonic-γ-lactone	0.43
D-Glucosaccharo-1,4-lactone	0.43	Ascorbic acid	0.57
D-Glucosaccharo-3,6-lactone	0.85	Isoascorbic (D-araboascorbic) acid	0.57
D-Galactonic acid	0.38		

compounds in these bands are water-soluble and the aqueous solutions when treated with the phenol–sulfuric acid reagent show some absorbance at 485 mμ. Hence, this colored material would therefore interfere with the colorimetric determination of carbohydrate compounds with the same R_F values. The other areas of the chromatoplate contain no water-soluble materials which interfere with the phenol–sulfuric acid assay and no blank is required for the determination of carbohydrate compounds located outside of the two areas of the colored bands referred to above.

The results of two separate determinations are as follows:

Experiment 1. Found: 3-O-Methyl-D-glucopyranose, 111.2 γ (0.575 μmole); 2,3,6-tri-O-methyl-D-glucopyranose, 43.2 γ (0.194 μmole); 2,3,4,6-tetra-O-methyl-D-glucose, 19.2 γ (0.0815 μmole). This corresponds to a molar ratio of 7.0:2.4:1.0.

Experiment 2. Found: 3-O-Methyl-D-glucopyranose, 176.0 γ (0.909 μmole); 2,3,6-tri-O-methyl-D-glucopyranose, 67.6 γ (0.304 μmole); 2,3,4,6-tetra-O-methyl-D-glucose, 30.0 γ (0.127 μmole). This corresponds to a molar ratio of 7.1:2.4:1.0. The molar ratio of the three sugars applied was 8.0:2.4:1.0.

Recovery of carbohydrates from silica gel is quantitative.

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SUMMARY

Thin-film silicic acid chromatography has been demonstrated to be a useful technique for the separation of a wide variety of partially and completely derivatized carbohydrates. The procedure can be applied to the quantitative separation and estimation of mixtures of O-methyl sugars.

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DÜNNSCHICHTCHROMATOGRAPHIE VON STRUKTUR- UND MOLEKULAREINHEITLICHEN *p*-KRESOL–FORMALDEHYD-KONDENSATEN

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Über die Anwendung der Dünnschichtchromatographie zur Trennung von Phenolen sind bereits einige Veröffentlichungen erschienen. So trennt STAHL[1] die in den ätherischen Ölen verschiedener Pflanzen enthaltenen Phenolderivate auf Kieselgelplatten mit Phenol als Laufmittel. PASTUSKA UND PETROWITZ[2] untersuchten das Verhalten zahlreicher Phenole, Phenolcarbonsäuren, Phenolaldehyde und Dimethylphenole.

Bei den vorliegenden Versuchen sollte festgestellt werden, ob sich Mehrkernverbindungen (Novolake) mit *p*-Kresol als Baustein[3] und verschiedene, verwandte Phenolalkohole durch Dünnschichtchromatographie trennen lassen. Tabelle I enthält die untersuchten Verbindungen.

Es wurden Trogkammern mit Kammersättigung benutzt und die von STAHL[4] angegebenen Standardbedingungen eingehalten. Die Kieselgel*-Platten waren mit Hilfe eines Streichgerätes** hergestellt. Die Kieselgelschichten hatten eine Stärke von 250 μ. Die Platten waren durch Erhitzen in einem Trockenschrank (30 Min. auf

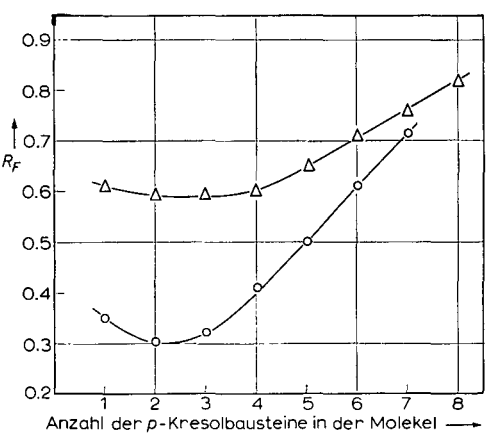


Fig. 1. Abhängigkeit der R_F-Werte von der Anzahl der *p*-Kresolbausteine in der Mehrkernverbindung bei Verwendung von Laufmittel I. Homologe Reihen der Struktur H-*n*-H (○) und Cl-*n*-Cl (△) (s. Tabelle I).

* Kieselgel G der Fa. Merck, Darmstadt.
** Desaga-Grundausrüstung der Fa. Desaga, Heidelberg.

TABELLE I

ZUSAMMENSTELLUNG DER UNTERSUCHTEN VERBINDUNGEN

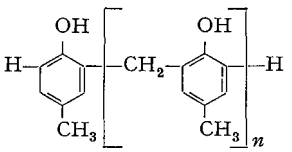
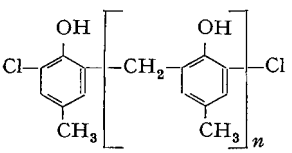
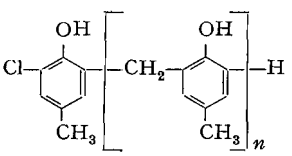
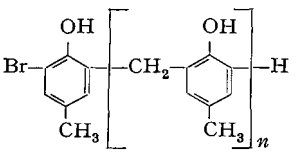
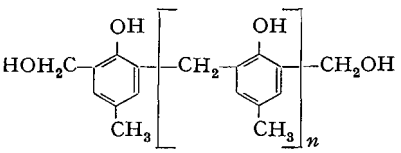
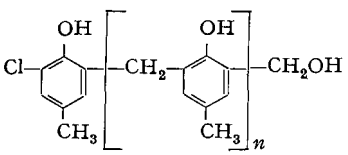
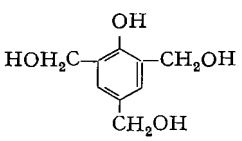
Strukturformel	*n*	*Kurzformel*
 H-*n*-H	0	H-1-H
	1	H-2-H
	2	H-3-H
	3	H-4-H
	4	H-5-H
	5	H-6-H
	6	H-7-H
 Cl-*n*-Cl	0	Cl-1-Cl
	1	Cl-2-Cl
	2	Cl-3-Cl
	3	Cl-4-Cl
	4	Cl-5-Cl
	5	Cl-6-Cl
	6	Cl-7-Cl
	7	Cl-8-Cl
	0	Cl-1-H
	1	Cl-2-H
	2	Cl-3-H
	0	Br-1-H
	1	Br-2-H
	2	Br-3-H
	0	$HOCH_2$-1-CH_2OH
	1	$HOCH_2$-2-CH_2OH
	2	$HOCH_2$-3-CH_2OH
	0	Cl-1-CH_2OH
	1	Cl-2-CH_2OH
		Phenoltrialkohol

TABELLE II

R_F-WERTE DER UNTERSUCHTEN *p*-KRESOL–FORMALDEHYD-KONDENSATE

Laufmittel: I. Benzol–Methanol–Eisessig (95:2,5:2,5, Vol.). II. Benzol–Methanol (75:25, Vol.). III. Chloroform–Methanol (96:4, Vol.). IV. Chloroform–Methanol–Wasser (95:4:1, Vol., Emulsion).

Verbindung Kurzformel	*Summenformel*	*(ber.) Mol. Gew.*	*Laufmittel*			
			I	*II*	*III*	*IV*
H-1-H	C_7H_8O	108	0.35	0.61	0.45	
H-2-H	$C_{15}H_{16}O_2$	228	0.30	0.65	0.52	
H-3-H	$C_{23}H_{24}O_3$	348	0.32	0.69	0.65	
H-4-H	$C_{31}H_{32}O_4$	469	0.41		0.74	
H-5-H	$C_{39}H_{40}O_5$	589	0.50		0.78	
H-6-H	$C_{47}H_{48}O_6$	709	0.61			
H-7-H	$C_{55}H_{56}O_7$	829	0.715			
Cl-1-Cl	$C_7H_6Cl_2O$	177	0.61	0.665	0.65	
Cl-2-Cl	$C_{15}H_{14}Cl_2O_2$	297	0.59	0.685	0.67	
Cl-3-Cl	$C_{23}H_{22}Cl_2O_3$	417	0.59	0.73	0.71	
Cl-4-Cl	$C_{31}H_{30}Cl_2O_4$	538	0.60		0.75	
Cl-5-Cl	$C_{39}H_{38}Cl_2O_5$	658	0.65		0.79	
Cl-6-Cl	$C_{47}H_{46}Cl_2O_6$	778	0.71			
Cl-7-Cl	$C_{55}H_{54}Cl_2O_7$	898	0.755			
Cl-8-Cl	$C_{63}H_{62}Cl_2O_8$	1018	0.82			
Cl-1-H	C_7H_7ClO	143	0.47		0.65	
Cl-2-H	$C_{15}H_{15}ClO_2$	263	0.37		0.69	
Cl-3-H	$C_{23}H_{23}ClO_3$	383	0.40		0.76	
Br-1-H	C_7H_7BrO	187	0.42		0.68	
Br-2-H	$C_{15}H_{15}BrO_2$	307	0.28		0.63	
Br-3-H	$C_{23}H_{23}BrO_3$	427	0.32		0.72	
HOH_2C-1-CH_2OH	$C_9H_{12}O_3$	168	0.01	0.35	0.16	0.13
HOH_2C-2-CH_2OH	$C_{17}H_{20}O_4$	288	0.03	0.44	0.21	0.22
HOH_2C-3-CH_2OH	$C_{25}H_{28}O_5$	409	0.05	0.46	0.26	0.32
Cl-1-CH_2OH	$C_8H_9ClO_2$	173	0.12	0.43		
Cl-2-CH_2OH	$C_{16}H_{17}ClO_3$	293	0.14	0.52		
Phenoltrialkohol	$C_9H_{12}O_4$	184	0.0	0.20		

110°) aktiviert. Ein Besprühen mit 20-% Antimonpentachloridlösung in Tetrachlorkohlenstoff diente zum Auffinden der Flecken, wobei manche Verbindungen erst nach Erwärmen auf 100° sichtbar wurden. Tabelle II enthält die mit verschiedenen Laufmitteln erhaltenen R_F-Werte.

Trägt man für die beiden homologen Reihen, deren Struktur durch die Kurzformeln H-*n*-H und Cl-*n*-Cl (n = 1,2,3,...) wiedergegeben werden kann, die für das Laufmittel I gefundenen R_F-Werte gegen die Anzahl der in den Verbindungen enthaltenen *p*-Kresolbausteine auf (siehe Fig. 1), so ist das Diagramm gleich dem Chromatogramm, das man erhält, wenn man jeweils die Gemische der beiden sich entsprechenden Mehrkernverbindungen auf der Startlinie aufträgt und diese als

Abszissenachse ansieht. Die R_F-Werte der beiden homologen Reihen geben Kurven, die bei der Zwei- bis Dreikernverbindung ein Minimum besitzen und von der Vierkernverbindung ab praktisch linear ansteigen.

Trägt man ein Gemisch der Verbindungen mit den Kurzformeln H-1-H bis H-7-H auf und entwickelt mit Laufmittel I, so geben Vier- bis Siebenkern völlig getrennte Einzelflecken. Dieses Laufmittel ist aber nicht imstande z.B. die Verbindungen mit den Strukturen H-1-H bis H-3-H und Cl-1-Cl bis Cl-4-Cl zu trennen. Als geeignetes Gemisch zur Trennung dieser Verbindungen erwies sich das Laufmittel III, wie man aus der Tabelle II durch Vergleich der Spalten I und III ersehen kann.

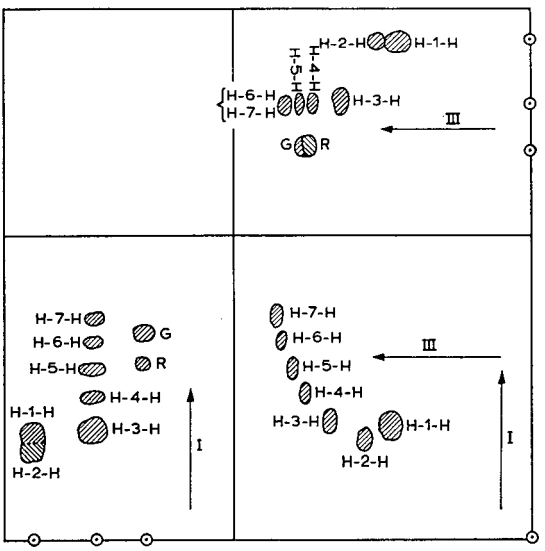


Fig. 2. Auftrennen eines Gemisches der Verbindungen H-1-H bis H-7-H (s. Tabelle I) mit Hilfe der zweidimensionalen Dünnschichtchromatographie. Kieselgel G, mit der Schichtdicke 250 μ; Trennstrecke 10 cm. Laufzeit: je Richtung 30 Min.; Auftragungs-(Start-)Punkte: ⊙; Eluiermittel I und III wie bei Tabelle II; die Pfeile bezeichnen die Entwicklungsrichtungen. G = Buttergelb; R = Sudanrot.

Mit Hilfe einer zweidimensionalen Trennung ist es nun möglich, ein Gemisch der Verbindungen H-1-H bis H-7-H in die einzelnen Komponenten zu zerlegen (s. Fig. 2). Man benutzt zunächst das Laufmittel I und dann nach Drehung um 90° das Laufmittel III.

Die einseitig halogenierten Verbindungen zeigen, wenn man mit Laufmittel I arbeitet, bei der Zweikernverbindung ebenso ein Minimum der R_F-Werte. Auch hier ist die Auftrennung von Verbindungen, die mit dem Laufmittel I ähnliche R_F-Werte zeigen, mit dem Laufmittel III möglich (s. Tabelle II).

Phenolalkohole wandern mit Laufmittel I sehr langsam oder gar nicht. Die Alkohole lassen sich dadurch leicht nachweisen, wenn sie als Verunreinigungen von der Synthese her in Verbindungen der homologen Reihen H-n-H oder Cl-n-Cl enthalten sind. Will man verschiedene Alkohole unterscheiden, so kann man Laufmittel II anwenden. Ein Auftrennen der Dialkohole ist vorteilhaft mit den Laufmitteln III oder IV.

ZUSAMMENFASSUNG

Bei zahlreichen Mehrkernverbindungen (Novolaken) aus *p*-Kresol und Formaldehyd und verschiedenen Phenolalkoholen wurden durch Dünnschichtchromatographie auf Kieselgelschichten R_F-Werte bestimmt. Es wurden verschiedenene Laufmittel angewendet. Es gelang, ein Gemisch einer homologen Reihe durch zweidimensionales Entwickeln endgültig zu trennen.

SUMMARY

The R_F values of numerous polynuclear compounds (novolacs) with *p*-cresol and formaldehyde as components and several phenol alcohols were determined by thin-layer chromatography on silica gel layers. Various solvent systems were used. A mixture of members of a homologous series could be separated by two-dimensional chromatography.

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SEPARATION AND IDENTIFICATION OF SULFONAMIDES BY THIN-LAYER CHROMATOGRAPHY

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Pure single sulfonamides can be easily identified by a number of reactions. When, however, several sulfonamides are present in a mixture it is generally necessary to perform a separation prior to the identification process. This is particularly true for those sulfonamides which do not have an analytically characteristic group that can be identified in a routine manner.

Paper chromatography[1–9] and electrophoresis[10] have proved to be very useful techniques for this purpose. Although paper chromatography has so far given very good results for many difficult separations, some combinations of sulfonamides have not yet been adequately resolved by this method. Furthermore, paper chromatography (strip and circular) is somewhat time consuming (6 to 24 h), which is a drawback, especially when used in routine work.

In recent years thin-layer chromatography (T.L.C.) has proved to be exceptionally useful for qualitative analysis and purity testing of many types of compounds, providing new facilities for their simple separation. The advantages of T.L.C. over paper chromatography have been emphasized in a number of papers[11–17].

With all this in mind the purpose of this paper was to elaborate a method for the rapid separation and identification of the most commonly used sulfonamides by this new technique. Some sulfonamides were separated by WOLLISH *et al.*[18] by T.L.C.

EXPERIMENTAL

Apparatus and reagents

All the experiments were carried out with the No. 600, Desaga, Heidelberg, thin-layer chromatography apparatus.

Adsorbent: Kieselgel G for thin-layer chromatography, Merck No. 7731.

Solvents: Chloroform p.a., methanol p.a., ether pro narcosi, redistilled.

Reagents: All reagents used were of p.a. purity grade.

Reference substances: Sulfacetamide-sodium, Sulfadiazine, Sulfaguanidine, Sulfamerazine, Sulfamethazine, Sulfamethoxypyridazine, Sulfanilamide, Sulfathiazole-sodium, Sulfathiazole, Sulfisoxasole, Acetylsulfisoxasole, Plisulfan. Purity grade: B.P. 1958 and U.S.P. XVI resp.

Procedure

Prepare Kieselgel G layers on glass plates (20 × 20 cm) by the technique used by STAHL[19] in such a way that the time from the addition of water to the adsorbent

to its spreading on the glass plates is 5 min. Activate the plate by drying in the oven for 30 min at 105°. Mark the starting line (15 mm from the lower edge of the plate) and the front (10 cm from the start) with a thin glass rod and apply 1 μl (= 0.4 μg of each sulfonamide) of the ethanol solution of the sulfonamide mixture to be tested, and of the respective solution of reference substances, by means of a micropipette, along the starting line. Insert the plate in the chamber (21 × 9 × 21 cm) containing 100 ml of the solvent and saturated with a filter paper sheet, dipped in the solvent and cover the chamber with the lid. When the solvent reaches the front, which, with the chloroform–methanol mixture, takes about 30 min, and with ether about 45 min, take out the plate and allow it to dry for a few minutes. Spray the plate with a freshly prepared 1 % solution of sodium nitrite in 0.1 *N* hydrochloric acid, dry in the oven at about 100° for 5 min and spray with 0.2 % β-naphthol solution in 0.1 *N* sodium hydroxide. Evaluate the chromatogram by comparing the spots of the sulfonamides in the sample mixture with those of the reference substances.

The degree of activity of the adsorbent was checked by means of the Desaga color test, using benzene as solvent (Sudan Red: R_F 0.18, Butter Yellow: R_F 0.45, Indophenol: R_F 0.07).

DISCUSSION AND RESULTS

From the various studies carried out so far on the paper chromatographic separation of sulfonamides it was believed that good separation of these substances would also be achieved on the inorganic adsorbent Kieselgel G on the basis of partition, using alkaline solvent systems. Results, however, of separations obtained in preliminary experiments with solvent systems such as *n*-butanol–ammonia–water, cyclohexane–chloroform–diethanolamine, chloroform–methanol–ammonia, *n*-butanol–ammonia–water were unsatisfactory. The same was found to be true for acid solvent systems, *e.g.* *n*-butanol–acetic acid–water.

Assuming that less or more hydrophobic compounds could be separated on inorganic plates on the basis of adsorption with hydrophobic solvents, experiments were carried out using a series of solvents such as chloroform, ether, cyclohexane, benzene, acetone, as well as their mixtures in various proportions.

As can be seen in Fig. 1 optimal possible separation effects for most of the sulfonamides were obtained when ether alone was used as solvent. Satisfactory separation conditions were obtained also with the solvent mixture chloroform–methanol (100:10) (Fig. 2).

The reproducibility of R_F values in T.L.C. has up to now been criticised considerably, but it is generally agreed that the R_F reproducibility can be considered satisfactory within the limits $\pm$ 0.05.

In our experiments special attention was paid to establishing optimal conditions which render the R_F values reproducible within these limits, and it was confirmed that when the given conditions of work are strictly adhered to, the reproducibility of R_F values is very good. In spite of this fact we consider that when carrying out an identification test standard sulfonamides or sulfonamide mixtures should be run on each plate with the sample to be tested.

In Table I the limits of R_F values of 12 sulfonamides tested are given.

As can be seen from Figs. 1 and 2, the technique proposed gives sharp round spots without tailing and with a satisfactory sensitivity. With the diazo reagent as

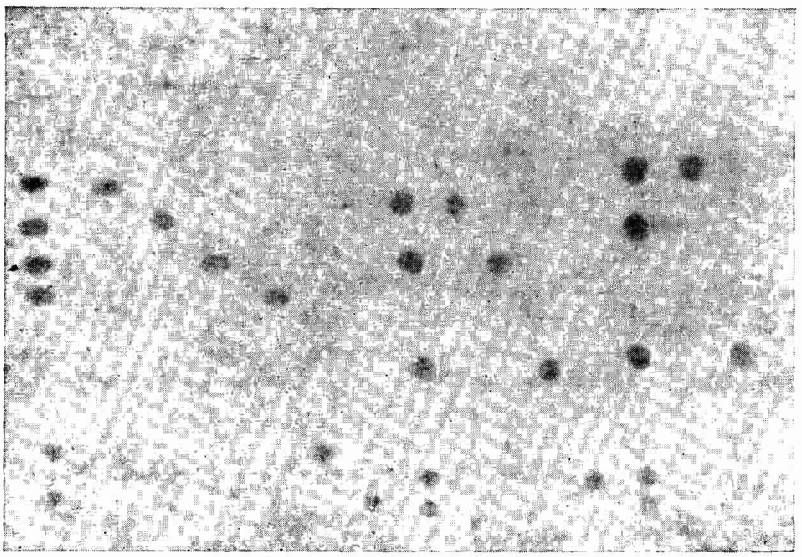

Fig. 1. Solvent: ether. M_1 = mixture of sulfonamides 12, 5, 4, 2, 8 and 3. M_2 = mixture of sulfonamides 11, 7, 6, 9 and 3. M_3 = mixture of sulfonamides 10, 5, 1, 9 and 3. For explanation of numbers, see Table I.

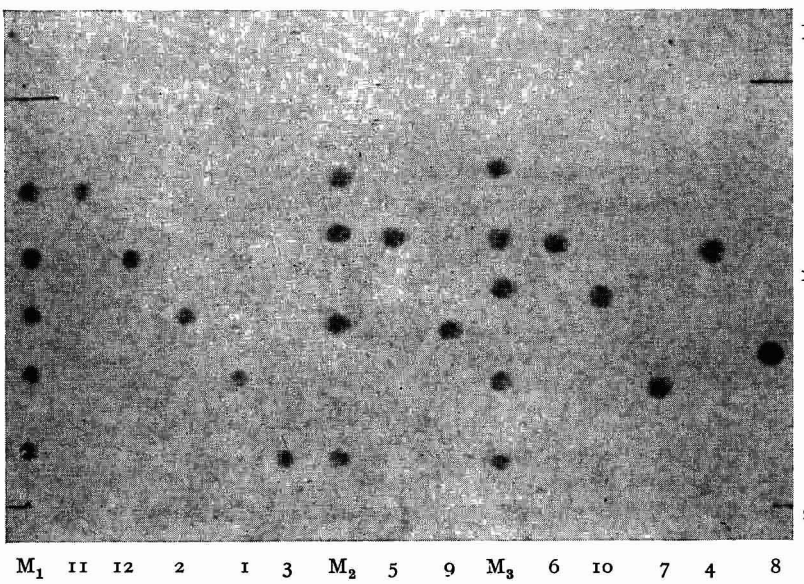

Fig. 2. Solvent: chloroform–methanol (100:10). M_1 = mixture of sulfonamides 11, 12, 2, 1 and 3. M_2 = mixture of sulfonamides 11, 5, 9 and 3. M_3 = mixture of sulfonamides 11, 6, 10, 7 and 3. For explanation of numbers, see Table I.

well as *p*-dimethylaminobenzaldehyde, 0.25 μg of the sulfonamide could still be easily detected (paper chromatography: 5 μg). The technique was applied to the identification of commercial preparations, *e.g.* Sulfacombin (Sulfadiazine, Sulfadimidine and Sulfathiazole) and Trisulfon (Sulfadiazine, Sulfathiazole and Sulfamerazine).

Work is in progress on the quantitative determination of sulfonamides and their mixtures and will be described in a subsequent communication from this laboratory.

TABLE I

LIMITS OF R_F VALUES OF VARIOUS SULFONAMIDES

Solvents: 1 = ether; 2 = chloroform–methanol (100:10).

No. (Figs. 1 and 2)	*Sulfonamide*	*R_F values in solvent*	
		1	*2*
1	Sulfacetamide sodium	0.34–0.38	0.34–0.39
2	Sulfadiazine	0.51–0.55	0.48–0.52
3	Sulfaguanidine	0.03–0.05	0.13–0.18
4	Sulfamerazine	0.58–0.62	0.56–0.62
5	Sulfamethazine	0.70–0.74	0.61–0.66
6	Sulfamethoxypyridazine	0.36–0.40	0.66–0.70
7	Sulfanilamide	0.59–0.63	0.34–0.38
8	Sulfathiazole	0.12–0.16	0.36–0.40
9	Sulfathiazole sodium	0.09–0.12	0.45–0.48
10	Sulfisoxasole	0.79–0.83	0.49–0.53
11	Acetylsulfisoxazole	0.74–0.77	0.79–0.83
12	Plisulfan	0.78–0.81	0.63–0.67

SUMMARY

By means of thin-layer chromatography on Kieselgel G layers it is possible to separate a number of sulfonamides in 30–45 min, using ether or a mixture of chloroform–methanol (100:10) as solvent. When using the diazo reagent or *p*-dimethylaminobenzaldehyde, as little as 0.25 μg of the sulfonamide can be easily detected.

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SEPARATION OF 2-BUTYNE-1,4-DIOL, ITS MONOESTERS AND DIESTERS

A COMPARISON OF GLASS-PAPER CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

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Glass-paper chromatography and thin-layer chromatography are two chromatographic techniques, recently introduced, which employ adsorption on a micro scale. Both techniques have been applied to the separation of lipids and related compounds. DIECKERT[1,2] and coworkers employed glass-paper chromatography to separate lipids into classes while HOLMAN[3], MANGOLD[4], and others[5,6] have applied thin-layer chromatography to the same problem.

To follow stepwise the synthesis and purification of esters of 2-butyne-1,4-diol, glass-paper and thin-layer chromatography methods for separating the individual ester mixtures have been developed for the first five ester homologs. A successful separation of the diol, monoesters, and diesters was achieved by chromatographing the mixtures on treated glass paper or on silica-gel-coated plates. Following development, the spots were detected with alkaline permanganate.

EXPERIMENTAL

Glass paper strips

Sheets of glass paper**, 25 cm × 38 cm, were cut (perpendicular to the grain of the paper) into quarters 9 1/2 cm × 25 cm. Each quarter[7] was immersed in 200 ml of a 2 % solution of sodium silicate*** (10.5 ml sodium silicate, Be 40° and 190 ml distilled water), dried in an oven at 100–110° for 1 h, then immersed in 300 ml of 4 *N* hydrochloric acid for 5 min. Following the acid bath, the paper was washed in 200 ml of distilled water for 5 min six successive times[2]. The paper was dried at 100–110° for 2 h then marked vertical to the base with a grease-free lead pencil at 3, 21, and 24 cm. Finally, each quarter of paper was cut into six strips, 1.5 cm × 25 cm. The individual strips were washed with the developing solvent to the 24 cm mark, ascending technique, dried over a hot plate, and spotted at the 3 cm mark on the smooth side of the paper. To determine the best conditions for separation, two mixtures of esters and diol: 2-butyne-1,4-diol, monoacetate, and diacetate, and 2-butyne-1,4-diol, the monovalerate, and divalerate were chromatographed.

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* National Science Foundation, Undergraduate Research Participant, 1961–62.
** Reeves Angel, No. 934-AH.
*** Philadelphia Quartz, lot No. 60948P.

Development

Glass columns 40 cm × 4.1 cm served as the developing chamber. The columns were equilibrated with developing solvent at 27° ± 1° for 4 h prior to the insertion of the spotted paper. To determine the solvent front, the chromatograms were viewed with indirect light. The chromatograms were developed to the 21 cm line, dried over a hot plate, then sprayed with the detecting reagent. All R_F values reported are the average of three determinations.

Conditions studied

The effect of each of the following conditions on the R_F of the esters was investigated:

1. Concentration of silicate coating: 0.25, 0.5, 1.0, and 2.0 %.
2. Solvent composition: isooctane–isopropyl ether: 100:0, 95:5, 90:10.
3. Heat-cleaned glass paper* as the silicic acid matrix *versus* glass paper not heat cleaned.
4. Variation of the sodium silicate from batch to batch.
5. Variation of the glass paper from sheet to sheet and lot to lot.

Thin-layer silica gel plates

Ten glass plates, 6 cm × 20 cm, were coated with a mixture of 20 g silica gel, Brockmann G** in 40 ml of distilled water, air-dried for 30 min, then activated at 100–110° for 1 h. The plates were stored in a desiccator until required for use. The starting points (3 cm from the lower edge of the plate and at a distance of 1 cm from each other) and a fine line at a distance 15 cm from these points were marked on the plate with a needle. Each plate was spotted with the five ester mixtures. Solvent was placed in the bottom of a glass tank (12 in. × 12 in. × 3 in.) to a height of 1 cm and adsorbent paper moistened with the solvent was placed on the sides of the tank. The tank was equilibrated 1 h before use. Individual plates were placed in the tank and development carried out until the solvent front reached the marked line. The temperature was maintained at 27° ± 1° throughout. Six solvent systems were investigated.

Solvents

Isopropyl ether, washed three times with 4% ferrous ammonium sulfate, three times with water, dried over calcium chloride and distilled, b.p. 67–69°. Isooctane washed three times with concentrated sulfuric acid, three times with water, dried over calcium chloride and distilled, b.p. 98–99°. Each of the following predried solvents was distilled: benzene, b.p. 79–81.5°; chloroform, b.p. 61–62°; ethyl acetate, b.p. 76–77°. Diethyl ether, Mallinkrodt, anhydrous, reagent grade.

Ester solutions

Five solutions, each a mixture of equal parts of 2-butyne-1,4-diol, monoester, and diester were prepared. The composition of the solutions was:

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* Sheets of glass paper were heat cleaned in a muffle furnace for 1 h at 600° prior to impregnating.

** Batch No. 62551.

2-butyne-1,4-diol, monoformate and diformate
2-butyne-1,4-diol, monoacetate and diacetate
2-butyne-1,4-diol, monopropionate and dipropionate
2-butyne-1,4-diol, monobutyrate and dibutyrate
2-butyne-1,4-diol, monovalerate and divalerate.

The synthesis of the esters will be reported elsewhere.

Concentration. 1 % w/v with respect to each component in benzene. 2 μl of each solution was spotted on the individual paper strip or plate.

Detection of spots on glass paper and silica-gel plates

After development the paper or plate was sprayed with a solution containing 0.5 % potassium permanganate in 2 % sodium hydroxide. On the glass paper the compounds first appeared as green spots on a lavender background. After a few minutes the spots turned yellow. It is essential to mark the spots promptly because the background of the strip changes from lavender to yellow within a short time after spraying. In the case of the silica-gel plates yellow spots appeared on a lavender background. These spots faded less rapidly.

RESULTS AND DISCUSSION

Glass-paper chromatography

Results of the preliminary investigation to determine the conditions necessary for separating the esters of 2-butyne-1,4-diol are summarized in Table I. In this investigation, the greatest source of variability affecting the R_F values was the glass paper. There was variation from sheet to sheet and from lot to lot. Also, there was some variation in commercial silicate solutions from one batch to another. Reproducibility of the R_F value for a given compound separated from a mixture on treated glass paper was good only if all conditions were carefully maintained and the strips from the same sheet of glass paper were used. Equilibrating the paper (2 to 24 h) after spotting and prior to development adversely affected the size of the spots. In such cases the R_F values varied considerably and the spots were diffuse and of poor quality. Some development by the vapors of the solvent probably accounts for the erratic behavior. Equilibrating the columns for 4 h before inserting the paper in the developing chamber greatly improved the quality of the spots.

Results with glass paper not heat-cleaned. Paper treated with 0.25% sodium silicate solution was very soft and the R_F values for the esters separated on this paper with isooctane–isopropyl ether (95:5) were high. There was little difference in the R_F values of the components separated on 0.5*, 1.0, or 2.0% silicate-treated paper with isooctane–isopropyl ether (95:5) as the developing solvent. Only with 2% silicate coated paper was the migration of the divalerate slow enough to keep the compound off the solvent front.

With isooctane as the developing solvent and 2% silicate-treated paper, the R_F values determined were low and the spots long and elliptical. The monoester spots averaged 3.5 cm in length and the diester spots 4.3 cm. With isooctane–isopropyl ether (90:10) the divalerate was always located on the solvent front.

* With all three concentrations the treated paper was hard.

TABLE I

RESULTS OF AN INVESTIGATION OF CONDITIONS FOR SEPARATING ESTERS OF 2-BUTYNE-1,4-DIOL BY GLASS PAPER CHROMATOGRAPHY

Condition varied	$R_F \times 100$						*Time*[a] *(min)*
	Acetate esters			*Valerate esters*			
	Diol	*Mono*	*Di*	*Diol*	*Mono*	*Di*	
Conc. of coating soln.[b]							
0.25% Sodium silicate	2 ± 0	28 ± 2	84 ± 1	0 ± 0	60 ± 3	96 ± 2	35 ± 2
0.5% Sodium silicate	0 ± 0	14 ± 2	63 ± 3	0 ± 0	45 ± 2	95 ± 1	31 ± 2
1.0% Sodium silicate	0 ± 0	13 ± 1	64 ± 4	0 ± 0	44 ± 1	98 ± 2	33 ± 1
2.0% Sodium silicate	0 ± 0	12 ± 0	61 ± 1	0 ± 0	42 ± 1	94 ± 2	30 ± 1
Developing solvent[c]							
Isooctane	0 ± 0	9 ± 1	34 ± 6	0 ± 0	14 ± 2	61 ± 3	33 ± 4
Isooctane, isopropyl ether 95:5	0 ± 0	17 ± 1	60 ± 1	0 ± 0	32 ± 2	89 ± 1	33 ± 4
Isooctane, isopropyl ether 90:10	3 ± 0	39 ± 0	79 ± 2	2 ± 1	55 ± 1	96 ± 1	29 ± 3
Other factors[d]							
Paper heat-cleaned	0 ± 0	30 ± 2	77 ± 1	0 ± 0	46 ± 0	92 ± 0	30 ± 1
Paper not heat-cleaned	2 ± 0	33 ± 1	80 ± 1	1 ± 0	49 ± 1	94 ± 1	33 ± 3
Another batch of silicate[e]	3 ± 0	38 ± 2	84 ± 3	2 ± 0	60 ± 3	97 ± 0	37 ± 3
Reproducibility of R_F values							
Stock sheet 1	0 ± 0	12 ± 0	61 ± 1	0 ± 0	42 ± 1	94 ± 2	30 ± 1
Stock sheet 2	0 ± 0	17 ± 1	60 ± 1	0 ± 0	32 ± 2	89 ± 1	33 ± 4
Stock sheet 3	2 ± 0	33 ± 1	80 ± 1	1 ± 1	49 ± 1	94 ± 1	33 ± 3
Stock sheet from another lot of paper	0 ± 0	20 ± 3	69 ± 2	0 ± 0	36 ± 0	91 ± 0	30 ± 2

The following conditions apply to all the above determinations except as noted: glass paper (same lot), not heat-cleaned, coated with 2% sodium silicate. Developing solvent: Isooctane–isopropyl ether (95:5). Temperature 27° ± 1°. Solvent height 18 cm. All R_F's the average of 3 determinations.

