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INFRARED MICROSPECTROMETRY OF CAPILLARY GAS CHROMATOGRAPHY EFFLUENTS AND OF GAS-PHASE FREE RADICALS

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The most useful single method of analysis of a volatile covalent substance is by infrared spectrometry. Due to the weakness of the interaction between molecules and infrared quanta, however, the absorption of a measurable fraction of the incident radiation requires sizable samples[1,2]. Samples as small as the vapor bands eluted from capillary columns, or free radicals produced in gases, could, however, be monitored by a microspectrometer which obtains their infrared spectra instead by measuring the effect of infrared radiation on molecular beams of such samples.

DIFFERENTIAL INFRARED MICROSPECTROMETER

A molecular-beam electric-resonance apparatus permits the observation of rotational spectra of polar molecules[3–8]. The spectrum is obtained by plotting the wavelength of the microwave radiation against the resulting molecular-beam intensity at the detector. The substitution of infrared radiation should likewise yield rotation–vibration spectra, since, according to the selection rules, the capture of vibrational energy by a molecule is likely to be accompanied by a rotational transition. A differential infrared microspectrometer, based on this principle, has been discussed[9]. Its sensitivity is limited by the precision with which the intensity of the molecular beam can be measured and by the intensity of the available infrared radiation. An electron bombardment detector can measure an attenuation of 10^{-4} of a molecular beam[10]. Very intense infrared radiation of continuously variable wavelength is emitted continuously by junction diode lasers. Alternatively, thermal radiation combined with a monochromator can be used; the following calculation offers an example involving a minimum emittance required from the radiant heater.

MATHEMATICAL TREATMENT OF INFRARED ABSORPTION BY A BEAM OF HCl MOLECULES

For HCl, the absorption intensities of the separate lines of the fundamental infrared band have been determined experimentally[11]. The following mathematical treatment, which is due to one of us (H.R.), is restricted to the case of diatomic molecules such as HCl.

After the molecular beam passed through the velocity selector and the rotational state selector, all the HCl molecules remaining in it are assumed to have a

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velocity w and to be in the quantum state with vibrational number $v'' = 0$ and rotational number $J'' = 1$. If infrared radiation from a thermal source is perpendicularly directed at a longitudinal length l of the HCl beam, then the exposure time (τ) of the molecules is given by $\tau = w/l$. Let the wave numbers of the allowed transitions from $v'' = 0$; $J'' = 1$ to $v' = 1$; $J' = 2$ and to $v' = 1$; $J' = 0$ be $\nu_{1,2}$ and $\nu_{1,0}$, respectively. The mean absorption cross-sections $\bar{\sigma}_{1,2}(\Delta\nu)$ and $\bar{\sigma}_{1,0}(\Delta\nu)$ for these transitions are assumed to be constant over an arbitrary width $\Delta\nu$ around both $\nu_{1,2}$ and $\nu_{1,0}$. If $Z(\nu_{1,2}; \Delta\nu)$ and $Z(\nu_{1,0}; \Delta\nu)$ are defined as the numbers of photons, within the same widths $\Delta\nu$ around $\nu_{1,2}$ and $\nu_{1,0}$, which traverse the length l of the molecular beam during the time τ, then the fraction $\Delta n/n$ of irradiated molecules which experience vibrational and thus rotational transitions is:

$$\Delta n/n = Z(\nu_{1,2}; \Delta\nu)\cdot\bar{\sigma}_{1,2}(\Delta\nu) + Z(\nu_{1,0}; \Delta\nu)\cdot\bar{\sigma}_{1,0}(\Delta\nu) \quad (1)$$

Calculation of the numbers of photons $Z(\nu_i; \Delta\nu)$

The following arbitrary numerical values meet the minimum radiation requirement of the differential infrared microspectrometer. Thermal radiation corresponding to the emittance of a surface area of 1 cm² of a black body at an absolute temperature T is focused on a 2-cm long section of the molecular beam. For HCl molecules with a velocity $w = 500$ m/sec, the exposure time then is $\tau = 4 \times 10^{-5}$ sec. The numbers of photons $Z(\nu_i; \Delta\nu)$ within a width $\Delta\nu = 0.1$ cm^{-1} by which the molecules are thus irradiated during the exposure time τ can be evaluated by Planck's radiation laws. Some numerical values resulting for these conditions are listed and specified in Table I.

TABLE I

NUMBERS OF PHOTONS $Z(\nu_i; \Delta\nu)$

Radiation		*Black body temperature (°K)*		
Wave length (μ)	*Wave number* (cm^{-1})	*2000*	*3000*	*4000*
3	3333	$92\cdot10^{10}$	$235\cdot10^{10}$	$480\cdot10^{10}$
5	2000	$93\cdot10^{10}$	$186\cdot10^{10}$	$285\cdot10^{10}$
10	1000	$71\cdot10^{10}$	$122\cdot10^{10}$	$174\cdot10^{10}$

Derivation of the absorption cross-sections $\bar{\sigma}_{1,2}(\Delta\nu)$

Let $I^\circ(\nu_{1,2})$ and $I(\nu_{1,2})$ be the radiation intensities at wave number $\nu_{1,2}$ before and after traversing a layer of x centimeters of the absorbing gas. In this gas, let the number of molecules in the quantum state $v'' = 0$; $J'' = 1$ be N_1 per cm³. Then the absorption cross-section $\sigma_{1,2}$ for the transition from $v'' = 0$; $J'' = 1$ to $v' = 1$; $J' = 2$ is defined by:

$$I(\nu) = I^\circ(\nu)\cdot\exp(-k_\nu\cdot x) = I^\circ(\nu)\cdot\exp(-\sigma_{1,2}\cdot N_1\cdot x) \quad (2)$$

where k_ν is the absorption coefficient. The fraction N_1/N, where N is the total number of molecules per cm³, is given by the Boltzmann-distribution expression. The ex-

perimentally determined quantity is the integral of the absorption coefficient ($S_{1,2}$), which is given by:

$$S_{1,2} = \int_0^\infty k_\nu \cdot d\nu = N_1 \int_0^\infty \sigma_{1,2} \cdot d\nu \tag{3}$$

for the single rotational line of the fundamental band of $H^{35}Cl$ corresponding to this transition. $S_{1,2}$ is proportional to N, so that $S_{1,2} = S^\circ_{1,2} \cdot N$. For $H^{35}Cl$ the experimental values are $S^\circ_{1,2} = 24 \cdot 10^{-20}$ and $S^\circ_{1,0} = 32 \cdot 10^{-20}$ cm/molecule. Introducing the mean absorption cross-section over the width $\Delta\nu$ by the relation:

$$\bar{\sigma}_{1,2}(\Delta\nu) \cdot \Delta\nu = \int_0^\infty \sigma_{1,2} \cdot d\nu \tag{4}$$

one obtains:

$$\bar{\sigma}_{1,2}(\Delta\nu) = \frac{S^\circ_{1,2} \cdot N}{\Delta\nu \cdot N_1} \tag{5}$$

For $J'' = 1$ and $T = 300°K$, $N/N_1 = 10$. Substituting expression (5) in equation (1), it follows that:

$$\Delta n/n = Z(\nu;\Delta\nu) \cdot \frac{N}{N_1} \cdot \frac{(S^\circ_{1,2} + S^\circ_{1,0})}{\Delta\nu} \tag{6}$$

Selecting the value of $Z(\nu;\Delta\nu)$ for $T = 3000°K$ and $\nu = 3333$ cm^{-1} in Table I, the attenuation of the molecular beam becomes:

$$\Delta n/n = 235 \times 10^{10} \times \frac{10}{1} \times \frac{(24 + 32) \times 10^{-20}}{0.1} = 1.3 \times 10^{-4}$$

to which the detector still responds.

INTEGRAL INFRARED MICROSPECTROMETER

Infrared spectra could be obtained with infrared radiation of only moderate intensity by a molecular-beam electric-resonance apparatus using a specialized design of the rotational state selector which is analogous to the Toepler arrangement for schlieren optics[12]. This selector, which is partly shown in Fig. 1, renders an additional rotational state analyzer (as in the differential microspectrometer) unnecessary.

This more sensitive version of the microspectrometer will now be described in some detail. Its major components are the source, the velocity selector, the rotational state selector, and the detector.

The construction of the source of the molecular beam depends on whether stable molecules or free radicals are to be analyzed. For capillary gas chromatography, a bundle of parallel, closely-spaced source canals is employed to conserve material by producing a narrow shower of weak molecular beams. The vapor bands must be eluted at low pressure, so that the mean free path of the molecules exceeds

the length of the canals. For free radicals continuously produced in gases, a source aperture of nearly negligible width, followed by a collimating aperture, is adequate. The resulting beam of molecules and free radicals is passed through an inhomogeneous magnetic field which separates them by deflecting only the latter.

The mechanical velocity selector serves to prevent slow molecules with a high rotational quantum number from overlapping with fast molecules with a low rotational quantum number, which would experience the same deflection in the rotational state selector. In the case of gas chromatography, it also retains the macromolecular vapors from the stationary phase and facilitates selective evacuation of the molecular-beam apparatus by scattering the atoms of the helium used as carrier gas. In the microspectrometer, the mechanical velocity selector must be rapidly adjustable over a velocity range, and hence low inertia and little precision are required. A simpler device than the conventional Fizeau velocity selector, which chops the molecular beam by nine parallel slotted wheels rotating perpendicularly to it, should be used. A satisfactory design could be based on a paddle-wheel, installed between two collimating apertures, whose blades move tangentially in the direction of the molecular beam.

In the electrostatic rotational state selector an intense inhomogeneous electric field with a gradient perpendicular to the molecular beam deflects polar molecules. An asymmetric pair of electrodes suffices to produce a field with which molecules in one particular rotational state can be selected[3,4]. It was found advantageous to add to this field a focusing effect, similar to that of an electrostatic lens, by using electrodes consisting of four parallel metal rods arranged in a square and charged alternately positive and negative[5–8]. The resulting electric quadrupole field is weak near the axis and becomes stronger towards the electrodes outside.

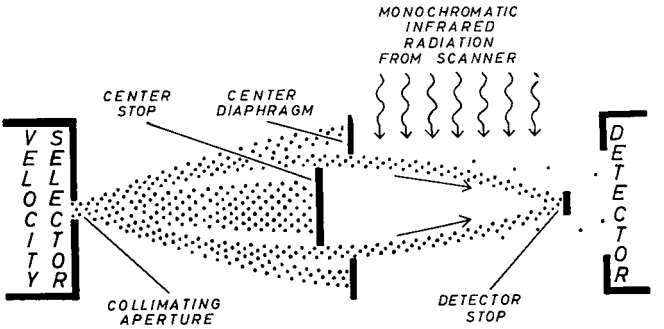

Fig. 1. Illustration of the paths followed by the molecular beam within the rotational state selector of the integral microspectrometer.

Fig. 1 illustrates the effect of the rotational state selector on the molecular beam, which in this case is defined by the collimating aperture together with the stop and the diaphragm that are placed at the center. The four electrodes (not shown in Fig. 1) are kept at such a low electric potential that only molecules in the lowest rotational state are focused; they follow a sigmoid path around the center-stop and fall on the stop placed before the detector. The inhomogeneous electric field acts only on molecules which traverse its gradient by entering it at an aperture angle large enough to prevent their collision with the center-stop. Molecules in higher rotational

states are not deflected sufficiently to avoid colliding with the center-diaphragm. The entire chamber containing this selector and a detector is cooled by liquid nitrogen which prevents the flow of less volatile molecules scattered from the beam and provides additional evacuation by cryogenic pumping.

The molecules in the segment of the molecular beam behind the center-stop and diaphragm do not reach the detector because they impinge on the detector-stop which retains them. This segment of the beam is irradiated by spectrally resolved radiation from a fast-scan infrared source. Molecules which capture photons, experiencing transitions in rotational state and moment of inertia, are not converged on the detector-stop and thus can reach the detector. The plot of wavelength of the infrared radiation *versus* detected molecular-beam intensity gives the spectrum of the beam material.

The combination of velocity selector and rotational state selector will resolve, without preliminary separation, only simple mixtures of free radicals.

The molecular-beam detector must be extremely sensitive to the small fraction of the beam which is analyzed, without responding to residual gases. Usually an electron bombardement detector is employed. It consists of an electron ionizer (adjustable so as not to ionize helium), a simple mass spectrometer such as a quadrupole mass filter[14,13], and finally an electron multiplier which counts positive ions. Its sensitivity is limited by the difficulty of removing positive ions from the electron space charge.

In a photoionization detector as used in gas chromatography[15], molecular beams could be ionized by ultraviolet radiation of very short wavelength without a space charge being created.

A fluorescence detector for beams of molecules absorbing in the near ultraviolet region could be made selective enough to dispense with a mass spectrometer. The molecular beam is irradiated by a collimated beam of ultraviolet radiation and the photons re-emitted in different directions are measured. In a collision-free beam, electronically excited molecules can dissipate their energy only by radiative processes. The residual gas is not excited when the radiation from a discharge lamp is filtered through a cell containing a gas mixture of the same composition.

A proportional detector for beams of not too small organic molecules could be constructed by combining pyrolysis and field ionization as follows. Organic vapors are cracked on tungsten filaments electrically heated to 1500°*K*, giving free radicals which are ionized (with extremely low efficiency) by the tungsten surface[16]. By field ionization, organic vapors are quantitatively ionized on the surface of tungsten wires with a diameter of 2.5 microns charged with a positive potential of 15000 V (ref. 17). The detector should hence consist of a network of parallel tungsten filaments, with diameter, temperature and potential as described, together with an electron multiplier. When the molecular beam falls on the incandescent charged tungsten filaments, each organic molecule will give rise to a burst of singly charged fragments. The resulting large pulses of the electron multiplier could easily be distinguished from those due to the residual gas.

The sensitivity of the integral infrared microspectrometer, unlike that of the differential version, depends not only on the intensity of the radiation but also on the intensity of the irradiated segment of the molecular beam. The latter can be evaluated by supposing that at a length of 50 cm from the source, for both selectors, a

molecular beam of $7 \cdot 10^{10}$ molecules/mm^2/sec contains $5 \cdot 10^{-4}$ molecules in the lowest quantum state which are converged 300-fold by the quadrupole field. If 1 % of the sample consists of the molecules or free radicals to be analyzed, some 10^8 of them per sec will be focused on a detector-stop with a surface area of 1 mm^2. Under the conditions of the above mathematical treatment of a beam of HCl molecules, 10^{-4} of them would capture photons, leaving a beam of 10^4 molecules or free radicals per sec to reach the detector. An electron bombardment detector can measure a molecular beam with a sensitivity of 2.5 % of the molecules[18]. In the chamber cooled by liquid nitrogen, which is evacuated by a mercury diffusion pump, there will be no interfering background of organic molecules.

Up to here it was assumed that the rotational state of a molecule which captures an infrared quantum is changed, permanently. However, a transition such as from $v'' = 0; J'' = 1$ to $v' = 1; J' = 1$ involves a change, not in rotational state but in moment of inertia of the molecule, which will thus also be discriminated by the molecular-beam electric-resonance apparatus. The lifetimes of most of the excited molecules suffice for their discrimination, and only a part of those molecules which reemit their vibrational energy too early will return to their original rotational state.