[a] Development time
[b] All strips from one sheet of stock paper (not heat-cleaned).
[c] All strips from one sheet of stock paper (not heat-cleaned).
[d] All strips from one sheet of stock paper.
[e] Philadelphia Quartz, sodium silicate no. 60347P.

Results with heat-cleaned glass paper. When the glass paper was heat-cleaned prior to coating with 2% sodium silicate solution, the R_F values were slightly lower and the quality of the spots improved. Presumably, some annealing of the paper occurs on heat-cleaning. This makes the paper more dense and compact in structure. The shape of the developed spot varied with the polarity of the compound; the diol was always small and compact, the monoesters formed elliptical spots averaging 3.3 cm long and 1 cm wide, while the diesters formed oval spots averaging 2.0 cm long and 1 cm wide.

Optimum conditions for separating these compounds are heat-cleaned glass paper treated with 2% sodium silicate. The solvent system, isooctane–isopropyl ether (95:5), provided good separation of the compounds without the divalerate ascending to the solvent front.

TABLE II

GLASS-PAPER CHROMATOGRAPHY
INCREASE OF R_F WITH INCREASED CONCENTRATION APPLIED

Ester	*Concentration*					
	1/8 %	*1/4 %*	*1/2 %*	*1 %*	*5 %*	*10 %*
Monoacetate	19 ± 0	20 ± 1	21 ± 3	31 ± 2	36 ± 2	36 ± 1
Diacetate	64 ± 1	65 ± 2	68 ± 1	71 ± 1	76 ± 2	78 ± 1

Heat-cleaned glass paper, coated with 2 % sodium silicate. Developing solvent: Isooctane–isopropyl ether (95:5). Solvent height 18 cm. Temperature 27° ± 1°. All R_F's the average of 3 determinations. 2 μl of solution applied in each case.

With the acetate–diol mixture, the minimum amount which can be detected on heat-cleaned, 2 % silicate-treated glass paper is 2.5 μg of each component while the range in concentration of components which can be separated and detected is 2.5 μg to 100 μg. With concentrations above 100 μg of each component the spots are so large that differentiation between the diol and monoester is lost. There is a distinct and stepwise increase in the R_F of both monoester and diester with increasing concentration of the mixture used to spot the paper (see Table II).

R_F values for all five homologs of the monoesters and diesters of 2-butyne-1,4-diol separated on strips of glass paper, 9 cm × 25 cm, which had been heat-cleaned at 600° for 1 h and treated with 2 % sodium silicate solution as previously indicated are summarized in Table III. The strips were developed with isooctane–isopropyl ether (95:5), ascending technique, in the same tank used for developing the thin-layer plates. The tank was equilibrated one hour before developing the spotted strips.

TABLE III

R_F VALUES × 100 OF ESTERS OF 2-BUTYNE-1,4-DIOL AT OPTIMUM SOLVENT COMPOSITION

Ester	*Glass-paper chromatography*[a]				*Thin-layer chromatography*[b]			
	Diol	*Mono*	*Di*	ΔR_F	*Diol*	*Mono*	*Di*	ΔR_F
Formate	0 ± 0	17 ± 7	66 ± 1	49	5 ± 0	23 ± 1	47 ± 4	24
Acetate	0 ± 0	23 ± 3	68 ± 2	45	5 ± 0	22 ± 1	36 ± 2	14
Propionate	0 ± 0	30 ± 2	77 ± 2	47	5 ± 0	29 ± 1	57 ± 2	28
Butyrate	0 ± 0	39 ± 1	84 ± 1	45	5 ± 0	34 ± 3	62 ± 2	28
Valerate	0 ± 0	42 ± 3	89 ± 1	47	5 ± 0	37 ± 3	65 ± 2	28
Development time in min			31 ± 2				37 ± 3	
Temperature			27 ± 1°				27 ± 1°	
Solvent height in cm			18				15	
Developing solvent	Isooctane–isopropyl ether (95:5) Paper-lined tank All R_F's the average of three determinations				Isopropyl ether Paper-lined tank All R_F's the average of three determinations			

[a] 2% Silicate-treated paper, heat-cleaned.
[b] Brockmann silica gel B batch No. 63131.

TABLE IV

$R_F \times 100$ OF ESTERS OF 2-BUTYNE-1,4-DIOL SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Compound	*Developing solvent*[a]				
	Benzene	*Chloroform*	*Isopropyl ether–isooctane (1:1)*	*Isopropyl ether*	*Ethyl acetate–isooctane (1:1)*
2-Butyne-1,4-diol	0[b] ± 0	0 ± 0	0 ± 0	5 ± 0	8 ± 1
Monoformate	0[b] ± 0	4 ± 0	7 ± 1	24 ± 1	28 ± 1
Diformate	12 ± 3	27 ± 0	24 ± 2	48 ± 1	51 ± 4
Monoacetate	0[b] ± 0	4 ± 0	7 ± 1	20 ± 1	27 ± 2
Diacetate	8 ± 1	22 ± 2	23 ± 2	41 ± 1	48 ± 3
Monopropionate	0[b] ± 0	4 ± 1	10 ± 1	28 ± 1	34 ± 2
Dipropionate	13 ± 1	27 ± 3	38 ± 2	59 ± 1	60 ± 1
Monobutyrate	3 ± 0	4 ± 1	13 ± 1	32 ± 1	38 ± 1
Dibutyrate	18 ± 2	36 ± 1	45 ± 3	64 ± 1	65 ± 0
Monovalerate	3 ± 0	5 ± 1	14 ± 1	35 ± 1	40 ± 1
Divalerate	23 ± 1	44 ± 4	51 ± 2	69 ± 0	69 ± 1
Developing time in min	45 ± 3	53 ± 2	48 ± 3	37 ± 1	48 ± 1

Developing height 15 cm. Temperature 27° ± 1°. All R_F's the average of three determinations. Paper-lined tank.

[a] Brockmann silica gel batch No. 62551.
[b] No separation of diol and monoester.

Thin-layer chromatography on silica-gel-coated plates

Model experiments were performed with five different three component mixtures of 2-butyne-1,4-diol, the monoesters, and diesters. Six different solvent systems were employed. The results are summarized in Table IV. The separation of the five mixtures increased with increasing polarity of the solvent. Ethyl acetate–isooctane (1:1) or isopropyl ether produced excellent separations. Diethyl ether was unsatisfactory as the developing solvent. With isopropyl ether as the solvent, the developing time was

TABLE V

SEPARATION OF ESTERS OF 2-BUTYNE-1,4-DIOL BY THIN-LAYER CHROMATOGRAPHY REPRODUCIBILITY OF R_F VALUES

Ester	*Silica gel G*[a]			*Silica gel G*[b]		
	Diol	*Mono*	*Di*	*Diol*	*Mono*	*Di*
Formate	5 ± 0	24 ± 1	48 ± 1	5 ± 0	23 ± 1	47 ± 4
Acetate	5 ± 0	20 ± 1	41 ± 1	5 ± 0	22 ± 1	36 ± 2
Propionate	5 ± 0	28 ± 1	59 ± 1	5 ± 0	29 ± 1	57 ± 2
Butyrate	5 ± 0	32 ± 1	64 ± 1	5 ± 0	34 ± 3	62 ± 2
Valerate	5 ± 0	35 ± 1	69 ± 0	5 ± 0	37 ± 3	65 ± 2
Developing time in min		37 ± 1			37 ± 3	

Developing solvent isopropyl ether. Solvent height 15 cm. Temperature 27° ± 1°. Paper-lined tank. All R_F's the average of 3 determinations.

[a] Brockmann batch No. 62551.
[b] Brockmann batch No. 63131.

considerably shorter. In all cases, except diethyl ether, the diol spots averaged 0.5 cm in diameter and the monoester and diester spots were 0.8 cm each in diameter. Reproducibility of R_F values was good and did not vary greatly from one batch of silica gel to another if care was taken to protect the activated plates from the atmosphere (see Table V). With the acetate mixture the range in concentration which can be separated and detected is 5 μg to 750 μg of each component. The R_F values did not vary significantly over the entire range in concentration. By lining the developing tank on both sides with adsorbent paper the developing time was cut from 75 $\pm$ 5 to 37 $\pm$ 1 min. A comparison of results obtained with treated glass paper and silica-gel-coated plates are summarized in Table III. The change in R_F with increasing chain length of the hydrocarbon group is shown in Fig. 1.

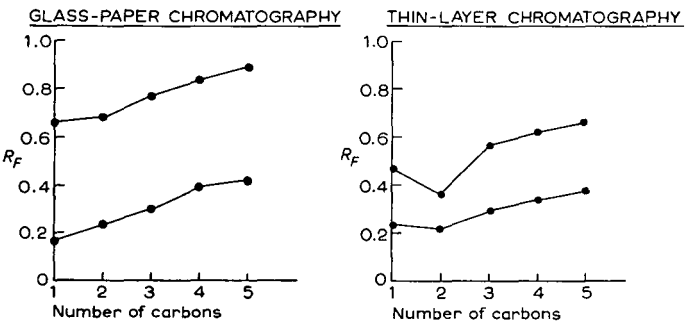

Fig. 1. Variation of R_F value with hydrocarbon chain length for the homologous series of monoesters and diesters of 2-butyne-1,4-diol. Data from Table III.

ACKNOWLEDGEMENTS

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SUMMARY

Excellent separations of the five glycol ester mixtures was achieved with treated glass paper and with silica-gel-coated plates. In the separation of 2-butyne-1,4-diol from its monoesters and diesters, thin-layer chromatography offers several advantages over glass-paper chromatography, specifically:

1. The spots are uniformly smaller and more compact.

2. No tailing was ever observed with silica-gel-coated plates, however, on treated glass paper all the monoesters tailed regardless of the conditions employed.

3. The reproducibility of R_F values from plate to plate and from one lot of silica gel to another is good, significantly better than the reproducibility achieved with different sheets of glass paper.

4. The time required to coat the plates is very short compared to the time required to treat glass paper.

5. The useful range for separating these esters by thin-layer chromatography is seven fold the useful range that obtained with treated glass paper.

6. The R_F values of the acetate esters separated on silica-gel-coated plates were reasonably constant and did not vary significantly with concentration. The R_F values

for the acetate esters separated on 2 % sodium silicate-treated glass paper gradually increased with increasing concentration of the solution applied to the paper. This observation suggests that the adsorption isotherm for ester separated on silica-gel plates is linear, whereas the adsorption isotherm for the esters separated on treated glass paper is convex.

7. There is significant interaction of both the monoacetate and diacetate with the silica-gel substrate on the silica-gel plates.

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AN INVESTIGATION OF THE INTERACTIONS BETWEEN MILK PROTEINS AND TEA POLYPHENOLS

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INTRODUCTION

The astringent taste of a black tea infusion has been attributed to the theaflavins[1] and the addition of milk to an infusion results in a reduction of the astringent taste. The present investigation was carried out to determine whether the reduction in astringency might be attributed to some interaction between milk proteins and the black tea polyphenols.

A tea infusion contains a complex mixture of phenolic compounds. ROBERTS[2] has published a review of the substances present in green tea and the changes they undergo during the manufacture of black tea. He has detected a series of polyphenolic substances characteristic of black tea which he has called A, B, C, D, P, Q, R, S_1, S_{1a}, S_{11}, X, Y and Z, and suggested that they are all derived from oxidation of *l*-epigallocatechin and its gallate. BHATIA AND ULLAH[3] have produced evidence that *l*-epicatechin gallate also takes part in the fermentation process. A, B and C are colourless compounds and are bisflavanols. P, Q and R are coloured compounds present in trace amounts. The most important polyphenols of black tea are the theaflavins (ROBERTS' compounds X and Y), and the thearubigins (ROBERTS' fractions S_1, S_{1a}, and S_{11}), which account for practically all the colour of a tea infusion[4]. The theaflavins are bright orange substances with very weak acidic properties which account for up to 3 % of the total dry weight of the tea. ROBERTS AND MYERS[5] have proposed structures for the theaflavins, and ROBERTS[6] and TEDESHVILI[7] have suggested different reaction mechanisms for further oxidation to the thearubigins, which are considered to be dimeric. VUATAZ AND BRANDENBERGER[8], however, have found evidence for the presence of nitrogen in part of the thearubigin complex, and suggest that thearubigins may be partly composed of substances akin to humic acids.

The major part of the analytical work on tea has been based on two-dimensional chromatography in butanol–acetic acid–water (4:1:2.2) and 2 % acetic acid[9]. For the simple colourless polyphenols this gives a good separation, but for the coloured compounds the separation is poor. Of the latter compounds only the theaflavins move in the second direction, and even these are not completely separated from the bulk of the coloured material which gives a long streak in the butanol–acetic acid–water direction. It is desirable that a better method of separation of the coloured compounds should be effected, and to this end we have investigated the use of paper electrophoresis.

Starch grain column electrophoresis has been used in this laboratory to achieve a

certain separation of the components of the protein system present in skim milk, and electrophoresis on cellulose acetate membranes has been used for the identification of the milk proteins in the resulting fractions. These methods were applied to the study of possible interactions between milk proteins and tea polyphenols.

EXPERIMENTAL

The teas used in the present work were an Assam tea, Rupai Pekoe Fannings, and a Ceylon tea which was used for the preparation of fractions S_1, S_{1a} and S_{11} by the method of ROBERTS *et al.*[9] The milk was reconstituted from a skim milk powder prepared from a milk which had been heated to 170°F for 15 sec before evaporating and spray drying.

100 ml of boiling water were added to 10 g of tea in a lagged vessel, the mixture was allowed to stand for 10 min and then filtered through glass wool to give a tea infusion containing *ca.* 3.5 % tea solids. A 1:1 mixture of this infusion with 20 % skim milk powder solution was used for the starch column electrophoresis. This mixture has the same proportion of tea to milk solids as that present in a cup of tea with milk at normal drinking strength, but the overall concentration is 7 times greater in the mixture than in the cup of tea.

1. *Starch column electrophoresis*

The apparatus was essentially as described by FLODIN AND PORATH[10]. Columns 2.4 cm × 50 cm were packed with potato starch in 0.05 *M* phosphate buffer pH 6.7. 2 ml of sample, not previously dialysed, were applied to the top of the column and subjected to electrophoresis with a current of 10 mA and an applied voltage of 300 V for 48–50 h at 6°C. The column was eluted over a fraction collector and 1.6 ml aliquots were collected. The optical densities of these aliquots at 280 mμ were measured and plotted against tube numbers.

2. *Membrane filter electrophoresis*

Electrophoresis on cellulose acetate strips was performed in a Shandon micro-electrophoresis tank at room temperature, essentially as described by KOHN[11]. Two 10 μl samples were applied to each 5 cm wide strip and electrophoresis was carried out in 0.05 *M* phosphate buffer pH 6.7 with a current of 2 mA per 5 cm width, and 6 V/cm length of strip for 4 h. Proteins were detected by staining with 0.005 % Nigrosine in 2 % acetic acid. β-Lactoglobulin (Light and Company) and α-lactalbumin (kindly presented by Dr. R. ASCHAFFENBURG*) were used as standards for the identification of protein bands.

3. *Paper electrophoresis*

Experiments were carried out in an LKB electrophoresis tank using Whatman 3 MM paper. Four 30 μl samples were usually applied across the 17.5 cm width of paper. Current was supplied from a constant current power pack. Proteins were detected by staining with 0.2% Lissamine Green in 2% acetic acid, and polyphenols with 1% $FeCl_3$–1 % $K_3Fe(CN)_6$ (1:1). Papers were also examined in U.V. light for the location

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of the polyphenols. Borate (pH 8.5) and molybdate (pH 4.9) buffers were used. The borate buffer was prepared by mixing 0.1 *M* H_3BO_3 and 0.025 *M* $Na_2B_4O_7$ (1:1). The molybdate buffer was made by dissolving 25 g hydrated sodium molybdate in 2,400 ml water and adjusting to pH 4.9 by addition of approx. 35 ml 5 *N* H_2SO_4.

RESULTS AND DISCUSSION

1. *The effect of tea on skim milk*

The elution diagram obtained after starch column electrophoresis of 10 % skim milk powder solution is shown in Fig. 1a. On the basis of relative mobility values[12], and further analysis of the fractions using paper, starch gel and membrane filter electrophoresis, peak I is considered to be due to the α-casein complex as defined by BRUNNER *et al.*[13], peak II to the major whey protein β-lactoglobulin and certain minor whey proteins such as blood serum albumin and proteose peptone, and peak III to β-casein and α-lactalbumin. Peak IV, which is considerably reduced in height when the sample is dialysed before electrophoresis, is considered to be mainly non-protein.

During the electrophoresis of milk to which tea infusion had been added the majority of the brown tea components were seen to move down the column as a sharp band and were eluted with the α-casein fraction. The elution diagram is shown in Fig. 1b. The absorbance of the eluate in tubes 1–20 was measured at 280 mμ where both protein and polyphenols exhibit absorption maxima, and also at 400 mμ in order to estimate the distribution of the coloured material present. Some coloured material remained at the starting zone and could not be eluted from the column.

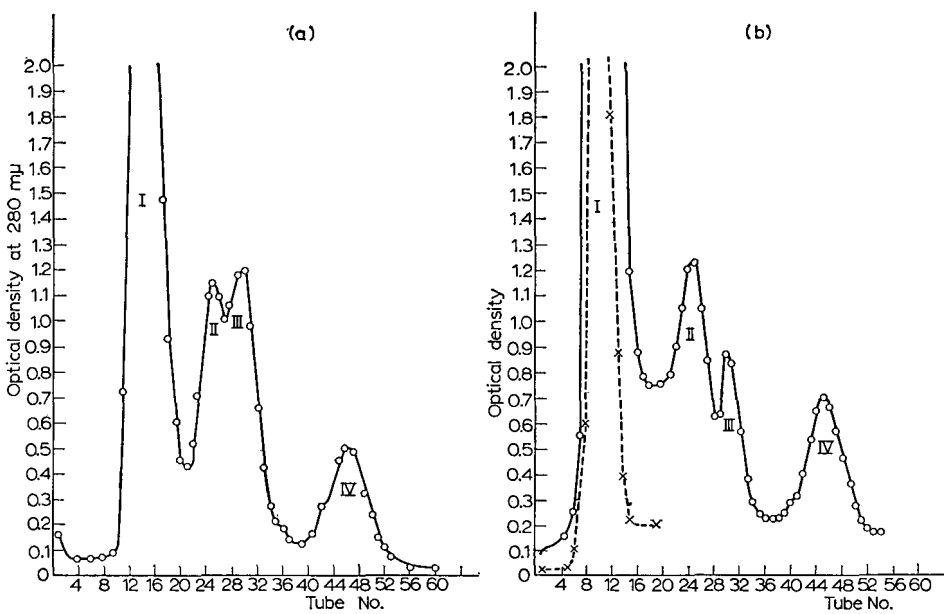

Fig. 1. (a) Elution diagram obtained from starch column electrophoresis of 2 ml 10 % skim milk powder solution. (b) Elution diagram obtained from starch column electrophoresis of a mixture (50:50) of 20 % skim milk powder solution and a strong tea infusion. O—O Optical density at 280 mμ; ×---× optical density at 400 mμ.

The concentration of milk proteins in the experiments represented by Figs. 1a and 1b was the same so that the elution patterns should be directly comparable with respect to protein distribution. Comparison of these two figures shows that:

(a) The majority of the brown tea polyphenols move with the α-casein complex represented by peak I.

(b) Peak II produced mainly by β-lactoglobulin is essentially unaltered by the presence of the tea polyphenols in the sample.

(c) Peak III due mainly to β-casein and α-lactalbumin is considerably reduced in height when the sample contains tea polyphenols.

(d) Peak IV is slightly increased in height when the sample contains tea polyphenols.

Membrane filter electrophoresis of the contents of certain tubes gave the protein patterns shown in Fig. 2. The α-casein complex isolated from milk by starch column electrophoresis gives a poorly defined protein pattern on membrane filters (Fig. 2a).

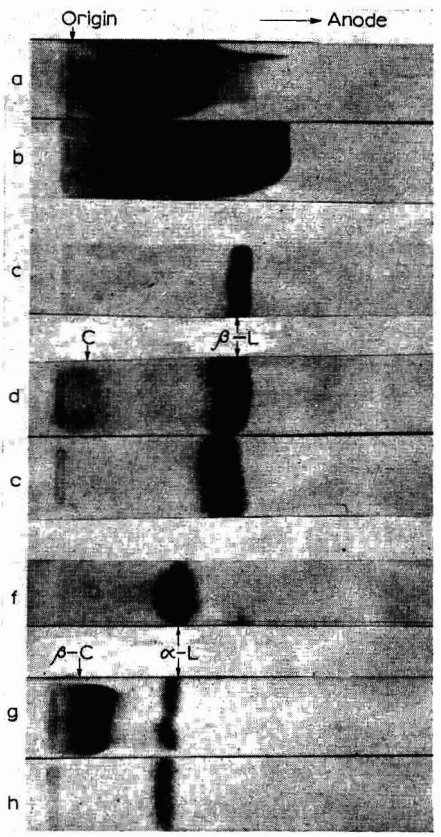

Fig. 2. Membrane filter electrophoretic patterns of milk proteins isolated from milk and milk + tea infusion samples by starch column electrophoresis. (a) α-Casein complex. Tube 14, milk sample. (b) α-Casein complex. Tube 10, milk + tea infusion sample. (c) 0.15% solution of crystalline β-lactoglobulin (β–L). (d) Tube 25, milk sample. (e) Tube 25, milk + tea infusion sample. (f) 0.1% solution of α-lactalbumin (α–L). (g) Tube 30, milk sample. (h) Tube 30, milk + tea infusion sample. C = casein; β–C = β-casein.

The α-casein complex isolated in a similar manner from the sample containing tea polyphenols gave a membrane filter protein pattern (Fig. 2b) which showed that the protein had migrated further along the strip than in pattern Fig. 2a, and had not been adsorbed to the same extent. A large part of the brown tea components was found to move with this protein band.

Fig. 2c and f show the membrane filter patterns given by solutions of crystalline β-lactoglobulin and of α-lactalbumin respectively. By comparison with these standard patterns membrane filter patterns of the fractions showed that tube 25 from the milk sample contained β-lactoglobulin and a little casein (Fig. 2d), whereas tube 25 from the sample of milk + tea infusion contained β-lactoglobulin but no contaminating casein (Fig. 2e). Similarly tube 30 from the milk sample contained α-lactalbumin and β-casein (Fig. 2g), whereas tube 30 from the tea infusion + milk sample contained α-lactalbumin only (Fig. 2h).

The membrane filter patterns shown in Fig. 3 further demonstrate the effects of the tea polyphenols on the milk proteins. When a strong tea infusion (*ca.* 3.5 % solids) is added in the proportions 1:2 to the α-casein complex isolated from milk by starch

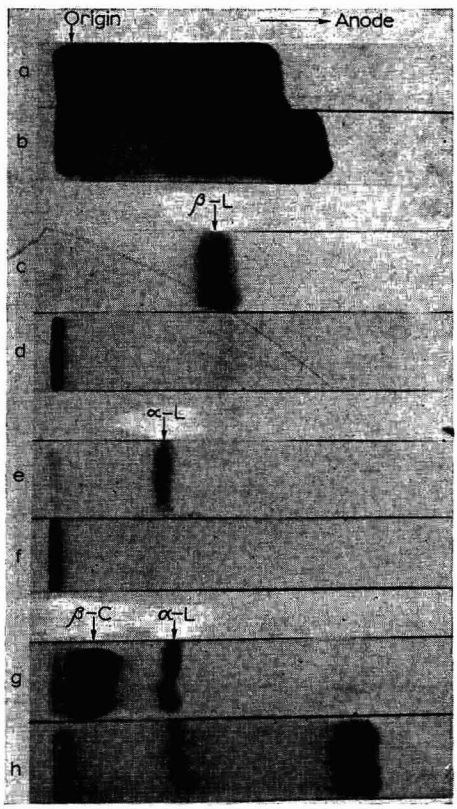

Fig. 3. Membrane filter electrophoretic patterns of milk proteins with and without added tea polyphenols. (a) α-Casein complex. Tube 14, milk sample. (b) α-Casein complex + tea infusion (2:1). (c) β-Lactoglobulin. (d) β-Lactoglobulin + tea infusion (2:1). (e) α-Lactalbumin. (f) α-Lactalbumin + tea infusion (2:1). (g) α-Lactalbumin + β-casein. Tube 30, milk sample. (h) α-Lactalbumin + β-casein + tea infusion.

column electrophoresis, and the resulting solution is subjected to membrane filter electrophoresis, the protein is found to move further along the strip in the presence of the tea polyphenols, and a large part of the brown tea components move with the protein (*cf.* Fig. 3a and b).

Solutions from tubes 25 and 30 obtained by starch column electrophoresis of milk + tea infusion, and which have been shown to contain β-lactoglobulin and α-lactalbumin respectively uncontaminated by casein (Fig. 2e and h) were treated with tea infusion in a similar manner to the α-casein complex. The membrane filter patterns showed that both the β-lactoglobulin and α-lactalbumin had been precipitated as shown by the immobilization of the protein at the starting zone, (*cf.* patterns (c) and (d), (e) and (f), Fig. 3). The formation of these precipitates was shown to be concentration dependent in the following manner. When a dilute (*ca.* 0.5 % solids) tea infusion was added to an 0.3 % solution of β-lactoglobulin in 0.05 *M* phosphate buffer pH 6.7, both solutions being at 70° to prevent creaming out of the tea, no precipitate was formed up to a protein: tea ratio of 2:1 in the resulting solution of pH 6.7, either at 70° or on cooling. When the experiment was repeated using a strong (*ca.* 3.5 % solids) tea infusion, no precipitate was formed in the resulting solution of pH 6.45, either at 70° or in the cold, until the amount of tea infusion added was such that the infusion to protein solution ratio was 1:1. A dark brown precipitate was then formed at 70°. The pH of the solution was 6.25. It would therefore appear that the formation of a precipitate, possibly an insoluble β-lactoglobulin–polyphenol complex, depends on the relative amounts of β-lactoglobulin and polyphenol present. Similarly, an 0.05 % solution of α-lactalbumin mixed with a strong tea infusion in proportions $> 7:1$ did not form a precipitate, but as the proportion of tea infusion present was increased up to and beyond this value a brown precipitate was formed.

A solution containing both α-lactalbumin and β-casein, obtained by starch column electrophoresis of the milk sample (tube 30), when mixed in proportion 2:1 with a strong tea infusion, on membrane filter electrophoresis gave the protein pattern shown in Fig. 3h. Comparison with pattern Fig. 3g shows that in the presence of the tea polyphenols the mobility of the β-casein was markedly increased, and that some of the α-lactalbumin had been precipitated. The β-casein band was accompanied by coloured tea polyphenols.

The foregoing experiments therefore indicate that on adding a tea infusion to milk both the α-casein and β-casein form soluble casein–polyphenol complexes, whereas the whey proteins, β-lactoglobulin and α-lactalbumin, in the presence of casein in the concentration used in these experiments, appear to be unaffected. In the absence of casein the whey proteins will form either soluble or insoluble protein–polyphenol complexes depending on the relative proportions of protein to polyphenol present.

2. *Paper chromatography*

Starch column and membrane filter electrophoresis of mixtures of tea infusions and milk proteins have shown that some of the coloured polyphenols, *i.e.* theaflavins and/or thearubigins, interact with the milk proteins. It is possible that some of the colourless phenolic substances also interact with the proteins.

Two-dimensional ascending paper chromatography of α-casein and β-casein respectively with tea infusions was carried out on Whatman No. 1 paper using

butanol–acetic acid–water (4:1:2.2) as solvent in the first dimension and 2 % acetic acid in water for the second dimension. The chromatograms were sprayed with ammoniacal silver nitrate solution and the phenolic spots identified by comparison of their positions with those given in the literature by ROBERTS *et al.*[9] and VUATAZ AND BRANDENBERGER[8]. Not all of the phenolic compounds listed by ROBERTS *et al.* were identified, but those colourless compounds of black tea which gave the strongest spots, *viz.* gallic acid, theogallin and the bisflavanols B and C, as well as other strong spots which were not correlated with ROBERTS' findings, were unaffected by the presence of the proteins. A larger part of the coloured polyphenols was immobilized at the origin in the presence of the proteins, but some coloured material did move in the butanol–acetic acid–water direction. There was a significant decrease in the theaflavin area in the presence of the proteins.

3. *Paper electrophoresis*

In view of the rather unsatisfactory nature of the separation of coloured tea compounds by paper chromatography, the use of paper electrophoresis was investigated. Strong tea infusions containing about 3.5 % solids were used.

Low voltage (*ca.* 6 V/cm) paper electrophoresis in 0.1 *M* acetate buffer pH 5.1, 0.05 *M* phosphate buffer pH 6.7 and 0.1 *M* borate buffer pH 6.8 gave essentially the same results, with no satisfactory movement of the coloured compounds.

However, paper electrophoresis of tea infusions in borate buffer at pH 8.5, with an applied voltage of 13.5 V/cm and a current of 9 mA for one hour, resulted in the movement of a large part of the brown coloured material towards the anode, and the separation of two yellow bands which moved more slowly. Inspection of the paper in U.V. light revealed other components which fluoresced strongly. The full pattern of bands is described in Table I. While this method is unsatisfactory for investigating the nature of the coloured components in tea because the colours of the borate complexes formed at this pH probably bear no relation to the colours of the free tea components, it is of value for the comparison of polyphenol fractions isolated from tea.

Paper electrophoresis was also carried out in molybdate buffer at pH 4.9. Again the method is open to the objection that the molybdate complexes probably differ in colour from the original polyphenols. However, this method has the advantage that electrophoresis is performed at the pH of a tea infusion. The pattern obtained in molybdate buffer consisted of a general brown background up to about 6 cm from the origin. Bands could sometimes be seen superimposed on the background, but the ease of detecting these bands depended on the amount of sample applied. If too much sample is applied the background is too strong to show the superimposed bands. Coloured bands at 12.0, 9.4, 7.5, and 6.0 cm from the origin could be clearly distinguished (Table II). In U.V. light none of the bands showed the bright coloured fluorescence observed on the borate patterns.

Group separation of the coloured polyphenols was undertaken in an attempt to identify some of the coloured bands obtained by paper electrophoresis.

The methods of ROBERTS *et al.*[9] and of VUATAZ AND BRANDENBERGER[8] were used. The paper electrophoretic patterns of ROBERTS' fractions S_1, S_{1a} and S_{11} are given for borate and molybdate buffers in Tables I and II respectively: those for VUATAZ AND BRANDENBERGER's polyphenol fractions 1, 2 and 3 are for molybdate buffer in Table II. Reference to these tables shows that in borate buffer ROBERTS' fractions

TABLE I

PAPER ELECTROPHORETIC PATTERNS OF TEA INFUSIONS AND POLYPHENOL FRACTIONS IN BORATE BUFFER pH 8.5

Appearance of band		*Distance moved in cm towards the anode*				
Visible light	*U.V. light*	*Assam tea*	*Ceylon tea*	*Fractions from Ceylon tea*		
				S_1	S_{1a}	S_{11}
Purple	None	10.2	10.0			
None	Blue F	9.5	9.5			
Pale brown	Yellow F	8.4	8.4			
Brown	Dark brown	7.7	7.8	7.7 w	7.8	7.7
Pale brown	Strong blue F	7.1	7.1	7.1 w	7.1	
Brown	Brown	6.5	6.5	6.6	6.5	6.5 w
Pale brown	Orange F		6.1	6.1 w		
Reddish	Brown		5.8			5.8
None	Blue F		5.4			
Yellow	Yellow F	4.7	4.9	4.9	4.8	4.9
None	Blue F	4.1	4.2			
Yellow	Dark brown	3.2 vw	3.2	3.2 w	3.2 w	3.2 w
None	Blue F	2.2				
None	Bluish F	1.5	1.8			
Orange-pink	None	1.2				
Purplish red	None		1.3	1.1		
None	Yellow F	0.6	0.4			

F = Fluorescence; w = weak; vw = very weak.

give more or less the same patterns, variations in the intensities of the bands being the only differences. In molybdate buffer the major part of the patterns for S_1, S_{1a} and S_{11}, up to 6.0 cm is once again the same. These patterns were rather diffuse and did not give very clear cut bands. The polyphenol fractions prepared by the method of VUATAZ AND BRANDENBERGER gave better defined patterns, but were still mixtures and it was not possible to ascribe any definite bands to the theaflavins or individual thearubigins. The sensitivity of the colour of these polyphenols to pH changes and, probably, to complex formation, makes simple identification of theaflavins as orange-yellow substances unwise. However, in this experiment the bulk of the theaflavins seemed to be extracted in fractions 1 and 2, probably because of incomplete removal of ethanol from the initial extract, and there were indications, from the electrophoretic patterns, that the fastest moving bands corresponded to the theaflavins.

Light white soluble casein (BDH nutritional casein) a readily available source of whole casein in the form of soluble sodium caseinates, was used for investigating the interaction between casein and the tea polyphenol fractions. The addition of this casein to a tea infusion altered the electrophoretic pattern of the latter in both borate and molybdate buffers as follows.

In borate buffer the brown band at 7.7 cm (Table I) was reduced in intensity and that at 6.5 cm disappeared. A new brown band appeared at 5.0 cm and this also stained for protein. Light white soluble casein gives a band at 5.2 cm. The α-casein–polyphenol complex isolated from skim milk + tea infusion by starch column electrophoresis gave a very weak brown band at 6.3 cm, and a strong band at 5.0 cm which gave a positive protein reaction with Lissamine Green.

In molybdate buffer the brown band at 7.5 cm (Table II) in tea infusion, fraction

TABLE II

PAPER ELECTROPHORETIC PATTERNS OF TEA INFUSIONS AND POLYPHENOL FRACTIONS IN MOLYBDATE BUFFER pH 4.9

Appearance of band	*Distance moved in cm towards the anode*							
	Assam tea	*1*	*2*	*3*	*Ceylon tea*	S_1	S_{1a}	S_{11}
Colourless, brown in U.V.	12.0				12.1			
Orange-brown	9.4		9.4		9.5		9.2	
Orange-brown		8.0						8.3
Green					7.6			
Orange-brown	7.5		7.5				7.5	
Red-brown	6.1	6.0	6.5	6.1	6.0	5.8	6.2	
		4.9	5.0					
Brown	3.9	4.0					4.5	4.0
					3.5			
Yellow						2.7	3.0	3.1
Green					1.7			
Purple	1.0							
Orange							0.9	0.9
Brown	o w	o w	o w	o s	o w	o s	o s	o s

1, 2 and 3 are polyphenol fractions prepared from Assam tea by the method of VUATAZ AND BRANDENBERGER[8]; S_1, S_{1a} and S_{11} were prepared from Ceylon tea by the method of ROBERTS *et al.*[9]; w = weak; s = strong.

2 and S_{1a} disappears in the presence of casein. The reddish-brown band at 6.0 cm is considerably reduced in intensity but the orange-brown band at 9.4 cm is unaffected. The slower moving coloured bands cannot be easily distinguished but the intensity of colour in this region is not greatly reduced. More brown colour remains at the origin in the presence of the casein. The protein is also immobilized at the origin.

These results suggest that casein reacts selectively with the coloured tea polyphenols in the presence of molybdate buffer, because two of the easily distinguished bands disappear, whilst the third remains unaffected.

The fact that casein does interact with the coloured tea polyphenols was further demonstrated by the use of Sephadex (G.25). The application of this cross-linked dextran to the separation of complex mixtures of peptides, proteins and amino acids has been described by PORATH[14]. Attempts to separate the coloured tea polyphenols in water on a Sephadex column were not successful. All the coloured material remained in a band at the top of the column and was only eluted on addition of *N*/10 NaOH, and then much trailing occurred. Adsorption of aromatic and heterocyclic compounds on Sephadex has been reported in the literature[15]. Casein washes straight through a column of Sephadex because the molecules are too large to enter the pores of the gel. When light white soluble casein was added to the tea infusion (1:1), and this mixture (pH 5.3) applied to a Sephadex column, a large part of the brown colour (*ca.* 90%) passed straight through the column and was eluted as a reddish-brown liquid. Some orange and pink substances remained at the top of the column and were very slowly eluted as trailing bands.

4. *Investigation of the nature of the polyphenol–casein interaction*

The preceding experiments indicate that some coloured polyphenols and the casein of the milk proteins interact in solution to form soluble complexes.

CHAPON *et al.*[16] have studied a similar system, *viz.* beer polyphenols and proteins. These polyphenols have been shown to be condensation products of anthocyanins which are very similar, structurally, to the simple flavanols in tea. The authors concluded that beer polyphenols complex with proteins by the formation of hydrogen bonds. In view of the similarity of the tea polyphenol/milk protein system we investigated the possibility of hydrogen bond formation by performing electrophoresis on membrane filters in the presence of urea which is a well known agent for breaking hydrogen bonds. The absence of complex formation in the presence of urea would suggest a mechanism involving hydrogen bonds.

Membrane filter electrophoresis was carried out as before using 0.05 *M* phosphate buffer pH 6.7 and 7 *M* with respect to urea, on samples of tea infusions and tea infusion + α-casein, β-casein, α-lactalbumin and β-lactoglobulin, as well as on the milk proteins alone. The results are shown in Fig. 4, from which it can be seen that the protein patterns are unchanged by the presence of the tea polyphenols. In the presence of the urea there was no precipitation of α-lactalbumin or β-lactoglobulin on addition of the tea infusion, and in all cases none of the brown colour was seen to move with the protein.

When tea alone is examined by membrane filter electrophoresis in phosphate

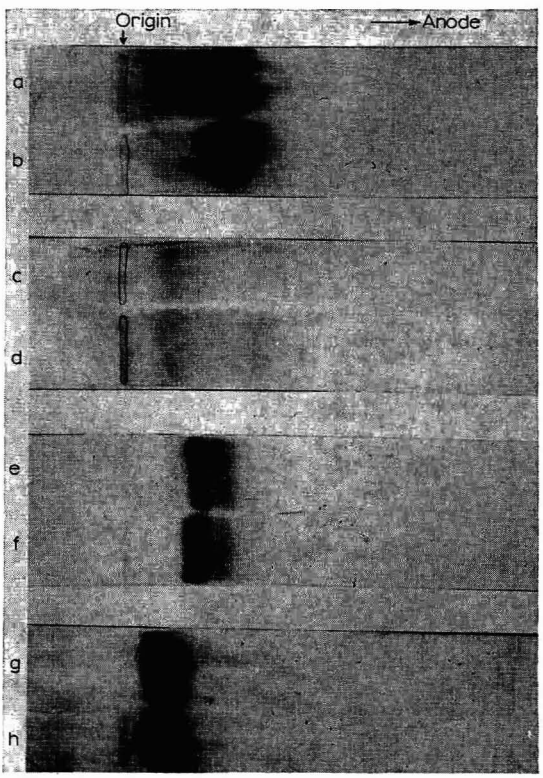

Fig. 4. Membrane filter electrophoretic patterns of milk proteins with and without added tea polyphenols in the presence of urea. (a) α-Casein complex. (b) α-Casein complex + tea infusion. (c) β-Casein. (d) β-Casein + tea infusion. (e) α-Lactalbumin. (f) α-Lactalbumin + tea infusion. (g) β-Lactoglobulin. (h) β-Lactoglobulin + tea infusion.

buffer 7 *M* with respect to urea the coloured material remains at the origin. A very faint yellow band moves to a position just ahead of that reached by the β-lactoglobulin used as standard. This tea pattern is not altered by mixing the tea with milk proteins.

The indications are that 7 *M* urea inhibits the interactions between milk proteins and the coloured tea polyphenols which suggests that these interactions are due, at least initially, to the formation of hydrogen bonds.

SUMMARY

Electrophoretic methods have been used to investigate the interactions in solution between coloured tea polyphenols and milk proteins. It has been found that:

1. When a tea infusion is mixed with milk in the proportions but not the overall concentration obtaining in a cup of tea with milk, the coloured tea polyphenols interact mainly with the α-casein complex and the β-casein of the milk to form soluble casein–polyphenol complexes. β-Lactoglobulin and α-lactalbumin, the main whey proteins, appear to be unaffected by the polyphenols in the presence of the casein at the concentrations used in this investigation.

2. In the absence of the casein both α-lactalbumin and β-lactoglobulin interact with the coloured tea polyphenols, forming either soluble or insoluble protein–polyphenol complexes, the type formed depending on the relative and overall proportions of protein and polyphenol present in the mixture.

3. Paper electrophoresis of tea infusion in borate buffer (pH 8.5) and in molybdate buffer (pH 4.9) is a useful method for the comparison of tea infusions and of polyphenol fractions isolated from tea.

4. Paper electrophoresis of tea infusion with added casein in molybdate buffer indicates that the casein interacts selectively with some of the coloured tea polyphenols in the presence of the molybdate complexing agent.

5. Membrane filter electrophoresis in phosphate buffer (pH 6.7) and 7 *M* with respect to urea indicates that the milk protein/tea polyphenol interactions are at least initiated by the formation of hydrogen bonds.

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STARCH–PEVIKON C-870 GEL AS A SUPPORTING MEDIUM IN ZONE ELECTROPHORESIS

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INTRODUCTION

Direct procedures for elution of proteins from starch gel have been discussed by SMITHIES[1]. More successful techniques allowing almost quantitative recoveries have been described[2–5], but appear to be rather time-consuming. Attempts to recover faster moving components by means of a fraction collector and the combined use of an electrostatic field and a buffer flow crossing a vertical gel have not given, so far, any significant results.

A preliminary account concerning the use of a gel formed by hydrolysed starch and a powdered co-polymer of polyvinyl chloride and polyvinyl acetate, "Pevikon C-870", has been reported[6]. The combination of starch with a non-swelling medium produced a gel with a high resolving power for protein mixtures and little tendency to adsorb proteins.

The present paper gives conclusive evidence that by means of starch–PVK* gel electrophoresis, serum proteins as well as soluble liver and brain proteins can be satisfactorily resolved; furthermore, data referring to recoveries of protein from starch–PVK gel and starch gel are presented and the procedure for elution of proteins from starch–PVK gel is described.

MATERIALS AND METHODS

Rabbit and human serum proteins were used throughout the experiments. Rabbit haemoglobin was prepared according to NYMAN[7].

Brain and liver of a heparinized rabbit (5 mg heparin/kg body-weight), after intravascular perfusion of warm saline, were cut up into small slices and homogenized with an equal amount of distilled water; the homogenates were frozen-thawed 7 times prior to centrifugation for 50 min at 34,000 × *g* in a Lourdes Model AX centrifuge. The whole procedure was carried out at 2–6°.

Protein solutions were labelled with carrier-free ^{131}I (The Radiochemical Centre) without oxidation, according to MCFARLANE[8]. Free iodide was removed by passing the solutions through anion exchange columns of De-Acidite (The Permutit Co. Ltd., London, W. 4) and then by dialysis against the buffer used for electrophoresis.

* Abbreviations: PVK = Pevikon C-870; TCA = trichloroacetic acid; Tris = tris-(hydroxymethyl)-amino-methane.

The small precipitates formed during dialysis were removed by centrifugation. ^{131}I-TCA soluble radioactivity as a percentage of the original radioactivity (after precipitation with TCA at a final concentration of 10 % in the presence of NaI and carrier rabbit serum proteins) was: brain, 18.1; liver, 5.3; haemoglobin, 6.1; serum, 2.4.

Starch–PVK gel was prepared as follows: starch (Connaught Medical Research Laboratories), 10.5 g, and PVK (Stockholms Superfosfat Fabriks Aktiebolag, Stockholm), 126.0 g, were thoroughly mixed with 100 ml Tris–citric acid buffer (POULIK[9]) and the suspension was heated as indicated by SMITHIES[10]. The mixture became almost solid at 50–60° but further heating and vigorous swirling of the flask produced a solution of suitable viscosity. In order to produce a homogeneous solution heating was prolonged for about 10 min to a point just short of boiling.

Starch gel was prepared at a concentration of 10.5 g of starch per 100 ml buffer.

A tray of 30.5 × 21.0 × 1.0 cm was usually used in horizontal position with a discontinuous buffer system according to POULIK[9]. The origin was at 7.5 cm from the cathodal end of the gel slab and the protein solutions were taken up in Ford's A 4 filter paper strips (about 0.065 ml per 1 cm^2 of filter paper). The solutions had the following protein concentration (mg/ml): brain, 21; liver, 45; haemoglobin, 33; serum, 72.

After the insertion of the sample a proper contact was insured by pushing the cathodal end of the gel towards the origin with a perspex strip and filling the space with wet filter paper. A voltage gradient of 6 V/cm was employed until the borate front reached the origin and then it was increased up to 19 V/cm (current of 80–90 mA); electrophoresis was considered complete when the borate front was about 12.5 cm from the origin towards the anode. Increasing the voltage gradient resulted in sharper and better protein separation and it shortened the run down to about 6.5 h. The electrophoretic run was performed at room temperature but the table, the polythene cushion resting on top of the gel and the borate bridge buffer[9] (3.7 l for each bi-compartmented vessel) were all cooled with running tap water.

Protein recoveries were measured as follows: the gel slab was sliced longitudinally and holes were made at 1.0 cm intervals using a needle. With the help of the needle holes protein bands were localized after staining and washing (SMITHIES[10]) the top slice of gel or, if the proteins were highly radioactive, by carrying out autoradiography directly on the lower slice of gel (Kodirex, X-Ray film). Selected segments (2.0 × 1.0 × 0.4 – 0.5 cm) of the lower slice were put in glass counting tubes and saline was added up to a volume of 3 ml. Some of the samples were kept at + 3° for different lengths of time, some were frozen at — 40° and thawed three times.

When the samples had been counted for total radioactivity in a well-type scintillator, proteins were eluted by squeezing the segments in a metal-glass syringe, a filter paper disc preventing the gel mass escaping. After centrifugation the clear supernatant was brought up to 3 ml and counted for calculating the percentage of recovered radioactivity. The standard deviation of counts recorded in this study did not exceed ± 1 %.

When proteins were to be eluted from large segments of gel the final procedure was as follows: saline was added to the starch–PVK gel so that it could be easily smashed with a spatula to a slurry consistency. The sample was counted for total radioactivity and, after centrifugation, the clear supernatant, having been brought to the original volume, was counted for measuring the percentage of recovered radioactivity. The

starch–PVK sediment could be washed in order to increase recoveries. For comparative purposes starch gel was treated in the same way except that the gel had to be smashed by an MSE homogeniser.

Proteins were concentrated by dialysis (Visking tubing 8/32 in.) under reduced pressure. The precipitated starch at the bottom of the bag, was eliminated by centrifugation.

In order to attempt the separation of soluble starch from proteins, a column (3.0 × 128.0 cm) with Sephadex G-100 (Pharmacia, Uppsala) was used for gel filtration[11] of eluates obtained from starch–PVK gel after electrophoretic separation of human ^{131}I-serum. A glass paper disc (Whatman GF/B) was placed on the top surface of the gel bed. Elution was carried out with 0.005 *M* NaCl in 0.005 *M* potassium phosphate buffer, pH 7.1. Elution rate: 26 ml/h.

Proteins were estimated from 280 mμ, 407 mμ and 540 mμ (GORNALL *et al.*[12]) absorption in a Unicam SP.500 spectrophotometer. A crystallized sample of bovine serum albumin (Sigma Co.) was used as a standard.

RESULTS

The starch–PVK gel was white and the mechanical strength slightly inferior to that of starch gel. Using a discontinuous buffer system starch–PVK gel shrank very little as the borate front progressed, thus causing only occasionally an imperfect contact at the origin.

Only 1.5 % of total ^{131}I-labelled proteins remained at the origin. In comparison with starch gel, proteins enter starch–PVK gel more slowly, and the albumin mobility is about 1/10 lower. Also with starch–PVK gel a sharpening of protein bands was observed when they were reached by the borate front.

Slicing of the gel presented no difficulties. Excess dye was more quickly washed out of starch–PVK gel due to the lack of adsorption of dye by the PVK. A useful property of starch–PVK gel is the absence of shrinkage after washing, thus making the localization of protein bands on the unstained gel easier and more precise.

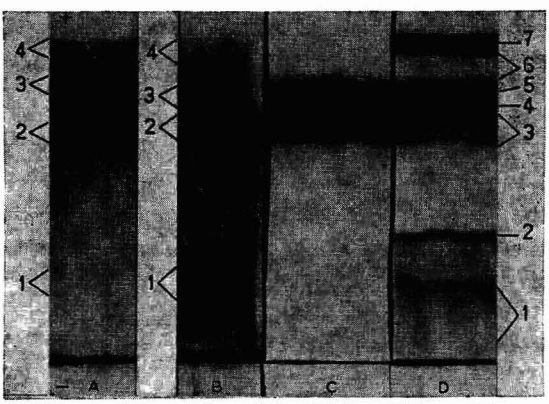

Fig. 1. Patterns of rabbit brain (A) and liver (B) soluble proteins, of haemoglobin (C) and serum proteins (D) obtained by starch–PVK gel electrophoresis. Nos. 1–4 (A,B) refer to the protein bands selected for elution (Table I). Numbers of (D) correspond to: (1) β-lipoprotein in γ-globulin region; (2) S α_2-globulin; (3) haptoglobins; (4) transferrin; (5) ceruloplasmin; (6) post-albumins; (7) albumin.

When using starch–PVK gel human serum protein recoveries increased up to 39 % and therefore it was thought worth while to investigate the degree of separation of tissue soluble proteins and to measure their recoveries.

Protein patterns as obtained by starch–PVK gel electrophoresis are shown in Fig. 1.

No attempts were made to identify the enzymatic activity of brain and liver soluble proteins and they were numbered arbitrarily from cathode to anode and recoveries of corresponding segments were measured from starch and starch–PVK gels. The results are listed in Table I.

TABLE I

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF RABBIT BRAIN AND LIVER ^{131}I-PROTEINS

Gel segments were frozen and thawed three times. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses.

*Protein fractions**	*Protein recovery*			
	Brain soluble ^{131}I-proteins		*Liver soluble ^{131}I-proteins*	
	Starch	*Starch–PVK*	*Starch*	*Starch–PVK*
1	11 (5.6)	40 (4.8)	17 (4.8)	39 (3.6)
2	33 (4.8)	61 (3.7)	31 (3.4)	52 (3.1)
3	55 (5.0)	66 (4.0)	50 (4.9)	68 (3.2)
4	75 (19.3)	76 (16.7)	78 (6.7)	81 (5.1)

* The numbers of the protein fractions refer to those in Fig. 1.

Protein recoveries were higher from starch–PVK gel and percentages of ^{131}I-TCA soluble radioactivity were lower than in starch gel.

Freezing may alter proteins and increase ^{131}I-TCA soluble radioactivity. In order to increase recoveries a very mild condition such as storing gel segments in saline at + 3° was tried.

The results showed (Table II) that higher protein recoveries were obtained when proteins diffused from gel into saline rather than freezing and thawing gels in order to disrupt their structure. Freezing of proteins resulted in consistently higher values of ^{131}I-TCA soluble radioactivity.

Protein recoveries, expressed as percentages of total radioactivity, increased considerably for both gels in the first 15 h and then progressively less until they reached the maximum after 90 h. Recoveries from starch gel segments left in saline as long as 90 h were, except for γ-globulin, only slightly lower when compared with corresponding starch–PVK gel segments, the eluates of the latter having, however, lower ^{131}I-TCA soluble radioactivity. Nevertheless from these results starch–PVK gel did not show, as far as protein recoveries were concerned, a striking advantage over starch gel.

Proteins diffused spontaneously from the gel because the saline became increasingly coloured when gel segments contained coloured proteins. From separate radioactivity measurements of saline and gel segments it appeared that squeezing the gel increased recoveries by no more than 10 %. Apparently free ^{131}I diffused from gel segments more rapidly than ^{131}I-proteins: ^{131}I-TCA soluble radioactivity appeared higher in the first 15 h than after 45 h.

TABLE II

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF RABBIT SERUM ^{131}I-PROTEINS AND OF RABBIT ^{131}I-HAEMOGLOBIN

Columns (A), (B), (C) and (D) refer to gel segments left in saline at + 3° for 2, 15, 45 and 90 h respectively. Columns (E) refer to gel segments kept frozen in saline at — 40° and thawed three times during 90 h. All segments came from the same gel slabs. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses. Percentages of calculated ^{131}I-radioactivity protein bound are underlined.

Protein fractions	*Protein recovery*									
	A		*B*		*C*		*D*		*E*	
	Starch gel	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*	*Starch gel*	*Starch–PVK gel*
γ-Globulin	9.0	15.6	14.0	35.0	29.4 (12.0) 25.9	46.6 (8.2) 42.8	34.3 (14.4) 29.4	57.8 (8.1) 53.1	14.6 (24.5) 11.0	44.5 (7.9) 41.0
Sα_2-Globulin*	8.4	25.7	26.1 (5.5) 24.7	42.0 (4.6) 40.1	44.8 (3.9) 43.1	63.0 (3.6) 60.7	55.4 (3.9) 53.2	69.1 (4.0) 66.3	11.7 (15.4) 9.9	44.8 (5.8) 42.2
Transferrin	17.1 (4.3) 16.4	29.3 (2.4) 28.6	40.4 (3.3) 39.1	59.4 (2.1) 58.2	60.4 (2.5) 58.9	74.3 (1.7) 73.0	73.5 (2.6) 71.6	85.5 (1.9) 83.9	43.4 (6.7) 40.5	79.6 (2.4) 77.7
Albumin	—	—	—	—	—	—	91.1 (2.7) 88.6	93.2 (2.4) 91.0	89.4 (2.9) 86.8	92.5 (2.4) 90.3
Haemo-globin	19.2 (19.4) 15.5	26.1 (7.2) 24.2	45.9 (11.3) 40.7	59.6 (7.0) 55.4	65.1 (13.5) 56.3	70.5 (5.3) 66.8	75.8 (10.9) 67.5	77.4 (5.8) 72.9	66.4 (15.1) 56.4	61.2 (7.5) 56.6

* This protein has been compared[13] to the human "slow" α_2-globulin.

TABLE III

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF HUMAN SERUM ^{131}I-PROTEINS

All proteins were eluted excluding only about 1 mm of gel at the origin. To both gels, saline equal to their volumes was added three times. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses.

Saline washings	*Protein recovery*	
	Starch gel	*Starch–PVK gel*
First	35 (2.6)	59 (2.5)
Second	18 (2.4)	24 (2.4)
Third	7	11 (2.5)

Finally the usefulness of using starch–PVK gel was mainly due to high protein recoveries being obtained with a very simple procedure (see Methods). The results are listed in Tables III and IV.

Starch–PVK gel allowed an overall increase of 34% in serum protein recoveries. This increase was quite consistent for all proteins except albumin. ^{131}I-Albumin which retained about 65% of total ^{131}I-serum radioactivity was always well recovered from both gels.

Protein fractions recovered from starch–PVK gel retained their original electrophoretic mobilities; moreover, judging from the absence of radioactive proteins at the origin, very little denaturation seemed to have occurred (Fig. 2). By autoradiographic estimation neither trailing nor tailing effects were observed after starch–PVK gel electrophoresis of ^{131}I-labelled protein fractions.

TABLE IV

PROTEIN RECOVERIES (AS PERCENTAGES) AFTER STARCH–PVK GEL AND STARCH GEL ELECTROPHORETIC SEPARATION OF HUMAN SERUM ^{131}I-PROTEINS

Proteins were eluted once, adding volumes of saline to one volume of gel as specified. Percentages of ^{131}I-TCA soluble radioactivity were estimated on the eluates and are given in parentheses

Protein fractions	*Gel: saline ratio*	*Protein recovery*		*Increase in recovery*
		Starch gel	*Starch–PVK gel*	
γ-Globulin	1:3	27 (12.3)	67 (6.8)	40
Sα_2-Globulin	1:3	44 (4.2)	63 (3.5)	19
Haptoglobins	1:3	50 (9.8)	75 (4.8)	25
Transferrin	1:4	61 (3.8)	76 (1.6)	15
Post-albumins	1:2	38	75	37
Albumin	1:5	73 (2.8)	77 (2.4)	4

Contents of soluble starch and protein contaminants from a blank experiment were measured and the results of a typical experiment are reported in Table V.

The amount of soluble starch eluted from a starch–PVK gel slab was lower than that eluted from a starch gel slab of identical volume. However, because starch–PVK gel contained 12 times as much PVK as starch the percentage of eluted starch was comparatively higher for starch–PVK gel. The amount of material reacting as protein by the method of GORNALL *et al.*[12] in eluates also obtained from gel slabs of identical

volume, were rather similar or equal, and the ^{131}I-protein bound radioactivity/protein amount (in mg) ratios were also equal or very similar for rabbit haemoglobin and albumin eluted from both gels.

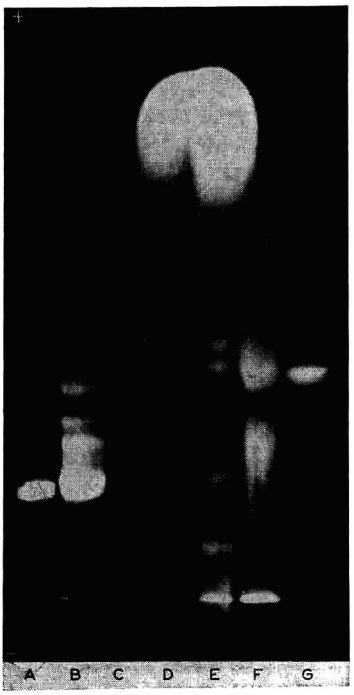

Fig. 2. Starch gel autoradiographs of ^{131}I-labelled human serum proteins: (A) $S\alpha_2$-globulin; (B) α_2-globulins obtained after PVK block electrophoresis[15] of serum proteins; (C) ceruloplasmin; (D) albumin; (E) whole serum; (F) β-globulins obtained as B; (G) transferrin. (A), (C) and (G) were obtained after starch–PVK electrophoresis of (B) and (F). (D) was obtained after starch–PVK gel electrophoresis of whole serum.

Gel filtration of protein eluates obtained from starch–PVK gel did not produce any separation of soluble starch from proteins with molecular weights higher than about 100,000: most of the soluble starch, detectable by addition of iodine to the

TABLE V

CONTENTS OF SOLUBLE STARCH AND PROTEIN (BLANK) IN STARCH GEL AND STARCH–PVK GEL SLABS AFTER ELECTROPHORESIS

Saline was added in equal volume twice and the eluates were pooled.

Samples	*Volume (ml)*	*Wet weight (g)*	*Dry weight (g)*	*Eluted soluble starch*		*Protein reacting material (mg)*
				as total amount (mg)	*as % of dry weight*	
Starch gel	21	23.0	2.17	210	9.7	27.5
Starch–PVK gel	21	25.6	15.60 (1.20)+	170	14.2	24.9

* Refers to the amount of starch present in starch–PVK gel.

samples, appeared between 250–259 ml of effluent together with a first protein peak namely γ-globulin and β-lipoprotein, whereas a second protein peak due either to transferrin or albumin (301–315 ml of effluent) still contained traces of undialysable starch. Recoveries of ^{131}I-proteins from a Sephadex G-100 column ranged between 87–95 %. However, the final recovery of transferrin after concentration by dialysis and elimination of most of the starch by gel filtration was only 51 % in comparison with 83 % as measured in the starch–PVK gel eluate.

DISCUSSION

Zone electrophoresis using starch–PVK gel as a supporting medium has proved useful for fractionating complex protein mixtures such as serum proteins and tissue soluble proteins. The resolving power of the method was improved by using a discontinuous buffer system in horizontal position and a voltage gradient of about 20 V/cm. Starch–PVK gel electrophoresis in a vertical direction could be troublesome if a tray with a removable glass plate was not available.

Recovery of proteins from PVK was found to be almost quantitative[14,15]: in order to reduce adsorption of proteins by the medium, PVK was mixed with hydrolysed starch in the hope of obtaining a gel that, besides high resolving power, allowed satisfactory protein recoveries. In fact with a very simple procedure for eluting proteins the overall recoveries increased up to 34 % in comparison with starch gel. Further advantage was obtained by avoiding the freezing-thawing procedure: ^{131}I-TCA soluble radioactivity was lower thus increasing the ^{131}I-protein bound radioactivity recoveries. When a high recovery was not essential undiluted protein fractions were obtained by just adding a few drops of saline to facilitate melting of the starch–PVK gel.

Minor advantages were: the possibility of readily detecting coloured proteins on a white background, the quick washing out of excess dye and the practical absence of shrinkage of the gel. Starch gel shrank about 14 % and this fact made localisation of proteins uncertain on the unstained gel.

Radioactive protein fractions did not show any trailing in starch–PVK gel autoradiographs and proteins travelled at the same rate throughout the thickness of the gel thus reducing the possibility of contamination and increasing protein recoveries. Although electrophoresis was performed at room temperature with a relatively high current, a vertical deformation of protein bands was avoided by applying a polythene cushion on top of the gel, which prevented evaporation and permitted cooling of the upper surface of the gel with running tap water.

Using the gel slab described, up to 120 mg of serum proteins were satisfactorily separated. The usefulness of the method was demonstrated in one experiment, when by using a slab of 31.0 × 1.2 × 24.0 cm as much as 360 mg of human serum proteins were separated after being taken up in two filter paper strips.

However, starch–PVK gel was not considered as a medium for preliminary protein fractionation but rather as a supporting gel suitable for final purification of simple protein mixtures. Resolution of protein components by either starch gel or starch–PVK gel one-dimensional electrophoresis was not feasible because of an overlapping of protein fractions. Albumin prepared in this way formed a single zone only when re-run in starch gel but it showed globulin contaminants when examined by cellulose acetate electrophoresis according to KOHN[16].

Two electrophoretic separations of rabbit serum carried out under different conditions were able to yield pure protein fractions, as was previously described[15]. Starch–PVK gel electrophoresis therefore can be employed as a final purification step, coupling the advantages of good resolving power with low adsorption for proteins.

A disadvantage of starch–PVK gel (shared by starch gel) was the contamination of proteins with rather large amounts of soluble starch that made any measurement of the carbohydrate content of proteins unreliable. Gel filtration of protein solutions did not separate soluble starch completely from albumin or transferrin as was, for example, possible by chromatography according to DE PAILLERETS *et al.*[17] or by electrophoresis[5].

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SUMMARY

A gel composed of hydrolysed starch and powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) is proposed as a supporting medium for separating serum proteins and soluble tissue proteins by electrophoresis. The procedure for eluting proteins from starch–PVK gel is very simple and recoveries are much higher than from starch gel. ^{131}I-trichloroacetic acid soluble radioactivity is consistently lower when proteins are recovered from starch–PVK gel. The presence of soluble starch does not affect the estimation of the specific activity of ^{131}I-labelled proteins.

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PAPER CHROMATOGRAPHY OF DYES

II. PAPER CHROMATOGRAPHY OF VAT AND SULPHUR DYES

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INTRODUCTION

Dyes insoluble in water, particularly the fast vat dyes and sulphur dyes occupy an outstanding place in the technology of dyeing and printing.

Vat dyes consist of any organic colouring matter (with the exception of basic and sulphur dyes) that is capable of undergoing a reversible reduction–oxidation cycle without serious colour loss or change of shade. Such dyes are the polycyclic and indigoid vat dyes (chemical derivatives of anthraquinone and polycyclic quinones with a larger number of condensed isocyclic and heterocyclic rings and derivatives of Indigo and Thioindigo). No general formula can be given for a vat dye. It is almost always a coloured organic compound containing two or more keto groups (>C = O) which are capable of being reduced by sodium hydrosulphite and alkali to give a leuco compound (⩾ C — ONa) which has affinity for cellulosic fibre.

The sulphur dyes are a class of highly coloured water-insoluble products reducible by dilute aqueous sodium sulphide to water-soluble derivatives which are substantive to cellulosic fibres; they are principally complicated thionated aromatic compounds of a rather high molecular weight, containing thiazole or thiazine or azine rings in the molecule.

The paper chromatography of these dyes is considerably more complicated than that of water-soluble dyes. Its success depends in the first place on the discovery of suitable solvents for the dye and secondly on the chromatographic technique itself. Despite these difficulties we were successful in elaborating a chromatographic method that would give reproducible and practical results[1–5].

Chromatography of vat and sulphur dyes is generally performed by the classical column method, while paper chromatography rarely finds application. The methods used are based on separating the dyes in their leuco forms by means of reducing solvents based on sodium hydrosulphite made alkaline with tetraethylenepentamine[6,7] or containing Cellosolve[8]. TAJIRI[9] has described the paper electrophoresis of vat and sulphur dyes in the presence of reducing agents; he concludes that normal paper chromatography is unsatisfactory; JUNGBECK[10] has stated that there is no reliable overall method of paper chromatography of these dyes, although JANICKA AND KAPRZAK[11] have described the separation of vat dyes on columns of cotton fibre. KOLŠEK, MLAKAR AND PERPAR[12] have described the paper chromatography of vat dyes in unreduced form. The vat dyes are dissolved in organic solvents.

Sulphur dyes present a complex problem, for usually they are not chemical individuals but a mixture of products of various sulphuration and condensation reactions. Thus, after fusion reactions with sulphur or polysulphides the final product contains various starting materials and intermediates, *e.g.* azines and carbazole. The literature contains few references to paper chromatography[1-5] and electrophoresis[9] of these dyes. Here again, reduction to the leuco form is essential.

EXPERIMENTAL

Vat dyes

Several methods were tried and it was found that the most consistent results were obtained by means of ascending chromatography at 80°, using an alkaline aqueous reducing solution of pyridine as eluent. The leuco compound was separated on the chromatogram after eluting for 2 h; Schleicher & Schüll 589[3] (blue ribbon) and Whatman No. 1 papers were found to be the most suitable.

Materials

The leuco compound of the dye, prepared according to normal commercial methods, is used in the form of a 1 % solution.

The reducing eluent has the composition:

Distilled water	32 ml
Turkey Red oil (tech.)–sodium alkylsulphonate, 1 % soln.	48 ml
Sodium hydroxide, 35 % soln.	4 ml
Sodium hydrosulphite	4 g
Pyridine	16 ml

When the solution is prepared, the components must be added in the order given above. For chromatograms 13 cm long, 10 ml of this solution are required, and for those 22 cm long, 15 ml. The solution should be freshly prepared for each determination.

Apparatus

The chromatographic test tubes have an inside diameter of 1.8 cm and are 20 cm long, and sufficient solvent is added to reach just below the starting point of the chromatogram. Four to eight test tubes are placed at the same time in a vertical position in a 3-l thermostat heated to 80° (Fig. 1).

The chromatogram is suspended from a hook in the rubber stopper of the test tube and small glass weights attached to its base keep it taut. During the separations the tubes must be completely immersed in the thermostat to prevent condensation of solvent vapours in the upper part of the test tubes and it is therefore essential that the rubber stopper fits tightly. If necessary it can be covered by a heavier rubber plug.

Method

The chromatograms generally used were 1 cm × 16 cm strips, the starting line being 2 cm from the lower edge and the distance from the starting line to the solvent front being 13 cm. For the separation of complex, artificial mixtures it is advisable to use 25 cm strips with a start–solvent front distance of 22 cm.