The theory of electrostatic focusing of polar molecules has only been worked out in detail for diatomic molecules. Polyatomic molecules are also deflected by an inhomogeneous electric field, but their rotational states lie much closer together. The selector will hence converge on the detector-stop polyatomic molecules in the lowest group of rotational states which will overlap due to the comparatively low resolution attainable at present.

APPENDIX: DEFINITION AND UNITS OF SYMBOLS

$I^\circ(\nu)$, $I(\nu)$ = radiation intensities at wave number ν before and after traversing a layer x of absorbing gas, respectively, arbitrary units

k_ν = absorption coefficient at wave number ν, cm^{-1}

l = length of molecular beam which is irradiated, cm

N = number of molecules per unit volume, cm^{-3}

N_1 = number of molecules per unit volume, which are in a specified quantum state, cm^{-3}

n = number of molecules irradiated by the $Z(\nu; \Delta\nu)$ photons, no units

Δn = number of molecules which experience rotational transitions, no units

$S_{1,2}$ = integral over absorption coefficient for rotational line corresponding to the transition $J'' = 1 \rightarrow J' = 2$ as defined by equation 3, cm^{-2}

$S^\circ_{1,2}$ = $S_{1,2}$ per number of molecules per unit volume N, cm

T = absolute temperature, °K

$v''; J''$ = vibrational and rotational quantum number in the lower energy level, no units

$v'; J'$ = the same quantum numbers in the upper energy level, no units

w = velocity of molecules in the beam, cm $\cdot$ sec^{-1}

x = thickness of the layer of irradiated absorbing gas, cm

$Z(\nu; \Delta\nu)$ = number of photons emitted per cm^2 of black body within a width $\Delta\nu$ around ν in time τ, no units

μ = wavelength, microns

ν = wave number, with subscripts referring to rotational quantum numbers, cm^{-1}

$\Delta\nu$ = arbitrary narrow width around the wave number ν, cm^{-1}

σ = absorption cross-section, with subscripts referring to rotational quantum numbers, cm^2

τ = time necessary for molecules to travel through the length l of the beam, sec

SUMMARY

Infrared spectra of samples, too small for conventional spectrometry which measures the effect of the sample on radiation, could instead be obtained by measuring the effect of radiation on the sample. The sample is passed through a proposed infrared microspectrometer in which it is converted into a molecular beam and irradiated. The sensitivity of the microspectrometer depends on the intensity of the available infrared radiation and on the intensity of the detected molecular-beam. The effect of the radiation intensity is calculated for one example, and new detectors for beams of organic molecules are suggested.

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STUDIES ON THE FLAME IONIZATION DETECTOR SIGNAL IN THE PRESENCE OF ORGANOSILICON COMPOUNDS

A METHOD FOR THE REGISTRATION OF THE DETECTOR PERFORMANCE

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INTRODUCTION

An outline has been given, in an earlier communication from this laboratory[1], of the observations made on the anomalous behaviour of the flame ionization detector (FID) in the presence of organometallic compounds. It was found that, under conditions which gave normal or even ideal detector performance for organic compounds, inversion of the gas chromatographic peaks occurred with organosilicon, organogermanium and organotin compounds. This phenomenon was observed with both types of detectors used in the experiments. The experiments reported in this paper were restricted to organosilicon compounds.

According to our experience, the inversion of peaks had no relation to the slight deposition of SiO_2 on the electrodes.

The inversion of the peak, *i.e.* the decrease of the ion current at increasing mass flow rates takes place when the silicon mass flow rate exceeds a critical value in the flame. On the basis of our previous experiments it has been stated that the critical values of the silicon mass flow rate, as well as the maximum ion current, depend on the following factors: detector geometry, detector voltage, the flow rates of the carrier-, fuel- and scavenging gases and the actual carbon mass flow rate in the flame, the extent of which is determined by the composition of the organosilicon compound tested. Except this last factor, these are the same as those which influence the sensitivity and the linearity range of an FID when measuring organic compounds. All experimental conditions being constant, the maximum height of the inverted peak depends only on the carbon/silicon molar ratio of the test substance.

In our previous communication[1], the application of an FID operating with a mixed hydrocarbon–hydrogen flame was also described. This arrangement detects organometallic or other volatile metallic compounds by a decrease of the ion current.

Following these observations, a more detailed investigation of the peak inversion seemed to be reasonable. A number of experiments was needed to afford the possibility of making a comparison between detector signal produced by organic and organosilicon compounds under different conditions.

Plotting of response curves, *i.e.* detector signal *vs.* mass flow rate, was chosen as the basis of the comparative examinations, as described by BRUDERECK *et al.*[2]. Con-

sequently the following terms will be used in the subsequent discussion for characterising the performance of the FID:

The response factor (r), the proportionality factor in the equation $I = r \cdot g(C) \cdot t^{-1}$. Since the numerical value of "r" is equal to the ion current (I) produced by the unit of carbon mass flow rate, MFC [$g(C) \cdot t^{-1}$] in the flame, it is a measure of the detector sensitivity. For convenience, "r" is usually expressed in millicoulombs/gram carbon.

The linearity range is defined by the constancy of the response factor. The value of the ion current and the carbon mass flow rate at the upper limit of the linearity range are designated as "I_{linear}" and "MFC_{linear}", respectively.

It is obvious from the foregoing discussion that under those circumstances when peak inversion takes place, the response curves display a maximum. For this reason, further characteristics of the detector performance had to be introduced in the case of organosilicon compounds: the value of the ordinate at the maximum of the curve is called the "*limiting ion current*" (I_{lim}), while the abscissa, called the "*limiting mass flow rate*", represents the mass flow rate at which the inversion of the peak takes place. This may be expressed either in terms of carbon mass flow rate (MFC_{lim}) or in silicon mass flow rate ($MFSi_{lim}$).

The dependence of these parameters on the operating variables of the FID had to be subjected to systematic investigation in order to explain the anomalies observed in the presence of organometallic compounds.

A method is presented in the first part of this paper, by means of which a record of the response curves for different substances may be obtained from a single chromatogram with great relative accuracy. In the second part a discussion of some experimental results illustrates the efficiency of this method.

PRINCIPLES OF THE AUTOMATIC RECORDING OF RESPONSE CURVES

The usual way of determining response characteristics of a mass flow rate sensitive detector is to measure the ion current at different known levels of continuous mass flow through the detector[3]. However, the application of a continuous mass flow proved to be impracticable in the case of organosilicon compounds, in consequence of the deposition of a considerable quantity of SiO_2 on the electrodes.

In the experiments already reported[1], the response curves were obtained by recording series of chromatograms. Increasing quantities of a mixture of known composition were injected and the values of the ion current at the peak maxima were plotted against the corresponding mass flow rates, calculated as follows.

At a first approach, the peak was assumed to be a triangle, the height of which—representing the maximum mass flow rate (MF_{max})—may be expressed by the equation:

$$MF_{max} = \frac{2\,G}{t}$$

If G represents the weight of the sample in micrograms and t the peak width at the base expressed in seconds, then MF_{max}, the mass flow rate at the peak maximum is obtained in microgram/seconds. As second approach, the MF_{max} values were then multiplied by a correction factor of 0.81, originating from the assumed Gaussian shape. The MFC terms used were derived from these corrected MF_{max} values.

This method was rather time-consuming and involved some uncertainties, partly arising from the limited accuracy of determining the sample quantity, but mainly from the distortion of the peaks, occurring with large sample quantities. This factor was rather considerable in the case of inverted peaks, so that in the very region of the anomalous responses the response curves became unreliable. The slight deviation of the shape of the peak from a Gaussian one, occurring at low sample quantities, may be regarded as the reason for uncertainties in determining MFC values in the linear range. The accuracy of the "r" values derived from these MFC values was ± 2.5 % when introducing small samples in the column.

In addition, the situation of the maximum of the response curves could only be determined by a fairly inaccurate graphical method as a consequence of the discontinuous method in plotting the curves.

The principles of the new method for the recording of the response curves may be summarized as follows.

A chromatographic peak is essentially a continuous recording of the ion current values, pertaining to mass flow rates varying from zero to a maximum value, defined by the quantity of the sample and the width of the chromatographic band. If the momentary values of the mass flow rate were known at any moment during the elution of the band, it would be possible to plot the response curve on the basis of a single chromatographic peak. Having, however, a detector which is supposed to function linearly throughout the whole range of mass flow rates occurring in the peak, the momentary values of the mass flow rates could be calculated at any point of the peak from the detector signal and the characteristic "r" value of the detector.

Providing that a perfectly synchronous change of the mass flow rate can be obtained in two FID-s with different performances, one of them being a reference detector (FID_{RX}) with constant response, and the other one the detector to be investigated (FID_{IY}), the signal of the latter may be recorded as a function of the reference detector signal by means of an X–Y recorder, the two channels of which are controlled by the signals of the FID_{RX} and the FID_{IY}, respectively.

The abscissa of the X–Y diagram thus obtained represents the ion current of the FID_{RX}. If the "r" factor in the FID_{RX} is known from independent measurements, the ion current data on the abscissa may be converted into mass flow rate values, and the diagram will represent the response curve of the FID_{IY}. In fact, the method employed earlier and already reported was the only way to derive the "r" factors needed.

APPARATUS AND TECHNIQUE

The experiments were carried out with a Carlo Erba Fractovap C type PAID/ff dual column gas chromatograph equipped with two flame ionization detectors. Some modification of the apparatus was needed for the special purpose of the present investigation.

The original detectors were of the parallel electrode type, connected with opposite polarity to the PAID/ff electrometer amplifier, in order to give difference signals. One of them was replaced by a detector in which the jet was functioning as anode, the cathode being a platinum cylinder of 8 mm diameter and 10 mm height. This arrangement served as reference detector. In the other detector the electrodes

were parallel platinum plates, situated laterally to the flame. Variation of the electrode gap was afforded by means of screws. This detector was disconnected from the circuit of the PAID/ff instrument and operated independently, in connection with a Vibron electrometer. The two channels of a Philips PR 2220 A/00 X–Y recorder were controlled by the signals of the PAID/ff electrometer and by the Vibron electrometer, respectively. In addition, the detector signals were fed also into two Speedomax G recorders, so the chromatograms and their X–Y diagram were recorded at the same time. The Vibron electrometer used for measuring the ion current of the FID_{IY} was supplemented with the necessary attenuating and compensating circuits in order to equalize the measuring ranges of the EA 1 and EA 2 electrometers. Thus it was possible to set the scales equal on both ordinates of the X–Y recorder. The schematic circuit diagram is shown in Fig. 1.

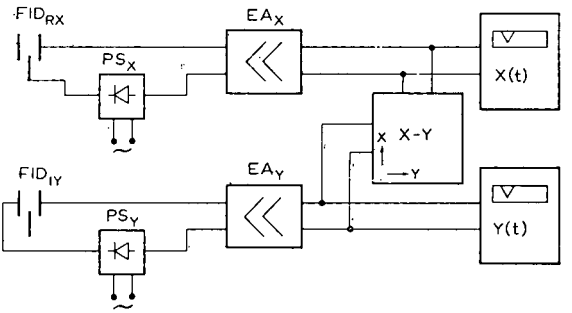

Fig. 1. Schematic circuit diagram. FID_{RX} = Reference detector (jet-electrode type); FID_{IY} = detector investigated (parallel-electrode type); PS_X = power supply of the Carlo Erba Chromatograph, 150 V; PS_Y = power supply, Solartron SRS 151 A, 20–500 V stab.; EA_X = Carlo Erba Electrometer Amplifier $5 \cdot 10^{-12}$–10^{-7} A.f.s.; EA_Y = Electrometer Amplifier Vibron Mod. 33C; A 33C 10^{-12}–10^{-7} A.f.s.; X(t) Y(t) Speedomax G Recorders 1 mV f.s.; 1 sec. X–Y: X–Y Recorder Philips PR 2220 A/00.

In order to obtain a synchronous change of mass flow rates in the two detectors, a single chromatographic column was used for the separation. The column effluent was divided into two equal streams. These were conducted through capillary resistances to the detectors. Uncoated stainless steel capillaries of about 50 cm length and 0.1 mm diam. were used for this purpose. The length of the capillaries was adjusted so that the dead volumes and flow resistances between the junction point and the burner jet were the same in both branches. Fig. 2 shows the schematic flow diagram of the system.

There were two main points which had to be ensured before starting the experiments: (1) the synchronisation of the mass flow rates in the two detectors, and (2) the linear performances of the FID_{RX}.

In order to verify that the mass flow rate of the sample eluted from the column was varying synchronously in both branches, the detectors were connected with opposite polarity to the same electrometer amplifier circuit and the difference of the two signals was recorded. The length of the capillary resistances was altered until a small symmetrical peak was obtained as difference-signal. If mass flow rates varied asynchronously in the two detectors, asymmetrical difference signals were recorded as shown in Fig. 3a–d. The small symmetrical peak shown in Fig. 3e indicates the

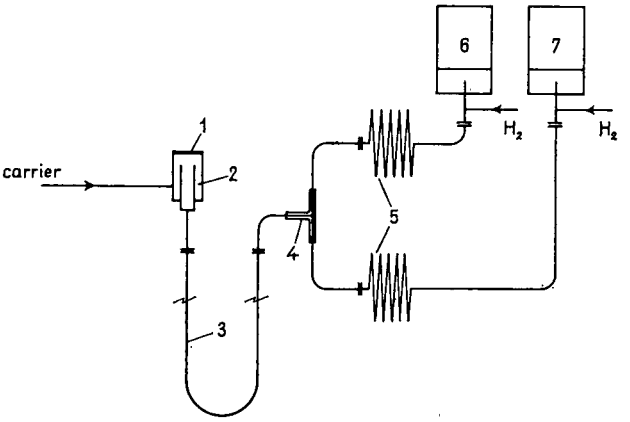

Fig. 2. Schematic flow diagram. 1 = Injection port; 2 = evaporator; 3 = chromatographic column; 4 = capillary splitter; 5 = capillary resistances; 6 = reference detector FID_{RX}; 7 = detector investigated FID_{IY}.

synchronous mass flow in two detectors having slightly different sensitivities. In the case of two detectors of the same sensitivity, the difference signal would be zero, of course. Another possibility was also furnished in the course of the experiments for controlling the synchronism of the mass flow rates; under these conditions, where both of the detectors were functioning linearly, the response curves recorded were straight lines starting from the origin.

The geometric features and other operating variables of the FID_{RX} were arranged so as to ensure its linear functioning up to the highest possible mass flow rates. For each component of the mixture used in these experiments the response curves of the FID_{RX} were plotted by means of the method described earlier. These curves proved to be linear up to mass flow rates as high as $1.3–1.5 \cdot 10^{-6}$ g C/sec for each component. Above these MFC values, a unidirectional deviation exceeding 2.5 % of the "r" factors was noticed. As the linear performance of the FID_{RX} is a requirement of great importance, the mass flow rates were always kept far below the linearity limit during the runs. 1.0 μl and in some cases 1.2 μl, quantities of the mixture tested were introduced in practice to ensure that the MFC values occurring in the flame should not exceed $1.0 \cdot 10^{-6}$ g C/sec.

In order to get information on the effect of the C/Si ratio of the sample detected, the mixture was made up in such a way as to contain several organosilicon compounds

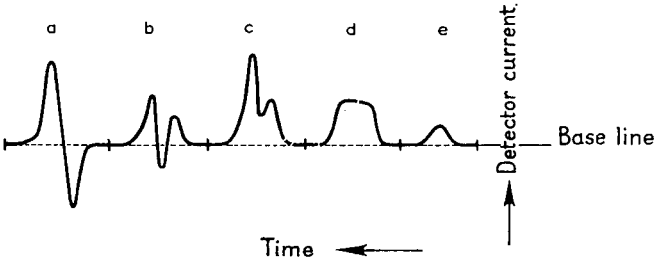

Fig. 3. Difference chromatograms of *n*-hexane.

of different C/Si ratio, and in addition a hydrocarbon, at the same time taking into account the requirement of a good separation. The components of the mixture are listed in Table I.

TABLE I

COMPOSITION OF TEST MIXTURE CONTAINING ORGANOSILICON COMPOUNDS

Number	*Compound*	*Concentration %*	*C/Si molar ratio*
1	Benzene	60.5	—
2	Trimethyl-butenylsilane	3.9	7
3	Hexamethyl-cyclotrisiloxane	13.7	2
4	Divinyl-tetramethyldisiloxane	11.4	4
5	*o*-Xylene	10.5	—

The numbers in Figs. 4–8 refer to the same compounds as in Table I. Benzene served only for the dilution of the mixture to the required extent, therefore neither the peaks on the chromatograms, nor the response curves referring to benzene were recorded.

The mixture was separated on a chromatographic column of 3 m length and 2 mm I.D., containing 6 % of SE-30 silicon elastomer on Chromosorb W, thermostated to 56°. Nitrogen was used as carrier gas, at a flow rate of 19.2 ml/min. The electrodes of both detectors were cleaned with hydrogen fluoride solution after every 2 or 3 runs, in order to minimize the effect of the slight deposition of SiO_2. The operating variables of the FID_{RX} were as follows:

Carrier gas: 9.6 ml/min,
Hydrogen: 19.5 ml/min,
Oxygen: 200 ml/min,
Detector voltage: 150 V.

Response factors of the FID_{RX} for the compounds used were calculated from series of chromatograms by the method described previously, and are listed in Table II.

As can be seen from the data in Table II, there is a pronounced difference between the individual response values. This fact has been taken into account when calculating the MFC values of the components in FID_{IY} from the ion current measured in the FID_{RX}.

TABLE II

RESPONSE FACTORS OF THE FID_{RX}

Compound	*"r" (mC per g carbon)*
Hexamethyl-cyclotrisiloxane	50.6
Xylene	47.0
Divinyl-tetramethyldisiloxane	47.5
Trimethyl-butenylsilane	46.4

The conversion of the FID_{RX} signal into MFC values, is based on "r" factors computed by the method used in our previous work. Consequently, the absolute values of carbon mass flow rates attributable to the abscissa of the X–Y diagrams as well as the "r" factors of the FID_{IY} may be assumed to have an error of $\pm 2.5\,\%$, this also being the accuracy in estimating the "r" factors of the FID_{RX}, when operating linearly. If, however, constancy of the reference detector response and synchronism of the mass flow rates in the detectors, are assured, then the distances on the abscissa of the X–Y diagram are exactly proportional to the mass flow rates, even in the non-linear region of the FID_{IY} response curve where the mass flow rates could not have been computed otherwise, owing to the inversion of the peaks. Consequently, the shape of the response curves recorded continuously is always correct, *i.e.* its relative accuracy is no longer charged with the uncertainties arising from the method in calculating FID_{RX} "r" factors.

During these experiments, the operating conditions of the FID_{RX} were kept constant, while those of the FID_{IY} were varied. Thus the influence of the operating variables on the characteristic parameters of the detector performance—I_{lim}, $MFCi_{lim}$, $MFSi_{lim}$ and "r"—could always be related to a fixed MFC scale on the abscissa. In this sense, the relative accuracy of these data was limited only by the precision of the electric equipment, which means 0.5 %.

This great relative accuracy does not refer, of course, to the comparison of the components in a chromatogram, considering that the individual "r" factors measured in FID_{RX} do not bear equally the uncertainties involved in the method of determination.

An indisputable advantage of the method is that the uncertainties arising from the graphical determination of the maximum on the plotted response curves are eliminated by the continuous recording.

TABLE III

CHARACTERISTIC DATA OF THE RESPONSE CURVES, OBTAINED AT VARIOUS OPERATING VARIABLES OF THE FID_{IY}

No.	*Detector voltage*	*% H_2*	*Electrode gap (mm)*	*Xylene*			*Hexamethyl-cyclotrisiloxane*			
				r (mcoul./gC)	*I linear ($mA \times 10^{-5}$)*	*MFC linear ($gC/sec \times 10^{-6}$)*	*r*	*I_{lim}*	*MFC'_{lim}*	*$MFSi'_{lim}$*
4	60	62	4.5	34.8	0.12	0.04	40.7	0.185	0.052	0.061
7	100	62	4.5	35.3	0.67	0.17	41.0	0.434	0.124	0.145
5	150	62	4.5	34.7	1.3	0.42	41.2	0.65	0.15	0.175
6	220	62	4.5	35.0	2.7	0.7	40.5	1.35	0.30	0.351
16	400	62	4.5	39.0	*	—	43.0	*	—	—
10	150	54	4.5	21.6	1.18	0.48	27.6	0.485	0.175	0.204
5	150	62	4.5	34.7	1.30	0.42	41.6	0.65	0.15	0.175
14	150	65.6	4.5	38.6	1.81	0.46	43.7	1.07	0.224	0.262
8	150	70	4.5	42.9	2.19	0.48	46.9	1.34	0.284	0.332
17	150	65.6	5.5	36.9	1.23	0.33	41.3	0.78	0.116	0.194
18	150	70.7	5.5	42.2	1.35	0.32	46.2	1.09	0.198	0.232
19	150	73.2	5.5	43.4	1.46	0.32	50.1	1.39	0.236	0.276

* Not reached under the experimental conditions.

The application of this procedure is not limited, of course, to the study of the anomalous behaviour of the FID. With an appropriate selection of the reference detector it provides a quick and reliable method for studying the sensitivity, linearity and the optimal operating conditions of a gas chromatographic detector of any kind.

EXPERIMENTAL RESULTS

Response curves of the parallel-electrode type detector were recorded in three series:

(1) The detector voltage was varied with constant electrode gap and constant gas flow rates.

(2) The flow rate of hydrogen gas was varied with constant electrode gap and detector voltage.

(3) Variation of the hydrogen gas flow rate was repeated at the same detector voltage, but with a different electrode gap from that employed in case (2).

The operating parameters of the detector and the characteristic data of the response curves are listed in Table III. The actual gas flow rates are characterized by the percentage of hydrogen in the mixture emerging from the burner jet, calculated from the volumetric flow rates of nitrogen and hydrogen. The flow rate of the carrier gas was maintained at 10 ml/min in all experiments and pure oxygen was always used as scavenging gas at a flow rate of 200 ml/min. In agreement with the observations of STERNBERG *et al.*[4], it was found that the response characteristics depend to a great extent upon the partial pressure of H_2 in the reaction zone of the flame.

Some of the response curves recorded are shown as examples in Fig. 5 I–VI, while Fig. 4 shows two chromatograms recorded synchronously, corresponding to

Trimethyl-butenylsilane				*Divinyl-tetramethyldisiloxane*			
r	I_{lim}	MFC'_{lim}	$MFSi'_{lim}$	r	I_{lim}	MFC'_{lim}	$MFSi'_{lim}$
32.0	0.294	0.114	0.038	~ 38	0.227	0.072	0.04
32.5	0.63	0.218	0.073	~ 38	0.535	0.143	0.083
31.4	0.97	0.32	0.107	~ 38	0.755	0.22	0.128
31.8	1.98	0.59	0.179	~ 38	1.58	0.415	0.242
38.0	*	—	—	~ 40	*	—	—
18.8	0.715	0.336	0.112	~ 24.5	0.585	0.236	0.137
31.4	0.97	0.32	0.107	~ 38	0.755	0.22	0.128
36.2	1.69	0.45	0.150	~ 41	1.3	0.299	0.174
40.7	2.27	0.58	0.19	~ 45	1.7	0.388	0.226
34.7	1.23	0.325	0.108	~ 39	0.98	0.232	0.135
40.0	1.72	0.385	0.128	~ 44	1.35	0.229	0.133
40.5	2.1	0.432	0.114	~ 46	1.67	0.318	0.185

run No. 18 in Table III. The measuring ranges of the EA1 and EA2 electrometers were set in such a way as to obtain a full-scale deflection of $8 \cdot 10^{-5}$ mA on the abscissa and of $4.21 \cdot 10^{-5}$ on the ordinate. (In Fig. 5: $20 \cdot 0.40 \cdot 10^{-5}$ mA and $10 \cdot 0.421 \cdot 10^{-5}$ mA, respectively.)

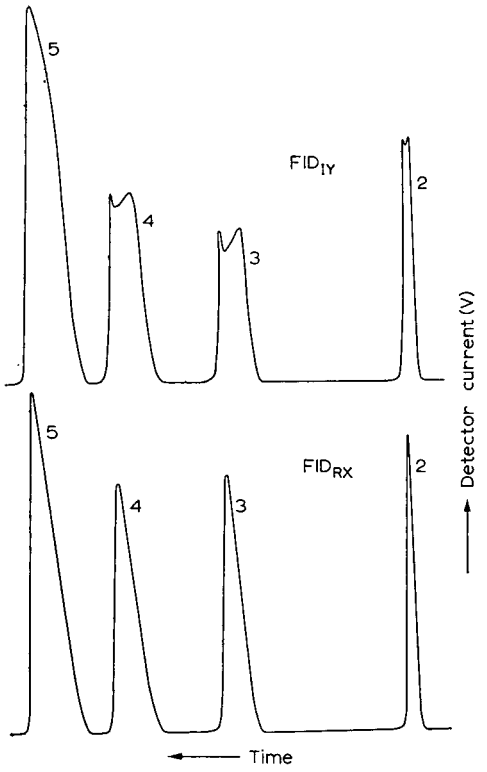

Fig. 4. Chromatograms referring to experiment No. 18. Sample quantity: 1.2 μl. Sample composition, see Table I. FID_{RX} operating variables, see text. FID_{IY} operating variables: detector voltage 150 V; electrode gap 5.5 mm; gas flow rates: N_2 10.1 ml/min; H_2 24.4 ml/min; O_2 200 ml/min.

Under the conditions corresponding to diagram I, the FID_{IY} was functioning linearly, similarly to the FID_{RX}. The relation of the two detector signals is represented by straight lines. The slightly different slopes of these lines are a consequence of the individual "r" factors of the test substances in FID_{IY}. A decrease of the detector voltage—other conditions being the same—resulted in the successive diminution of the linearity limit of the hydrocarbon component and in the appearance of peak inversion of the organosilicon components (see diagrams II and III). At the same time the slope of the initial linear part of the curves remained unaltered. The effect of the H_2 partial pressure becomes manifested by the comparison of diagrams IV and V. The limiting values—both MFC_{lim} and I_{lim}—are considerably higher in diagram V, corresponding to an increased hydrogen content. In contrast to the variation of the detector voltage, a variation of the percentage of H_2 also affected the response factors, *i.e.* the slopes of the linear parts of the curves. A comparison of diagrams V and

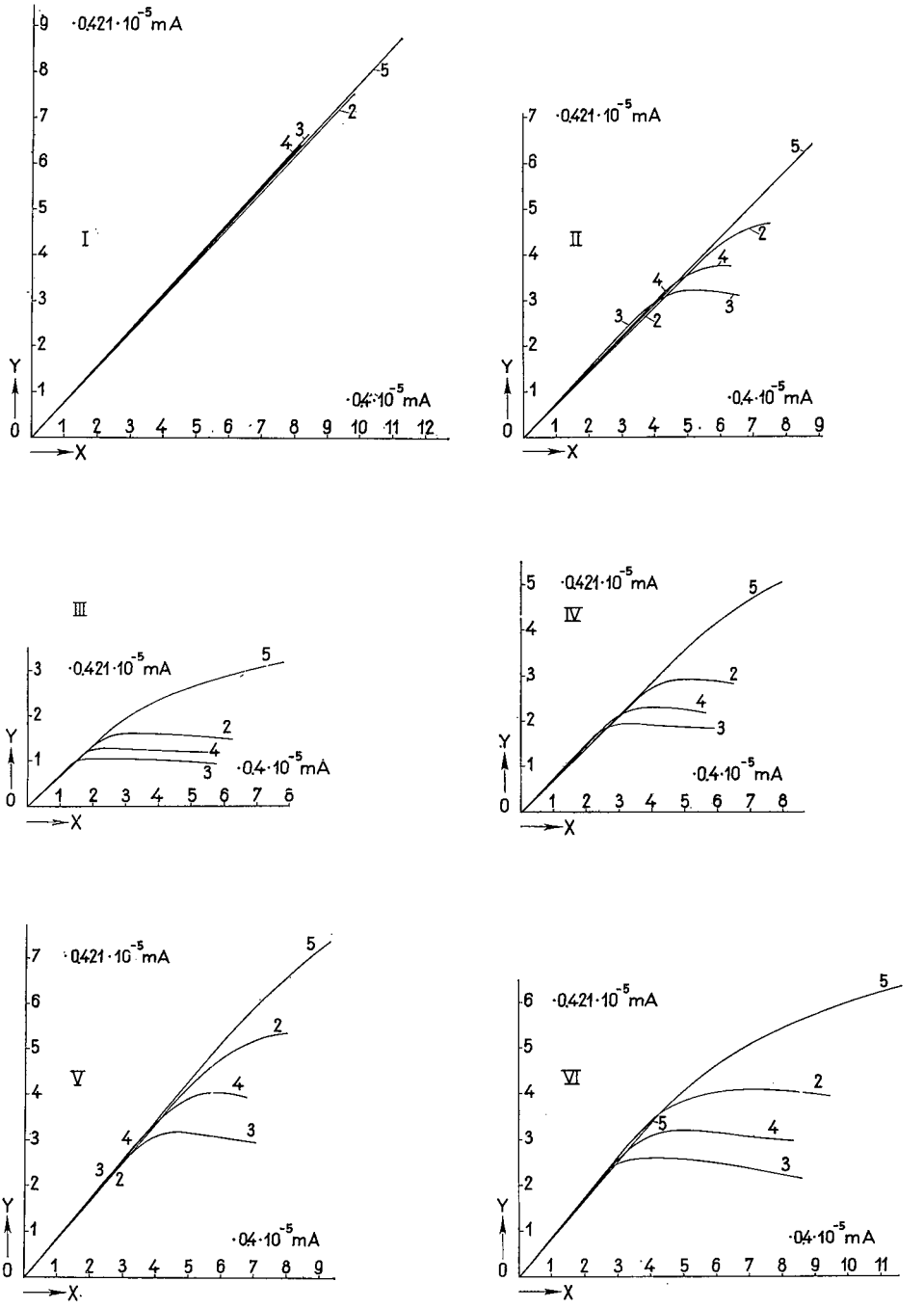

Fig. 5. Response curves, obtained with the "X–Y Recorder". Sample quantity: I and VI, 1.2 μl; II, III, IV and V, 1.0 μl. Sample composition, see Table I. FID_{RX} operating variables, see text. FID_{IY} operating variables, see Table III. Diagram I refers to Expt. 16; II to Expt. 6; III to Expt. 7; IV to Expt. 5; V to Expt. 8; VI to Expt. 18.