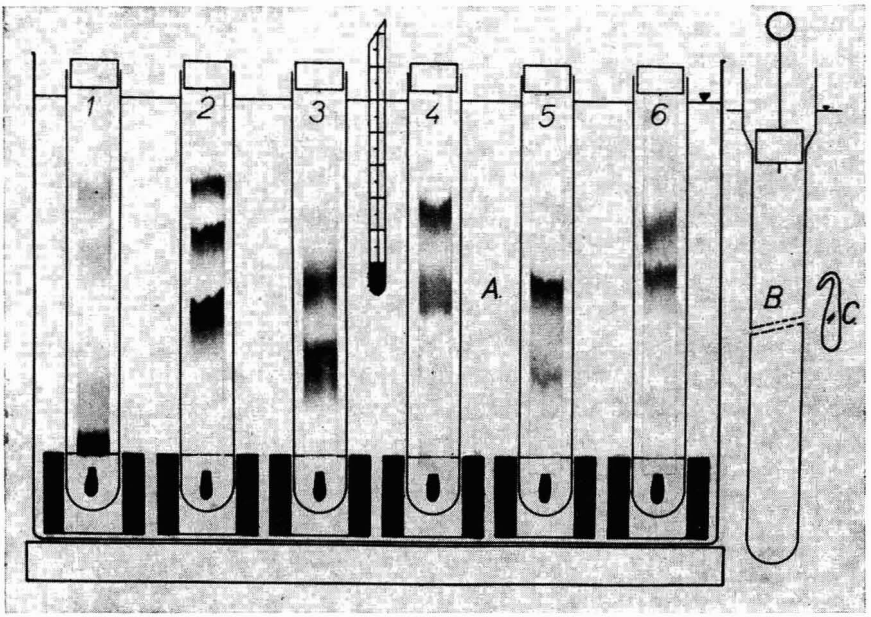


Fig. 1. Apparatus for chromatography of vat dyes with typical examples of the separation of their mixtures. A = thermostat; B = test tube for separation of complex mixtures; C = glass weight.

Chromatogram No.	*Commercial name*	*C.I. No.*
1.	Mixture of:	
	Indanthrene Khaki GG	71050
	Indanthrene Yellow 4GF	68420
	Indanthrene Brilliant Green B	59825
	Brilliant Indigo 4B	73065
2.	Mixture of:	
	Indanthrene Scarlet R	71140
	Indanthrene Red GG	71130
	Algol Scarlet B	71135
3.	Mixture of:	
	Indanthrene Orange RRT	59705
	Indanthrene Red Brown RR	59500
4.	Mixture of:	
	Helindon Yellow CG	56005
	Indanthrene Yellow 6GD	56080
5.	Mixture of:	
	Indanthrene Blue GCD	69815
	Indanthrene Orange F3R	69540
6.	Mixture of:	
	Indanthrene Cyanine B	60015
	Indanthrene Brilliant Green B	59825

The leuco vat dye (5–10 μl of 1 % solution) is applied by means of a micropipette to the starting line on the chromatogram, which is quickly placed (without being dried) in a test tube containing the solvent (10 ml for a 13 cm chromatogram, 15 ml for a 22 cm chromatogram) at 80°, so that the reducing solvent quickly begins to ascend the paper. Although some oxidation of the leuco compound occurs, the re-

ducing solvent quickly reduces the vat dye back to the leuco compound and separation then begins. The separation is complete after 2 h with the shorter chromatogram and after 4 h with the longer one. After the separation the chromatogram is exposed to the air and rinsed in water to remove residual solvent.

Sulphur dyes

Sulphur dyes are chromatographed under conditions identical to those used for vat dyes, the leuco compounds in this instance being obtained by dissolving the dye in sodium hydroxide and sodium hydrosulphite.

RESULTS AND DISCUSSION

Vat dyes

By means of the technique described above, all the important dyes of known constitution listed in the *Colour Index*[13], as well as some mixtures, have been examined. The results are reproducible and the method is sufficiently sensitive to permit separation of structurally similar compounds.

The rate of movement of the dye depends on the size of the molecule and its chemical structure. Large molecules travel slowly, owing to increased substantivity for the paper and decreased solubility in the mobile phase; the chemical groups present will affect the solubility of the dye, smaller molecules being most affected.

Such separations are particularly useful for testing the purity of dyes during manufacture and they also indicate the probable dyeing characteristics on cellulosic materials[14].

Vat dyes can be divided into five groups on the basis of their rate of movement on the chromatogram, *viz.*

(1) The main spot does not move, but forms a slight forward tail.

(2) The main spot moves slightly, but tails back to the origin.

(3) The dye moves up to half-way along the chromatogram.

(4) The dye moves up to two-thirds of the length of the chromatogram.

(5) The dye moves with the solvent front.

Fig. 2. is a representation of this classification.

The individual groups of vat dyes behave as shown in Table I.

From the results the following general conclusions concerning the chemical structure can be drawn.

(1) Substitution by $-CH_3$, $-OCH_3$ or benzoylamino reduces the rate of movement; substitution by halogens increases it.

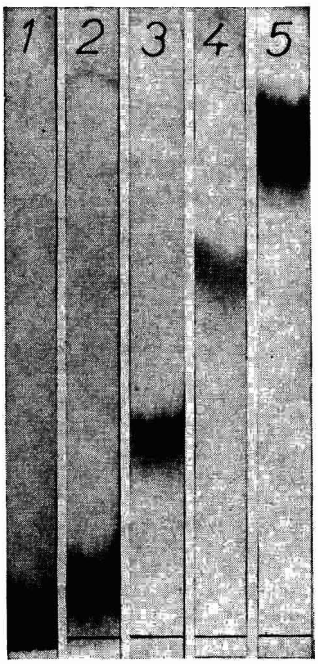
(2) Dyes containing a thiazole, oxazole, acridone, cyanuric or N,N′-dihydropyrazine ring travel more slowly than dyes without such a ring, the thiazole ring having the greatest effect.

(3) Increasing the number of keto groups in the dye progressively reduces the rate of movement.

(4) Dibenzanthrones travel faster than isodibenzanthrones.

(5) Thioindigoid dyes travel faster than indigoid dyes.

(6) Of naphthoylenebenziminazoles having *cis*- and *trans*-isomers, the *cis*-isomers travel most rapidly.



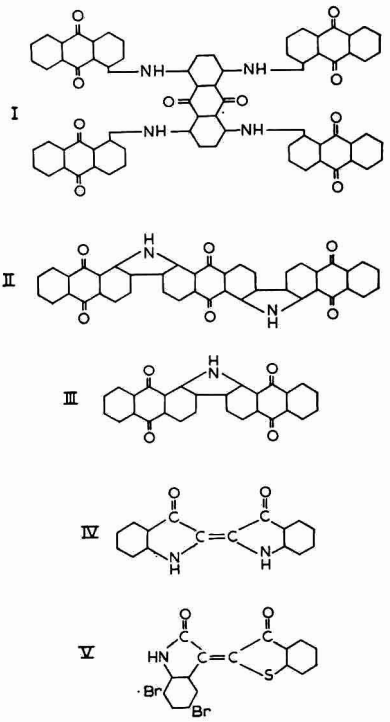


Fig. 2. Influence of molecular complexity on chromatographic behaviour of vat dyes.

Chromatogram No.	*Commercial name*	*Separation group*	*Structural formula*
1.	Indanthrene Khaki GG	1.	I.
2.	Indanthrene Yellow 3R	2.	II.
3.	Indanthrene Yellow FFRK	3.	III.
4.	Indigo	4.	IV.
5.	Ciba Red G	5.	V.

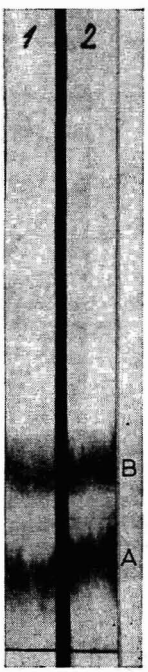
TABLE I

	Chemical type	*Separation group*
1.	Thiazine	4
2.	Sulphur	4
3.	Arylaminoquinones	4 (3)
4.	Acylaminoanthraquinones	2, 3, 4
5.	Anthraquinonylaminotriazines	2, 3, 4
6.	Anthraquinonylamines (Anthrimides)	2,3
7.	Anthraflavones	3
8.	Anthraquinonecarbazoles (Diphthaloylcarbazoles)	2, 3 (5)
9.	Benzanthronylaminoanthraquinone Benzanthrone "Acridone"	1, 2
10.	Anthraquinonethiazoles	1, 2
11.	Anthraquinoneiminazoles	1, 2

(continued on p. 529)

TABLE I (*continued*)

	Chemical type	*Separation group*
12.	Anthraquinoneoxazoles	1, 2
13.	Anthraquinoneacridones	1
	Anthraquinoneacridones (unmodified)	3
14.	Anthraquinonethioxanthenes	3
	Pyrazinoanthraquinones	
15.	N,N'-Dihydro-anthraquinone-azines (Indanthrones)	3
16.	Flavanthrones	3
17.	Pyranthrones	2, 3
18.	Anthrones, Dibenzopyrenequinones, Acedianthrones	1, 2, 3, 4
19.	Anthrapyrimidines	3,4
	Pyridoanthrones	
20.	Bispyrazoleanthrones	3
	Thiabenzanthrones	
21.	Dibenzanthrones, Violanthrones	(1) 2, 3, 4
22.	Isodibenzanthrones, Isoviolanthrones	2, 3, 4
23.	Benzanthronylpyrazoleanthrones	1, 2
24.	Perylenetetracarboxylic acid	2, 3, 4
25.	Naphthoylenebenziminazoles	2, 3
26.	Phthalocyanines	3
27.	Indigoid, Thioindigoid	4, 5
	Indole-thianaphthene	



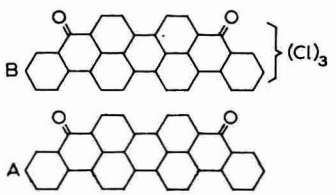


Fig. 3. Separation of a mixture of dyes of the dibenzanthrone type (Violanthrones). Chromatogram Nos. 1 and 2: Mixture of A and B.

	Commercial name	*C.I. number*
A.	Indanthrene Dark Blue BOA	59800
B.	Cibanone Navy Blue RA	59815

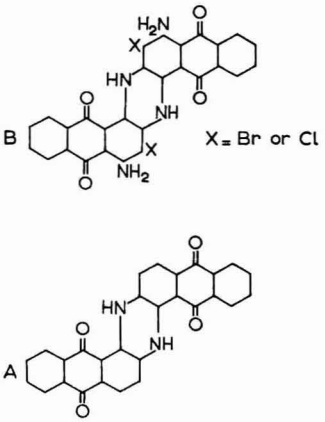
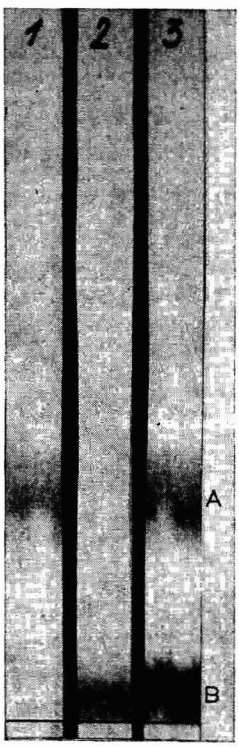


Fig. 4. Separation of a mixture of dyes of the indanthrone type (N,N'-dihydro-anthraquinoneazines).

	Commercial name	*C.I. number*
A.	Indanthrene Blue RSN	69800
B.	Indanthrene Green BB	69850



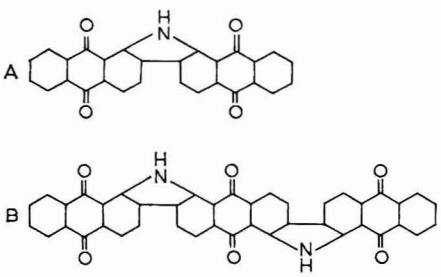
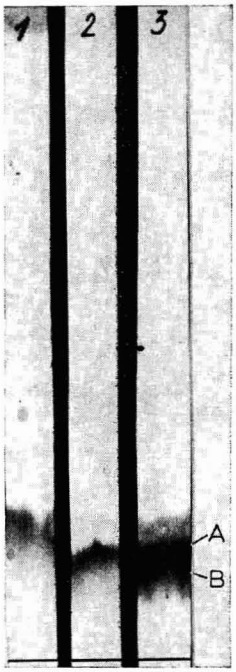


Fig. 5. Separation of a mixture of dyes of the anthraquinonecarbazole type.

Chromatogram No.		*Commercial name*	*C.I. number*
1.	A.	Indanthrene Yellow FFRK	69000
2.	B.	Indanthrene Yellow 3R	70805
3.		Mixture of A and B	



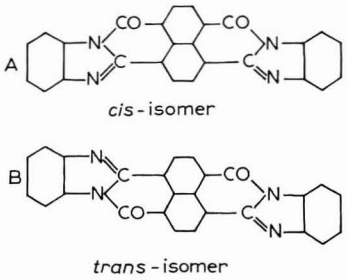
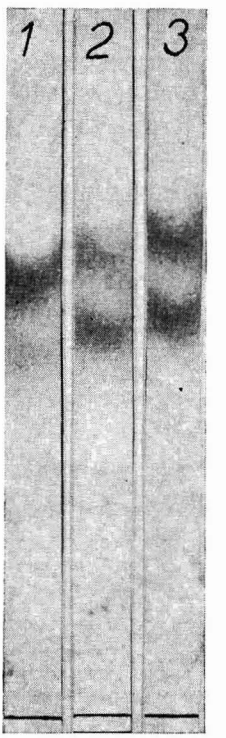


Fig. 6. Separation of *cis*- and *trans*-isomers (naphthoylene-benziminazole).

Chromatogram No.		*Commercial name*	*C.I. number*
1.	A.	Indanthrene Bordeaux HRR (*cis*-isomer)	71100
2.	B.	Indanthrene Brilliant Orange GR (*trans*-isomer)	71105
3.		Indanthrene Scarlet GG (an isomeric mixture of A and B)	71110



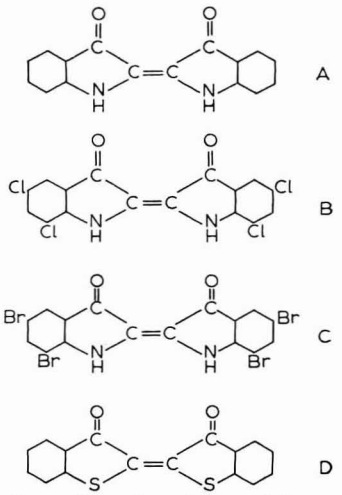


Fig. 7. Separation of a mixture of dyes of the indigoid and thioindigoid type.

Structural formula	*Commercial name*	*C.I. number*
A.	Indigo (synthetic)	73000
B.	Brilliant Indigo B	73040
C.	Brilliant Indigo 4 B	73065
D.	Thioindigo Red B	73300

Chromatogram No. 1 = mixture of A and B; No. 2 = mixture of A and C; No. 3 = mixture of A and D.

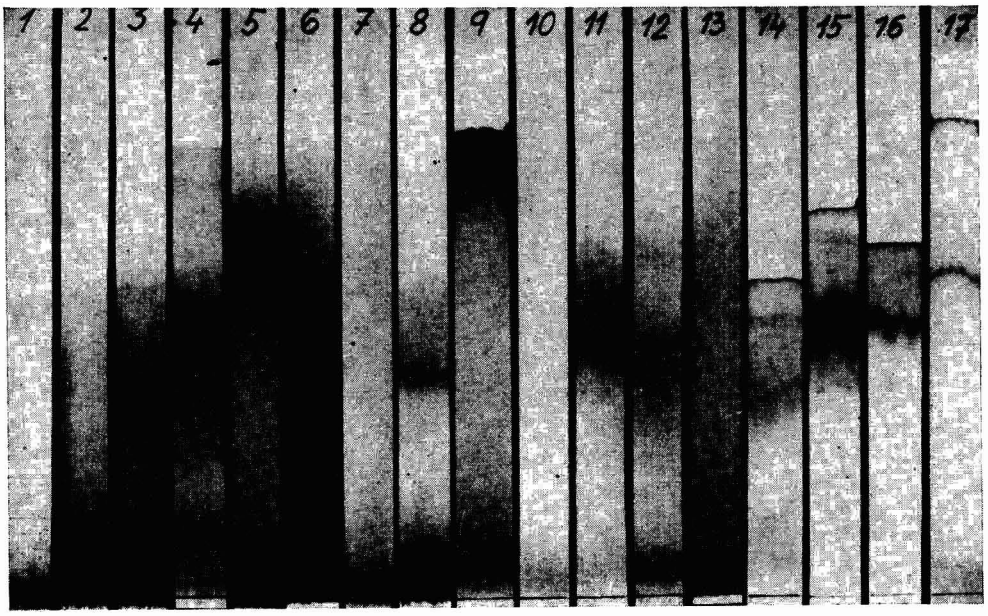


Fig. 8. Chromatograms of sulphur dyes. 1 = Sulfogen Yellow G; 2 = Sulfogen Orange G; 3 = Sulfogen Yellow R; 4 = Sulfogen Yellow Olive G; 5 = Immedial Yellow Brown GL (C.I. 53325); 6 = Immedial Catechu 4 RL"F" (C.I. 53320); 7 = Immedial Brown GGL (C.I. 53327); 8 = Sulfogen Red Brown 4 R; 9 = Sulfogen Brilliant Black B 4 R; 10 = Sulfogen Dark Blue 6 BZ; 11 = Immedial Fast Blue 6 GL (C.I. 53460); 12 = Sulfogen Green 2 G; 13 = Sulfogen Dark Green B; 14 = Sulfogen Green Blue CV ex; 15 = Sulfogen Green 3 G; 16 = Sulfogen Green MK; 17 = Sulfogen Green G.

The examples of the separation of mixtures of vat dyes given in Figs. 3, 4, 5, 6 and 7 demonstrate the influence of the molecular complexity and of substituents on the chromatographic behaviour of the vat dyes.

Evidence of configuration isomers can further be obtained in the case of analogues of Indanthrene Scarlet G G, that is with the chloro-derivative of Indanthrene Printing Brown 5R (C.I. 71115) and the ethoxy-derivative of Indanthrene Printing Brown B (C.I. 71120).

Sulphur dyes

Sulphur dyes are either (a) eluted only with difficulty from the starting point or (b) move a considerable distance. Dyes in group (a) must be eluted for 30–60 min and those in group (b) for 10–15 min. Group (a) includes thiazole dyes (yellows, oranges, and browns), mono- and binuclear-substituted amino and nitro compounds (C.I. 53005–53160), polycyclic compounds (C.I. 53320–53335), and acridine, azine, oxazone, and thiazone derivatives (C.I. 53680–53830) (browns, bordeaux and violets). Group (b) includes thiazine dyes (blues, greens, and blacks), substituted phenols and naphthalenes (C.I. 53165–53300), and indophenols (C.I. 53400–53640).

Because of the complex nature of the sulphur dyes, their chromatograms usually consist of a series of bands. This is particularly the case for dyes that fall within group (b). In Fig. 8 a chromatogram illustrating the separation of sulphur dyes is given.

SUMMARY

The paper chromatography of vat and sulphur dyes, using reducing solvents at 80°, has been carried out, the dyes being separated as their leuco compounds. In both cases ascending paper chromatography was applied. The relation between dye constitution and chromatographic behaviour has been examined.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART VIII. THE ACID HYDROLYSIS OF SODIUM TRIMETAPHOSPHIMATE WITH REFERENCE TO RING STABILISATION

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Recent investigations[1-3] into the mechanism of the hydrolysis of sodium trimetaphosphimate (TMPm), have shown that the replacement of the three imido-linkages by oxygen, takes place in a regular sequence, yielding the corresponding ring phosphates, *i.e.* diimido-trimetaphosphate (DITMP), monoimidotrimetaphosphate (ITMP), and trimetaphosphate (TMP). POLLARD, NICKLESS AND WARRENDER[3] have shown that the replacement of the individual imido-linkages by oxygen takes place without the intervention of intermediate unstable chain imidophosphates, and that the ring phosphate becomes increasingly stable as oxygen replaces the imido-groups.

Ion-exchange chromatographic separations of TMPm, DITMP, ITMP, and TMP have been developed to yield completely separated fractions of each phosphorus-bearing species. This paper reports the evaluation of the reaction kinetics of the hydrolysis reaction, and the relative stabilities of these closely related compounds.

EXPERIMENTAL

Sodium trimetaphosphimate

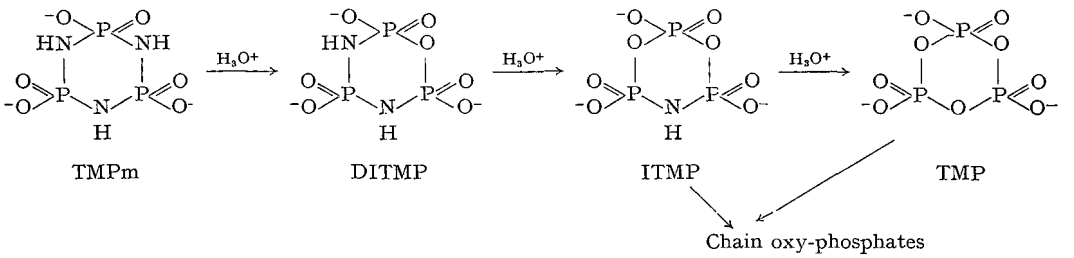
Preparation. Sodium trimetaphosphimate can be prepared by the hydrolysis of the trimeric phosphonitrilic chloride dissolved in diethyl ether, using sodium acetate solution as the aqueous hydrolytic medium, following the method described by STOKES[4]. However, on repetition of STOKES' method it was found to suffer the serious disadvantage that since diethyl ether and water are immiscible, the time of reaction has to extend to four or five days, and furthermore, reaction yields were found to be low.

To overcome these difficulties, dioxan was used as solvent for the trimeric phosphonitrilic chloride, and caustic soda solution was used as the hydrolytic medium. Since dioxan and water are miscible, the reaction time was decreased to about six hours. A typical preparation was as follows:

30 g of trimeric phosphonitrilic chloride was dissolved in 170 ml dioxan, and poured into a solution of 6 *N* caustic soda solution (150 ml). The mixture was gently agitated by stirring for six hours at room temperature, the contents of the reaction vessel gradually solidified. The products of the reaction, TMPm, sodium chloride, and sodium orthophosphate were filtered off, washed with a 60–40 % methanol–water solution, in which only TMPm is insoluble. After repeated washings, the prod-

uct was dissolved in water, and reprecipitated by slowly adding methanol. Filtration, and final washing with methanol, yielded a white compound which was pure sodium trimetaphosphimate. The salt was characterised by the normal methods, analysis, potentiometric titration, infra-red, and gave a single peak, the retention volume of which corresponded to TMPm.

Detection, and determination of hydrolysis products. It has been shown that in acid solution[3], TMPm breaks down to yield DITMP, which hydrolyses in turn to ITMP, which subsequently hydrolyses to TMP and chain oxy-phosphates.



Thus for elucidation of the kinetics of the reaction, a method of analysis for TMPm, DITMP, ITMP, TMPm and orthophosphate is required. POLLARD, NICKLESS AND WARRENDER[3], published such a separation but by refinement of the anion-exchange technique, using Dowex-1 resin (1 × 8) of mesh size 100–200, packed into a column 50 cm long, and 0.9 cm diameter, and eluting the phosphate species with a gradient chloride solution of 0.75 *M* potassium chloride pH 5 dropping into 0.075 M potassium chloride pH 5[3,5], the type of separation obtained is shown in Fig. 1. Each species was estimated using the phosphovanadomolybdate method for phosphorus analysis[6], after hydrolysis to orthophosphate[3].

The retention volumes (position of peak maximum) were:

Orthophosphate	70 ml
Trimetaphosphimate	270 ml
Diimidotrimetaphosphate	370 ml
Monoimidotrimetaphosphate	490 ml
Trimetaphosphate	730 ml

The sharpness of the elution peaks, enabling complete fractions to be isolated, was mainly due to the very slow flow-rates (30 ml/h) and the very steep chloride gradient. Higher flow rates and lower chloride gradients were both found to "spread" the peaks. The yield of phosphorus recovered from each peak was always within 1–2 % of the theoretical quantity when using known mixtures.

Kinetics of hydrolysis of TMPm at pH 3.62. The hydrolysis of TMPm was studied at two different temperatures, 52° and 65°.

Approximately 0.5 g of TMPm was dissolved in 50 ml of a sodium acetate–hydrochloric acid buffer pH 3.62, in a 100 ml graduated flask, containing a loosely fitting stopper. The flask was immersed in a water-bath, thermostatically controlled at the required temperature, at a noted time. At various time intervals, 5 ml samples of the hydrolysing solution were quickly removed from the reaction vessel and poured into a 10 ml graduated flask, containing 5 ml of ice cold 0.1 *N* caustic soda solution.

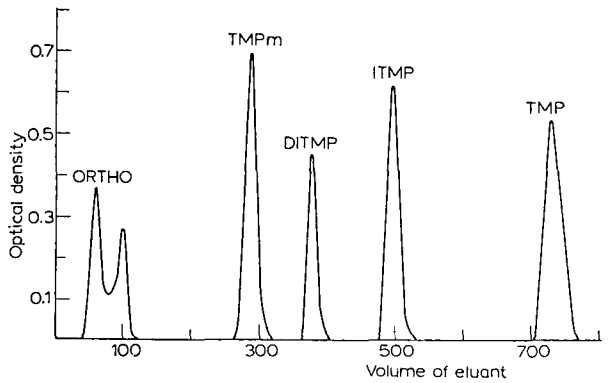


Fig. 1. Separation of orthophosphate, trimetaphosphimate (TMPm), diimidotrimetaphosphate (DITMP), imidotrimetaphosphate (ITMP), and trimetaphosphate (TMP).

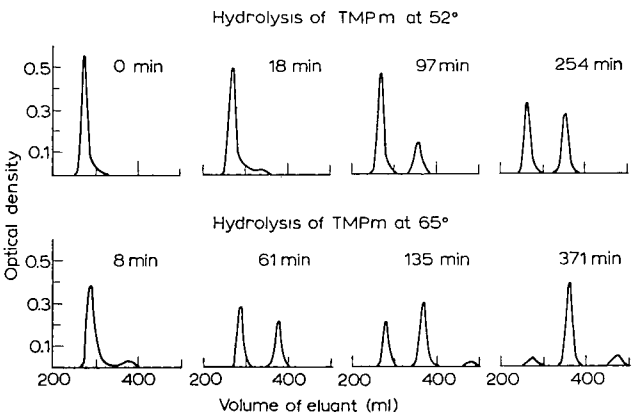


Fig. 2. Elution patterns for the hydrolysis of trimetaphosphimate at pH 3.6.

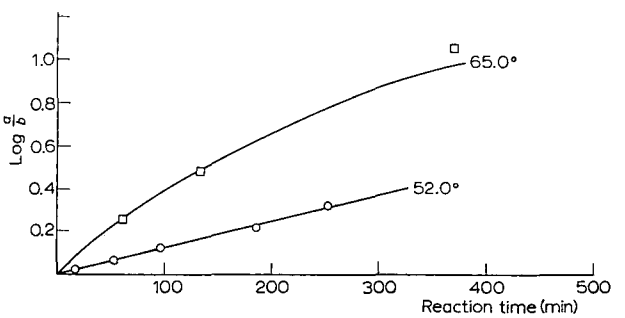


Fig. 3. Variation of log a/b with time for the hydrolysis of trimetaphosphimate at pH 3.6. a = concentration of TMPm at T = 0 min; b = concentration of TMPm at T = t min.

The flask was surrounded by water at 0°. This procedure "freezes" the reaction at the noted time interval, and the contents of the flask remain unreactive and at constant proportions for several days under these conditions.

To analyse the mixture, a 1 ml sample is pipetted on to the anion-exchange column, and the elution allowed to take place, in the manner as described previously. Fig. 2 shows the course of the reaction at 52° and at 65° respectively, using the scheme above.

TABLE I

VELOCITY CONSTANTS OF TMPm DEGRADATION

52°		65°	
Time of reaction (min)	$k \times 10^3$ *(min^{-1})*	*Time of reaction (min)*	$k \times 10^3$ *(min^{-1})*
19	2.25	62	9.41
47	3.05	136	8.01
97	2.81	371	6.49
187	2.61		
254	2.78		
$k_{52°} = 2.81 \cdot 10^{-3}$ min^{-1}		$k_{65°} = 8.91 \cdot 10^{-3}$ min^{-1} (using the initial rate of reaction)	

Results. As the total optical density of any one analysed phosphorus species is directly related to the quantity of phosphorus present, it is a simple matter to evaluate the reaction velocity constants (assuming first order kinetics), which are given in Table I and their variation in time in Fig. 3.

Sodium diimidotrimetaphosphate

Preparation and purification. The preparation of sodium diimido trimetaphosphate involving the hydrolysis of sodium trimetaphosphimate as described by STOKES[4] and DE FICQUELMONT[7] are both satisfactory in so far as the product is predominantly DITMP (up to 80%), but for kinetic studies, they are unsatisfactory as impurities of TMPm and ITMP are certain to occur.

From studies on the hydrolysis of TMPm, it is apparent that the maximum yield of DITMP is obtained after hydrolysing TMPm at pH 3.62, and 65° for 6 h. Lengthier hydrolysis would yield greater impurities of ITMP, whilst shorter hydrolysis times would give TMPm impurities.

It was realised, that the only practical method available of isolating pure DITMP from TMPm and ITMP impurities, was to employ in some manner, the ion-exchange separation. The method which was finally used is as follows:

TMPm, DITMP, ITMP have retention volumes of 270, 370, and 490 ml ($\pm$ 10 ml) respectively, under the conditions used for the analysis. It is assumed that the phosphate species remained adsorbed at the top of the ion-exchange column until a certain and specific "desorption" chloride concentration is reached, whereupon it exchanges rapidly with the resin and eluant. Thus it travels quickly down the column to be eluted from the column after 30 ml (the dead volume of the column) of the requisite chloride concentration is attained and passes down the column. Now for the different retention volumes of TMPm etc. the actual chloride concentration at these volumes of effluent can be evaluated using the equation:

$$M_t = M_I - (M_I - M_0)e^{-rt/v}$$

where M_0 = molarity of KCl solution in mixing bottle initially (moles/l)
M_t = molarity of KCl in mixing bottle at time t (min) (moles/l)
M_I = molarity of KCl in reservoir (moles/l)
r = rate of flow of eluant (ml/min)
v = volume of solution in mixing bottle (ml)

The various chloride concentrations calculated are shown in Table II.

0.5 g of a mixture of imido-phosphates containing approximately 14 % TMPm, 75 % DITMP, and 16 % ITMP was dissolved in 50 ml 0.226 M potassium chloride

TABLE II

Species	*Concentration of KCl (moles/l)*
TMPm	0.226
DITMP	0.278
ITMP	0.325

solution and adsorbed on to a column of Dowex-1 resin 50 cm long and 12 cm diameter.

The phosphate mixture was obtained by hydrolysing 0.5 g TMPm dissolved in 50 ml sodium acetate–hydrochloride acid buffer pH 3.62 at 65° for 6 h. The phosphate species were precipitated from solution using an ethanol–water solution (2:1), washed thoroughly with pure ethanol, and dried under vacuo. The column was previously equilibrated with 50 ml of 0.226 M potassium chloride solution buffered to pH 5.0 by a citric acid–caustic soda solution. After absorption of the imido-phosphates, a further 50 ml of 0.226 M potassium chloride solution was passed through the column to remove the TMPm. 120 ml of 0.278 M potassium chloride buffered to pH 5 were now passed through the column, and the effluent was allowed to drop directly into a large volume of methanol, whereupon the DITMP was precipitated out. Filtration, washing with ethanol, drying under vacuo produced a white powdery solid, which on examination was found to be DITMP only. Analysis, potentiometric titration, and especially anion-exchange showed only one phosphorus species to be present, with no detectable amounts of TMPm or ITMP (*i.e.* less than 0.5 %). The yield of pure DITMP was of the order of 45 %.

Kinetics of hydrolysis of DITMP at pH 3.62. Using exactly similar procedures to those used for the TMPm hydrolyses reactions, a study of the hydrolysis of DITMP

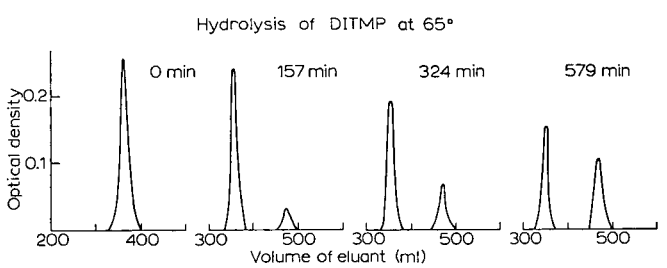


Fig. 4. Elution patterns for the hydrolysis of diimidotrimetaphosphate at pH 3.6 and 65°.

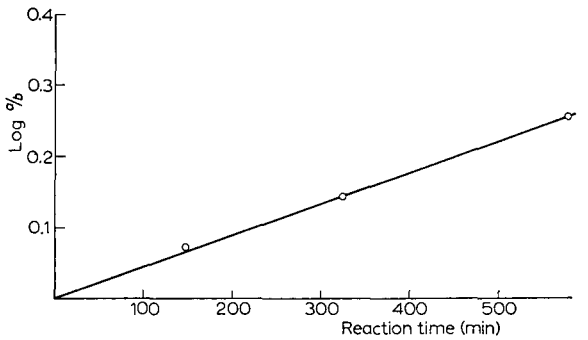


Fig. 5. Variation of log a/b with time for the hydrolysis of diimidotrimetaphosphate at 65° and pH 3.6. a = concentration of DITMP at T = 0 min; b = concentration of DITMP at T = t min.

was made, however, only at one temperature 65° because of the greater stability of DITMP as compared to TMPm. The results are shown in Figs. 4 and 5, and assuming first order kinetics, the velocity constants are given in Table III.

TABLE III

VELOCITY CONSTANTS OF DITMP DEGRADATION AT 65°

Time of reaction (min)	$k \times 10^{-3}$ *(min^{-1})*
157	1.05
324	1.01
579	1.01
1294	0.90

Sodium monoimidotrimetaphosphate

Preparation and purification. The preparation of sodium monoimidotrimetaphosphate by the acid hydrolysis of TMPm[8], yields a moderately pure product, but again, one which is contaminated by its immediate decomposition products, and by DITMP from which it is actually formed.