VI is representative of the effect of the electrode gap, an increase of which resulted in a decrease of the limiting values, while the response factors remained constant.

In general, the shape of the response curves draws attention to the fact that there is a fairly wide linearity range of the FID_{IV} signal with respect to organosilicons, under all operating conditions examined. Within this range the corresponding "r" factors do not differ considerably from the "r" values of a hydrocarbon. Hexamethyl-cyclotrisiloxane and divinyl-tetramethyl-disiloxane showed an "r" factor slightly higher, while trimethyl-butenyl-silane an "r" factor slightly lower than xylene. This observation indicates that the silicon does not exert any lowering effect on the ion current below a critical MFSi, in fact some increment of the ion current may be attributed to the Si–C bond.

As shown Fig. 5, the response curves of organosilicons may be divided into different regions. There is always an initial linear region followed by a non-linear one and, in some cases, this is also followed by a region of constancy. Finally, if conditions are favourable for peak inversion, a descending region is observed. The length of these regions as well as the slopes of the descending region depend to a great extent on the operating conditions.

The experimental results obtained hitherto by recording response curves are summarized in the diagrams of Figs. 6, 7 and 8 and in Table III.

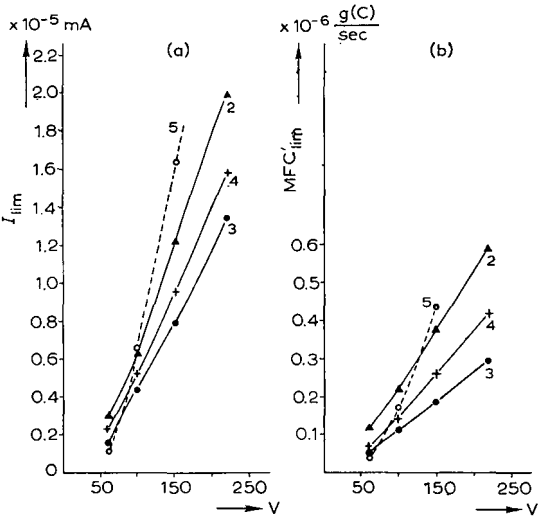

Fig. 6. Effect of detector voltage on (a) I_{11m} (solid lines) and linear I (dotted lines), and (b) MFC'_{11m} (solid lines) and linear MFC (dotted lines) at a constant electrode gap (4.5 mm) and constant gas flow rates (H_2:62.4 %). 2 = Trimethyl-butenyl-silane; 3 = hexamethyl-cyclotrisiloxane; 4 = divinyl-tetramethyl-disiloxane; 5 = *o*-xylene.

The following conventions were used for the evaluation of the characteristic data of the response curves listed in Table III.

As the determination of the abscissa of the maximum proved to be unreliable or even impossible in many cases because of the flat maximum or constant region of the curve, the initial linear region of the response curve was elongated and a horizontal line was drawn at the height of the maximum ion current (I_{11m}). The abscissa of

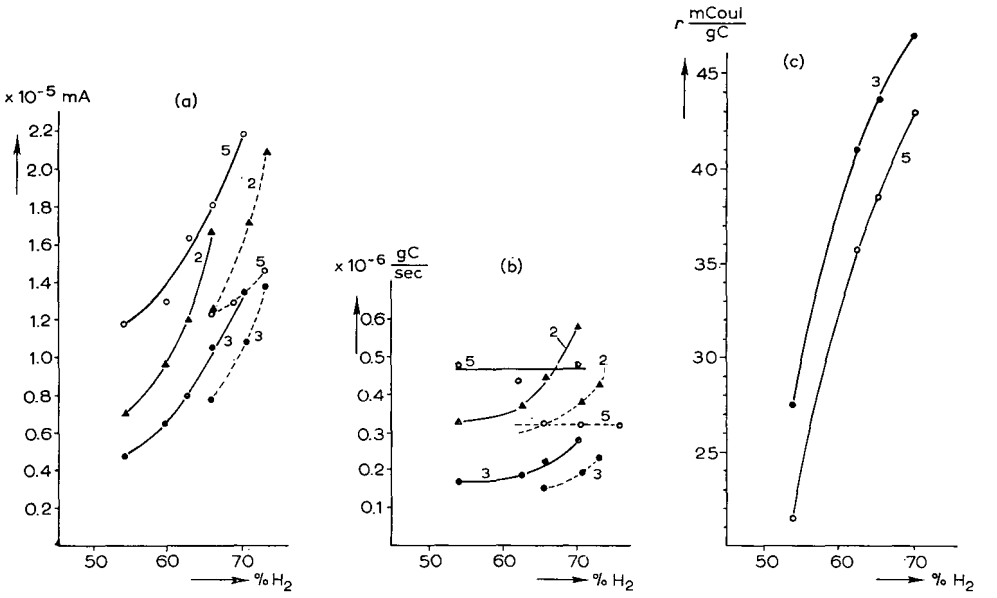

Fig. 7. Effect of the hydrogen percentage on I_{lim} and linear I (a), on MFC'_{lim} and linear MFC (b), and on "r" factors (c), with an electrode gap of 4.5 mm (solid lines) and 5.5 mm [dotted lines in (a) and (b)] and a constant detector voltage of 150 V. For an explanation of the numbers see Table I.

the intersection of the two straight lines is called the extrapolated limiting carbon mass flow rate (MFC'_{lim}). Presuming the constancy of "r" up to the maximum of the curve, this value would represent the MFC pertaining to the limiting ion current I_{lim}.

Another convention had to be used for the evaluation of the linearity limit on the response curves of xylene. Here again the linear section of the curve was extrapolated. That point of the curve where the deviation of the recorded curve from the elongated line reached 0.5 % of the actual ion current was considered to be the linearity limit, since the precision of the recorder was 0.5 %.

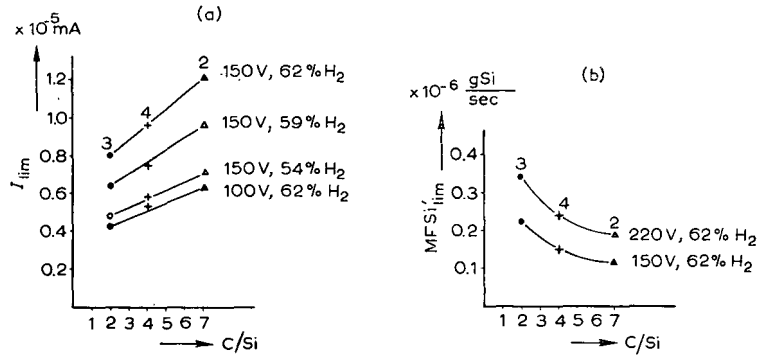

Fig. 8. Effect of the C/Si molar ratio of the test substance on the critical values I_{lim} (a) and $MFSi'_{lim}$ (b). For an explanation of the numbers see Table I.

In accordance with the data in the literature, these experiments show that the response factors both of hydrocarbons and organosilicons are independent of the detector voltage within the range of 60–400 V. The extrapolated limiting carbon mass flow rates of organosilicons show a linear increase with increasing detector voltage as illustrated in Fig. 6b. The "r" factor being constant, the same relation is also valid for the limiting ion currents (Fig. 6a). The effect of the detector voltage on the linearity limit of xylene is still more pronounced (see dotted lines in Figs. 6a and b).

An increase in the concentration of hydrogen, within the range of 50–70 % H_2, causes a rapid increase of the "r" factor both for xylene and for organosilicons (5 and 3 in Fig. 7c). At the same time an increase in the limiting mass flow rates—MFC'_{lim}—of the organosilicons is to be seen in Fig. 7b with increasing H_2 concentration. Consequently, the dependence of the limiting ion current—I_{lim}—on the hydrogen concentration shows a still more rapid increase within the range of the present measurements, as illustrated in Fig. 7a. Fig. 7b draws attention to a rather interesting fact: in contrast to the MFC'_{lim} values of organisilicons the linearity limit of the carbon mass flow rate (linear MFC) of xylene does not change with the percentage of hydrogen. Consequently, the increase of the linearity limit in ion current (linear I) is not so steep in the case of xylene as the increase of I_{lim} values in the case of organosilicons.

Figs. 7a and b also illustrate the effect of the electrode gap. The limits of both linearity and of inversion expressed in both MFC and ion current values, decrease with increasing electrode gap. Fig. 8a shows a relationship of practical importance: the limiting ion currents of organosilicons are varying linearly with the carbon/silicon molar ratio of the compound within the investigated range of 2–7 C/Si. The diagram in Fig. 8b demonstrates that the $MFSi'_{lim}$ values are as a first approximation inversely proportional to the actual MFC values.

CONCLUSIONS

The experiments reported do not afford sufficient data to explain the anomalous response of the FID for organometallic compounds. However, it seems unquestionable that these anomalies have no relation to the deposition of solid combustion products on the electrodes.

Deviations from the linear relationship between the ion current and carbon mass flow rate always imply the process of ion recombination. The dependence of the recombination rate upon the electric field strength and carbon mass flow rate in hydrogen flames containing small amounts of organic material has been the subject of detailed studies[4,5].

The combustion of organometallic (organosilicon) compounds introduces a new parameter which must be taken into account as a cause of recombination. According to our observations this parameter proved to be a critical metal concentration in the flame, the extent of which is a function of the actual carbon mass flow rate, besides the operating variables of the detector. The rate of recombination is affected much more markedly by this factor than by all the others. It is remarkable that the increase of the metal concentration does not result in a continuous diminution of the ionization efficiency. Below the critical mass flow rate the "r" factor is of the

same magnitude or even higher than in the case of a hydrocarbon. The relatively elevated response factors of hexamethyl-trisiloxane and divinyl-tetramethyldisiloxane may be quoted as examples. A possible explanation is given by the higher ionizability of the Si–C bond than that of the C–C bond.

The observation that the recombination is unaffected by small amounts of silicon may be explained if it is assumed that the recombination takes place on the surface of solid combustion products floating in the reaction zone, and that the formation of solid particles of sufficient size requires a critical silicon concentration. This assumption is also supported by an experiment where small amounts of Freon were added to the fuel gas, in order to prevent the formation of solid SiO_2 in the flame. In this experiment peak inversion did not occur. However, the dependence of the critical silicon mass flow rate upon the actual carbon mass flow rate is inconsistent with this simplified theory.

Apart from these still unsolved problems, the relationship between I_{11m} and the C/Si ratio is a valuable result of the experiments reported. This relationship affords the possibility of estimating the C/Si ratio of an unknown compound from the maximal height of its inverted peak. Further investigations are still needed in order to make statements concerning the accuracy and practicability of information of this kind.

SUMMARY

A method is described by means of which the response curves of a flame ionization detector may be recorded on the basis of a single chromatogram. The column effluent is divided into two equal streams which simultaneously enter a reference detector and the detector being examined. The relation between the two detector signals is recorded with the aid of an X–Y recorder. Providing that the performance of the reference detector is linear, and by determining its response factor, the abscissa of the X–Y diagram represents a scale of mass flow rates, and the diagram itself the response curve of the detector tested. The method was used for the investigation of the FID performance when detecting organosilicon compounds. Inversion of the gas chromatographic peaks takes place and the response curves display a maximum under certain operating conditions. The shape of the response curves, the height and situation of the maximum depend on the detector voltage, the electrode gap, the flow rate of gases and the carbon/silicon ratio of the compound detected. The estimation of the carbon/silicon ratio on the basis of the maximum ion current is suggested.

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DERIVATOGRAPHIC STUDIES OF GAS-CHROMATOGRAPHIC PARTITION LIQUIDS. I.

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The number of partition liquids used in the chromatography of gas–liquid systems is increasing every year. One of the most important characteristics of the partition liquids is the upper temperature limit of their applicability.

Different values for the upper temperature limits of the partition liquids have been given by different workers owing to the diverse methods used for the determination. Therefore these values should be used with reservation, because in some cases the partition liquid leaves the carrier in considerable amounts at much lower temperatures than those given in the literature as the temperature limit of use. This has an adverse effect on the operation of the detector and also changes the characteristic parameters of the column to an uncertain extent.

On the basis of previous experiments with the thermobalance[1] we have found the derivatographic method[2] suitable for studying gas-chromatographic problems concerning temperature limits of usability of partition liquids.

Our experiments were carried out with a PAULIK-PAULIK-ERDEY ORION-GYEM 676 Type derivatograph[3].

The thermoanalytical measurements were carried out with samples of about 300 mg, in air, or in a nitrogen atmosphere of varying purity, at a heating rate of 3°/min.

For the evaluation of the data obtained the experimental conditions of the derivatographic measurements should also be taken into consideration. In the case of programmed temperature gas chromatography the thermal conditions are completely similar (if the rates of heating during the programming and during the derivatographic measurements are equal), consequently the results obtained really give the upper temperature limit of applicability for partition liquids.

The results obtained under dynamic conditions can only be used for columns operating under isothermal conditions with certain reservations. In these cases, it has been found as a result of experience that it is expedient to give temperature data some 20–30° lower than the decomposition temperatures found by the dynamic method. The temperature at which the vapour pressure of the partition liquid reaches 10 mm Hg is usually given as the upper limit of applicability[4]. The upper temperature limit can be characterized by the point on the derivatographic DTG curve—which is more sensitive to changes than the TG curve—where it deviates from the base line.*

* TG = thermogravimetric; DTG = differential-thermogravimetric; DTA = differential-thermoanalytic.

First we wished to decide whether the pure partition liquid or the prepared packing is more suitable for the determination of the temperature limit. In practice, however, the study of the latter is more important. The experiments, which include a derivatographic study of the support, will also show whether or not any interaction occurs between the partition liquid and support. Fig. 1 shows the derivatograms of a

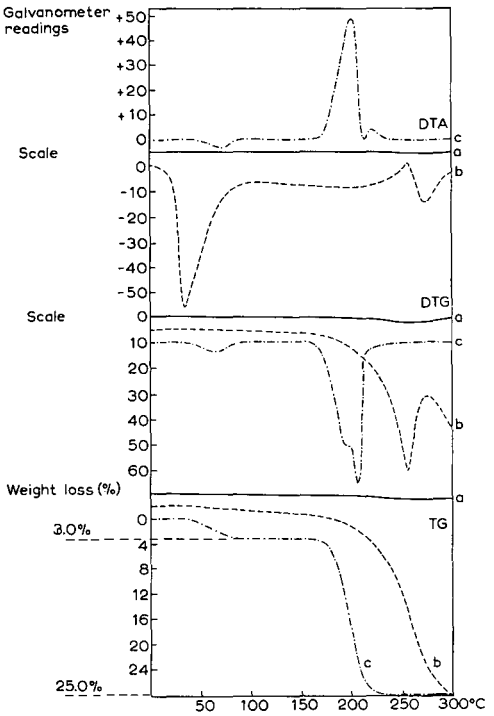

Fig. 1. Derivatograms of Celite (a), polyethylene glycol (b), and Celite packing freshly wetted with 25 wt. % polyethylene glycol (c).

pure support, Celite, of a pure partition liquid, polyethylene glycol, and of a column filling prepared freshly with both of them, (a), (b) and (c) respectively. It was found that, up to 400°, Celite showed no considerable weight change while the pure partition liquid decreased in weight with increasing temperature. At 250° an exothermic decomposition process, probably oxidation, takes place, after which the rate of decomposition decreases. The DTA curve also shows that the polyethylene glycol melts at about 40°. This was not detectable in the curve for the prepared packing. This curve shows that the packing had a constant weight up to 170° apart from the bleeding of a small amount of solvent. This bleeding of the solvent can only be observed with fresh fillings. Above 170°, it loses the polyethylene glycol in an exothermic process at a higher rate than that due to the bleeding process. So the thermal decomposition of the pure partition liquid and of the prepared support proceeds differently.

This can be explained by the fact that two opposing processes take place between the support and the partition liquid as a result of the rising temperature.

The partition liquid is adsorbed on the surface of the support; this process results in bleeding of the liquid not taking place until higher temperatures. On the other hand, the high specific surface area of the support has the effect of increasing the surface of the liquid phase, and therefore makes it easier for the latter to leave the support than the pure liquid phase. The derivatograph measures the result of the two effects, and shows the second to be predominant: the partition liquid is therefore removed from the support at lower temperatures than the pure liquid phase.

Besides establishing the upper temperature limit for the use of column packings, the derivatographic measurements can also be used for determining or checking the degree of wetting of the fillings and the degree of exhaustion.

In order to approach the conditions of the removal of the partition liquid from the column as an effect of heating during the measurements, experiments were also carried out in a flow of nitrogen used as carrier gas in the gas chromatograph. It was observed that the experiments in nitrogen atmosphere taken from a bomb yield practically the same results as those in air. Using lamp-pure nitrogen and ensuring the total exclusion of oxygen from the inside of the furnace, an essential difference was found. Fig. 2 shows the derivatograms taken of Celite coated with 25 % Apiezon grease under the above described conditions (a). No change of the column filling can be observed up to 175° either in presence of air or bomb nitrogen. At this point a small

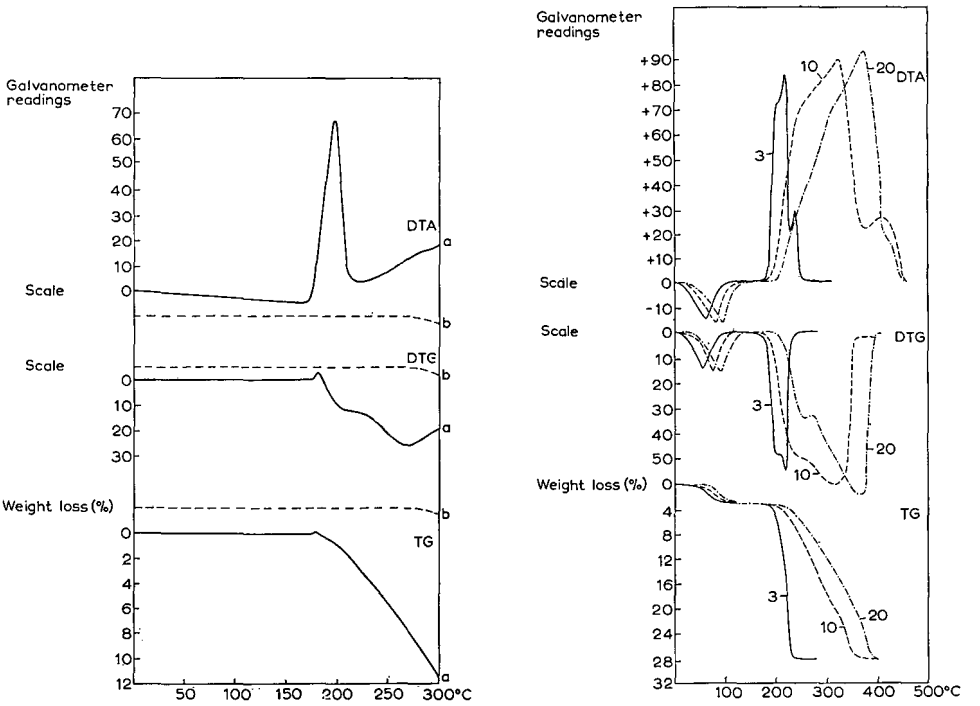

Fig. 2. Derivatogram of Celite packing containing 25 wt. % Apiezon fat in a stream of bomb nitrogen (a) and in an oxygen-free atmosphere (b).

Fig. 3. Derivatograms of Celite packing wetted with 25 wt. % polyethylene glycol, at heating rates of 3, 10 and 20°/min, respectively.

but distinct weight increase can be observed, after which the partition liquid begins to leave the carrier. Simultaneously with the weight increase or little after it a distinct, sharp exothermic peak appears on the DTA curve, which can be explained by oxidation. The DTA curve remains in the exothermic region also after the peak, which means that the substance burns off under the given experimental conditions.

In the experiments carried out with the exclusion of oxygen the process is different. No weight or enthalpy change occurs up to 280°, and the partition liquid begins to leave the column only above that temperature in an endothermic process (b). A similar phenomenon was observed with other partition liquids. It can be stated that the purity of the nitrogen used has an important effect on the upper temperature limit of the usefulness of the column filling. In the absence of oxygen the upper temperature limit is higher. In air and in impure nitrogen an oxidation process takes place: the oxidation of the partition liquid.

Fig. 3 presents the thermoanalytical decomposition curves of Celite wetted with 25 % polyethylene glycol, the rate of heating being 3, 10 and 30°/min in air. The fact, well known by thermoanalysts, that the curves vary according to the heating rate is demonstrated in Fig. 3. At a lower heating rate the oxidation of polyethylene glycol starts at lower temperatures, while at a higher heating rate it starts only at higher temperatures. The partition liquid for programmed (heating) gas chromatographic measurements should be chosen with consideration for this fact.

The derivatographic method also seems to be suitable for the examination of mixed partition liquids. Our experiments concerning this are in progress.

It can be stated also from the results given here that the derivatograph can be successfully used in a new field, for the investigation of problems concerning the upper temperature limit for the use of partitions liquids.

ACKNOWLEDGEMENT

The authors wish to thank Professor Dr. L. ERDEY for his continued interest and help.

SUMMARY

The upper temperature limit of applicability of gas chromatographic partition liquids and problems concerning this were studied by means of the derivatograph. It has been found that the instrument cannot only be used for the determination or checking of the upper temperature limit, but also for the measurement of the extent of wetting and exhaustion.

It was shown that the upper temperature limit for the application of column fillings also depends on the experimental conditions. An ong others we have studied the dependence of the upper temperature limit of the partition liquid on the heating rate. The method seems to be suitable for the examination of the interactions between the support and the liquid phase.

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GAS CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME SYMPATHOMIMETIC AMINES IN USE IN ANOREXIGENIC DRUGS

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INTRODUCTION

Some papers have already been published on the gas chromatographic analysis of sympathomimetic amines[1,2]; their separation is not easy to perform. This is mainly attributable to the high basicity and polarity of these compounds, as a result of which adsorption on the supports used may occur. The adsorption phenomenon manifests itself as a pronounced tailing of the peaks, the result being that the retention times become dependent on the quantity of amine injected and the separating power of the column declines considerably[3]. This makes it difficult to identify sympathomimetic amines in drugs, especially in a mixture.

It is therefore of great importance that inert supports, such as Teflon, be used, or that the support be treated with, *e.g.*, KOH in methanol or with hexamethyldisilazane, etc. in order to inactivate the adsorptive sites at the surface. Further improvement may be achieved by applying a high percentage of stationary phase to the support.

A second problem is decomposition of the amines during separation. This process is promoted by the high injector and detector temperatures. Because of this, a gas chromatograph with glass columns and on-column injection has to be used. It also appears from data from the literature and our own research that some stationary phases promote this phenomenon[5]; however, it is possible to stabilize the amines by converting them into other compounds, such as acetates[6–8].

EXPERIMENTAL

An F & M gas chromatograph, Model 400, with a flame ionization detector was used. The column consisted of glass; length about 105 cm, and diameter 25 mm. The injector-heater zone of the column was filled with 60–80 mesh glass beads and closed with a plug of glass wool. Nitrogen was used as carrier gas.

A preliminary investigation showed that the best results were obtained if a silanized support was used.

The following column packings were tested on a Diatoport S* (60–80 mesh) support: (1) 18.8 % Apiezon N; (2) 20.6 % Versamid 900; (3) 12.3 % triethanolamine;

* Diatomaceous earth specially treated and silanized with dimethyldichlorosilazane, supplied by F & M Scientific Corporation.

TABLE I

SYMPATHOMIMETIC AMINES INVESTIGATED

No.	*Name**	*Chemical name*
1	Amphetamine	α-Methylphenylethylamine
2	Methamphetamine	N,α-Dimethylphenylethylamine
3	Isopropylhexedrine	N,β-Dimethylcyclohexane-ethylamine
4	Phenylpropanolamine	α-Methyl-β-hydroxy-phenylethylamine
5	Diethylpropion	α-Diethylaminopropiophenone
6	Methylphenidate	α-Phenyl-2-piperidineacetic acid methyl ester
7	Phenmetrazine	3-Methyl-2-phenylmorpholine
8	Phendimetrazine	3,4-Dimethyl-2-phenylmorpholine
9	Chlorphentermine	*p*-Chloro-α,α-dimethylphenylethylamine
10	Phentermine	α,α-Dimethylphenylethylamine

* WHO name where possible.

(4) 19.7 % Hyprose; and (5) 22.5 % Carbowax 20M. Carbowax 20M was not further used because of decomposition of the amines No. 3, 4, 9 and 10 in Table I.

1–2 μl of a 2.5 % solution of the amine bases in chloroform was injected on to the column by means of a 10 μl Hamilton syringe. This was followed by a mixture of butanol-1, octanol-1 and nonanol-1, a mixture of *n*-alkanes with an even carbon number, C_6 to C_{16} inclusive, as reference, and finally a mixture of all amine bases in chloroform.

Prior to identification by the gas chromatograph, the sympathomimetic amines were extracted from a basic solution of the drug containing them with ether or chloroform. If the amine was present in the form of an HCl salt, an extract could be made direct by boiling the drug with ethanol in the case of solid drugs, as the retention times of the free bases and the HCl salts proved to be the same.

So as to be less dependent on minor changes in the carrier gas velocity the retention times found, after correction for the column dead time, were divided by the retention time of nonanol-1.

TABLE II

COLUMNS USED AND ASYMMETRY FACTOR

Column temperature = approx. 140°, see Table III.

Name	*Column No.*			
	1	*2*	*3*	*4*
Amphetamine	1.5	1.4	2.3	1.3
Methamphetamine	1.5	1.3	1.1	1.8
Propylhexedrine	1.6	1.4	1.2	2.8
Phenylpropanolamine	1.4	—	—	—
Diethylpropion	1.1	1.0	0.9	1.1
Methylphenidate	—	—	—	—
Phenmetrazine	1.1	1.2	—	1.2
Phendimetrazine	1.3	1.1	0.9	1.1
Chlorphentermine	1.1	1.4	1.0	1.2
Phentermine	1.3	1.2	1.1	1.3
Nonanol-1	1.3	1.1	1.1	1.2
Hexadecane	1.1	1.1	1.1	—

TABLE III

RELATIVE RETENTION VALUES WITH RESPECT TO NONANOL-1 OF THE SYMPATHOMIMETIC AMINES INVESTIGATED

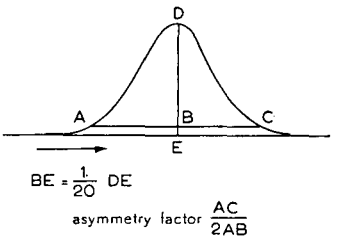
Name	*Column*									
	18.8 % Apiezon N			*20.6 % Versamid 900*			*12.3 % triethanolamine*		*19.7 % Hyprose*	
Amphetamine	0.99	1.00	1.07	0.48	0.62	0.58	1.60	1.68	1.28	1.15
Methamphetamine	1.42	1.47	1.48	0.48	0.64	0.81	0.99	1.04	0.97	0.87
Isopropylhexedrine	1.46	1.41	1.39	0.42	0.49	0.63	0.29	0.37	0.54	0.39
Phenylpropanolamine	3.18	2.77	2.55	—	—	—	—	—	—	—
Diethylpropion	10.14	7.15	5.57	3.02	3.08	2.98	1.95	2.06	2.80	2.53
Phenmetrazine	8.18	6.03	5.07	3.46	3.69	3.56	—	14.90	7.65	9.51
Phendimetrazine	9.11	6.71	5.57	2.73	3.03	2.98	4.53	4.59	4.07	3.87
Chlorphentermine	5.07	4.21	3.59	2.08	2.15	2.18	5.90	5.36	3.28	3.71
Phentermine	1.27	1.29	1.34	0.56	0.67	0.83	1.42	1.46	1.13	1.04
Ethanol	—	—	—	—	—	—	0.03	0.04	—	—
Butanol-1	—	—	—	0.04	0.04	0.13	0.08	0.11	0.12	0.05
Octanol-1	0.49	0.56	0.66	0.52	0.59	0.68	0.61	0.66	0.66	0.58
Octane	—	—	—	0.06	0.09	—	—	—	—	—
n-Decane	0.42	0.47	0.54	0.09	0.16	0.20	—	—	—	—
n-Dodecane	1.73	1.47	1.39	0.23	0.30	0.37	0.02	—	—	0.03
n-Tetradecane	8.26	4.64	3.48	0.85	0.76	0.79	0.05	0.10	0.15	0.10
n-Hexadecane	—	14.17	8.75	3.02	2.10	1.69	0.12	0.24	0.35	0.31
Retention time of nonanol (min)	7.30	1.70	0.56	18.3	3.70	1.20	15.90	5.61	2.02	14.40
Gas velocity at outlet (ml/min)	80	80	90	60	50	60	100	90	70	70
Column temperature (°C)	101	138	180	99	138.5	180	80	100	142	100
Injector temperature (°C)	236	265	257	242	247	247	203	205	247	220
Detector temperature (°C)	235	257	265	238	246	255	216	210	258	223
Pressure at inlet (atm.)	1.55	1.7	1.8	1.5	1.6	1.6	1.8	1.6	1.8	1.8
Weight of column packing (g)	2.5021			2.6416			2.0503		2.2440	

RESULTS

Peak tailing

The columns chosen do not entirely suppress the tailing. Nevertheless, it is possible to achieve a reasonably small asymmetry factor even with amphetamine (Table II).