ITMP was prepared by dissolving 10 g of sodium trimetaphosphimate in a 5:1 vol./vol. water–glacial acetic acid solution, and heating the solution to 50° for 150 h. Paper chromatographic analysis of the reaction solution showed the presence of orthophosphate, ITMP and TMP. The ITMP was precipitated from solution with ethanol, leaving the orthophosphate and TMP in solution. The product was washed thoroughly with ethanol, redissolved in a minimum (20 ml) of water, filtered and re-precipitated with ethanol. The dried product, when examined by anion-exchange chromatography, showed the presence of traces of TMP, DITMP and orthophosphate, therefore it was necessary to purify the ITMP. The calculated chloride concentration for desorption from the exchange column is 0.325 M potassium chloride solution. Using this value and an exactly similar procedure to that described for the DITMP purification, a pure sample of ITMP was prepared. Ion exchange and paper chromatographic analysis showed the final product to be 100 % pure ITMP.

Kinetics of hydrolysis of ITMP at pH 3.62. Identical methods of procedure were used for studying the hydrolysis of ITMP as was used for the hydrolysis of DITMP.

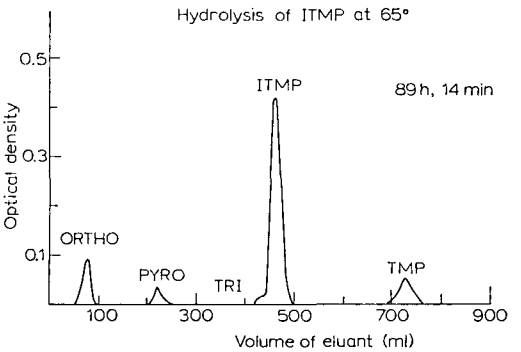


Fig. 6. Elution pattern for the hydrolysis of monoimidotrimetaphosphate at 65° and at pH 3.62 after nearly 3 days reaction time.

The results are shown in Fig. 6. ITMP is hydrolysed by two mechanisms[3]:

(i) Replacement of the final –NH– group linkage by oxygen.

(ii) Degradation of the ring structure to chain oxy-phosphates.

Assuming first order kinetics for both processes the following results are obtained and are shown in Table IV.

TABLE IV

VELOCITY CONSTANTS OF ITMP DEGRADATION AT 65°

Time of reaction (min)	*Velocity constant*	
	Chain species (min^{-1})	*Ring species (min^{-1})*
469	No reaction	
1,025	$4.6 \cdot 10^{-5}$	Undetected
3,720	$4.3 \cdot 10^{-5}$	$2.4 \cdot 10^{-5}$
5,354	$4.2 \cdot 10^{-5}$	$2.1 \cdot 10^{-5}$

DISCUSSION

The evaluation of the k_{TMPm} velocity constants for the hydrolysis of TMPm, at 65°, and 52°, leads to an estimation of the activation energy E^*_{TMPm} (Fig. 7). The point at $I/T = 0.0030$ is from the data of POLLARD, NICKLESS AND WARRENDER[3]. $E^*_{TMPm} = 19.5$ kcal/mole^{-1}. Assuming now that the constant A in the equation:

$$\log k = \log A - \frac{E^*}{RT}$$

is constant for the closely related series of compounds TMPm, DITMP and ITMP, then the activation energies of the latter two compounds can also be calculated and are given in Table V.

The replacement of one imido-linkage in a six-membered ring, by an oxygen atom stabilizes the ring by 1.45 kcal/mole. It might be logically deduced, that the ring phosphate by the further replacement of an imido-linkage by oxygen would be stabilized by a further factor of 1.5 kcal/mole, and this is in fact shown by the figures. Furthermore, kinetic studies on the hydrolysis of trimetaphosphate in acid solution[9], evaluate E^*_{TMP} to be 24.0 kcal/moles^{-1}, a stabilisation increase of 1.5 kcal/mole over

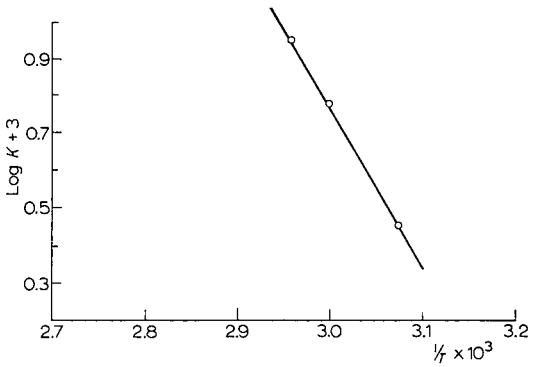


Fig. 7. Variation of reaction velocity K for the hydrolysis of trimetaphosphimate at pH 3.62 with temperature.

ITMP. Thus there appears to be a remarkably consistent stabilisation factor of 1.5 kcal/mole in the series trimetaphosphimate to trimetaphosphate, as each imido-linkage is successively replaced by oxygen.

TABLE V

REACTION CONSTANTS OF IMIDOMETAPHOSPHATES

Species	*Velocity constant at 65° (min⁻¹)*	*E** *(kcal/mole)*	*Differences (kcal/mole)*
TMPm	$8.91 \cdot 10^{-3}$	19.5	1.45
DITMP	$1.02 \cdot 10^{-3}$	20.9(5)	1.45
ITMP	$2.30 \cdot 10^{-5}$	22.5	

ACKNOWLEDGEMENT

The authors wish to thank the Department of Scientific and Industrial Research for a Research Studentship to A. M. BIGWOOD, during the tenure of which the research was carried out.

SUMMARY

A kinetic study of the hydrolysis of the series trimetaphosphimate, diimidotrimetaphosphate, monoimidotrimetaphosphate is described, where the products of reaction are determined by a combination of gradient elution anion exchange chromatography and colorimetry. Reaction velocity constants are determined, and observations concerning the ring stabilisation are discussed.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART X. THE CHROMATOGRAPHIC SEPARATION AND AUTOMATIC FLAME SPECTROPHOTOMETRIC DETERMINATION OF THE ALKALINE-EARTH METALS

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A previous communication[1] described a separation of the alkaline-earth metals by cation-exchange chromatography, and the diluted effluent fractions were estimated by flame spectrophotometry. Due to the sensitivity of the flame spectrophotometer, the dilution steps in this method were somewhat tedious, work was carried out to render this step unnecessary, and it has been found possible to trace directly the elution patterns of the separated species using the flame spectrophotometer as a column effluent detector. The possibility of developing a continuous and automatic method of analysis became apparent and the method has thus been developed which is rapid, reasonably accurate and avoids the slow stages of classical methods of analysis, and yet is suited for routine analysis.

The chromatographic technique in this method differs only slightly from the previous method[1], in that a more rapid separation of the alkaline-earth metals has been achieved, complete separation and determination may be executed in under twelve hours.

EXPERIMENTAL

The flame spectrophotometer used was a Unicam SP 900 Mark I instrument as described previously[2]. Using conventional cation-exchange chromatography techniques of gradient elution, micro- and milli-gram quantities of alkaline-earth metals were separated and their concentration in the effluent (diluted to a constant volume of water by means of a simple constant volume burette[3]) was determined by direct aspiration into the flame. The area of the elution peaks so obtained was a measure of total alkaline-earth metal concentration. The accuracy of the method was found to be within 5% at all levels.

Apparatus and general technique

The chromatographic procedure was similar to that previously recommended[1] except that the resin bed length was decreased to 60 cm, and that Dowex-50W X8, 100–200 mesh replaced the Dowex-50W X8, 200–400 mesh resin. The gradient employed throughout was obtained by dropping 5 *M* ammonium lactate (instead of 3 *M* ammonium lactate) into 1 *M* ammonium lactate, and the pressure head was increased

to about 50 cm height. These differences in procedure considerably curtailed the elution times, thus allowing automatic detection feasible.

A constant volume delivery burette used as shown in Fig. 1, was modified from that described by GUILT AND ROBERTSON[3]. Such a device is necessary to enable

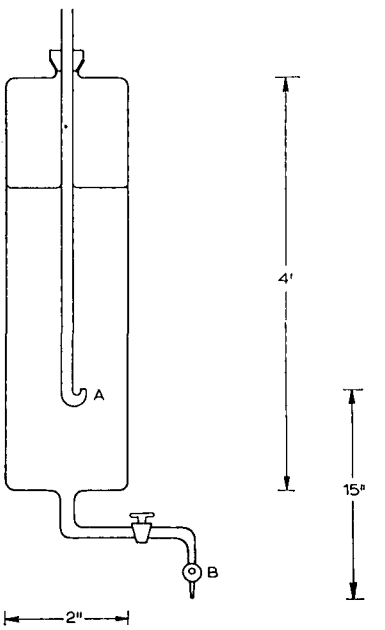

Fig. 1. Constant volume delivery burette (not to scale). A. Fine air bleed-in. B. Fine control burette tap—diaphragm type.

chromatographic elution to occur into a constant volume of solution since the column flowrate is much less than the aspiration rate of the flame spectrophotometer.

Purity of water and reagents

As in previous work, all water used was deionized and all the reagents were of AnalaR grade, the alkaline-earth content of which was negligible.

Reference solutions for chromatography

Standard barium solution	$BaCl_2 \cdot 2H_2O$ in water containing 1 mg/ml of barium.
Standard calcium solution	$CaCO_3$ in 0.01 *N* hydrochloric acid containing 1 mg/ml of calcium.
Standard magnesium solution	$MgCO_3$ in 0.01 *N* hydrochloric acid containing 1 mg/ml of magnesium.
Standard strontium solution	$SrCO_3$ in 0.01 *N* hydrochloric acid containing 1 mg/ml of strontium.

The column was loaded with 0.5, 1.0, and 2.0 ml aliquots of these solutions for calibration purposes.

The quantitative chromatogram

The resin was generated into the ammonium form by passage of 3 *M* ammonium chloride down the column followed by copious washing with water. The alkaline-earth metal bearing solutions were loaded into the column by washing on the requisite volumes of solution with distilled water. Elution was then carried out with 1 *M* ammonium lactate solution until the magnesium had been eluted, when after which gradient elution was started by dropping 5 *M* ammonium lactate solution into 500 ml of the 1 *M* lactate solution. Gradient elution was continued in the conventional manner until both the calcium and strontium were removed from the column. The column was then washed with water, and the barium eluted with 0.1 *M* disodium ethylenediaminetetraacetic acid (ENTA). Regeneration after elution, with ENTA was necessary, since it was found that the resin was converted to the sodium form, which interfered with both the sequence of the next elution, and the flame spectrophotometric estimation.

Automatic detection of the alkaline-earth metals

The column effluent was dropped into 25 ml of water kept at constant volume, which was stirred continuously. This solution was aspirated directly into the flame of the instrument, which was set at the correct wavelength to detect the alkaline-earth metal being eluted. Since the aspiration rate into the flame is greater than the flow-rate of the column, water was added from the constant volume delivery burette to keep the 25 ml of water at constant volume. In this way, the concentration of alkaline-earth metal present in the solution is directly proportional to the concentration of metal in the column effluent. By setting the flame spectrophotometer at a constant wavelength to detect the alkaline earth metal being eluted, and coupling the output of the flame spectrophotometer to a potentiometric recorder, the pattern of the eluted metal will be drawn. Under standard conditions, the area under the elution curve is proportional to the total alkaline-earth metal eluted from the column.

For the most consistent results, and the smoothest elution curves, care must be taken to observe the following points:

(a) efficient stirring of the solution aspirated into the flame;

(b) the outlets of both the ion-exchange column and the constant volume delivery burette must be just below the surface of the constant volume liquid, so that the solutions enter in a continuous stream instead of dropwise;

(c) the potentiometric recorder slightly under-damped;

(d) the dropping rate of the ion-exchange column must be constant from one run to another;

(e) the optimum wavelength for detection of each alkaline-earth metal must be found and used for maximum sensitivity and linearity of the calibration graph.

Calibration curves using 0.5 mg, 1.0 mg, 2.0 mg samples of the alkaline-earth metals were found for each element, and graphs of area *vs.* concentration were drawn.

Unknown solutions prepared from mineral samples were then eluted down the column, and the alkaline-earth content determined from the prepared calibration graphs.

Flame spectrophotometry

The SP 900 was operated under the following conditions: British Oxygen Co. cylinder

gases with 3 in. pressure of commercial acetylene, using a manometer containing dibutyl phthalate, and 30 p.s.i. of compressed air.

Unfortunately since no standards can be eluted immediately prior to determinations, the gain settings are fixed and the acetylene pressure is varied slightly from day to day to give a set deflection with a standard solution. This gives a day to day check on sensitivity and improves reproducibility of results over a period of time.

The potentiometric recorder was a Sunvic RSP. 2, 0–10 mV F.S.D., and 3 in./h chart speed.

The most critical factor affecting the estimations was the wavelength settings used for each alkaline-earth metal. Many runs were carried out using 2.0 mg of each metal to find the relationship between the peak area of the elution pattern and the wavelength of detection. These results are shown in Fig. 2; each run in this series was carried out under identical conditions. Optimum conditions are given in Table I for

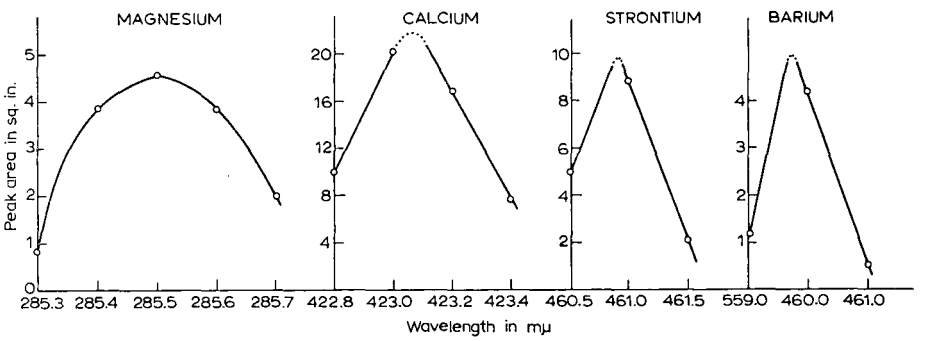

Fig. 2. Variation of peak area and wavelength for alkaline-earth metals using 2 mg samples.

each element. These settings are not absolute optimum and no doubt can be improved upon, and the used wavelength represents the optimum wavelength at which accurate location was possible.

The gain settings were kept constant, since day to day variation of the instrument was offset by variation in the acetylene pressure.

The shape of the elution patterns is given in Fig. 3. It appears from this, that the magnesium has a flattened peak, probably due to variation of maximum emission wavelength with concentration of the solution[4]. Little tailing is apparent, with barium a side peak or ledge was always noticed, and since the curve does not return to the baseline zero position, it appears that this is due to the enhancement caused by the ENTA background. In this case the peak area was taken from the base of the side peak to the tail of the peak. All areas were measured using a planimeter.

TABLE I

SPECIAL CONDITIONS USED TO RECORD THE FLAME SPECTRA

Conditions	*Magnesium*	*Calcium*	*Strontium*	*Barium*
Slit width (mm)	0.08	0.04	0.04	0.05
Wavelength (mμ)	285.5	423.0	461.0	560.0
Gain	4.0	2.2	2.2	4.5

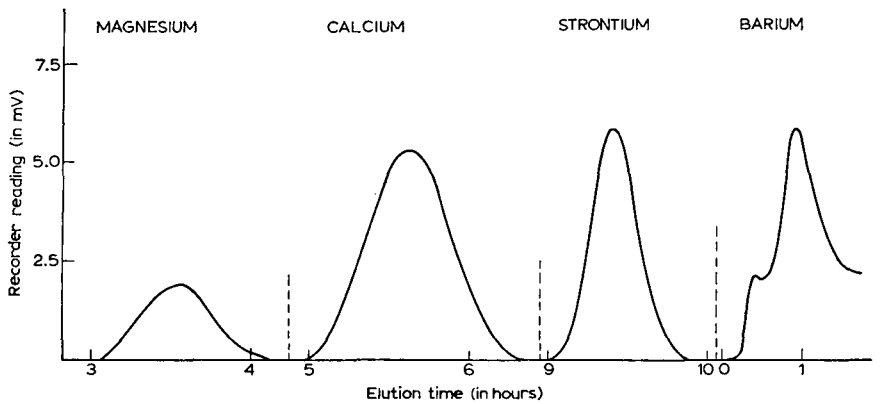

Fig. 3. Elution curves for alkaline-earth metals. Magnesium, calcium and strontium are eluted with ammonium lactate solution, while barium is eluted with ENTA.

RESULTS AND DISCUSSION

Some results obtained for calibration are given in Table II.

These results showed that the relationship between area of elution curve and quantity of alkaline-earth metal eluted from column was linear for calcium strontium and barium, but not so for magnesium as shown in Fig. 4. The result of non-linear calibration for magnesium may be due to not setting on an optimal wavelength,

TABLE II

CALIBRATION RESULTS FOR THE ALKALINE-EARTH METALS

	Peak areas (sq. in.)											
	Magnesium			*Calcium*			*Strontium*			*Barium*		
Amount (mg)	*0.5*	*1.0*	*2.0*	*0.5*	*1.0*	*2.0*	*0.5*	*1.0*	*2.0*	*0.5*	*1.0*	*2.0*
	1.18	2.60	3.10	1.09	2.16	4.54	2.1	4.47	8.62	—	2.24	4.50
	1.12	2.40	3.00	1.16	2.15	4.70	—	4.36	9.00	—	2.21	4.25
	1.16	2.40	3.20	1.06	2.13	4.70	2.1	4.48	8.90	—	2.10	4.20
	1.23	2.53	3.25	1.07	1.92	4.52	—	4.40	8.70	—	2.25	4.25
Mean	1.15	2.48	3.14	1.10	2.09	4.44	2.1	4.43	8.81	—	2.20	4.30

but it is most likely due to the changing wavelength of maximum intensity. If the latter is the explanation, then the magnesium calibration will never be linear using such an automatic process.

The process was devised for use as a routine form of analysis which once the calibration curves are established, they can be used at any time; provided that the conditions used are standardised and are repeatable. The method should be applicable to all types of flame spectrophotometers since the constant volume of water used may be varied to enable the concentrations of the alkaline-earth metals aspirated into the flame to be altered to within the range of the instrument. Concentrations of alkaline-earth metals less than 1 mg are used; the method can be accelerated by using a shorter

column length, and a faster dropping rate, which enables all four metals to be determined in under eight hours. However, if the column length is decreased below about 55 cm, there is incomplete separation of the magnesium and calcium.

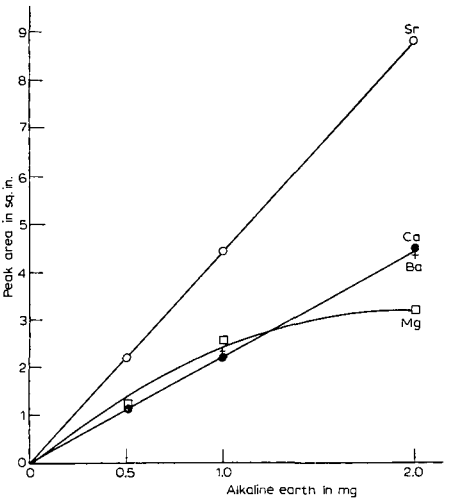

Fig. 4. Calibration curves for the alkaline-earth metals. □ magnesium; ● calcium; O strontium; + barium.

An indication of the applicability of this method may be judged from the analysis of some mineral samples. The results are given in Table III.

TABLE III

ANALYSIS OF MINERAL SAMPLES

		Found %	*Found previously by chromatography*[1] %	*Found by classical analysis* %
Dolomite	magnesium	5.8	5.90	5.78
	calcium	30.3	31.6	31.1
Celestine	strontium	47.3	47.5	45.6
Barytes	calcium	16.7	16.6	16.8
	barium	34.7	32.2	35.4

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SUMMARY

A method of separation of microgram and milligram quantities of alkaline-earth metals using cation-exchange chromatography has been developed, using a flame spectrophotometer as a continuous automatic detector. The accuracy is within 5 %.

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Notes

Paper chromatography using liquid ion exchangers

The use of paper impregnated with liquid ion exchangers as a vehicle for the separation of various species of compounds has been described[1,2], and the separation of amino acids *via* paper chromatography using an ascending technique in a variety of solvent systems is a widely used technique[3]. In the present study, a combination of these procedures, *i.e.* using a liquid ion exchanger[4] as the ascending solvent on filter paper, resulted in an effective separation of several amino acids. In addition, unlike the problems observed with several of the commercially available ion exchange-impregnated papers, using a dilute solution of the ion exchanger in an organic phase as the ascending solvent permitted a direct application of ninhydrin reagent to develop the color of the amino acid spots without appreciable paper discoloration.

Experimental

Preparation of solvent and paper. For those data reported in Table I, the ascending solvent was prepared by mixing one volume of a 5 % solution of Amberlite LA-2* in

TABLE I

R_F VALUES OF SOME AMINO ACIDS IN AN LA-2–*n*-BUTYL ALCOHOL SYSTEM

Solvent system: a sample of 5 ml of Amberlite LA-2 liquid ion exchanger was dissolved in 95 ml of *n*-butanol, and the resulting solution was saturated with 100 ml of 0.5 *M* phosphate buffer, pH 6. The organic phase was decanted and used as the ascending solvent. The chromatograms were developed with 0.2 % ninhydrin reagent dissolved in acetone, and allowed to dry at room temperature.

Amino acid	R_F	*Amino acid*	R_F
Alanine	0.13	Isoleucine	0.46
Arginine	0.01	Leucine	0.50
Asparagine	0.04	Lysine	0.01
Aspartic acid	0.42	Methionine	0.33
Cysteine	0.03	Phenylalanine	0.53
Cystine	0.03	Proline	0.17
Glutamic acid	0.51	Serine	0.09
Glutamine	0.05	Threonine	0.14
Histidine	0.06	Tryptophan	0.28
Glycine	0.08	Tyrosine	0.23
		Valine	0.31

n-butanol and one volume of 0.5 *M* phosphate buffer, pH 6. After a brief equilibration period, the phases were separated and the organic layer freed of water droplets by filtration through paper. Two microliters of 0.01 *M* solutions of the various DL-amino

* N-Lauryl-N-trialkylmethylamines.

TABLE II

EFFECT OF pH ON THE R_F VALUES OF SOME AMINO ACIDS

Solvent mixture: 50 ml of appropriate 0.5 *M* phosphate buffer, 47.5 ml of *n*-butanol, and 2.5 ml Amberlite LA-2 liquid ion exchanger.

pH of buffer	*R_F of amino acid*			
	Glutamic acid	*Glutamine*	*Aspartic acid*	*Asparagine*
3	0.48	0.06	0.49	0.04
4	0.54	0.08	0.47	0.06
5	0.55	0.07	0.49	0.05
7	0.39	0.04	0.27	0.03
8	0.12	0.04	0.09	0.03
9	0.03	0.03	0.03	0.03

acids in water were applied to the origin (1 in. from the bottom edge) of rectangles of Whatman No. 1 filter paper (the particular size of the paper was determined by the number of samples to be assayed concurrently, and the desired distance of travel of the solvent front). The paper was made into a cylinder by means of stapling the long edge of the paper, and it was then placed in a jar containing a sample of the organic phase described above and allowed to develop at about 30° using the general technique of WILLIAMS AND KIRBY[3]. The amino acid spots were visualized on the dried chromatograms by dipping the paper into a 0.2 % solution of ninhydrin in acetone, and finally allowing the paper to dry at room temperature for several hours.

Study of operational variables. For purposes of determining the effect of pH and resin concentration on the R_F of the amino acids as determined in this ion exchange solvent system, glutamic acid, aspartic acid, and the corresponding amides were used as model compounds. Using the procedure described above for obtaining the organic phase to be used as the solvent, the effects upon the R_F in several solvent systems are presented in Tables II and III.

TABLE III

EFFECT OF ION-EXCHANGE RESIN CONCENTRATION ON THE R_F VALUES OF SOME AMINO ACIDS

Solvent mixture: 50 ml of 0.5 *M* phosphate buffer (pH 6), and 50 ml of the appropriate concentration of Amberlite LA-2 liquid ion exchanger in *n*-butanol

Concentration of resin in butanol, %	*R_F of amino acid*			
	Glutamic acid	*Glutamine*	*Aspartic acid*	*Asparagine*
0	0	0.04	0.01	0.03
2	0.40	0.05	0.34	0.05
4	0.54	0.06	0.45	0.06
6	0.62	0.08	0.45	0.06
10	0.56	0.09	0.51	0.06
20	0.53	0.09	0.44	0.07

Results and discussion

These studies were initially undertaken in an effort to find an efficient and reproducible paper chromatographic solvent system which could be used to distinguish between

the α-amino-dicarboxylic acids and their corresponding amides. In preliminary experiments, the distribution coefficients of aqueous solutions of glutamic acid were determined in the presence of a number of organic solutions of the liquid anion exchanger Amberlite LA-2. The solvents used included aliphatic, chlorinated-aliphatic and aromatic hydrocarbons, and aliphatic alcohols. The larger distribution coefficients observed were with aliphatic alcohols in the C_4–C_6 range, and *n*-butanol was accepted as the solvent of choice. Subsequently, a number of studies concerning the effect of resin concentration, pH, buffer concentration, height of solvent front, and temperature of the system on the R_F values of the amino acids were carried out. In general, the apparent optimum conditions lay over a relatively broad range of values; thus, minor errors in preparing the solvent system should not greatly affect the values observed. However, as is true in most paper chromatographic systems, standard samples should always be assayed concurrently with the unknown material to eliminate minor variations in R_F values.

From the data presented in the experimental section, a single set of conditions was chosen as being representative of this system, and the R_F values of 21 amino acids were then determined for comparative purposes. These R_F values were not appreciably affected by a change in temperature of operation between 7° and 35°, nor by increasing the height of travel of the solvent front from 15 to 30 cm. This type of solvent system appears to possess certain advantages over that of the typical organic–aqueous procedures[3] in that certain biologically comparable derivatives, such as the α-amino-dicarboxylic acids and their corresponding amides, are significantly separated from one another. Of further interest is that this solvent system also separates various dipeptides in an efficient manner, and further studies with these types of compounds are presently in progress.

Acknowledgements

The authors are grateful to Professor WILLIAM SHIVE for his interest throughout the course of this study. They are deeply indebted to JOAN BECKER, TURNER BRITTON and JOSEPH COTROPIA for carrying out the many replicate experiments in order to establish the data presented, and to The Rohm and Haas Company, Philadelphia, Pennsylvania for the generous gift of Amberlite LA-2 used in these studies.

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Multiple column chromatography of wheat gluten proteins

In studies on the chromatography of wheat gluten proteins it became necessary to perform chromatographic analyses on a relatively large number of protein samples. This paper describes apparatus that has been assembled for the simultaneous chromatography of up to six protein samples on columns of carboxymethyl-cellulose using salt gradient and step-wise salt and pH elution according to the procedure described by SIMMONDS AND WINZOR[1].

Columns (1.5 × 25 cm) were packed with carboxymethyl-cellulose (Whatman powder CM 70) and equilibrated with 0.005 *M* sodium acetate adjusted to pH 4.1 with acetic acid. The protein samples were applied to the columns in 0.005 *M* acetate buffer, pH 4.1, and prior to protein elution, the columns were equilibrated with 0.005 *M* sodium acetate containing 1 *M* dimethyl formamide (DMF) (also adjusted to pH 4.1 with acetic acid). The elution system is shown in Fig. 1. The gradient apparatus

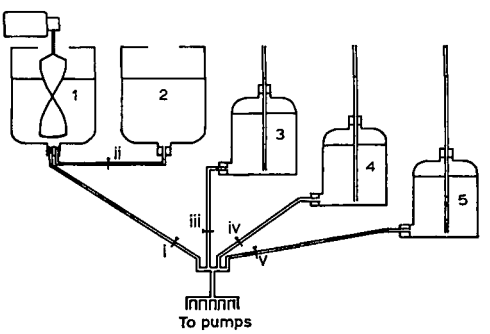

Fig. 1. Solvent-dispensing system for gradient and step-wise elution chromatography. i, ii, iii, iv and v are clamps on the delivery tubes of Solvents 1, 2, 3, 4 and 5 respectively.

consisted of two similar polyethylene bottles each with a hole drilled in the bottom. The two bottles, used in the inverted position, were joined by tubing passing through rubber bungs inserted in the necks of the two bottles. As solvent drained from the left hand bottle (originally containing Solvent 1, 0.005 *M* acetate–1 *M* DMF), Solvent 2 (0.2 *M* NaCl–0.005 *M* acetate–1 *M* DMF) entered from the right hand vessel and was mixed with the bulk of the solution by means of a perspex blade (5 × 15 cm twisted through 90°). A rotation speed of 80 rev./min ensured complete mixing without the formation of a vortex.

Following gradient elution, successive step-wise elutions with Solvents 3, 4 and 5 (0.5 *M* NaCl–acetate–DMF, 0.005 *M* phosphate–0.5 *M* NaCl–1 *M* DMF and 0.1 *N* NaOH, respectively) were produced by three bottles set up as shown in Fig. 1. The bottles were placed so that the lower opening of each Mariotte tube, passing through the stopper of the bottle, was below the opening of the delivery tube of the previous bottle. (Although the delivery tubes are shown entering by an opening at the bottom of each bottle, entry via the neck of the bottle is equally satisfactory.) Fine tubing is recommended for Mariotte and delivery tubes, but for the filling procedure to be effective the volume of the delivery tube must exceed the volume of the Mariotte tube.

With all clamps closed the bottles were filled with the required volumes of the respective solvents and the stoppers carrying the Mariotte tubes were inserted. After filling the delivery tubes with the respective solvents, the clamps were opened in the order v, iv, iii, i, allowing time for the solvent heights to come to equilibrium. As the system was drained, virtually all the solvent came initially from the two gradient bottles. When the liquid level dropped below the lower opening of the Mariotte tube of bottle 3, virtually pure Solvent 3 drained from the system, and so on through the succession of solvents. As the liquid levels fell in the Mariotte tubes, a small amount of solvent drained from each of the delivery tubes. By filling the tubes in the prescribed manner, this latter solvent had the same composition as the main solvent being drawn off at any one time. Contamination of later solvents by earlier solvents draining from the delivery tubes cannot be avoided, but can be minimised by using small diameter tubing. Large fluctuations in temperature should be avoided in the Mariotte bottle system as the resulting change in volume of the airspace displaces solvent from the bottle.

The above system is also useful for the automatic regeneration of the columns with step-wise solvent changes in the order, distilled water, 0.05 *M* acetate buffer, 0.005 *M* acetate buffer. The solvent dispensing apparatus is similar to that described by ANDERSON *et al.*[2]. However, the use of Mariotte tubes in standard reagent bottles saves costly construction of the special containers recommended by these authors, allows the bottles to be used only partly filled and also permits closer spacing of the bottles with respect to height.

Of a number of methods tested for the collection of fractions, the only method found to be satisfactory was the combination of a metering pump with a timer-operated fraction collector (Fig. 2). A four-channel peristaltic pump, supplied by Sigmamotor Inc., Middleport, New York (Model T8), was coupled to a variable speed changer (Zero-max Co., Minneapolis, Model 142 X). The pumping unit was modified so as to take an additional two channels, and a screw adjustment was fitted to the speed change lever to facilitate regulation of flow rate. The modified unit was capable of delivering up to 15 ml/min to each of six channels, with accuracy at 1 ml/min of $\pm$ 0.01 ml/min, using Tygon tubing of 1/16 in. internal diameter. For fraction collection, two LKB RadiRac Automatic Fraction Collectors (LKB-Produkter AB, Stockholm) were used in conjunction with a pair of times (E. Dold u. Söhne, Furtwangen, Baden, Germany, Models ZR1U 712 f, 40 sec and ZR1U 701 f, 60 min). The advantage of the common solvent system for multiple column chromatography has been pointed out previously by MARR AND GILBO[3], but the fraction collection system described above avoids the tedious measuring of individual fractions performed by these workers.

The efficiency of the solvent-dispensing system was tested by substituting increasing concentrations of the dye auramine for Solvents 1–5. The system was drained at the rate of 1 ml/min by each of the six channels and 10-min fractions were collected from one of the solvent streams. The absorbancy at 430 mμ of the fractions (Fig. 3) indicated that a linear gradient was produced, that the change-overs between step-wise elutions were sharp and that the emergent concentrations of each of Solvents 3, 4, and 5 were unchanged from each of the original concentrations.

The chromatographic profiles obtained by the simultaneous chromatography of the gluten proteins of six wheat varieties are shown in Fig. 4. The gluten proteins were extracted from the crushed wheat samples using 0.05 *M* acetic acid, after preliminary

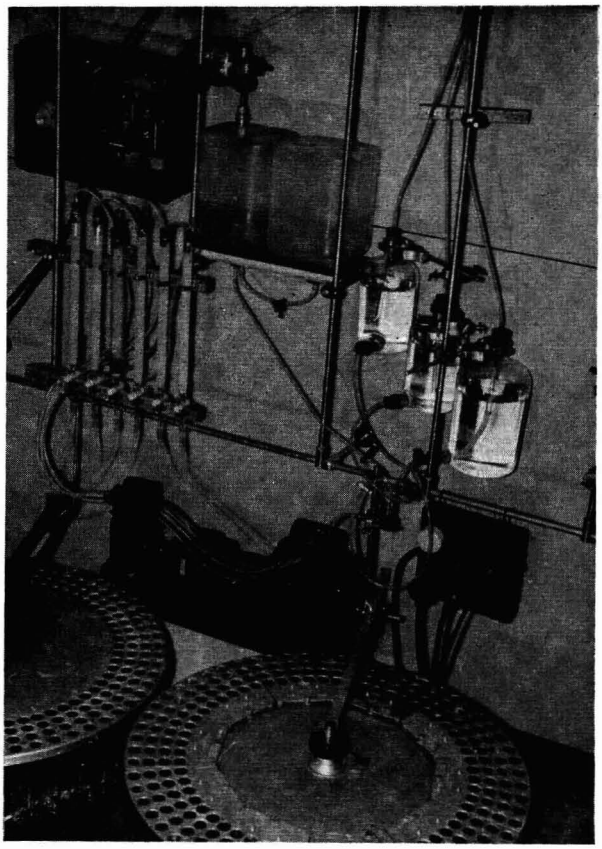

Fig. 2. Apparatus for the simultaneous chromatography of six protein samples.