An asymmetry factor of less than 1 indicates overloading of the column (see Fig. 1).



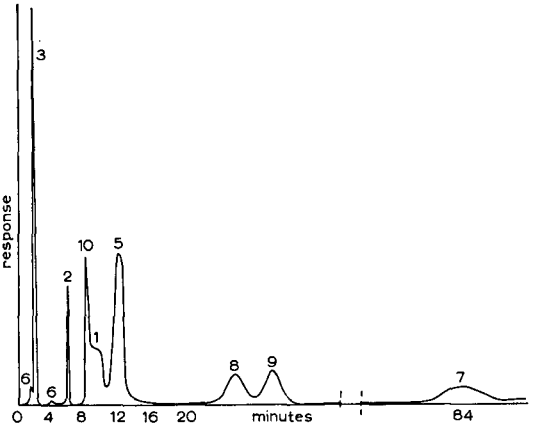


Fig. 1. Peak shape defined by the asymmetry factor.

Fig. 2. Gas chromatogram, on a triethanolamine column of a mixture of 10 sympathomimetic amines as described in Table III. Column temperature: 100°. Numbers above peaks, see Table I.

Relative retention (Table III and Figs. 2 and 3)

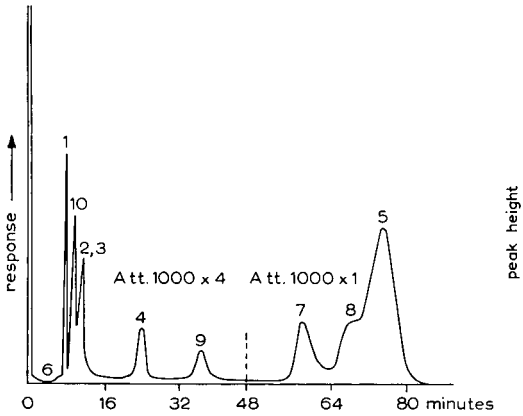
Methylphenidate did not give a clear response on any of the columns; total decomposition occurred in almost all cases. This phenomenon was also noticeable with phenylpropanolamine, though it was not as pronounced.

In the case of the Apiezon column, the calculated Kovats indices[9] were found

TABLE IV

THE KOVATS INDEX AND EFFECT OF COLUMN TEMPERATURE ON THE APIEZON COLUMN

Name	*Column temperature*		
	180°	*138°*	*100°*
Amphetamine	1145	1136	1120
Methamphetamine	1214	1200	1172
Propylhexedrine	1200	1192	1176
Phenylpropanolamine	1333	1369	1278
Diethylpropion	1502	1501	1426
Phenmetrazine	1482	1485	1399
Phendimetrazine	1502	1495	1417
Chlorphentermine	1406	1381	1338
Phentermine	1192	1176	1156
Nonanol-1	1530	1332	1122



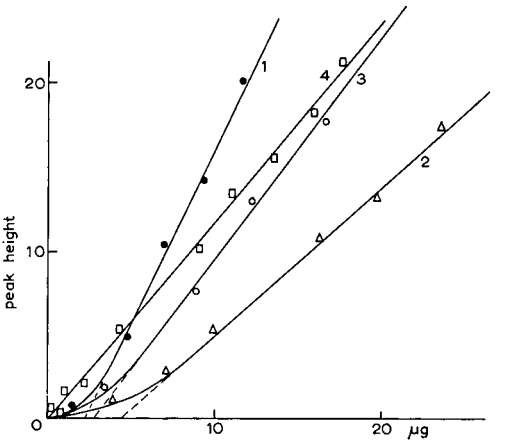


Fig. 3. Gas chromatogram on Apiezon N column, of a mixture of 10 sympathomimetic amines as described in Table III. Column temperature: 101°. Numbers above peaks, see Table I.

Fig. 4. Detector response *vs.* injected quantity of amphetamine. 1 = Apiezon column; 2 = Versamid column; 3 = Hyprose column; 4 = Hyprose column with amphetamine–acetone derivative.

to be the least dependent on temperature (Table IV). However, the separation of all the amines, except phenylpropanolamine, was better on the triethanolamine column. The retention times were not very dependent on the quantity of amine injected if this was not less than 1 μg (5 μg for Column 1) or not more than 100 μ.

Response

As the columns tested will be used in future for the quantitative analysis of sympathomimetic amines in anorexigenic drugs, it was necessary to determine in what region the relation between quantity injected and peak height is linear for amphetamine. Amphetamine was chosen for this investigation because the peak in the gas chromatogram of this substance tails more strongly than that of the other amines. The line representing the relation between peak height and quantity injected was found to be straight above 5 μg; below this quantity it curves away towards the origin of the system of coordinates. The minimum detectable quantity was still fairly small (Table V).

TABLE V

MINIMUM DETECTABLE QUANTITY OF AMPHETAMINE

Column No.	*1*	*2*	*3*	*4*	*4*
Minimum quantity (μg)	0.5	0.2	2.5	0.15	0.02*
Column temperature (°C)	138	140	100	142	140
Injector temperature (°C)	250	242	205	220	245
Detector temperature (°C)	255	245	210	237	253
Gas velocity at outlet (ml/min)	90	80	100	70	80

* A much better result was obtained by boiling amphetamine base with acetone. The linear relation between peak height and injected quantity was then maintained down to the minimum detectable quantity of 0.02 μg.

DISCUSSION

It is possible to identify which sympathomimetic amine has been used in an anorexigenic drug by analysing a chloroform or ethyl alcohol extract with a column consisting of 18.8% Apiezon N on Diatoport S 60–80 mesh. Only the peaks for methamphetamine and isopropylhexedrine coincide. Although the latter two drugs are separated on a column of 12.3% triethanolamine on Diatoport S, there is no response here, however, to phenylpropanolamine.

In quantitative analysis, it is advantageous to react the primary amines, especially those where considerable peak tailing occurs, with acetone. Further details of the quantitative analysis of sympathomimetic amines in drugs will be reported in the future.

SUMMARY

The identification and separation of some sympathomimetic amines which are used in anorexigenic drugs is described. These compounds can be successfully separated with Apiezon N on Diatoport S, with the exception of methamphetamine and isopropylhexedrine. The latter two can be separated with one of the other columns described.

It was found impossible to analyse methylphenidate. A detailed study of the minimum detectable quantity and the linearity of the peak height, plotted against quantity injected, was carried out on one of the amines, *viz.* amphetamine.

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APPLICATION OF AN ELECTRON CAPTURE DETECTOR TO THE DETERMINATION OF DIBROMOETHANE IN GASOLINES

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INTRODUCTION

The electron capture detector (ECD) is one of a number of ionisation detectors widely used for monitoring the effluent from gas chromatographic columns[1]. The range of response of an ECD, on a weight basis, covers several orders of magnitude, being very low for saturated hydrocarbons and extremely high, in particular, for polyhalogenated species. In this respect the ECD differs strikingly from other GC detectors which are (within a factor of 2 or 3) insensitive to compound type. Use of an electron capture detector is thus attractive (i) for the determination of small quantities of strongly electron capturing species and (ii) as a means of obtaining qualitative information about components separated by GC. Published applications of electron capture detection relate mainly to the determination of lead alkyls in gasolines[2,3] and of highly halogenated insecticides[4,5]. Although the potential of this detector for qualitative purposes has been noted[1,6] no direct applications have been reported to date.

Dibromo- and dichloroethane are frequently added to gasolines as scavengers, to aid in the removal of lead compounds (arising from the lead alkyl anti-knock agents in gasoline) from engines. All of these gasoline additives can be determined, in principle, using the electron capture phenomenon and the alkyl leads have been determined as mentioned above. However, the relative sensitivity of an ECD to alkyl leads and also to dichloroethane is very much less than to dibromoethane. In attempting to use an electron capture detector difficulties were experienced due to such effects as varying sensitivity and optimum voltage for the same compound from day to day. As a result it has proved difficult to devise a repeatable method for the determination of substances for which the ECD sensitivity is relatively low, such as dichloroethane and alkyl leads.

This paper presents a method for the determination of 1,2-dibromoethane in gasolines. It illustrates the quantitative application of an ECD, and describes some of the experimental problems encountered in its use.

APPARATUS

The oven unit, electron capture detector and their associated electronic equipment were supplied by Gas Chromatography, Ltd., Maidenhead, England. The electron capture detector itself is shown in Fig. 1, the tritium source strength being about

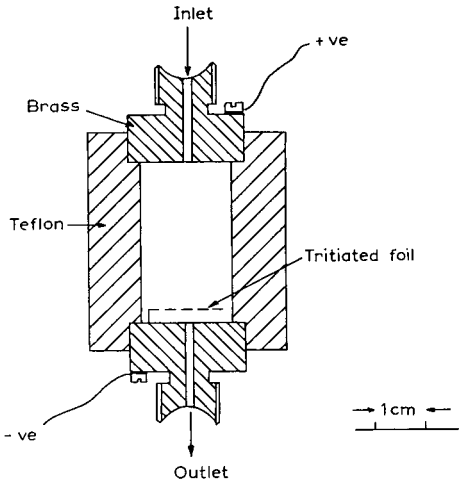


Fig. 1. An electron capture detector.

200 mC. A special "Electron Capture Control Box" permitted the application of any d.c. potential between O and 100 V (to ± 0.1 V) across the cell.

A 10' by $^1/_4$" inner diameter column packed with 5 % Apiezon "L" plus 0.5 % polyethylene glycol 4000 on "Embacel" was used. The temperature was held at 95°, and the nitrogen carrier gas flow rate was 100 c.c.s. min^{-1}. The carrier gas flow was maintained permanently through column and detector. At the column exit the carrier gas flow was split between detector and waste in the ratio 15:85.

OUTLINE OF THE TECHNIQUE

The determination of dibromoethane (DBE) in gasolines by ECD is a comparatively easy task since the detector response to DBE is many times greater than to any other volatile components present in a gasoline, including the lead alkyls. Indeed the main problem is to reduce the amount of DBE reaching the detector to a level such that the ECD is not completely "saturated".

Gasoline samples were diluted between 25- and 250-fold to bring the DBE content to the range 10–50 p.p.m. by weight, the diluent being 60–80 petroleum ether. A calibration blend of 1,2-dibromoethane in 60–80 petroleum ether, containing about 40 p.p.m. by weight of DBE was prepared and various known volumes of this solution were injected into the chromatograph using a Hamilton syringe (10 μl capacity). A calibration curve of DBE peak height *versus* total amount of DBE injected was thus obtained. The DBE content of unknown samples could then be deduced from the DBE peak height given by a known volume of the petroleum ether diluted sample and its relation to the calibration curve.

DISCUSSION OF THE APPARATUS AND TECHNIQUE

Important parameters to be considered when using d.c. electron capture detectors for quantitative work are:

(a) the potential across the detector
(b) the carrier gas flow rate
(c) the temperature of the detector
(d) the total amount of electron capturing material present in the detector at any instant
(e) possibility of detector contamination.

Detector potential

The variation in detector response with applied potential is shown in Fig. 2. At very low voltages the detector does not respond at all. Above what might be called the "threshold" voltage response increases rapidly to a maximum, falls fairly rapidly to zero and then becomes negative as cross-section, and other, ionisation phenomena take over. Voltages for maximum sensitivity and for the cross-over to

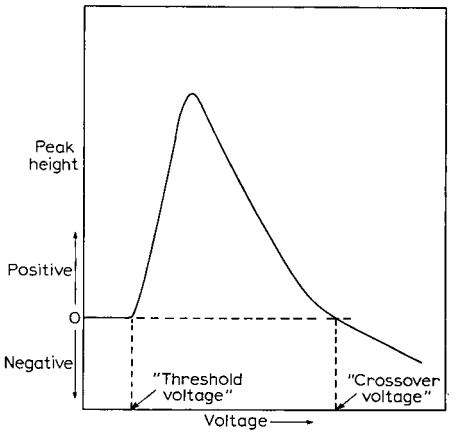


Fig. 2. Electron capture detector. Variation in response with voltage. Temperature 95°.

negative response vary somewhat with compound type. For the present purpose and with the Gas Chromatography Ltd. detector (in which the inter-electrode instance was about 5 cm) a potential of 4 V gave approximately maximum sensitivity to DBE. The very small amount of hydrocarbons of similar boiling point (and, therefore, of similar elution times) present in petroleum ether diluted samples were quite undetectable under these conditions. If, however, there had been interference between DBE and other hydrocarbon components, for example if the DBE levels had been 2 or 3 orders of magnitude lower, it would have been possible to operate the detector at a rather higher voltage (about 7 V). At this voltage hydrocarbons are at, or close to, their "cross-over" point (response becoming negative) and even very large quantities (*i.e.* 10^{-5} moles·sec^{-1}) give zero response whereas DBE is still below its cross-over point, though sensitivity is reduced.

Carrier gas flow

When carrier gas alone is passing through an ECD a steady, standing current flows. The standing current tends to increase with increasing carrier gas flow rate, particularly at low temperatures (Fig. 3). Since entry into the detector of an electron

capturing species leads to a fall in the current the sensitivity and dynamic range of the detector to electron capturing species is likely to increase with increasing standing current. It is therefore useful to operate the detector with a high gas flow rate. In some cases where a low flow rate is needed (*e.g.* for a capillary column) it is desirable to add further nitrogen to the column effluent before it reaches the detector.

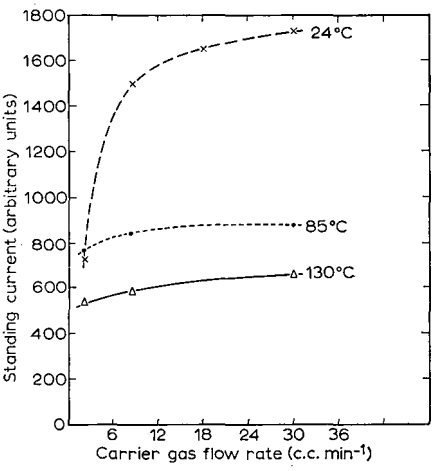


Fig. 3. Electron capture detector. Variation in standing current with gas flow rate.

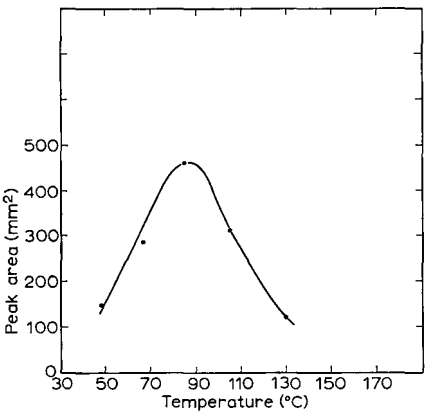


Fig. 4. Electron capture detector. Variation in response with temperature. Sample: 1,2-dibromoethane; voltage: 4 V.