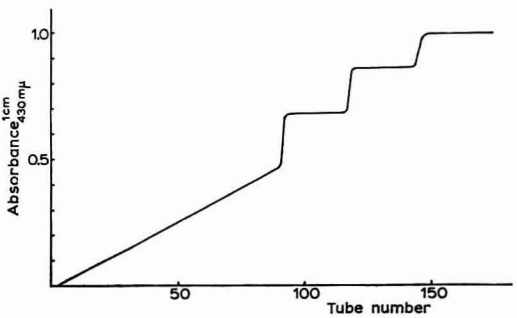

Fig. 3. Absorbance at 430 mμ of fractions delivered from the solvent-dispensing system substituting increasing concentrations of the dye auramine for Solvents 1–5. The original absorbance values of Solvents 1–5 were 0.00, 0.48, 0.68, 0.86 and 1.02 respectively.

extraction with 0.01 *M* sodium pyrophosphate, pH 7.0, as previously described[4]. Included in the six extracts chromatographed is an extract of the wheat variety Gabo, which is routinely used as a standard. The appearance of the profile that is characteristic of this variety provides a check on the correct functioning of the procedure. The Gabo profile further serves as a standard for estimating the distribution of protein between the chromatographic peaks. The eluted fractions corresponding to each peak were pooled in the standard manner for the Gabo extract and the fractions obtained

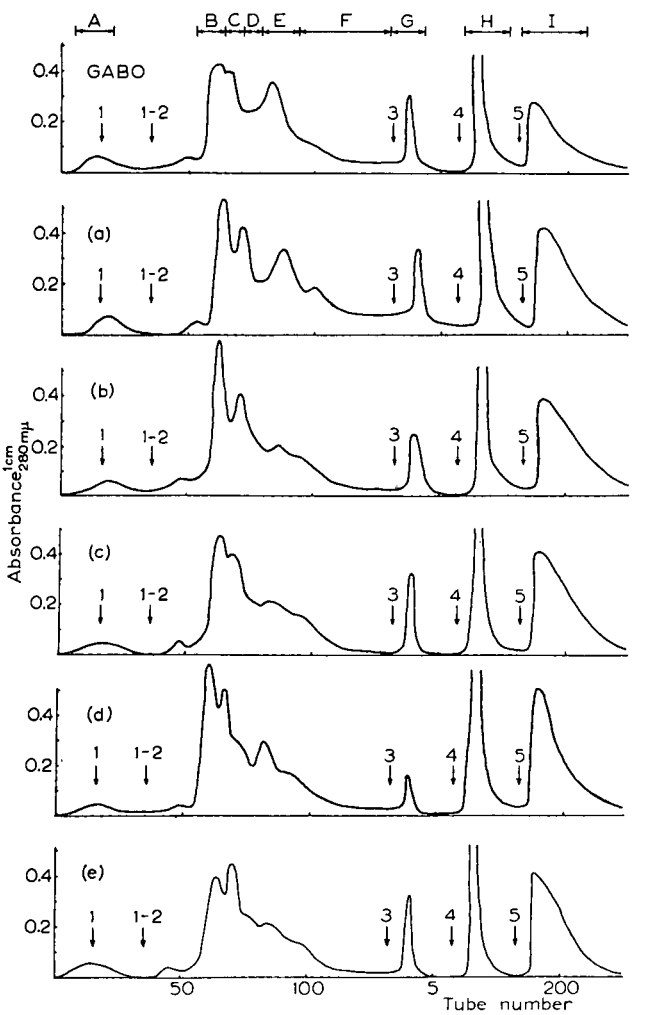

Fig. 4. Chromatographic profiles resulting from the simultaneous chromatography of the gluten extracts of six wheat varieties using the apparatus described in this paper. Column loadings (mg N) were as follows: Gabo, 65.0; (a) 61.4; (b) 58.3; (c) 52.4; (d) 67.8; (e) 55.3. The manner of pooling the tubes that correspond to each of the peaks A to I is indicated at the top of the figure. Changes of solvents are indicated as follows: (1) 0.005 *M* acetate containing 1 *M* DMF (pH 4.1); (1)–(2) gradient from 0 to 0.2 *M* NaCl in acetate–DMF; (3) 0.5 *M* NaCl in acetate–DMF; (4) 0.005 *M* trisodium phosphate–1 *M* DMF–0.5 *M* NaCl, pH 12; (5) 0.1 *N* NaOH. Flow rate, 1 ml/min for each column. Fraction size, 10 ml.

from the other five extracts were pooled in an identical manner preparatory to colorimetric protein determination[5]. Pooling tubes according to a standard profile thus allows comparison of protein distribution between corresponding peaks from one set of chromatographic analyses to another.

The apparatus described provides a system whereby up to six protein samples can be eluted simultaneously from chromatographic columns under identical conditions by the automatic introduction of a succession of solvents. The routine inclusion of a standard protein sample provides a check on the reproducibility of the analysis, thus allowing comparison between sets of chromatographic analyses.

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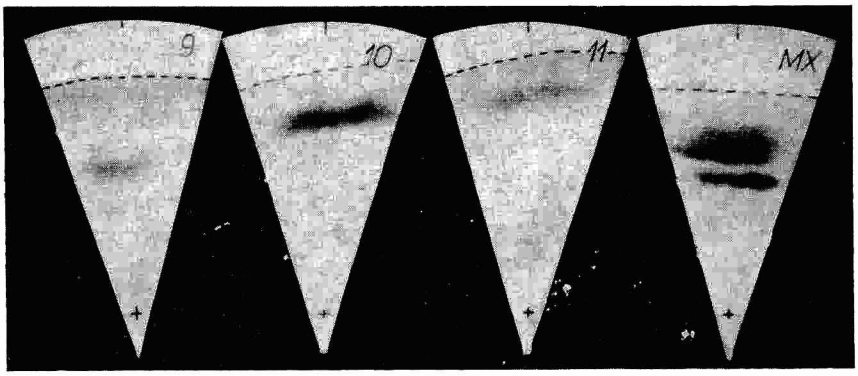
Some remarks on the paper "Centrifugally Accelerated Chromatography of Steroids"

In a recent paper by MATTHEWS AND CERVANTES[1] on centrifugal chromatographic separation of steroids, velocities of 200–250 r.p.m. were used and it was found that the steroids did not move from the line of application. These authors thought that "this is probably because at high velocities the solvent travels too fast for partitioning to occur". However, the present authors consider that the unsatisfactory results of separation were due to an unsuitable arrangement of the centrifugal chromatography, for the following reasons.

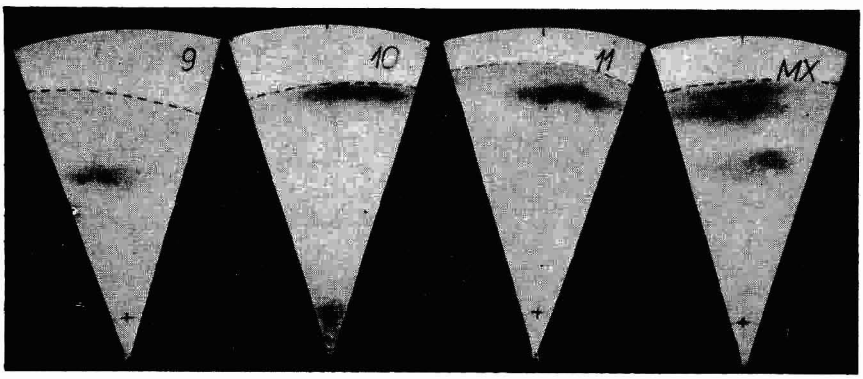
(1) The velocity of 200–250 r.p.m. is not sufficiently high to cause a substantial acceleration of the mobile phase flow. According to our own experience the run will take about 30 min instead of 40, which is the necessary time for developing a standard circular chromatogram.

(2) The velocity necessary to establish the partition equilibrium is much higher than the velocity of the solvent flow. Even a velocity of the mobile phase which amounts to *ca.* 1,700 r.p.m. will not interfere with the partition.

No reason can be seen why these compounds (hydrocortisone, cortisone and 11-desoxy-17-hydroxycorticosterone) should not be separated by centrifugal chromatography under high velocities and actual separations are shown in Fig. 1. These were performed at 300, 600, 900 and 1,200 r.p.m. Paper preparation and sample application were the same as described by MATTHEWS AND CERVANTES, but Whatman paper No. 3



(a)



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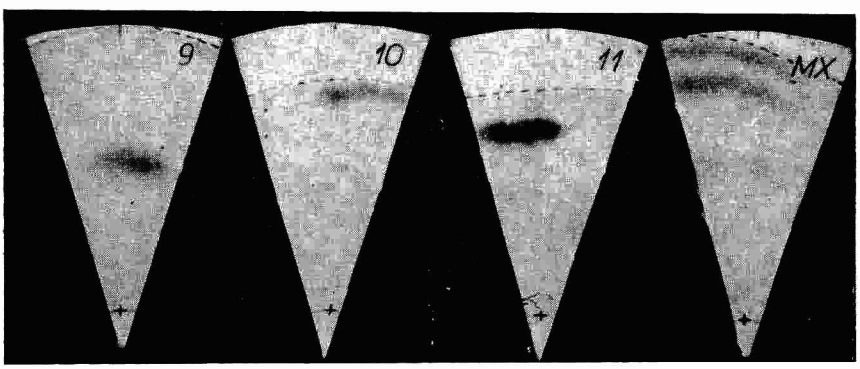

(c)

Fig. 1. Chromatographic separation of hydrocortisone, cortisone and 11-desoxy-17-hydroxycorticosterone in a centrifugal field. (a) 600 r.p.m.; (b) 900 r.p.m.; (c) 1,200 r.p.m. Samples: 9 = hydrocortisone; 10 = cortisone; 11 = 11-desoxy-17-hydroxycorticosterone.

was used in these experiments. Detection was performed by applying triphenyltetrazolium chloride. For the chromatographic separation the apparatus according to PAVLÍČEK *et al.*[2] was used.

In centrifugal chromatography it is necessary to apply an accurate quantity of the mobile phase to the paper, otherwise the mobile phase is not able to saturate the chromatographic paper and it forms a thin layer of solvent that flows over the surface of the paper (see Figs. 2a, b). In this case the separated compounds either form diffuse zones or they remain at the origin. These effects are very common if papers with low

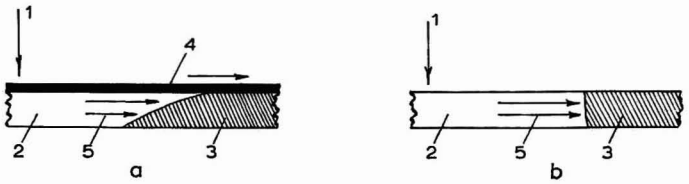

Fig. 2. Schematic representation of mobile phase flow during chromatography in a centrifugal field. (a) Chromatographic separation with an excess of the mobile phase. (b) Chromatographic separation with an adequate quantity of the mobile phase. Symbols: 1 = mobile phase inlet; 2 = chromatographic paper saturated with the mobile phase; 3 = chromatographic paper saturated with the stationary phase; 4 = excess of the mobile phase forming a thin layer of the mobile phase on the surface of the chromatographic paper; 5 = direction of the centrifugal force.

capacity like Whatman No. 1 or 4 are used and because it is difficult to achieve a perfect flow rate for the mobile phase; this would account for the low mobility of the steroids obtained by MATTHEWS AND CERVANTES.

Summarizing, it can be said that rapid and perfect separations of cortisone, hydrocortisone and 11-desoxy-17-hydroxycorticosterone by means of centrifugal chromatography are obtained under the following conditions: (a) Complete saturation of the chromatographic chamber by the vapour of the mobile phase, (b) accurate control of the solvent flow rate (mobile phase inlet), and (c) use of a chromatographic paper with a large capacity such as Whatman No. 3 or Ederol 225. In the case of Whatman No. 3 paper the mobile phase inlet is about 0.8 ml/min. It differs a little according to the construction of the apparatus for centrifugal chromatography. The separation is more rapid at higher velocities and even a velocity of 1,700 r.p.m. does not interfere with the partition process.

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Permanent record and scanning of disc electrophoresis patterns*

With the advent of disc electrophoresis comes the need for a simple and inexpensive scheme for converting the patterns in acrylamide gels to a permanent record. We have developed a technic which provides such a record together with a means of scanning with any densitometer.

The gels from disc electrophoresis, although containing significantly more bands than either paper or cellulose acetate, give rise to certain problems. If they are to be stored, the ever-increasing number of individually stoppered and labeled tubes soon clutters laboratory shelves. If they are to be scanned, an investment must be made in a special densitometer, the cost of which may be prohibitive in many laboratories.

Method

The key to our scheme is a special photographic technic: a Polaroid camera fitted with a +6 close-up lens is loaded with type 46-L projection film. The stained gels are placed in glass tubes which have been carefully bent in such a manner that the center third forms an arc with a radius of curvature of $6^1/_4$ in. Four such tubes are positioned with their centers $6^1/_4$ in. below the front of the nearest accessory lens with the camera distance scale set at $3^1/_2$ ft.

A white background about 6 in. below the tubes is illuminated by two photoflood lamps. Using new No. 2 lamps, 12 in. from the center of the background at an angle of 30° from the horizontal, a shutter setting of 15 provides correct exposure with uniform background**. The physical relationship of all components is shown in Fig. 1.

Each transparency provides a record of four gels and can be cut into four strips. These, in turn, can be scanned with any standard densitometer. In Fig. 2 a transparency of four human serum protein patterns is shown. Fig. 3 is a graph obtained with our method by scanning the bottom pattern of Fig. 2.

Results

We have proven to our satisfaction that graphs obtained by our method are comparable with those obtained by the commercial apparatus specifically designed for scanning electrophoresis patterns in gel cylinders. In Fig. 4 scans*** obtained with the commercial device and by our method have been superimposed. There is satisfactory agreement between these two rabbit serum protein patterns.

Our method can be used for gels stained with different dyes used to demonstrate protein, lipoprotein, or glycoprotein fractions, for serum, spinal fluid, nutrient media or other material. In the case of protein, if one stains with bromphenol blue, a No. 546 orange filter may be attached to the camera to increase contrast.

Discussion

Consider the photographic set-up depicted in Fig. 5, in which a gel cylinder 5 mm in

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* Supported by grant HE-02534-07SI of the National Heart Institute, N.I.H., U.S.P.H.S.

** These figures are given only as a point of departure for those wishing to duplicate our set-up; even illumination as well as careful alignment, focusing, and exposure are essential.

*** Provided by Canalco, 4935 Cordell Avenue, Bethesda 14, Maryland, U.S.A.

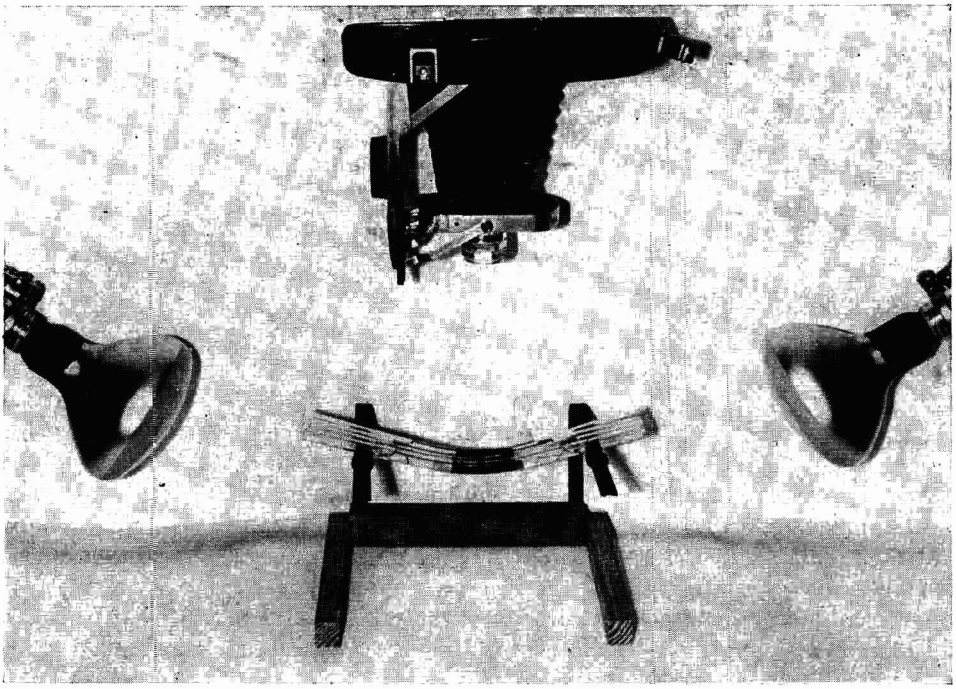

Fig. 1. Photographic set-up for preparing transparencies.

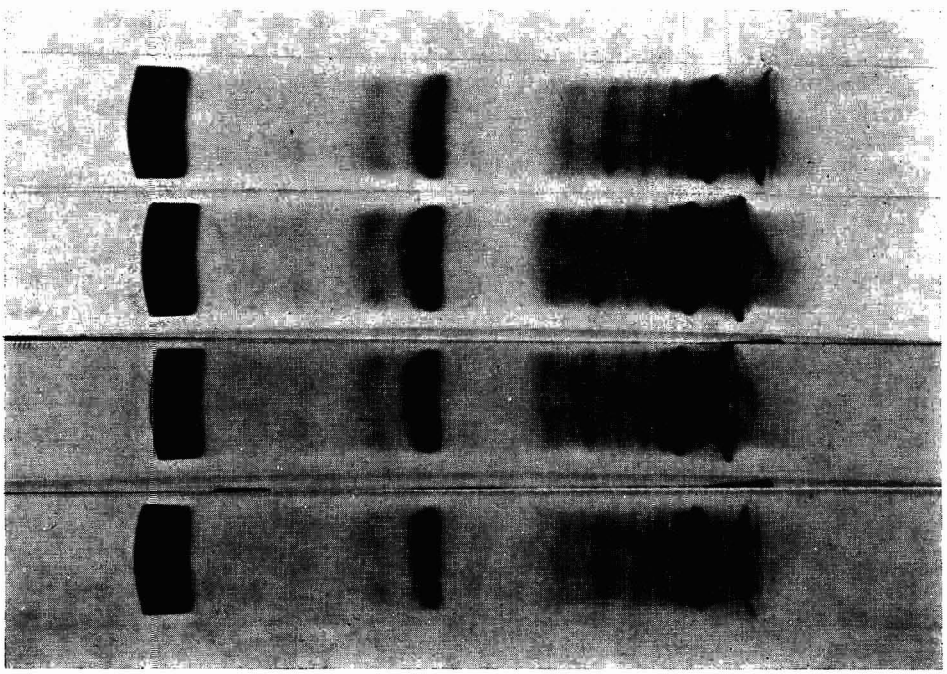

Fig. 2. Transparency with four patterns.

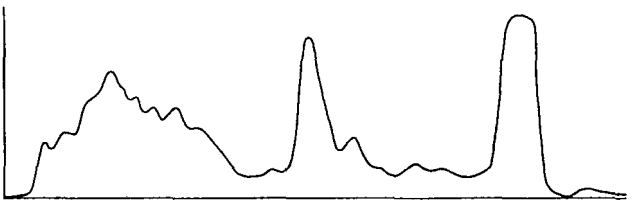

Fig. 3. Densitometric trace of human serum protein pattern.

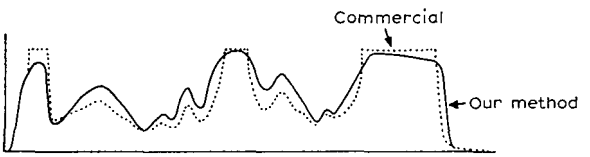

Fig. 4. Comparison of densitometric tracings of rabbit serum protein patterns.

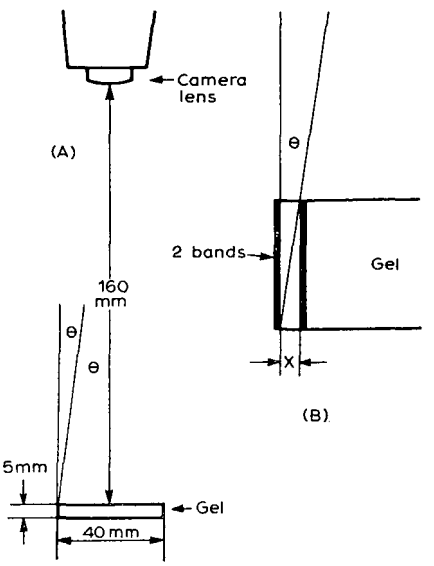

Fig. 5. (a) Diagrammatic representation of photographic set-up employing straight gel-containing tube. (b) Enlargement of left end of gel. See "Discussion" for explanation.

diameter and 40 mm long is 160 mm from the camera lens. Examination reveals the following trigonometric relationships:

$$\tan\theta = \frac{20}{160}, \tan\theta = \frac{x}{5}, \text{ and } x = \frac{5}{8}$$

When the separated discs are in parallel planes, as when the gel-containing tubes are straight, those near the end of a 5 × 40 mm gel separated by less than 5/8 mm will not be resolved. Since it is not at all uncommon for bands (discs) to be closer together than this, resolution would be impaired and some components might go undetected.

For complete resolution the smallest allowed separation of two bands, x, is given by the formula:

$$x = \frac{LW}{2d}$$

L = length of gel, W = width of gel, and d = lens-to-gel distance. Consequently, one should decrease the gel length, decrease the gel width, or increase the lens-to-gel distance; none of these concessions is desirable.

Alternately, a scanning device wherein both gel and film are in motion could provide for the camera "seeing" each disc "edge on". Such a complicated mechanism is not only expensive but unnecessary if the gel-containing tubes are bent into an arc, the radius of curvature of which equals the lens-to-gel distance.

The characteristics of photographic emulsions are not unchanging. We chose Polaroid film type 46-L rather than the higher contrast type 146-L to minimize this effect. Contrast may then be increased by the use of appropriate filters. Also, the background is no less uniform than that of filter paper or cellulose acetate on which analysis by scanning is an accepted procedure.

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A systematic, simultaneous analysis of steroid sapogenins by thin-layer chromatography

As a preliminary to the study on the constituents of plants, 20 kinds of pure steroid sapogenins were simultaneously submitted to thin-layer chromatography, and the relationship between structure and adsorptivity of the compounds was examined.

Liquid column chromatography[1], paper chromatography[2,3] and gas chromato-

graphy[4] have been utilized for the separation of steroid sapogenins. A few papers on thin-layer chromatography of steroid sapogenins have also been published[5–7], but systematic and simultaneous analysis has not yet been reported.

TABLE I

LIST OF COMPOUNDS STUDIED

No.	*Compound*	*Configuration at C-25*	*Configuration of rings A/B and position of double bonds*	*Position of*	
				OH group(s)	*CO group*
1	Luvigenin	D	$\Delta^{1,3,5(10)}$, 4-Me	—	—
2	Neometeogenin	L	$\Delta^{1,3,5(10)}$, 1-Me	11α	—
3	Meteogenin	D	$\Delta^{1,3,5(10)}$, 1-Me	11α	—
4	Sarsasapogenin	L	*cis*	3β	—
5	Diosgenin	D	Δ^{5}	3β	—
6	Tigogenin	D	*trans*	3β	—
7	Pennogenin	D	Δ^{5}	3β,(17α?)	—
8	Gentrogenin	D	Δ^{5}	3β	12
9	Hecogenin	D	*trans*	3β	12
10	Convallamarogenin	—	*cis*	1β,3β	—
11	Isorhodeasapogenin	D	*cis*	1β,3β	—
12	Rhodeasapogenin	L	*cis*	1β,3β	—
13	Nogiragenin	D	*cis*	3β,11α	—
14	Heloniogenin	D	Δ^{5}	3β,12α	—
15	Yonogenin	D	*cis*	2β,3α	—
16	Gitogenin	D	*trans*	2α,3β	—
17	Tokorogenin	D	*cis*	1β,2β,3α	—
18	Metagenin	D	*cis*	2β,3β,11α	—
19	Kitigenin	D	*cis*	1β,3β,4β,5β	—
20	Kogagenin	D	*cis*	1β,2β,3α,5β	—

In the present study, a mixture of 20 kinds of steroid sapogenins listed in Table I were chromatographed on thin layers with 25 different solvent systems consisting of commonly available solvents. The R_F values of steroid sapogenins in some solvent systems which give good separations, are shown in Table II. Systematic and simultaneous analysis of these compounds was successfully carried out with a few exceptions.

The following findings were obtained in this series of experiments: (i) adsorptivity is enhanced as the number of hydroxyl groups is increased, *e.g.* No. 4, 10, 17 and 20; (ii) R_F values of Δ^5-ene and 5α derivatives are similar, *e.g.* No. 5 and 6, and 8 and 9; (iii) the separation of two sapogenins differing in configuration at C_{25} (neo- and iso-derivatives) is difficult in a mixture, *e.g.* No. 2 and 3, and 11 and 12; (iv) the sequence of R_F values varies depending upon the solvent system, *e.g.* No. 8, 9, and 10.

For developing the color of the compounds on the chromatogram, antimony trichloride[8], as widely used in paper chromatography, conc. sulfuric acid, acetic anhydride–conc. sulfuric acid and chlorosulfonic acid–acetic acid (1:2)[9] were used. Each sapogenin exhibited a characteristic color in visible and ultraviolet light after spraying with one of the above mentioned reagents and heating at 80–90° for 10–15 min. A reliable qualitative analysis of steroid sapogenins is thus possible by this sensitive color reaction. The procedure can be made quantitative for the compounds, if combined with densitometry or photometry.

TABLE II

R_F VALUES OF STEROID SAPOGENINS BY THIN-LAYER CHROMATOGRAPHY

The R_F value of each substance was determined by simultaneous analysis of a mixture. The adsorbent used was WAKOGEL (silica gel for thin-layer chromatography, Wako Pure Chemicals Co., Tokyo), coated by Stahl's method, dried at 130° for 60 min; thickness: 250 μ; activity: R_F value of Butter Yellow: 0.65; Indophenol: 0.11. Solvent: benzene. Developing distance: 15 cm. Quantity of each sample: 0.1 to a few γ (10^{-9}–10^{-8} mole).

No.	R_F in solvent system					
	Chloroform–EtOH (95:5)	*Chloroform–Me_2CO (9:1)*	*Benzene–Me_2CO (85:15)*	*Benzene–MeOH (92:8)*	*n-Hexane–EtOAc (1:1)*	*n-Hexane–Me_2CO (8:2)*
1	0.87	0.76	0.80	0.85	0.80	0.78
2	0.81	0.52	0.63	0.66	0.64	0.48
3	0.81	0.52	0.63	0.66	0.64	0.48
4	0.65	0.39	0.46	0.51	0.51	0.42
5	0.59	0.35	0.41	0.42	0.46	0.34
6	0.59	0.35	0.39	0.39	0.46	0.29
7	0.53	0.26	0.30	0.42	0.35	0.22
8	0.49	0.24	0.25	0.32	0.26	0.16
9	0.46	0.22	0.22	0.37	0.21	0.16
10	0.39	0.21	0.24	0.30	0.28	0.22
11	0.39	0.18	0.25	0.38	0.28	0.22
12	0.37	0.18	0.25	0.32	0.28	0.22
13	0.27	0.11	0.15	0.20	0.18	0.15
14	0.26	0.07	0.13	0.16	0.16	0.11
15	0.21	0.11	0.11	0.17	0.13	0.09
16	0.19	0.07	0.11	0.16	0.09	0.11
17	0.09	0.02	0.02	0.12	0.03	0.04
18	0.06	0.01	0.01	0.09	0.02	0.01
19	0.04	0.01	0.01	0.10	0.00	0.01
20	0.03	0.01	0.01	0.08	0.00	0.00

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A systematic analysis of bile acids and their derivatives by thin-layer chromatography

Although a few articles dealing with the separation of bile acids and their derivatives by means of thin-layer chromatography have been reported[1-3], no systematic and simultaneous analysis has been described as yet.

In order to separate natural bile extract components and to detect new components, about sixty of these acid derivatives were submitted to thin-layer chromatography and the relationship between structure and adsorptivity (R_F value) was examined. This method was found to be far better than some of the known methods[4-7]. With few exceptions, systematic analysis of these compounds was successful.

Various combinations of developing solvents were examined and the best separation was found to be effected with the benzene–ether system for methyl esters of bile acids, with the ether–acetic acid system for free acids, and with the chloroform–methanol system for bile alcohols.

The relationship between adsorptivity on silica gel and the polarity, position and configuration of functional groups is discussed below. The R_F values obtained are given in Table I.

TABLE I

R_F VALUES IN THIN-LAYER CHROMATOGRAPHY

R_F values were determined by the separation of each component from a mixture. Glass plates: 20 × 20 × 0.3 cm. Adsorbent: Wakogel (Silica gel for thin-layer chromatography, Wako Pure Chemicals Co. Ltd. Tokyo), coated by Stahl's method, dried at 130° for 60 min; thickness: 250 μ; activity: R_F value of Butter Yellow = 0.46, Indophenol = 0.15 (benzene). Developing distance: 15 cm; time: 45–90 min; temperature: about 20°. Quantity of each compound: a few μg (10^{-9} to 10^{-8} mole). Colouring agent: conc. H_2SO_4, chloroform solution of $SbCl_3$ and 60% $HClO_4$; heating (conc. H_2SO_4 gives the most sensitive colour). Compounds marked with an asterisk (*) are found in nature.

		Solvent system (v/v)		
		Benzene–Et_2O (8:2)	*Hexane–EtOAc (8:2)*	*Hexane–EtOAc (7:3)*
	Methyl cholanates			
1	7-oxo-5β-	0.89	0.90	
2	6,7-seco-5β-, 6,7,24-trioate	0.87	0.85	
3	A-nor-3-oxo-5β-	0.85	0.84	
4	12α-hydroxy-5β-	0.82	0.83	
5	7α-hydroxy-5β-	0.82	0.83	
6	3-oxo-5β-	0.82	0.83	
7	3α,12α-diacetoxy-5β-	0.77	0.72	
8	3α-acetoxy-12-oxo-5β-	0.77	0.67	
9	3β-hydroxy-5β-	0.36	0.54	
10	3β-acetoxy-6-oxo-5α-	0.66	0.52	
11	3α-acetoxy-6-oxo-5α-	0.61	0.52	
12	3α-hydroxy-5β-	0.32	0.41	
13	6α-acetoxy-3-oxo-5α-	0.58	0.41	
14	12α-acetoxy-3-oxo-5β-	0.45	0.41	
15	6β-acetoxy-3β-formyloxy-5α-hydroxy-	0.45	0.41	0.45
16	3α,7α,12α-triacetoxy-5β-	0.54	0.32	0.32
17	3,6-dioxo-5α-	0.45	0.26	0.32

(continued on p. 566)

TABLE I (*continued*)

		Solvent system (v/v)		
		Benzene–Et_2O (8:2)	*Hexane–EtOAc (8:2)*	*Hexane–EtOAc (7:3)*
18	3α,7α-diacetoxy-12-oxo-5β-	0.45		0.32
19	3,7-dioxo-5β-	0.41		0.32
20	3β-formyloxy-5α-hydroxy-6-oxo-	0.26	0.18	0.30
21	3α-acetoxy-7,12-dioxo-5β-	0.36		0.20
22	7α,12α-diacetoxy-3-oxo-5β-	0.23		0.16
23	3α,7α-diacetoxy-12α-hydroxy-5β-	0.20		0.16
24	7α-acetoxy-3,12-dioxo-5β-	0.18		0.11
25	3,7,12-trioxo-5β-	0.14		0.09
26	7α-acetoxy-3α-hydroxy-12-oxo-5β-	0.02		0.04
*27	7α,12α-dihydroxy-3-oxo-5β-	0.00		0.03
28	7α-acetoxy-3α,12α-dihydroxy-5β-	0.00		0.02
*29	3α,12α-dihydroxy-7-oxo-5β-	0.00		0.01
30	3α,7α,12α-trihydroxy-5β-	0.00		0.00

		Et_2O–AcOH (99.6:0.4)	*Benzene–AcOH (95:5)*
	Cholanic acids		
31	5β-3-enic	1.00	0.23
32	5α-	1.00	0.18
33	7-oxo-5β-	0.95	0.14
*34	3β-hydroxy-5β-	0.89	
35	7,8-seco-8-oxo-5β-, 7,24-dioic	0.89	
36	3,4-seco-5-oxo-A-nor-, 3,24-dioic	0.89	
37	3,4-seco-5β-, 3,4,24-trioic	0.86	
*38	3α-hydroxy-5β-	0.84	
39	12-oxo-3α-succinoxy-5β-	0.81	
40	12α-hydroxy-3α-succinoxy-5β-	0.78	
41	2,3-seco-5β-, 2,3,24-trioic	0.75	
*42	3α-hydroxy-12-oxo-5β-	0.65	
43	3,7,12-trioxo-5β-	0.48	
44	3β,6β-dihydroxy-5β-	0.44	
*45	3α,6β-dihydroxy-5β-	0.44	
*46	3α,12α-dihydroxy-5β-	0.44	
*47	3α,7α-dihydroxy-5β-	0.44	
48	3β,6β-dihydroxy-5α-	0.41	
*49	3α-hydroxy-7,12-dioxo-5β-	0.37	
*50	3β,6α-dihydroxy-5β-	0.24	
*51	3α,6α-dihydroxy-5β-	0.24	
52	3α,7α-dihydroxy-12-oxo-5β-	0.24	
53	3β,6α-dihydroxy-5α-	0.22	
*54	3α,7α,12α-trihydroxy-5β-	0.06	

		$CHCl_3$–MeOH (9:1)
	Cholanes	
55	3α,24-dihydroxy-5β-	0.82
56	3α,12α,24-trihydroxy-5β-	0.34
57	3α,7α,12α,24-tetrahydroxy-5β-	0.10

(1) *Polarity of the functional groups*

Adsorptivity increases in the order –OAc, =O, and –OH, and also with their increasing numbers (example: Nos. 16, 23, 26, 28, 30, etc.).

(2) *Position of the functional groups*

Adsorption is affected greatly by the positions of the functional groups, and their presence in 3- and 6-positions was shown to effect greater adsorption than those in 7- and 12-positions. The order of adsorptivity of various functional groups is as follows:

3α-OH, 3β-OH > 7α-OH, 12α-OH (example: Nos. 4, 5, 9, 12)

6α-OH > 7α-OH, 12α-OH (example: Nos. 46, 47, 51)

3 =O > 7 =O (example: Nos. 1, 6)

3 =O = 7α-OH, 12α-OH (example: Nos. 4, 5, 6)

(3) *Effect of configuration*

(i) The effect of the configuration of 6-OH is greater than that of 3-OH.

3α-OH (eq.) ≧ 3β-OH (ax.) (5β) (example: Nos. 9, 12; 44, 45; 50, 51)

6α-OH (eq.) ⩾ 6β-OH (ax.) (5β) (example: Nos. 44, 50; 45, 51)

(ii) A/B-*trans* > A/B-*cis* (example: Nos. 31, 32).

(4) *Dioic and trioic acids*

These show little adsorptivity in spite of their greater number of carboxyl groups (example: Nos. 35, 36, 37, 39, 40, 41).

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Simple chromatographic separation of 5-hydroxytryptophan from tryptophan

The biosynthesis of 5-hydroxytryptamine from tryptophan has been extensively studied by several groups of workers (*e.g.* UDENFRIEND, TITUS, WEISSBACH AND PETERSON[1]) and 5-hydroxytryptophan has been established as the immediate precursor of 5-hydroxytryptamine. The enzymic hydroxylation of tryptophan to 5-hydroxytryptophan has been difficult to demonstrate. Three groups of workers, COOPER AND MELCER[2], FREEDLAND, WADZINSKI AND WAISMAN[3] and RENSON, WEISSBACH AND UDENFRIEND[4], have recently claimed to have isolated enzyme systems capable of carrying out this step. However, they were not able to isolate the immediate reaction product of the hydroxylation, but estimated the increase of total 5-hydroxyindoles in the reaction mixture. As no method has so far been described to separate 5-hydroxytryptophan from tryptophan, the following simple procedure has been devised, which may prove useful in future studies on tryptophan-5-hydroxylase.

Experimental

Amberlite resin CG-50, 400 mesh, was stirred for 1 h with 2 *N* NaOH, washed with water and stirred with 4 *N* HCl for 1 h. Fines were removed by repeated suspension in distilled water, the resin washed free of acid and poured into glass columns (1 cm internal diameter) to the requisite height (27 cm or 56 cm). The column was washed further with distilled water until the optical density of the eluate, measured spectrophotometrically, was less than 0.005 at 275 mμ. The column was then ready for use.

5-Hydroxytryptophan and tryptophan were each dissolved in 0.001 *N* HCl. Equal portions of each solution were mixed and quantitatively transferred to the column. The amino acids were then eluted with distilled water and the optical density of each fraction was measured at 275 mμ. The fractions containing each compound were pooled and quantitatively analysed spectrophotometrically against standards, in order to assess the recovery of each acid.

The pooled samples from each "peak" were chromatographed on Whatman No. 1 paper using butanol–acetic acid–water (12:3:5) as the solvent system and stained with Ehrlich's reagent.

Results

Figs. 1 and 2 show the separation of the two amino acids by column chromatography using columns of two different heights.

Each of the two "peaks" ran as a single spot on a paper chromatogram. These chromatograms showed that the first "peak" contained 5-hydroxytryptophan and the second, tryptophan.

In each case the recovery of the individual amino acid was greater than 90%.

The same two columns (Figs. 1 and 2) were used to determine whether flow rate (0.2 ml/min to 5 ml/min), volume of amino acid solution transferred (0.4 ml to 1.0 ml) or the total amount of amino acids transferred (1.04 mg to 4.1 mg), affected resolution, or recovery of the individual amino acid. Within the limits stated, this was found not to be the case.

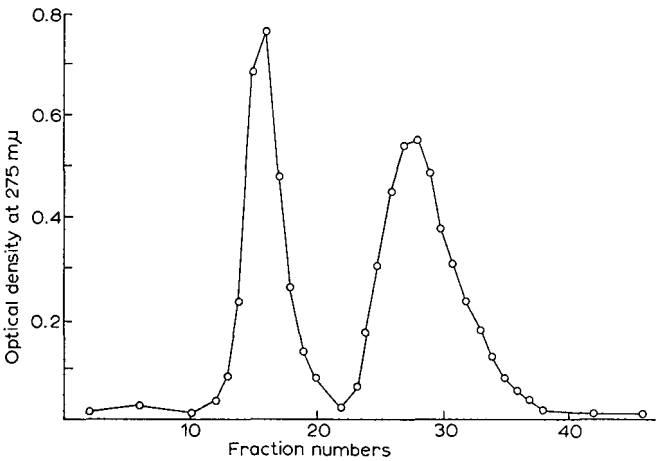

Fig. 1. Chromatographic separation of 5-hydroxytryptophan from tryptophan on Amberlite CG-50 resin, 400 mesh, H^+ form. Column length = 27 cm. Flow rate = 1 ml/min. 3 ml fractions collected. A mixture of tryptophan (0.56 mg in 0.2 ml of 0.001 *N* HCl) and 5-hydroxytryptophan (0.48 mg in 0.2 ml of 0.001 *N* HCl) was quantitatively transferred to the column. Recovery of tryptophan = 97.4 % and of 5-hydroxytryptophan = 95.5 %.

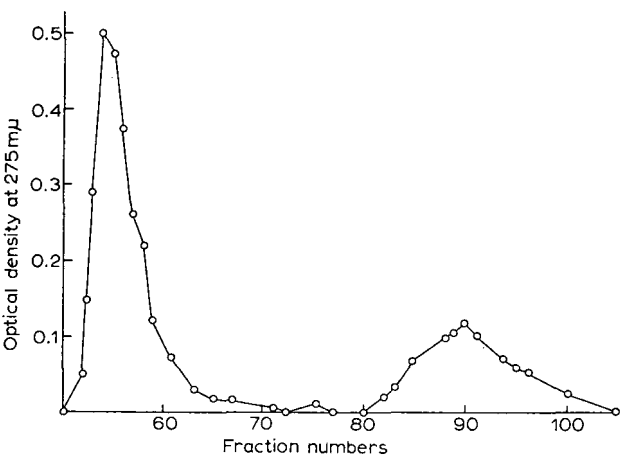

Fig. 2. Chromatographic separation of 5-hydroxytryptophan from tryptophan on Amberlite CG-50 resin, 400 mesh, H^+ form. Column length = 56 cm. Flow rate = 0.2 ml/min. 3 ml fractions collected. A mixture of tryptophan (2.0 mg in 0.2 ml of 0.001 *N* HCl) and 5-hydroxytryptophan (2.1 mg in 0.2 ml of 0.001 *N* HCl) was quantitatively transferred to the column. Recovery of tryptophan = 90.5 % and of 5-hydroxytryptophan = 92.4 %.

Discussion

BUCHANAN AND MARKIW[5] showed that tryptophan, tyrosine and phenylalanine could be separated with 70 to 78 % recoveries using IRC-50 resin and water as the eluant. As the pK_a of 5-hydroxytryptophan, by virtue of its phenolic hydroxyl group, is greater than the pK_a of tryptophan at pH 7.0, a carboxylic resin such as IRC-50 in the H^+ form, would tend to retain tryptophan to a greater extent than 5-hydroxytryptophan. The technique of BUCHANAN AND MARKIW[5] was therefore tried, and

though a tendency to separation between the two compounds was observed, resolution was not satisfactory. As these authors had used a coarse grade of resin, a finer grade (400 mesh) was used in this study and good resolution and recoveries of the two compounds was secured.

Comparison of Figs. 1 and 2 shows that though the longer column (Fig. 2) gave a better resolution of the two amino acids, a column of half this length was sufficient to obtain a separation, and gave better recoveries of the amino acid.

The use of water as an eluant, apart from its simplicity and convenience, provides mild conditions of operation, preventing destruction of labile compounds such as 5-hydroxytryptophan. Regeneration of the column was found to be unnecessary when the two pure compounds were being separated. The same columns were used repeatedly without affecting their performances.

This method can obviously be extended to the separation of 5-hydroxytryptamine, 5-hydroxytryptophan and tryptophan, with simultaneous desalting, from biological fluids containing a mixture of these substances. 5-Hydroxytryptamine and cations would be absorbed on the resin, the former being subsequently recovered quantitatively by elution with 4 *N* acetic acid as in the method of AWAPARA, DAVIS AND GRAHAM[6].

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CHROMATOGRAPHIC DATA

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TABLE 1

R_F VALUES OF SOME N^{ε}-TOSYL-L-LYSINE PEPTIDES

(J. D. CIPERA, *J. Org. Chem.*, 26 (1961) 206)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, upper phase).
S_2 = Butan-2-ol–3% NH_4OH (3:1).
Paper: Not specified.
Detection: Ninhydrin.

Compound	R_F	
	S_1	S_2
N^{ε}-Tosyl-L-lysine	0.75	0.53
N^{ε}-Tosyl-L-lysyl-N^{ε}-tosyl-L-lysine	0.90	0.65
Glycyl-N^{ε}-tosyl-L-lysyl-L-lysine	0.91	0.85
N^{ε}-Tosyl-L-lysyl-L-valine	0.89	

TABLE 2

R_F VALUES OF MONO-, DI- AND TRIPHOSPHATES OF *myo*-INOSITOL

(S. E. KERR AND G. A. KFOURY, *Arch. Biochem. Biophys.*, 96 (1962) 347)

Solvents: S_1 = Methanol–formic acid–water (16:3:1).
S_2 = Propan-2-ol–glacial acetic acid–water (3:1:1).
S_3 = Butan-1-ol–glacial acetic acid–water (2:1:1).
S_4 = Methanol–0.5 *N* NH_4OH (7:3).
S_5 = Propan-1-ol–14 *N* NH_4OH–water (5:4:1).
S_6 = Propan-2-ol–14 *N* NH_4OH–water (5:4:1).
Paper: Whatman No. 1 (descending).
Temperature of run: 25° (approx.).
Detection: D_1 = Molybdate reagent for phosphate compounds (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).
D_2 = $AgNO_3$–NaOH–NH_4OH reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444).

Compound*	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
A_{alk}(In-P)**	0.42	0.14	0.12	0.34	0.41	0.58
C_{acid}(In-diP)**	0.49	0.07	0.05	0.13	0.28	0.49
C_{alk}(In-diP)**		0.09	0.04	0.15	0.30	0.49
D_{acid}(In-triP)**	0.54	0	0.02	0→0.04	0.19	0.41
L-*myo*-Inositol 1-mono-P		0.15	0.12		0.38	0.55
myo-Inositol 2-mono-P	0.45	0.15	0.12	0.33	0.40	0.57
Inositol 3-P		0.20	0.15		0.43	0.56
Inositol		0.28	0.24		0.60	
α-Glycero-P	0.65	0.45	0.25 0.33	0.47	0.48	0.64
P_i	0.73	0.53	0.39	0.39	0.34	0.47
Chloride	0.62	0.41	0.32		0.50	0.56

* P_i = inorganic phosphate; In-P = *myo*-inositol monophosphate; In-diP = *myo*-inositol diphosphate; In-triP = *myo*-inositol triphosphate. Various isomers are covered by abbreviations.
** Origin of compound: "alk" or "acid" means from alkaline or acid hydrolysates of phytic acid.

TABLE 3

R_F VALUES (THIN LAYER) OF FAT-SOLUBLE DYESTUFFS

(J. W. COPIUS PEEREBOOM, *Chem. Weekblad*, 57 (1961) 625)

Solvents: S_1 = Hexane–ethyl acetate (90:10).
S_2 = Chloroform.
S_3 = Petroleum ether–ether–acetic acid (70:30:1).
S_4 = Petroleum ether–ether–ammonia (70:30:1).
S_5 = Hexane–ethyl acetate (98:2).
S_6 = Cyclohexane.

Thin-layer carrier: C_1 = Silica gel G.
C_2 = Aluminium oxide G.
C_3 = Kieselguhr G.

Detection: Visible light.

Dyestuff	*Colour Index 1956*	R_F					
		C_1				C_2	C_3
		S_1	S_2	S_3	S_4	S_5	S_6
Sudan I	12055	0.68	0.60	0.77	0.70	0.56	0.63
Sudan II	12140	0.72	0.58	0.78	0.67	0.62	0.44
Sudan III	26100	0.56	0.52	0.68	0.61	0.41	0.15
Sudan IV	26105	0.56	0.53	0.68	0.61	0.38	0.15
Bixin	75120	0	0	0.23	0	0	0
Martius Yellow	10315	0	0	0.28	0	0	0
Chlorophyll	75810	0	0.08	0.21	0	0	0
β-Carotene	75130	0.88	0.92	1.0	1.0	1.0	1.0
Dimethylazobenzene (Butter Yellow)	11020	0.68	0.62	0.68	0.57	0.59	0.85
Ceres Red G	12150	0.18	0.46	0.30	0.36	0.19	0.16
Ceres Orange GN	11920	0.14	0.24	0.36	0.37	0	0
Ceres Yellow	12700	0.54	0.61	0.74	0.75	0.56	0.54
Yellow XP	12740	0.60	0.64	0.81	0.80	0.68	0.40
Yellow OB	11390	0.27	0.82	0.50	0.49	0.27	0.87
Yellow AB	11380	0.25	0.80	0.46	0.41	0.22	0.88

TABLE 4

R_F VALUES OF ASPIDIN, ALBASPIDIN AND PHLOROPYRON

(A. PENTTILÄ AND J. SUNDMAN, *Acta Chem. Scand.*, 15 (1961) 839)

Solvent: Chloroform–benzene (1:1) (see A. PENTTILÄ AND J. SUNDMAN, *J. Pharm. Pharmacol.*, 13 (1961) 531).

Paper: Schleicher & Schüll 2043b buffered filter paper (see A. PENTTILÄ AND J. SUNDMAN, *loc. cit.*).

Detection: Aqueous tetrazotized bis-*o*-anisidine, spray (see A. PENTTILÄ AND J. SUNDMAN, *loc. cit.*).

Compound	R_F
Aspidin	0.73
Albaspidin	0.56
Phloropyron	0.28

TABLE 5

R_F VALUES (THIN LAYER) OF SOME STEROIDS

(A. A. ACHREM AND A. I. KUZNETSOVA, *Dokl. Akad. Nauk SSSR*, 138 (1961) 591)

Solvents: S = Cyclohexane–ethyl acetate mixtures (see table for composition).
Thin-layer adsorbents: Silica gel (195–200 mesh), Mark KSK (6.9 g) with binder.
Binder: Plaster of Paris (medical standard; 0.35 g) with 18 ml H_2O (dist.).
Plate: 11 × 17.8 × 0.2 cm; final drying: 100–105°, then desiccator over silica gel.
Time of run: 30 min–1.5 h.
Length of run: 9–10 cm.
Detection: D_1 = Conc. H_2SO_4; (a) visible light, (b) U.V. light.
D_2 = $SbCl_3$ reagent; (a) visible light, (b) U.V. light.
Also:
D_3 = Phosphomolybdic acid reagent.
D_4 = 2,4-Dinitrophenylhydrazine reagent.

Compound	*S*	R_F	*Colour*			
			D_1		D_2	
			(a)	*(b)*	*(a)*	*(b)*
Dehydroepiandrosterone	2:1	0.28 ± 0.03	+		+	
Dehydroepiandrosterone acetate	2:1	0.59 ± 0.02	+r		+v	
17α-Ethynyl-Δ^5-androstene-3β,17β-diol	2:1	0.35 ± 0.02	+r	+	+	
17α-Ethynyl-Δ^5-androstene-3β,17β-diol 3-acetate	2:1	0.55	+r			
17α-Ethynyl-Δ^5-androstene-3β,17β-diol 3,17-diacetate	2:1	0.75 ± 0.7	+r	+	+v	+
5,21,21-Tribromopregnane-3β,6,17β-triol 3,17-diacetate	2:1	0.27 ± 0.03	+b weak	+	—	+
5α,6α-Epoxyepiandrosterone acetate	2:1	0.34				
	1:1	0.66 ± 0.03	+ *	—		
5β,6β-Epoxyepiandrosterone acetate	2:1	0.38				
	1:1	0.59 ± 0.03	+ *	—		
5α,6α-Epoxy-17α-ethynyl-androstane-3β,17β-diol 3-acetate	1:1	0.80 ± 0.01	+r			
5β,6β-Epoxy-17α-ethynyl-androstane-3β,17β-diol 3-acetate	1:1	0.69 ± 0.01	+r			
Cortisone	2:1	0.78	+b	+		
Pregnine 17α-acetate	2:1	0.51	+b**	+		
	2:3	0.86	+b	+		
17-Acetoxy-21,21-dibromo-Δ^4-pregnen-17β-ol-3,20-dione	2:1	0.39				
	2:3	0.75 ± 0.05	—	—		
17-Acetoxy-Δ^4-pregnene-3,20-diol	2:1	0.35				
	2:3	0.68 ± 0.03	—	—		
Δ^4-Androstene-3,17-dione	2:1	0.34				
	2:3	0.65 ± 0.03	—**	—		

+ = positive; — = negative; r = rose; b = blue; v = violet.
* D_3 positive.
** D_4 positive.

TABLE 6

R_F VALUES OF SOME STEROIDS

(P. KABASAKALIAN AND J. M. TALMIDGE, *Anal. Chem.*, 34 (1962) 273)

Solvents: S_1 = Chloroform–formamide (Fischer Scientific Co., Fair Lawn, N. J., stabilised).
S_2 = Benzene–formamide.
S_3 = Toluene–propylene glycol.
S_4 = Ligroin–propylene glycol.
S_5 = Heptane–methylcellosolve
S_6 = Heptane–phenylcellosolve.
(See P. KABASAKALIAN AND A. BASCH, *Anal. Chem.*, 32 (1960) 458)

Paper: See P. KABASAKALIAN AND A. BASCH, *loc. cit.*).

Compound	R_F*					
	S_1	S_2	S_3	S_4	S_5	S_6
11β,17α,21-Trihydroxypregna-1,4-diene-3,20-dione	0.10					
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	0.16					
11β,17α,21-Trihydroxypregna-1,4-diene-3,20-dione 21-acetate		0.12				
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione 21-acetate		0.24				
17α,21-Dihydroxypregna-1,4-diene-3,11,20-trione 21-acetate		0.41	0.32			
17α,21-Dihydroxypregn-4-ene-3,11,20-trione 21-acetate		0.47	0.36			
9α-Fluoro-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-acetate			0.17			
17α,21-Dihydroxypregn-4-ene-3,20-dione			0.20			
17α,21-Dihydroxypregn-4-ene-3,20-dione 21-acetate			0.45			
21-Hydroxypregn-4-ene-3,20-dione			0.69	0.10		
21-Hydroxypregn-4-ene-3,20-dione acetate				0.33		
Pregn-4-ene-3,20-dione				0.57	0.31	
17β-Hydroxypregn-4-ene-3,20-dione acetate					0.17	
3β-Hydroxypregna-5,16-dien-20-one					0.18	
16α,17α-Oxidopregn-4-ene-3,20-dione					0.21	
3β-Hydroxypregna-5,16-dien-20-one acetate					0.55	
5β-Pregnane-3,20-dione					0.42	0.12
3β-Hydroxy-5α,22α,25D-spirost-9(11)-en-12-one acetate						0.23
5α,22α,25D-Spirostan-3β-ol					0.66	0.30
Stigmasta-4,22-dien-3-one						0.70
9α-Fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione	0.19					
16α-Methyl-11α,17α,21-trihydroxypregna-1,4-diene-3,20-dione	0.21					
17α,20β,21-Trihydroxypregna-1,4-diene-3,11-dione	0.30					
16α-Methyl-11α,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-acetate		0.13				
9α-Fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-acetate		0.21				
9α-Bromo-16α-ethyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-acetate		0.28				

(continued on p. D5)

TABLE 6 (*continued*)

Compound	R_F*					
	S_1	S_2	S_3	S_4	S_5	S_6
Estra-1,3,5(10)-triene-3,17β-diol			0.12			
17α-Methyl-17β-hydroxyandrost-4-en-3-one			0.56	0.24		
16α-Methyl-9β,11β-oxido-17α,21-dihydroxypregna-1,4-diene-3,20-dione 21-acetate			0.65	0.37		
16α-Methyl-3β-hydroxy-5α-pregnan-20-one					0.30	0.18
16α-Methyl-17α,20α-oxido-5α-pregnane-3β,20β-diol diacetate						051

R_F is actually $(R_F)_P$ = distance moved by leading edge of solution band/distance moved by solvent (*cf.* R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224).

TABLE 7

R_F VALUES (THIN LAYER) OF SOME 18-SUBSTITUTED HYDROXY-STEROIDS AND RELATED COMPOUNDS
(L. LÁBLER AND F. ŠORM, *Collection Czech. Chem. Commun.*, 27 (1962) 276)

Solvents: S_1 = Ether–2 % ethanol.
S_2 = Benzene–ether (1:1).
S_3 = Benzene–ether (75:25).
Thin-layer carriers: C_1 = Alumina, neutral; Grade III–IV.
C_2 = Alumina, Grade IV; impregnated with morin (H. BROCKMANN AND F. VOLPERS, *Chem. Ber.*, 80 (1947) 77); layer 1 mm thick (*cf.* V. ČERNÝ, J. JOŠKA AND L. LÁBLER, *Collection Czech. Chem. Commun.*, 26 (1961) 1658).
Detection: D_1 = U.V. light; 240 mμ (Chromatolite, Hanovia).
D_2 = Philora (Philips) (*cf.* V. ČERNÝ *et al.*).

Compound	R_F			Detection	
	S_1	S_2	S_3	D_1	D_2
(20*R*)-20-Hydroxy-3-oxopregn-4-en-18-oic acid (18 → 20)-lactone	0.65			+	
(20*R*)-7ξ,20-Dihydroxy-3-oxopregn-4-en-18-oic acid (18→20)-lactone (I)	0.10–0.40			+	
(20*R*)-11α,20-Dihydroxy-3-oxopregn-4-en-18-oic acid (18→20)-acetone (II)	0.10–0.40			+	
11α-Acetoxy derivative of (II)	0.50			+	
(20*R*)-20-Hydroxy-3-oxopregna-4,6-dien-18-oic acid (18→20)-lactone	0.67–0.75			+	
(20*R*)-Hydroxy-3,11-dioxopregn-4-en-18-oic acid (18→20)-lactone mono-2,4-dinitrophenylhydrazone		0.58			
(20*R*)-Hydroxy-3,11-dioxopregn-4-en-18-oic acid (18→20)-lactone			0.1–0.26	+	
(20*R*)-3-Ethylenedioxy-20-hydroxy-11-oxopregn-5-en-18-oic acid (18 → 20)-lactone			0.34–0.53		+

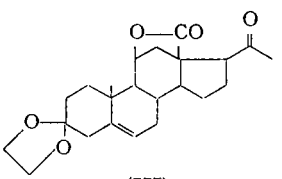
TABLE 8

R_F VALUES OF SOME 18-SUBSTITUTED HYDROXY-STEROIDS AND RELATED COMPOUNDS

(L. LÁBLER AND F. ŠORM, *Collection Czech. Chem. Commun.*, 27 (1962) 276)

Solvents: S_1 = Formamide/benzene (Zaffaroni type systems) (A. ZAFFARONI, *Recent Progr. Hormone Res.*, 8 (1953) 51).
S_2 = Formamide/cyclohexane–benzene (2:1).
S_3 = Formamide/cyclohexane.
Paper: Whatman No. 1 (descending).
Temperature of run: 21° (*cf.* S. A. SIMPSON *et al.*, *Helv. Chim. Acta*, 37 (1954) 1163).
Detection: D_1 = Fluorescent screen for compounds absorbing at 240 mμ; (also Chromatolite and Philora U.V. sources).
D_2 = Lactic acid reagent for ketals (dry chromatogram sprayed with 50% lactic acid; infra-red lamp heating for 20 min); liberated α,μ-unsaturated ketones detected by D_1.
D_3 = 2,4-Dinitrophenylhydrazine reagent (*cf.* R. NEHER, *J. Chromatog.*, 1 (1958) 205).

Compound	R_F		
	S_1	S_2	S_3
(20*R*)-20-Hydroxy-3-oxopregn-4-en-18-oic acid (18 → 20)-lactone	0.95		
(20*R*)-7ξ,20-Dihydroxy-3-oxopregn-4-en-18-oic acid (18 → 20)-lactone (I)	0.40	0.18	
(20*R*)-11α,20-Dihydroxy-3-oxopregn-4-en-18-oic acid (18 → 20)-lactone (II)	0.40	0.23	
7ξ-Acetoxy derivative of (I)			0.19–0.25
11α-Acetoxy derivative of (II)			0.07–0.15
(20*R*)-3-Ethylenedioxy-20-hydroxy-11-oxopregn-5-en-18-oic acid (18 → 20)-lactone		0.65	
(18 → 11)-Lactone isomer (III)		0.41	
11β-Hydroxy-3,20-dioxopregn-4-en-18-oic acid (18 → 11)-lactone (IV)	0.35–0.50		



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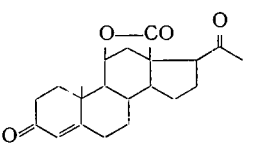

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TABLE 9

R_F AND RELATIVE R_F VALUES OF *Solanum* ALKALOIDS

(L. H. BRIGGS, R. C. CAMBIE AND J. L. HOARE, *J. Chem. Soc.*, (1961) 4645)

Solvents: S_1 = Ethyl acetate–acetic acid–water (11:2:1.85) (*cf.* V. A. PASESHNICHENKO AND A. R. GUSEVA, *Biokhimiya*, 21 (1956) 585).
S_2 = Benzene–chloroform (1:2) saturated with formamide (stabilised with 1% v/v of light petroleum (*cf.* J. TUZSON, *Naturwiss.*, 43 (1956) 198).
Paper: Whatman No. 1 (equilibrated 12 h before use with solvent system).
Impregnation: I = Formamide–acetone (3:7) (*cf.* J. TUZSON, *Naturwiss.*, 43 (1956) 198).
Detection: D_1 = Iodine in light petroleum solution.
D_2 = 25% $SbCl_3$ in chloroform.
(Both spray reagents.)

Compound	$R_{\alpha S}$*	R_F	
	S_1	S_1	S_2I
Solanine	1.0		
Solasonine	1.3		0.0
Solamargine	2.3		
Solasodine glucosylgalactoside	2.5		
Solasodine rhamnosylglucoside	5.0		
Solasodine glucoside	10.0	0.40	0.0
Solasodine	15.0	0.76	0.5
Solanidine	14.0	0.73	0.3
α-"Solanigrine"	0.2		
β-"Solanigrine"	0.5		

* $R_{\alpha S}$ = R_F compound/R_F α-solanine.

TABLE 10

R_F VALUES OF TOCOPHERYL-*p*-QUINONE-^{14}C

(A. S. CSALLANY H. H. DRAPER AND S. N. SHAH, *Arch. Biochem. Biophys.*, 98 (1962) 142)

Solvents: S_1 = Cyclohexane.
S_2 = Acetonitrile–water (90:10).
S_3 = Acetonitrile–water (50:50).
S_4 = Ethanol.
S_5 = Ethanol–water (75:25).
S_6 = Ethanol–water (25:75).
S_7 = Ethanol–water (50:50).
Paper: P_1 = Zinc carbonate impregnated (J. GREEN, S. MARCINKIEWICZ AND P. R. WATT, *J. Sci. Food Agr.*, 6 (1955) 274).
P_2 = Silicone coated (J. A. BROWN, *Anal. Chem.*, 25 (1953) 774).
P_3 = Paraffin coated (P. W. R. EGGITT AND L. D. WARD, *J. Sci. Food Agr.*, 4 (1953) 176).
P_4 = Glass (not specified).
Detection: ^{14}C technique.

Compound	R_F*							
	S_1P_1	S_2P_2	S_3P_3	S_4P_3	S_5P_3	S_6P_4	S_6P_4	S_7P_4
Tocopheryl-*p*-quinone	0.60	0.95	0.00	0.95	0.60	1.00	0.00	Spread

* Only one peak observed in each case.

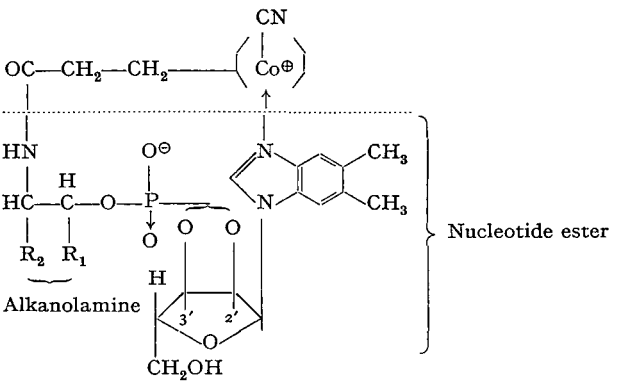
TABLE 11

R_F VALUES OF CERTAIN SYNTHETIC VITAMIN B_{12} ANALOGUES

(W. FRIEDRICH, H. C. HEINRICH, E. GABBE, C. NARDIN AND P. RIEDEL, *Biochem. Z.*, 333 (1961) 554)

Solvents: S_1 = Water satd. *sec.*-butanol–acetic acid–10% HCN (100:1:0.1, v/v) (J. E. FORD, E. S. HOLDSWORTH AND S. K. KON, *Biochem. J.*, 59 (1955) 86).
S_2 = *sec.*-Butanol–water–25% NH_4OH–10% HCN (50:18:7:0.1, v/v) (J. E. FORD *et al.*, *loc. cit.*).
S_3 = Water satd. *sec.*-butanol–10% HCN (100:0.02, v/v).
S_4 = $Na_2CO_3 \cdot 10H_2O$–H_2O–isoamyl alcohol (27 g:100 ml:100 ml) (R. BONNETT, J. G. BUCHANAN, A. W. JOHNSON AND A. TODD, *J. Chem. Soc.*, (1957) 1168).
S_5 = 5% Na_2HPO_4–isoamyl alcohol (100:50) (R. J. BLOCK, R. LESTRANGE AND G. ZWEIG, *Paper Chromatography*, Academic Press, New York, 1952, p. 118).
S_6 = 5% $(NH_4)HCO_3$–isoamyl alcohol (100:50).

Paper: Not specified.
Detection: Not specified.



B_{12} analogue					*$R_{B_{12}}$* B_{12} analogue*			*R_F Nucleotide ester*		
Type No.	R_1	R_2	*P-ribose bond*	*Alkanol-amine*	S_1	S_2	S_3	S_4	S_5	S_6
1	H	H	3′		0.91	1.03	0.91			
2	H	H	2′		0.9	1.06	1.02			
3	H	CH_3	3′	L	1.0	1.06	1.0			
4	H	CH_3	2′	L	1.02	1.13	1.17			
5	Ph**	H	3′	DL	1.4	1.15	1.42	0.50	0.64	0.59
6	Ph**	H	2′	DL	1.31	1.37	1.36	0.63	0.64	0.79
7	H	Bz**	3′	DL	1.31	1.23	1.32	0.34	0.60	0.70
8	H	Bz**	2′	DL	1.2	1.23	1.21	0.46	0.68	0.78

* $R_{B_{12}} = R_F$ compound/R_F B_{12}
** Ph = phenyl ; Bz = benzyl.

TABLE 12

R_F VALUES OF LONG-CHAIN ALKYL SULPHATES

(J. BORECKÝ, *Chem. Ind. (London)*, (1962) 265)

Solvents: S_1 = Methanol–ammonia (1:1).
S_2 = Methanol–ammonia–85% formic acid (50:50:1).
S_3 = Methanol–ammonia–85% formic acid (10:10:1).
Paper: Whatman No. 3.
Impregnation: 5% lauryl alcohol in benzene.
Temperature of run: 35–36°.
Detection: 0.05% aq. Pinacryptol Yellow and U.V. viewing (J. BORECKÝ, *J. Chromatog.*, 2 (1959) 612).

Alkyl sulphate	R_F			*Colour**
	S_1	S_2	S_3	
Stearyl	0.16	0	0	Yf
Oleyl	0.33	0	0	B-Y
Cetyl	0.38	0.07	0	Yf
Myristyl	0.65	0.23	0.08	Yf
Lauryl (dodecyl)	0.82	0.51	0.28	Yf
Decyl	0.92	0.78	0.64	B-O
Octyl	**	**	**	G-Yf

* B = brownish; G = golden; O = orange; Y = yellow; f = fluorescence; pale bluish-green background.
** Runs with front.

TABLE 13

R_F VALUES OF GLUTAMIC AND γ-HYDROXYGLUTAMIC ACIDS

(S. -I. HATANAKA, *Acta Chem. Scand.*, 16 (1962) 513)

Solvents: S_1 = Butan-1-ol–glacial acetic acid–water (630:100:270).
S_2 = Phenol–water/NH_3 (1000:365).
Paper: Whatman No. 4 (descending).
Detection: Ninhydrin.

Compound	R_F	
	S_1	S_2
Glutamic acid	0.11	0.30
γ-Hydroxyglutamic acid	0.07	0.17

TABLE 14

R_F VALUES OF SOME DINITROPHENYLAMINO ACIDS

(N. A. TIUNOVA, *Tr. Gl. Botan. Sada, Akad. Nauk SSSR*, 8 (1961) 113)

Solvents: S_1 = Benzyl alcohol–ethanol (10:1) saturated with phthalate buffer, pH 6.
S_2 = Butanol–butyl acetate–1% NH_4OH (1:2:3).
Paper: Not specified.
Detection: Not specified.
(See F. SANGER, *Biochem. J.*, 39 (1945) 507; R. R. PORTER AND F. SANGER, *Biochem. J.*, 42 (1948) 287; K. R. RAO AND H. A. SOBER, *J. Am. Chem. Soc.*, 76 (1954) 1328.)