Temperature

It has been found that electron capture detector response is influenced by temperature. Data for dibromoethane are plotted in Fig. 4. Similar response curves have been obtained for other substances. Ideally an ECD should be operated in a thermostated enclosure separate from the column oven.

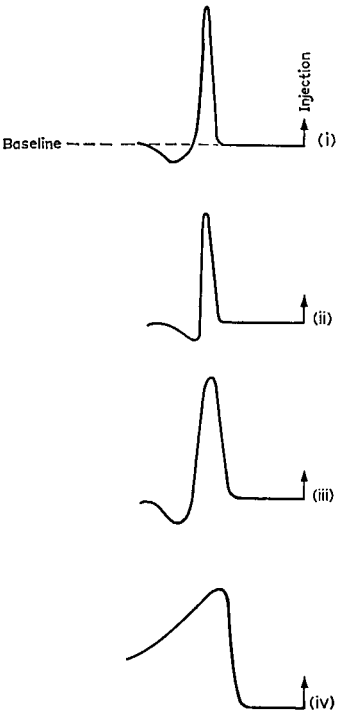
Quantity of electron capturing material

The ECD functions because electron-capturing materials reduce the standing current. The quantity of electron capturer present in a detector can be so high that all electrons may be captured and no current flows. This occurs if one μl of 1,2-dibromoethane is injected into an ECD, and the detector standing current takes many minutes to recover to its original level. Qualitatively if successively larger quantities of, for example, DBE are passed into an ECD, the peaks obtained have the form shown in Fig. 5. Peak broadening and tailing are seen to be functions of the detector. Peak heights are reckoned from the base line indicated in Fig. 5 (i). In the present state of knowledge it would seem safest to use an ECD only in the range where peak width is constant and peaks are symmetrical. Because of the exponential nature of the capture process calibration is essential.

Possibility of detector contamination

Deposition of relatively involatile material on the tritiated foil of the detector would lead to a serious loss of β-particle emission and consequent loss of sensitivity.

In the analysis of samples which contain relatively involatile components use of a pre-column, with back-flushing facility, is advisable to prevent such components reaching the detector.



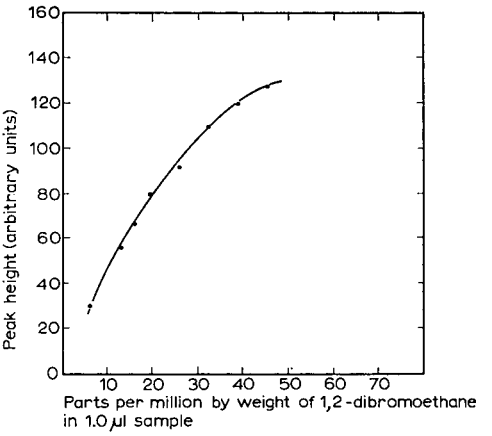


Fig. 5. Influence of quantity on peak shape. The quantity of electron capturing species increases from (i) to (iv).

Fig. 6. Calibration curve for 1,2-dibromoethane.

RESULTS

In the foregoing sections the influence of some variables on the determination of 1,2-dibromoethane has been considered. The actual conditions used are set out in Table I. Under these conditions the limit of detection of 1,2-dibromoethane was about $4 \cdot 10^{-13}$ moles$\cdot$sec^{-1}.

A calibration curve obtained for DBE is given in Fig. 6. In Table II some actual response measurements are given, as measured on a number of days, to show the variation in detector response. When the voltage across an ECD is changed, in the low potential range 1–10 V, the detector takes some time to re-equilibrate as can be seen from the data in Table III. At least 2 h should be allowed before attempting to use a detector after changing the applied potential. The precision of the sample charging (1.0 μl sample size), peak height measurement and detector repeatability operations over a 3-hour period was found, from 12 experiments, to be ± 3 p.p.m. at the 32.4 p.p.m. (by weight) level. It is believed that the precision limiting step in this analysis is the sample charging operation.

TABLE I

OPERATING CONDITIONS FOR DETERMINATION OF 1,2-DIBROMOETHANE

Detector temperature: 95°.
Detector voltage: 4 V.
Carrier gas flow rate:
Column: 100 c.c. min^{-1}.
Electron capture detector: 15 c.c. min^{-1}.
Column: 10′ 5% Apiezon "L"—0.5% P.E.G. 4000 on 100–120 mesh "Embacel".
Elution time of dibromoethane: 6 min.
Sample size: 1.0 μl of 60–80 petroleum ether diluted sample, so that DBE concentration is between 1 and 50 p.p.m.

TABLE II

DAY-TO-DAY REPEATABILITY OF DIBROMOETHANE CALIBRATION

Dibromoethane in sample (p.p.m.)	*Peak heights (mm)*				
	Feb. 6	*Feb. 7*	*Feb. 10*	*Feb. 11*	*Feb. 17*
6.5	27	36	34	29	30
13.0	52	54	—	—	56
16.2	62	71	—	—	66
19.5	—	61	77	59	80
25.9	100	96	—	—	92
32.4	110	121	118	105	110
45.4	149	—	—	—	128

TABLE III

"EQUILIBRATION" TIME OF AN ELECTRON CAPTURE DETECTOR

Response given by an electron capture detector to a fixed sample charge of DBE (precision of operation ± 10% at 2σ level).
Carrier gas flow and temperature had been constant for 5 days but voltage across the cell was changed from 7 to 4 at time zero.

Time zero plus (min)	*Peak height (mm)*
60	80
105	101
120	110
140	110

SUMMARY

In this paper a method for the determination of 1,2-dibromoethane in gasolines is discussed. The method utilises an electron capture detector which is much more sensitive to dibromoethane than to other gasoline components. The importance of voltage, temperature, total quantity of dibromoethane in the detector and carrier gas flow rate on the performance of the detector is considered. The sensitivity of the detector is $4 \cdot 10^{-13}$ mole·sec^{-1} for 1,2-dibromoethane. The repeatability of the method is $\pm$ 3 p.p.m. at the 30 p.p.m. level.

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THEORY OF SORPTION CHROMATOGRAPHY

II. NUMERICAL CALCULATIONS

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SYMBOLS

f = solute concentration in mobile phase
h = solute concentration in stationary phase
c = concentration of sorbent
k_1 = rate constant for sorption
k_2 = rate constant for desorption
v = translational velocity of mobile phase
D_1, D_2 = diffusion coefficients in mobile and stationary phase respectively
V_1, V_2 = volumes per interphase area of mobile and stationary phase respectively
$f^{m_{ij}}$ = matrix element representing f
$h^{m_{ij}}$ = matrix element representing h
A_i = ith moment of the concentration distribution
μ = mean of the concentration distribution
μ_2 = variance of the concentration distribution
M = mode of the concentration distribution
τ = duration of equilibration step
γ = partition coefficient
v = peak velocity
D = spreading coefficient
ω = velocity of concentration front in frontal analysis

$$\alpha = \frac{2 D_1}{\tau v^2}$$

$$\eta = \frac{\gamma V_2}{V_1 + \gamma V_2}(1 - e^{-m\tau})$$

$$m = \frac{2 D_2}{V_2}\left(\frac{\gamma}{V_1} + \frac{1}{V_2}\right)$$

INTRODUCTION

The theoretical treatment of sorption chromatography in the preceding article[1] has been supplemented by numerical calculations performed on a digital computer. As the basis of the calculations the following equations were used:

$$\frac{\partial f}{\partial t} + \frac{1}{V_1}\frac{\partial h}{\partial t} = D_1\frac{\partial^2 f}{\partial x^2} - v\frac{\partial f}{\partial x} \tag{1}$$

$$\frac{\partial h}{\partial t} = k_1 (c - h) f - k_2 h \tag{2}$$

These equations were solved by a finite difference approximation method, leading to eqns. (40)–(42) in the Appendix. The numerical treatment of the problem followed the general outlines of the procedure in partition chromatography[2,3]. The results were obtained in the form of the matrixes $(f^m{}_{ij})$ and $(h^m{}_{ij})$ representing concentration distributions in the mobile and stationary phases respectively of the chromatographic column. The data were abstracted from the computor in the form of a few selected columns of a matrix, representing the concentration distribution at different times. The zeroth, first and second moment with respect to the origin, with the cell width as unit length, were also calculated for every column. For the jth column they are defined as follows:

$$A_0 = \sum_i f_{ij} \tag{3}$$

$$A_1 = \sum_i i\, f_{ij} \tag{4}$$

$$A_2 = \sum_i i^2 f_{ij} \tag{5}$$

with corresponding definitions for the h-matrix.

For a characterization of the concentration distributions the reduced moments, the mean μ and the variance μ_2 were used. They are defined as follows:

$$\mu = \frac{A_1}{A_0} \tag{6}$$

$$\mu_2 = \frac{A_2}{A_0} - \mu^2 \tag{7}$$

In addition the mode M, defined as the location of the maximum of the smoothed distribution curve, was also determined.

The primary results of the calculations are in the following given in terms of the parameters A_0, M, μ and μ_2.

METHOD OF CALCULATION

In the present calculations the characteristic parameters of column operation were varied in order to determine their effect on the chromatographic process.

From the form of eqns. (1) and (2) it follows that not all parameters need be varied independently. The following transformations are seen to leave the equations unchanged:

$$\left(c, \frac{a}{V_1}\right) \rightarrow \left(ac, \frac{1}{V_1}\right) \tag{8}$$

$$(c, af, k_1) \rightarrow \left(\frac{c}{a}, f, ak_1\right) \tag{9}$$

$$(at, k_1, k_2, D_1, v) \rightarrow (t, ak_1, ak_2, aD_1, av) \tag{10}$$

where a is an arbitrary constant.

Each of these transformations makes it possible to change the value of one of the parameters via corresponding changes in some other parameters. In the calculations therefore only the parameters c, D_1, k_1 and k_2 were varied, the others being kept constant and, when not otherwise stated, had the values:

$$v = 0.01 \text{ (cm sec}^{-1}\text{)} \tag{11}$$

$$V_1 = 0.004 \text{ (cm)} \tag{12}$$

$$f = 100 \tag{13}$$

$$\tau = 5 \text{ (sec)} \tag{14}$$

The value of V_1 was chosen to represent a column filling consisting of tightly packed spherical beads with a radius of approximately 0.01 cm. The value of τ may be fixed arbitrarily, but is related to the values of other variables by formula (10). (D_1, k_1 and k_2 enter the calculations in form of the combined parameters $\alpha = 2\,D_1/\tau v^2$, $\tau/m\ k_1$ and $\tau/m\ k_2$). The value in (14) may be used for convenience, as it provides realistic operational conditions for the column. It gives a cell width $\tau v = 0.05$ cm.

All the matrixes were of the order $n = 200$ and in all cases the value $m = 5$ was used.

The calculations were carried out with the following initial conditions:

$$f^0_{i1} = \begin{cases} 100 \text{ for } i = 1 \\ 0 \text{ for } i = 2, \ldots, 200 \end{cases} \tag{15}$$

$$f^0_{1j} = \begin{cases} 100 \text{ for } j = 1, \ldots, n \\ 0 \text{ for } j = n+1, \ldots, 200 \end{cases} \tag{16}$$

$$f^m_{0j} = \begin{cases} 100 \text{ for } j = 1, \ldots, n-1 \\ 0 \text{ for } j = n, \ldots, 199 \end{cases} \tag{17}$$

$$h^m_{i0} = 0 \text{ for } i = 1, \ldots, 200 \tag{18}$$

In the case of isolated peaks in general the value $n = 5$ was used, though for matrixes 22, 24, 25 and 26 the value of n was 2, 10, 15 and 20, respectively. In the case of frontal analysis, for matrixes 21 and 22, the value of n was 200.

The values of the characteristic parameters for the different matrixes are listed in Table I, and the primary results of the calculations are given in Tables II and IV.

In Table II, the matrixes may be grouped together according to the following scheme. In 1, 2 and 3 the longitudinal diffusion coefficient is varied; in 4, 5, 2 and 6 the equilibrium constant is varied; in 7, 8, 9, 10, 2 and 11 the concentration of the sorbent is varied; in 12, 13, 14, 2, 15 and 16 the reaction rate is varied and in 17, 18, 2 and 19 the feed concentration is varied. Finally, the matrixes 20 and 21 represent frontal

TABLE I

VALUES OF CHARACTERISTIC PARAMETERS

Matrix No.	f	c	α	$\tau/m\,k_1$	$\tau/m\,k_2$
1	100	0.4	0	0.005	0.05
2	100	0.4	0.1	0.005	0.05
3	100	0.4	0.2	0.005	0.05
4	100	0.4	0.1	0.005	0.001
5	100	0.4	0.1	0.005	0.005
6	100	0.4	0.1	0.005	0.25
7	100	0	0.1	0.005	0.05
8	100	0.02	0.1	0.005	0.05
9	100	0.1	0.1	0.005	0.05
10	100	0.2	0.1	0.005	0.05
11	100	0.7	0.1	0.005	0.05
12	100	0.4	0.1	0.0005	0.005
13	100	0.4	0.1	0.001	0.01
14	100	0.4	0.1	0.002	0.02
15	100	0.4	0.1	0.007	0.07
16	100	0.4	0.1	0.009	0.09
17	20	0.4	0.1	0.005	0.05
18	50	0.4	0.1	0.005	0.05
19	400	0.4	0.1	0.005	0.05
20	100	0.4	0	0.0005	0.005
21	100	0.4	0.8	0.0005	0.005
22	100	0.4	0.08	0.005	0.05
23	100	0.4	0.2	0.002	0.02
24	100	0.4	0.4	0.001	0.01
25	100	0.4	0.6	0.00067	0.0067
26	100	0.4	0.8	0.0005	0.005

analysis with constant feed concentration, and there the longitudinal diffusion coefficient is varied.

RESULTS AND DISCUSSION

We will first consider isolated peaks. From the results in Table II it appears that in sorption chromatography steady state conditions are approached much more slowly than in partition chromatography. Therefore, under ordinary conditions, plots of μ and μ_2 against time yield curved lines and hence the peak velocity v and spreading coefficient D are variable quantities. However, if the sorption isotherm has a finite slope at the origin, as is the case with Langmuir isotherm, the conditions of partition chromatography are approached as a limit. We will therefore first study the asymptotic behaviour of isolated peaks.