DNP-Amino acid	R_F	
	S_1	S_2
DNP-Aspartic acid	0.06	0
DNP-Glutamic acid	0.10	0
DNP-Serine	0.28	0.21
DNP-Threonine	0.38	0.31
DNP-Glycine	0.40	0.26
DNP-Alanine	0.52	0.41
Dinitrophenol	0.58	0.52
DNP-Valine	0.70	0.58
DNP-Phenylalanine	0.74	0.66
DNP-Leucine	0.76	0.66
Dinitroaniline	0.89	0.90

TABLE 15

ELECTROPHORETIC MOBILITIES OF HISTIDINE, CARNOSINE AND HOMOCARNOSINE

(D. ABRAHAM, J. J. PISANO AND S. UDENFRIED, *Arch. Biochem. Biophys.*, 99 (1962) 210)

Electrolyte: 0.1 *M* borate buffer (pH 10).
Paper: Not specified.
Potential applied: 700 V (19.5 V/cm).
Time of run: 5 h.
Mobility: *M* (cm from origin).
Detection: Diazotised *p*-nitroaniline spray (I. SMITH, in I. SMITH (Editor), *Chromatographic Techniques*, Heinemann Medical Books, London, 1958, p. 195).

Compound	*M*
Histidine	16.7
Carnosine	12.2
Homocarnosine	6.0

TABLE 16

R_F VALUES (RELATIVE) OF METHYL ETHERS OF 6-DEOXYHEXOSES

(A. P. MACLENNAN, *Biochem. J.*, 82 (1962) 394)

Solvent: Butan-1-ol–ethanol–water (4:1:5, v/v; upper phase).
Paper: See A. P. MACLENNAN, H. M. RANDALL AND D. W. SMITH, *Biochem. J.*, 80 (1961) 309.
Detection: See A. P. MACLENNAN *et al.*, *loc. cit.*

Compound	$R_{Rhamnose}$*
3-O-Methylfucose	1.18
6-Deoxytalose	1.24
2-O-Methylfucose	1.32
3-O-Methylrhamnose	1.42
2-O-Methylrhamnose	1.48
3-O-Methyl-6-deoxytalose	1.70
2,3-Di-O-methylrhamnose	1.90
2,4-Di-O-methylrhamnose	1.96
3,4-Di-O-methylrhamnose	2.04

* $R_{Rhamnose}$ = R_F compound/R_F rhamnose.

TABLE 17

R_F VALUES (RELATIVE) OF OLIGOSACCHARIDES OBTAINED BY PARTIAL ACETOLYSIS OF GANGLIOSIDE G_2

(R. KUHN, H. EGGE, R. BROSSMER, A. GAUHE, P. KLESSE, W. LOCHINGER, E. RÖHM, H. TRISCHMANN AND D. TSCHAMPEL, *Angew. Chem.*, 72 (1960) 805)*

Solvent: Pyridine–glacial acetic acid–ethyl acetate–water (5:1:5:3).
Paper: Not specified.
Detection: Not specified.

Oligosaccharide	*Units***	R_L***
Tetrasaccharide	L,Ac-Gal,Gal,Gl<	0.25
Trisaccharide	Ac-Gal,Gal,Gl<	0.48
Trisaccharide (3'-lactaminic acid-lactose)	L,Gal,Gl<	0.41
Disaccharide (lactose)	Gal,Gl<	1.00
Disaccharide	Ac-Gal,Gl<	1.22

* Also in *Angew. Chem., Intern. Ed.*, sample issue, May (1961) 19.
** L = lactaminic acid; Ac-Gal = N-Acetyl-D-galactosamine; Gl = glucose.
*** R_L = R_F compound/R_F lactose.

TABLE 18

ELECTROPHORETIC MOBILITIES OF FOUR URONIC ACIDS AND GLUCOSE

(A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 15 (1961) 1395)

Electrolytes: E = 0.01 *M* borax solution, pH 9.2, x *M* with respect to $CaCl_2$. E_1, $x = 0$; E_2, $x = 0.0001$; E_3, $x = 0.0003$; E_4, $x = 0.0005$; E_5, $x = 0.001$; E_6, $x = 0.002$; E_7, $x = 0.005$; E_8, $x = 0.007$.
Paper: Schleicher & Schüll, 2043b.
Current: 0.5 mA/cm.
Apparatus: LKB.
Mobility: M_m = mobility relative to mannuronic acid.
Time of run: Not specified.
Detection: 2.5% aniline trichloroacetate in glacial acetic acid; dipped; then heated to 100°, 2–3 min; viewed in U.V. light.

Compound	M_m							
	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8
Guluronic acid	0.93	0.93	0.92	0.85	0.84	0.85	0.85	0.81
Mannuronic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Galacturonic acid	0.97	0.97	1.01	0.98	1.00	1.09	1.18	1.16
Glucuronic acid	1.05	1.05	1.12	1.13	1.19	1.28	1.44	1.40
Glucose	0.71	0.76	0.80	0.88	1.00	1.01	1.14	1.17

TABLE 19

ELECTROPHORETIC MOBILITIES OF FOUR URONIC ACIDS IN ACID MEDIUM

(A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 15 (1961) 1395)

Electrolyte: Phthalate buffer, pH 3.15.
Paper: Schleicher & Schüll 2043b.
Current: 0.5 mA/cm.
Apparatus: LKB.
Mobility: M_m = mobility relative to mannuronic acid.
Time of run: Not specified.
Detection: 2.5% aniline trichloroacetate in glacial acetic acid; dipped, then heated to 100°, 2–3 min; viewed in U.V. light.

Compound	pK_s	M_m
Glucuronic acid	3.20	1.10
Mannuronic acid	3.38	1.00
Galacturonic acid	3.42	0.95
Guluronic acid	3.65	0.88

TABLE 20

R_F VALUES (COLUMN) OF VARIOUS PHENOLS

(B. SMITH, *Acta Chem. Scand.*, 16 (1962) 843)

Solvent: Benzene (crystallisable) (elution continued until the solvent just reaches the bottom of the tube).
Adsorption support: Silica gel (100–200 mesh).
Column dimensions: 6 mm outer diameter, 200 mm long.
Detection: 0.01 mole $KMnO_4$ in 1000 ml 0.5 % H_2SO_4 (in capillary previously inserted down the centre of the tube which is slowly withdrawn during ejection of the reagent).

Compound	R_F*	*Compound*	R_F*	*Compound*	R_F*
Phenol	0.17	*m*-Chlorophenol	0.28	2,4-Dihydroxybenzaldehyde	0.07
m-Cresol	0.17	*p*-Chlorophenol	0.25	3,4-Dihydroxybenzaldehyde	0.01
p-Cresol	0.16	*p*-Chloro-*m*-cresol	0.27	*p*-Hydroxyacetophenone	0.03
p-*tert*.-Butylphenol	0.17	*o*-Chlorophenol	0.42	*p*-Hydroxypropiophenone	0.02
3,4-Xylenol	0.17	2-Chloro-5-methylphenol	0.42	*o*-Hydroxyacetophenone	0.22
3,5-Xylenol	0.19	2,4-Dichlorophenol	0.45	2,4-Dihydroxyacetophenone	0.02
o-Cresol	0.26	2,4,5-Trichlorophenol	0.46	*m*-Hydroxybenzoic acid	0.04
2,3-Xylenol	0.25	*o*-Bromophenol	0.46	*p*-Hydroxybenzoic acid	0.02
2,4-Xylenol	0.26	2,4,6-Tribromophenol	> 0.70	Salicylic acid	0.06
2,5-Xylenol	0.27	*m*-Nitrophenol	0.04	3,4-Dihydroxybenzoic acid	0.02
2,3,5-Trimethylphenol	0.25	*p*-Nitrophenol	0.06	Gallic acid	0.01
Carvacrol	0.30	*o*-Nitrophenol	0.40	*m*-Hydroxymethylphenol	0.01
Thymol	0.34	Picric acid	0.20	*p*-Hydroxymethylphenol	0.02
2,4-Di-*tert*.-butyl-*m*-cresol	0.70	*p*-Hydroxydiphenyl ether	0.17	*o*-Hydroxymethylphenol	0.04
2,6-Xylenol	0.43	*o*-Hydroxydiphenyl ether	0.35	2,6-Dimethyl-4-hydroxymethyl-phenol	0.01
2,4,6-Trimethylphenol	0.39	Resorcinol monomethyl ether	0.12	2,5-Dimethyl-4-hydroxymethyl-phenol	0.05
2,6-Di-*tert*.-butyl phenol	1.00	Hydroquinone monomethyl ether	0.08	Resorcinol	0.03
2,2-Bis-(*p*-hydroxyphenyl)-propane	0.07	Guaiacol	0.30	Hydroquinone	0.02
o-Allylphenol	0.41	Eugenol	0.30	Catechol	0.06
p-Hydroxydiphenyl	0.19	Isoeugenol	0.30	Phloroglucinol	0.02
o-Hydroxydiphenyl	0.39	2,4-Dihydroxyanisole	0.07	Pyrogallol	0.04
o,*o*'-Dihydroxydiphenyl	0.11	*m*-Hydroxybenzaldehyde	0.05	*p*-Aminophenol	0.02
α-Naphthol	0.25	*p*-Hydroxybenzaldehyde	0.04	*o*-Aminophenol	0.02
β-Naphthol	0.20	*o*-Hydroxybenzaldehyde	0.30		

* R_F value calculated from distance of middle of colour zone to upper surface of gel layer.

TABLE 21

R_F VALUES OF 3-O-METHYLADRENALINE AND RELATED COMPOUNDS

(O. KRAUPP, H. BERNHEIMER AND D. PAPASTIS, *Clin. Chim. Acta*, 6 (1961) 851)

Solvents: S_1 = Propan-2-ol–25% NH_3–water (40:1:9).
S_2 = Butan-1-ol–glacial acetic acid–water (4:1:1).
S_3 = Benzene–propionic acid–water (300:220:13).
Paper: Whatman No. 1 (25 cm × 25 cm; ascending).
Detection: Diazotised *p*-nitroaniline spray. Spray A: 1.5 g *p*-nitroaniline, 40 ml conc. HCl and water to 1000 ml; 1.5 ml 5% $NaNO_2$ is added to 50 ml mixture shortly before spraying. Spray B: 20% Na_2CO_3. Spray B follows spray A.

Compound	R_F			*Colour**
	S_1	S_2	S_3	
3-O-Methyladrenaline	0.71	0.51	0.18	V
3-O-Methylnoradrenaline	0.56	0.42	0.10	V
3-O-Methyldopamine	0.75	0.52	0.44	GGr
p-Sympathol	0.84	0.58	0.13	R
m-Sympathol	0.85	0.57	0.13	C
Phenylethylamine	0.88	0.74	0.72	C
Phenylethanolamine	0.83	0.70	0.49	C
Tyramine	0.78	0.65	0.19	V
Hordenine	0.97	0.61	—	V

* C = carmine; G = gray; Gr = green; R = red; V = violet.

TABLE 22

R_F VALUES OF 2,6-DIIODOHYDROQUINONE AND OTHER THYROXINE PRECURSORS

(J.-G. LJUNGGREN, *Acta Chem. Scand.*, 15 (1961) 1772)

Solvents: S_1 = Heptane–propan-1-ol–acetic acid–0.001 *M* sodium thiosulphate (100:50:1:100, v/v; organic phase).
S_2 = Petroleum ether (60–80°)–acetone–0.001 *M* sodium thiosulphate (3:1:3, v/v; organic phase).
S_3 = Benzene–0.001 *M* sodium thiosulphate (1:1, v/v; organic phase).
S_4 = Acetone–acetic acid–0.001 *M* sodium thiosulphate (8:1:60, v/v).
S_5 = Propan-1-ol–acetic acid–0.001 *M* sodium thiosulphate (10:1:60, v/v).
Paper: Whatman No. 1.
Temperature of run: 20°.
Detection: D_1 = Diazotised sulphanilic acid (B. N. AMES AND H. K. MITCHELL, *J. Am. Chem. Soc.*, 74 (1952) 252).
D_2 = Alkaline $AgNO_3$ for diiodohydroquinone (R. B. BURTON, A. ZAFFARONI AND E. H. KEUTMANN, *J. Biol. Chem.*, 188 (1951) 763).
D_3 = 0.1 *N* ferric nitrate + 3% H_2O_2 for iodide.

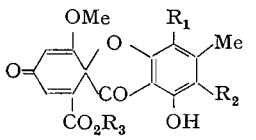
Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
2,6-Diiodohydroquinone	0.73	0.47	0.60	0.51	0.52
Iodide	0	0	0	0.84	0.84
L-Tyrosine	0	0	0	0.80	0.79
3-Monoiodo-L-tyrosine	0	0	0	0.70	0.71
3,5-Diiodo-L-tyrosine	0	0	0	0.59	0.59
L-Thyroxine	0	0	0	0	0
3,5,3′-Triiodothyronine	0	0	0	0	0

TABLE 23

R_F VALUES OF GEODIN, ASTERRIC ACID AND RELATED METABOLITES

(A. RHODES, M. P. MCGONAGLE AND G. A. SOMERFIELD, *Chem. Ind. (London)*, (1962) 611)

Solvents: S_1 = Benzene–cyclohexane–methanol–water (5:5:6:4) (A. RHODES, B. BOOTHROYD M. P. MCGONAGLE AND G. A. SOMERFIELD, *Biochem. J.*, 81 (1961) 28).
S_2 = Benzene–acetic acid–water (6:7:3) (L. A. GRIFFITHS, *Nature*, 180 (1957) 1373).
S_3 = Butan-1-ol–ethanol–water (4:1:5) and 0.5% acetic acid in upper layer.
Paper: Not specified.
Detection: D_1 = U.V. light.
D_2 = Diazotised sulphanilic acid.



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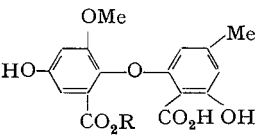

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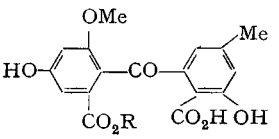

(III)

Compound		R_F S_1	R_F S_2	R_F S_3	*Colour** D_1	*Colour** D_2
Geodin (I) $R_1 = R_2 = Cl$; $R_3 = Me$	A_1	0.92–0.94			Pu(q)	Y
	F	0.90–0.96	0.90–0.95		Pu(q)	Y
"Dechlorogeodin" (I) $R_1 = R_2 = H$; $R_3 = Me$	A	0.72–0.78			Pu(q)	O
	F	0.70–0.78	0.90–0.95		Pu(q)	O
"Intermediate G" (I) $R_1 = R_2 = Cl$, H or HCl; $R_3 = Me$**	F	0.83–0.87	0.90–0.95		Pu(q)	P
"Intermediate E" ("dechloroerdin") (I) $R_1 = R_2 = R_3 = H$**	F	0.07–0.09	0.66–0.72		Pu(q)	O
Asterric acid (II) R = Me	A	0.46–0.49, 0.53***	0.68		GBl(f)	O
	F	0.42–0.55, 0.52***	0.68–0.78		GBl(f)	O
Demethylasterric acid	A	0.04			Bl(f)	O
	F	0.04			Bl(f)	O
"Intermediate S" ("demethyl sulochrin") (III) R = H**	F		0.10–0.15	0.92–0.98	Pu(q)	O
	Os		0.14	0.94		OB
Sulochrin (III) R = Me	A		0.34	0.95	Pu(q)	O
	F		0.32–0.38	0.95–0.98	Pu(q)	O
	Os		0.32–0.38	0.94–0.99	Pu(q)	O

A = authentic specimen; A_1 = crystalline sample; F = *Aspergillus terreus* fermentation; Os = *Oospora sulphurea-ochracea* fermentation.

* B = brown; Bl = blue; G = green; O = orange; P = pink; Pu = purple; Y = yellow; f = fluorescent; q = quenching.

** Tentative identification.

*** Crystalline samples on same paper at same loading.

TABLE 24

R_F VALUES OF FUSARIC, ALTERNARIC AND GIBBERELLIC ACIDS

(G. F. PEGG, *Ann. Botany (London)*, 26 (1962) 219)

Solvents: S_1 = Butan-1-ol saturated with water.
S_2 = Butan-1-ol–NH_4OH (sp. gr. 0.88) (100:3).
S_3 = Butan-1-ol–glacial acetic acid–water (12:3:5).
S_4 = Propan-2-ol-NH_4OH (sp. gr. 0.88)–water (10:1:1).
S_5 = Butan-1-ol–NH_4OH (sp. gr. 0.88)–water (200:6:36).
Paper: Whatman No. 1 (25.4 cm², spot at origin; acetone washed; equilibrated for 4 h in solvent-saturated atmosphere; run in paper-lined tanks, ascending; dried on frame in forced draft, off frame at 25° for 2 h (S_4) or 5 h (S_3).
Time of run: 10 h (S_1–S_3, S_5); 11 h (S_4).
Temperature of run: 21°.
Detection: D_1 = 0.04% aqueous bromocresol green.
D_2 = 0.5% aqueous $KMnO_4$.

Compound	R_F					Colour*	
	S_1	S_2	S_3	S_4	S_5	D_1	D_2
Fusaric acid (5-*n*-butylpicolinic acid)	0.46	0.50	0.85	—	—	Y(S_1), st(S_2)**, h(S_3)	
Alternaric acid	0.18	—	0.90	0.41	—		stB(S_1, S_4), dB(S_3)
Gibberellic acid	—	—	—	0.52	0.24		dB(S_4), B(S_5)

* Y = yellow; P = pink; B = brown; st = streaking; h = horseshoe spot; d = discrete spot.
** Characteristic horseshoe spot tailing, yellow centre with pink halo.

TABLE 25

R_F VALUES OF LUTEOLINIDIN AND ITS 5-MONO- AND DIGLUCOSIDES

(G. BENDZ, O. MÅRTENSSON AND L. TERENIUS, *Acta Chem. Scand.*, 16 (1962) 1183)

Solvents: S_1 = Butan-1-ol–acetic acid–water (6:1:2).
S_2 = Water–conc. HCl (97:3).
S_3 = Butan-1-ol–2 *N* HCl (1:1, upper phase).
S_4 = Acetic acid–conc. HCl–water (15:3:82).
S_5 = Acetic acid–conc. HCl–water (30:3:10).
(*cf.* J. B. HARBORNE, *Biochem. J.*, 74 (1960) 262.)
Paper: Whatman No. 3 MM.
Detection: Visible light.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Luteolinidin	—	0.02	—	—	0.63
Luteolinidin-5-glucoside	0.41	0.13	0.31	0.37	—
Luteolinidin-5-diglucoside	0.32	0.44	0.52	0.68	—

TABLE 26

R_F VALUES OF RARE EARTH METAL IONS

(N. S. POLUEKTOV AND M. P. NIKONOVA, in D. I. RYABCHIKOV (Editor), *Rare-Earth Elements (Extraction, Analysis, Applications)*, Academy of Sciences, U.S.S.R., Moscow, 1959; translated from Russian, published for the National Science Foundation, Washington D.C. and the Department of Commerce by the Israel Program for Scientific Translation, 1960, p. 227)

Solvents: Mixtures of acetone–isobutanol–ether being 0.3 *N* with respect to HNO_3 and equilibrated twice with a saturated solutions of NH_4NO_3 (5 vol. solvent: 1 vol. NH_4NO_3).
Paper: Ashless "white band" chromatographic cardboard dipped into a 20% NH_4NO_3 solution and dried.

Ratio of acetone–iso-butanol–ether	R_F						
	La	*Ce–Sm*	*Gd*	*Y*	*Yb*	*Sc*	*Th*
1:1:1	0.03	0.06–0.12	0.18	0.2	0.28	0.42	0.84
1:2:2	0.01	0.03	0.05	0.09	0.16	0.31	0.76
2:1:1	0.07	0.1	—	0.24	0.37	0.43	0.75

TABLE 27

R_F AND RELATIVE R_F VALUES OF SOME ORGANIC MERCURY COMPOUNDS

(J. N. BARTLETT AND G. W. CURTIS, *Anal. Chem.*, 34 (1962) 80)

Solvent: Butan-1-ol–95% ethanol–28% aq. NH_4OH (8:1:3).
Paper: Whatman No.1 (A = ascending, 12 × 14 cm, no equilibration; D = descending, 17 cm × 54 cm, 4 h equilibration).
Temperature of run: 23° ± 1°.
Length of run: A = 10 cm; D = 40 cm.
Time of run: A = 1 h; D = 13–14 h.
Detection: 0.005% dithizone in $CHCl_3$ spray.

Compound	R_F		R_S*
	A	*D*	*D*
Phenylmercuric chloride	0.38	0.39	—
Tolylmercuric chloride	0.48	0.49	1.22
o-Chloromercuriphenol	0.28	0.22	0.61
p-Chloromercuriphenol	0.07	0.07	0.19
Di-*p*-tolylmercury		0.94	
N-(γ-Carboxymethylmercaptomercuri-β-methoxy)-propylcamphoramic acid disodium salt (Mercaptomerin sodium)	0.16	0.22	0.38
N-(β-Hydroxymercuri-γ-methoxy)-propyl-N′-succinylurea (Mercuhydrin)	0.17	0.15	0.28
o-[(3-Hydroxymercuri-2-methoxypropyl)-carbamyl]-phenoxyacetic acid sodium salt (Mercurital)	0.43	0.41	
Sodium ethylmercurithiosalicylate (Merthiolate)	0.70	0.75	2.02
Salyrgan (= Mercurital)	0.40	0.39	

* R_S = R_F compound/R_F phenylmercuric chloride.

TABLE 28

R_F VALUES OF *cis*- AND *trans*-ACENAPHTHENE-1,2-DIOL

(R. P. HOPKINS, C. J. W. BROOKS AND L. YOUNG, *Biochem. J.*, 82 (1962) 457)

Solvents: S_1 = Methanol.
S_2 = Methanol–carbon tetrachloride (9:1, v/v).
S_3 = Methanol–cyclohexane (9:1, v/v).
Paper: Whatman No. 1 (ascending).
Length of run: 30–35 cm.
Detection: U.V. light after spraying with ethanol–conc. H_2SO_4–water (18:1:1, v/v) and heating (95–100°, 1 min).

Compound	R_F		
	S_1	S_2	S_3
cis-Acenaphthene-1,2-diol	0.64, 0.65	0.70	0.68, 0.72
trans-Acenaphthene-1,2-diol	0.72	0.76, 0.77	0.76, 0.80

TABLE 29

R_F VALUES OF COMPOUNDS SYNTHESISED FROM HYDROGEN CYANIDE IN AQUEOUS AMMONIA

(J. ORÖ AND A. P. KIMBALL, *Arch. Biochem. Biophys.*, 96 (1962) 293)

Solvents: S_1 = Butan-1-ol, saturated with water.
S_2 = Butan-1-ol–acetic acid–water (4:1:1).
S_3 = Butan-1-ol–diethylene glycol–water (4:1:1).
S_4 = Propan-1-ol–*N* NH_4OH (3:1).
S_5 = Phenol saturated with water.
Paper: Whatman No. 3 MM (ascending).
Detection: D_1 = *p*-Dimethylaminobenzaldehyde.
D_2 = Ferrocyanide–nitroprusside reagent.
D_3 = Diazotised *p*-nitraniline.
D_4 = Diazotised sulphanilic acid.
D_5 = Ninhydrin reagent.

Compound	$R_F \times 100$				
	S_1	S_2	S_3	S_4	S_5
Adenine	42	58	54	50	—
4-Aminoimidazole-5-carboxamidine	15	28	44	17	—
4-Aminoimidazole-5-carboxamide	27	50	56	21	—
Glycinamide	20	19	44	40	—
Glycine	10	23	28	23	—
Formamidine	11	43	46	—	—
Formamide	44	61	67	—	—
Alanine	—	37	35	—	58
Aspartic acid	—	23	17	—	9

TABLE 30

R_F VALUES OF SOME PYRIDINE-CARBOXYLIC ACIDS

(I. MORIMOTO AND K. FURUTA, *Anal. Chem.*, 34 (1962) 1033)

Solvent: Butan-1-ol–acetic acid–water (4:1:2, v/v).
Paper: Tôyô Roshi No. 51 (corresponding to Whatman No. 1) (ascending).
Time of run: 10–12 h.
Length of run: 25 cm.
Temperature of run: 23–25°.
Detection: D_1 = New reagent. A = methanol; B = 1% aq.soln. Mohr's salt–10% aq. $NH_2OH \cdot HCl$–pyridine (20:2:1, v/v). Mix A and B (1:1,v/v); spray reagent; pyridine-carboxylic acids (α-CO_2H) give yellow or red spots which fade in 1–3 h. (Detection limit: 0.5–1 μg for picolinic acid and pyridine-2,6-dicarboxylic acid; 1–2 μg for other pyridine-carboxylic acids).
D_2 = 2% ethanolic 2,4-dinitrofluorobenzene spray, heated 15 min at 110°; then sprayed with 5% NaOH (for nicotinic and isonicotinic acid).

Compound	R_F	*Colour**	
		D_1	D_2
Picolinic acid	0.56	Y	
Nicotinic acid	0.73	R-B	+
Isonicotinic acid	0.61	V	+
Pyridine-2,3-dicarboxylic acid	0.46	O	
Pyridine-2,4-dicarboxylic acid	0.54	R-Y	
Pyridine-2,5-dicarboxylic acid	0.53	R-Y	
Pyridine-2,6-dicarboxylic acid	0.43	R-Y	
Pyridine-2,4,5-tricarboxylic acid	0.40	SP	
Pyridine-2,4,6-tricarboxylic acid	0.25	pPu	

* B = brown; O = orange; P = pink; Pu = purple; R = reddish; S = salmon; V = violet; Y = yellow; p = pale.

TABLE 31

R_F VALUES (THIN LAYER) OF ASCORBIGEN AND RELATED COMPOUNDS

(E. PIIRONEN AND A. I. VIRTANEN, *Acta Chem. Scand.*, 16 (1962) 1286)

Solvents: S_1 = Butan-1-ol satd. with water.
S_2 = Benzene–ethanol (2:1).
Thin-layer carrier: Paper (?).
Detection: Ehrlich reagent.

Compound	R_F	
	S_1	S_2
Ascorbigen	0.83	0.59
3-Hydroxymethylindole	0.89	0.67
3,3'-Di-indolylmethane	0.94	0.80

TABLE 32

R_F VALUES OF INOSINE AND DEAMIDO-DIPHOSPHOPYRIDINE NUCLEOTIDE

(J. A. M. WHITAKER AND W. W. UMBREIT, *Arch. Biochem. Biophys.*, 96 (1962) 541)

Solvents: S_1 = Butan-1-ol–acetic acid–water (62.5:15:62.5).
S_2 = Isobutyric acid–acetic acid–water (100:1:50).
S_3 = Propan-1-ol–NH_4OH–water (60:30:10).
S_4 = 95% ethanol–1 *M* ammonium acetate (70:30 v/v) adjusted to pH 5.
S_5 = Propan-2-ol–HCl–water (170:41:39).
S_6 = Propan-2-ol–NH_4OH–water (85:1.3:15).
Paper: Whatman No. 1 (descending: S_1,S_{4-6}; ascending: S_2,S_3).
Detection: U.V. light (absorbing substances).

Compound	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
Inosine	0.35	0.29	0.23	0.61	0.36	0.17
Deamido-DPN	0.11	0	0	0.17	0.11	0

TABLE 33

R_F VALUES (THIN LAYER) OF NUCLEOTIDE DI- AND TRI-PHOSPHATES

(K. RANDERATH, *Nature*, 194 (1962) 768)

Solvent: S_1 = 0.02 *N* HCl.
S_2 = 0.03 *N* HCl.
S_3 = 0.04 *N* HCl.
Thin-layer carrier: DEAE-cellulose (slurry from 10 g powder shaken vigorously for 30–45 sec with 60–70 ml dist. water with detergent prior to spreading. Layers spread as usual and air-dried overnight, covered jar not required).
Time of run: 5–10 min.
Detection: U.V. light (used to follow course of chromatography; final positions of spots traced on "Cellophane").

Compound	R_F		
	S_1	S_2	S_3
ADP	0.48	0.68	
ATP	0.11	0.20	0.56
GDP	0.27	0.51	
GTP	0.07	0.14	0.41
CDP	0.53		
CTP	0.13	0.31	0.64
UDP	0.15	0.25	
UTP	0.04	0.08	0.18

TABLE 34

R_F VALUES OF SOME PYRAZOLO-[3,4-*d*]-PYRIMIDINE DERIVATIVES, RELATED COMPOUNDS AND FUNGAL METABOLIC PRODUCTS

(P. J. CURTIS AND D. R. THOMAS, *Biochem. J.*, 82 (1962) 381)

Solvents: S_1 = Butan-1-ol–aq.NH_4OH (sp.gr. 0.88)–water (4:1:5, by vol.).
S_2 = Butan-1-ol–acetic acid–water (4:1:5, by vol.).
S_3 = Acetone–water (9:1, v/v).
S_4 = Phenol satd. with water.
S_5 = Ethanol.
S_6 = Water.

Paper: Whatman No. 1.

Detection: D_1 = 0.4% Bromophenol Blue in acetone–aq. 2% $AgNO_3$ (1:1); dip; blue spots after water wash; for pyrimidine, thiazole and imidazole derivatives (T. WOOD, *Nature*, 176 (1955) 175).
D_2 = 10% CCl_3COOH spray; heated (105°; 15 min) to hydrolyse; then NH_3 atmosphere followed by D_1; for ribosides.
D_3 = 0.5 *N* HNO_3 spray; then heated (105°); for 5-acetamido-2-oxobenzimidazoline (yellow spot).
D_4 = $Hg(NO_3)_2$ spray; for 5-amino-2-oxobenzimidazoline (E. VISCHER AND E. CHARGAFF, *J. Biol. Chem.*, 168 (1947) 781).
D_5 = Aniline hydrogen phthalate spray reagent; then heated (105°; 10 min) (S. M. PARTRIDGE, *Nature*, 164 (1949) 443).

Compound	R_F*					
	S_1	S_2	S_3	S_4	S_5	S_6
4-Aminopyrazolo-[3,4-*d*]-pyrimidine riboside	0.31	0.42	0.53	—	—	—
4-Hydroxypyrazolo-[3,4-*d*]-pyrimidine riboside	0.10	0.39	0.57	—	—	—
4-Aminopyrazolo-[3,4-*d*]-pyrimidine	0.63	0.55	0.55	—	—	—
4-Hydroxypyrazolo-[3,4-*d*]-pyrimidine	0.23	0.58	0.55	—	—	—
Ribose	0.18	0.31	0.45	0.59	—	—
2-Amino-6-acetamidobenzothiazole	0.73	0.78	—	—	0.90	—
5-Acetamido-1,2-dimethylbenzimidazole	0.79	0.78	—	—	0.7	0.2–0.5
5-Acetamido-2-oxobenzimidazoline	0.64	0.67	—	—	—	0.35
2,6-Diaminobenzothiazole	0.52	—	—	—	—	—
5-Amino-1,2-dimethylbenzimidazole	0.58	—	—	—	—	—
5-Amino-2-oxobenzimidazoline	0.44	—	—	—	—	—

* The R_F values varied considerably between chromatograms but were within 0.02 on a single chromatogram.

TABLE 35

R_F VALUES OF Δ^5-STEROIDS AND Δ^5-STEROID SULPHATES

(L. STÁRKA, J. ŠULCOVÁ AND K. ŠILINK, *Clin. Chim. Acta*, 7 (1962) 309)

Solvents: S_1 = Butan-1-ol–toluene–3 % ammonia (1:1:2) (J. J. SCHNEIDER AND M. L. LEWBART, *Recent Prog. Hormone Res.*, 15 (1959) 201).
S_2 = Bush B5 system (I. E. BUSH, *Biochem. J.*, 50 (1952) 370).
S_3 = Benzene.
S_4 = CCl_4.
S_5 = Petroleum ether.
(S_3–S_5, see L. STÁRKA, *J. Chromatog.*, 4 (1960) 334.)
Paper: Whatman No. 1 (presumed).
Impregnation: I=Triethylene glycol (L. STÁRKA, *loc. cit.*).
Detection: D_1 = $SbCl_3$ reagent ($CHCl_3$ satd. with $SbCl_3$–acetic anhydride (4:1)) at 90° for 2–3 min.
D_2 = Zimmermann reagent.

Compound	R_F					*Colour**	
	S_1	S_2	S_3I	S_4I	S_5I	D_1	D_2
Dehydroepiandrosterone sulphate	0.77					R	+
7-Hydroxydehydroepiandrosterone sulphate	0.30					B	+
7-Hydroxydehydroepiandrosterone disulphate	0.12					B	+
7-Oxodehydroepiandrosterone sulphate	0.59					—	+
7-Hydroxydehydroepiandrosterone sulphate (from urine)	0.30					B	+
Δ^5-Androsten-3β-ol-17-one		0.93	0.66	0.62	0.22		
Δ^5-Androstene-3β,7α-diol-17-one		0.42	0.10	0.04	0.01		
Δ^5-Androsten-3β-ol-7,17-dione			0.36	0.12	0.05		
Δ^5-Adrostene-3β,7α,17β-triol		0.08	0.02	0.00	0.00		
$\Delta^{5,7}$-Androstadien-3β-ol-17-one		0.85	0.61	0.52	0.16		

* B = blue; R = red; + = positive; — = negative

TABLE 36

ELECTROPHORETIC MOBILITIES OF SOME Δ^5-STEROID SULPHATES

(L. STÁRKA, J. ŠULCOVÁ AND K. ŠILINK, *Clin. Chim. Acta*, 7 (1962) 309)

Electrolyte: 0.1 *N* veronal buffer, pH 9.2.
Paper: Whatman No. 1.
Potential: 20 V/cm.
Time of run: 2 h.
Mobility: *M* = Mobility relative to *p*-nitrophenol (= 100).
Detection: D_1 = $SbCl_3$ reagent ($CHCl_3$ satd. with $SbCl_3$–acetic anhydride (4:1) at 90° for 2–3 min.
D_2 = Zimmermann reagent.

Compound	*M*	*Colour**	
		D_1	D_2
Dehydroepiandrosterone sulphate	60	R	+
7-Hydroxydehydroepiandrosterone sulphate	62	B	+
7-Hydroxydehydroepiandrosterone disulphate	96	B	+
7-Oxodehydroepiandrosterone sulphate	61	—	+
7-Hydroxydehydroepiandrosterone sulphate (from urine)	63	B	+

* B = blue; B = red; + = positive; — = negative.

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