In a column of infinite length the spreading of a peak will cause the concentration in the peak to decrease indefinitely. Thus, as f tends to zero eqn. (2) takes the asymptotic form:

$$\frac{\partial h}{\partial t} = k_1 c f - k_2 h = -k_2 \left(h - \frac{k_1 c}{k_2} f \right) \tag{19}$$

Eqns. (1) and (19) may be compared with those of partition chromatography, eqns. (1) and (2) in ref. 6. To make a direct comparison possible we delete the term for

longitudinal diffusion in the stationary phase in the latter equations and put $V_2 = 1$. Then, by identity:

$$k_2 = 2\,D_2 \tag{20}$$

and

$$\frac{k_1 c}{k_2} = \gamma \tag{21}$$

It then becomes possible to use the exact expressions for peak velocity and peak spreading, which were derived for the partition case, eqns. (36) and (39) in ref. 6. With proper values of the parameters ($V_2 = 1$ and $D_2 = 0$ in the last term in the expression for D) we get:

$$v = \frac{1}{1 + \dfrac{k_1 c}{k_2 V_1}} \tag{22}$$

and

$$D = \frac{D_1}{1 + \dfrac{k_1 c}{k_2 V_1}} + \frac{k_1 c v^2}{k_2^2 V_1 \left(1 + \dfrac{k_1 c}{k_2 V_1}\right)^3} = D_1 v + \frac{v^2 \gamma^2 (1 - v)}{k_2} \tag{23}$$

These relations are amenable to simple physical interpretations. Thus, v is equal to the fraction of solute in the mobile phase, and is independent of the rate of the sorption reaction (k_1/k_2 is the equilibrium constant). D, on the other hand, is strongly dependent on the reaction rate. For an infinitely fast reaction the chromatographic dispersion vanishes, and the spreading is solely due to longitudinal diffusion in the mobile phase. The spreading coefficient then equals the diffusion coefficient times the fraction of solute in the mobile phase.

In order to show the deviation from asymptotic conditions for different column characteristics, v and D values were calculated for the matrixes in Table II according to eqns. (22) and (23), and from finite differences of the data in Table II, according to:

$$v = \frac{\Delta\mu}{\Delta t}, \quad D = {}^1/_2 \frac{\Delta\mu_2}{\Delta t} \tag{24}$$

The results for the mobile phase are listed in Table III. They are expressed in local units (τ and $v\tau$ as units of time and length respectively) and refer to the mid-points of the respective intervals.

The data in Table III show that v generally is rather close to its asymptotic value, whereas for D pronounced deviations occur. The deviations are small if the initial concentration is low, as in matrixes 17 and 18. Also, in the case of large D values the asymptotic conditions are rapidly approached. Then the peak spreads out rapidly and its concentration falls to a level where asymptotic conditions prevail. This is the case in matrixes 12, 13 and 14 where the reaction rate is low and hence D is large. In cases when the concentration in a peak remains high, usually pronounced deviations from asymptotic conditions occur. This happens when the column is overloaded, matrix 19, and also when the reaction rate is high, matrixes 15 and 16.

TABLE II

PRIMARY RESULTS OF THE CALCULATIONS

For each matrix the rows, reading from top to bottom, give the values of A_0, M, μ and μ_2.

Matrix No.	*Column No.*								
	Mobile phase					*Stationary phase*			
	10	*50*	*100*	*150*	*200*	*10*	*50*	*100*	*200*
1	126.111	68.588	61.152	58.091	56.317	1.4956	1.7257	1.7554	1.7747
	4.8	12.60	19.81	26.45	32.65	4.6	12.54	19.75	32.61
	4.0224	10.600	17.034	22.989	28.708	3.6475	10.286	16.714	28.380
	1.6984	9.7520	18.301	26.474	34.470	2.3889	11.446	20.652	37.722
2	123.498	67.282	60.086	57.133	55.424	1.4952	1.7110	1.7397	1.7583
	4.7	12.36	19.54	25.98	32.18	4.5	12.25	19.50	32.12
	3.9773	10.508	16.892	22.806	28.491	3.6522	10.227	16.602	28.190
	1.6973	9.6061	18.594	26.995	35.218	2.4484	11.579	21.003	38.552
3	120.103	65.994	59.038	56.192	54.547	1.4980	1.6963	1.7239	1.7418
	4.6	12.07	19.17	25.63	31.74	4.4	11.98	19.10	31.70
	3.9362	10.420	16.755	22.630	28.280	3.6648	10.171	16.492	28.005
	1.7265	9.9579	18.932	27.578	36.053	2.5214	11.728	21.397	39.451
4	9.6332	2.4568	2.0591	1.8304	1.6769	1.9613	1.9882	1.9879	1.9866
	4.9	4.1	4.5	4.6	4.8	2.6	3.3	3.7	4.5
	4.4815	3.6217	3.8065	3.9852	4.1539	3.0292	3.2932	3.5202	3.8943
	1.2111	1.9654	2.3040	2.5824	2.8276	2.2075	2.7091	3.0882	3.6873
5	31.436	13.033	10.493	9.4131	8.7810	1.8723	1.9368	1.9427	1.9465
	4.6	5.71	7.14	8.47	9.55	3.5	5.50	6.87	9.34
	3.7826	4.8012	5.9842	6.9985	7.9256	3.1137	4.5336	5.7071	7.6316
	1.6851	3.5698	5.1717	6.5948	7.9360	2.2635	4.5542	6.4001	9.5094
6	238.848	192.418	183.839	180.226	178.117	1.0265	1.2103	1.2446	1.2675
	5.2	23.51	43.43	62.54	81.22	5.2	23.52	43.42	81.22
	4.8280	21.532	40.470	58.845	76.939	4.7332	21.362	40.294	76.759
	2.0536	15.664	32.885	50.054	67.224	2.7010	17.416	35.405	70.835
7	495.000	495.000	495.000	495.000	346.638				
	7.7	48.00	98.03	148.05	198.05				
	8.0707	48.071	98.071	148.071	195.758				
	2.6273	6.6209	11.631	16.681	10.682				

8	459.080	428.881	406.426	392.063	382.909	0.14402	0.26448	3.5430	4.4836
	7.5	46.64	93.42	137.79	180.03	7.4	46.39	92.69	178.65
	7.7654	43.972	86.474	129.942	166.145	6.5889	39.852	81.307	160.879
	2.6059	27.424	94.473	183.724	282.373	7.2454	59.010	146.728	359.992
9	341.762	212.619	188.169	178.755	173.412	0.61478	1.1295	1.2273	1.2864
	6.8	33.58	56.14	76.56	95.88	6.7	32.84	55.49	95.19
	6.6890	28.259	48.312	66.822	84.607	5.6525	26.360	46.496	82.818
	2.3631	45.099	99.778	152.654	204.668	5.5739	56.221	115.985	227.796
10	239.843	124.792	110.947	105.362	102.145	1.0246	1.4808	1.5362	1.5714
	6.1	21.75	35.06	47.12	58.58	6.0	21.50	34.76	58.29
	5.6326	18.219	30.019	40.928	51.406	4.7835	17.374	29.184	50.569
	2.1894	24.483	48.157	70.817	93.000	4.0784	29.070	54.653	102.094
11	66.190	38.656	34.757	33.170	32.255	1.7337	1.8266	1.8411	1.8510
	2.8	7.13	11.22	14.94	18.54	2.7	7.13	11.22	18.54
	2.6526	6.4143	10.106	13.538	16.884	2.6119	6.3409	10.017	16.742
	0.9285	4.5136	8.5470	12.4624	16.3148	1.4144	5.3026	9.6288	17.801
12	148.374	54.145	51.625	50.428	49.706	1.3986	1.7681	1.7750	1.7813
	6.3	10.28	15.59	20.87	26.08		5.6	11.78	22.60
	6.8402	12.942	18.265	23.425	28.490	3.9451	9.8471	15.202	25.428
	7.3502	47.812	80.931	113.328	145.359	5.2846	41.351	75.968	142.318
13	105.427	58.387	54.346	52.599	51.567	1.5722	1.7489	1.7629	1.7737
	6.0	10.02	16.03	21.74	26.84	1.8	8.2	14.47	25.81
	5.4799	10.559	16.238	21.644	26.910	3.6630	9.2225	14.903	25.563
	3.6926	22.351	40.353	57.860	75.124	4.0726	22.681	41.689	77.903
14	104.221	63.012	57.398	55.035	53.653	1.5757	1.7287	1.7504	1.7654
	5.0	10.94	17.59	23.70	29.59	3.6	10.33	17.00	29.06
	4.3586	10.108	16.159	21.836	27.327	3.5571	9.5346	15.578	26.735
	2.6085	11.405	24.394	35.308	46.041	3.0368	14.664	26.745	49.516
15	127.997	67.781	60.344	57.317	55.571	1.4765	1.7090	1.7386	1.7577
	4.7	12.60	19.78	26.35	32.57	4.5	12.58	19.76	32.56
	3.9732	10.627	17.047	22.982	28.683	3.6854	10.359	16.768	28.395
	1.5896	9.6680	18.326	26.632	34.756	2.3806	11.435	20.760	38.107

(*continued on p. 46*)

TABLE II (*continued*)

Matrix No.	*Column No.*								
	Mobile phase					*Stationary phase*			
	10	*50*	*100*	*150*	*200*	*10*	*50*	*100*	*200*
16	129.716	67.868	60.371	57.331	55.581	1.4693	1.7087	1.7385	1.7577
	4.7	12.65	19.85	26.46	32.63	4.5	12.64	19.84	32.63
	3.9775	10.670	17.095	23.032	28.735	3.6988	10.401	16.816	28.446
	1.5544	9.6618	18.334	26.645	34.767	2.3597	11.444	20.779	38.142
17	59.979	50.061	48.469	47.804	47.415	1.7729	1.7804	1.7861	1.7903
	1.8	6.24	11.39	16.37	21.29	1.7	6.21	11.36	21.26
	2.0412	6.3548	11.326	16.188	20.998	2.065	6.343	11.307	20.974
	0.8577	4.8799	9.8704	14.842	19.802	0.9468	5.1401	10.250	20.349
18	81.953	57.285	53.381	51.756	50.810	1.6695	1.7512	1.7665	1.7768
	2.8	8.60	14.08	20.17	25.63	2.7	8.55	14.54	25.59
	2.7495	7.9634	13.531	18.839	24.020	2.7135	7.8897	13.446	23.926
	1.1320	6.1853	12.177	18.068	23.901	1.4214	6.9305	13.235	25.392
19	318.544	107.617	84.652	76.372	71.761	0.7083	1.5496	1.6414	1.6930
	7.0	29.2	41.3	51.1	60.3	7.0	29.4	41.5	60.5
	6.6133	21.789	31.219	39.316	46.792	5.3824	19.744	29.339	44.953
	1.8307	43.651	78.864	109.377	137.944	5.5842	54.906	93.526	157.288
20	556.007	2597.37	5214.34	7833.34	10452.4	1.77597	9.61053	19.1426	38.1905
21	560.996	2579.01	5193.57	7812.39	10431.4	1.88860	9.82940	19.3712	38.4197

However, it should be noted that the use of finite differences in the calculations involves an approximation which becomes less satisfactory at high reaction rates (see Appendix). The deviations in the latter case are therefore exaggerated.

TABLE III

VALUES OF RELATIVE PEAK VELOCITIES AND SPREADING COEFFICIENTS

For each matrix ν is given in the first row and D in the second.

Matrix No.	*Time (in units of τ)*				
	30	*75*	*125*	*175*	∞
1	0.1644	0.1287	0.1191	0.1144	0.09091
	0.1006	0.0855	0.0817	0.0799	0.03005
2	0.1633	0.1277	0.1183	0.1137	0.09091
	0.0909	0.0899	0.0840	0.0822	0.03460
3	0.1621	0.1267	0.1175	0.1130	0.09091
	0.1029	0.0897	0.0865	0.0848	0.04114
4		0.003695	0.003576	0.003373	0.001996
		0.00339	0.00278	0.00245	0.000895
5	0.02546	0.02366	0.02029	0.01854	0.009901
	0.01884	0.01752	0.01423	0.01342	0.004378
6	0.4176	0.3788	0.3675	0.3619	0.3333
	0.1701	0.1722	0.1717	0.1717	0.07592
7	1.0000	1.0000	1.0000		1.0000
	0.05000	0.05000	0.04999		0.05000
8	0.9052	0.8500	0.8093	0.7841	0.6667
	0.3102	0.6705	0.8925	0.9865	0.6260
9	0.5393	0.4010	0.3702	0.3557	0.2857
	0.5342	0.5468	0.5288	0.5201	0.2476
10	0.3147	0.2360	0.2182	0.2096	0.1667
	0.2787	0.2367	0.2266	0.2218	0.1009
11	0.09405	0.07384	0.06863	0.06613	0.05405
	0.04481	0.04033	0.03915	0.03882	0.01376
12	0.1526	0.1064	0.1032	0.1013	0.09091
	0.5058	0.3312	0.3240	0.3203	0.3051
13	0.1270	0.1136	0.1081	0.1053	0.09091
	0.2332	0.1800	0.1751	0.1726	0.1548
14	0.1437	0.1210	0.1135	0.1098	0.09091
	0.1100	0.1299	0.1091	0.1073	0.07968
15	0.1664	0.1284	0.1187	0.1140	0.09091
	0.0808	0.0866	0.0831	0.0813	0.02600
16	0.1673	0.1285	0.1188	0.1140	0.09091
	0.1013	0.0867	0.0831	0.0812	0.02124
17	0.1078	0.0994	0.0973	0.0962	0.09091
	0.0503	0.0499	0.0497	0.0496	0.03460
18	0.1303	0.1114	0.1062	0.1036	0.09091
	0.0632	0.0599	0.0589	0.0583	0.03460
19	0.3764	0.1886	0.1619	0.1495	0.09091
	0.5228	0.3521	0.3051	0.2857	0.03460

Peak asymmetry

The form of the concentration peaks was found to be rather similar in all cases studied. From the data in Table II it appears that generally $\mu < M$. Thus, the peaks exhibit negative skewness (according to Pearson's measure $S = (\mu - M)/\sqrt{\mu_2}$). This behaviour can be explained as an effect of the nonlinear sorption isotherm, which has the tendency to compress the leading boundary of a peak. This effect is reduced when asymptotic conditions are approached. It is realized from eqns. (22) and (23) that in the limit of partition chromatography the operational conditions are symmetric, as the equations are invariant under the reversal of the velocity of the mobile phase. Under these conditions an originally symmetric peak will remain symmetric. Some typical peaks are reproduced in Figs. 1, 2 and 3. In Fig. 1 the peaks both in the mobile and stationary phases are shown, whereas in Figs. 2 and 3 the variation of shape with time is shown.

Convergence of the numerical solutions

In order to investigate the dependence of the numerical solutions on the size of the finite differences, some calculations were carried out in which the number of cells for a given length of column was varied. Thus, in the matrixes 22, 23, 24, 25 and 26, the initial peak is confined to 2, 5, 10, 15 and 20 cells, respectively, and the operational conditions of the corresponding chromatographic columns are identical if τ is assigned the values 10, 4, 2, $^4/_3$ and 1 sec, respectively. The results for the mobile phase are listed in Table IV in the form of $\tau\mu$ and $\tau^2\mu_2$ values for two columns of each matrix,

TABLE IV

CONVERGENCE OF THE NUMERICAL SOLUTIONS

Matrix No.	τ (*sec*)	*Column No.*	$\tau\mu$	$\tau^2\mu_2$	v	D
22	10	10	28.336	126.952	0.1333	0.5867
		20	41.665	244.283		
23	4	25	26.834	115.947	0.1347	0.4923
		50	40.308	214.411		
24	2	50	26.633	116.946	0.1346	0.4893
		100	40.092	214.814		
25	$^4/_3$	75	26.595	117.672	0.1345	0.4913
		150	40.041	215.939		
26	1	100	26.583	118.116	0.1344	0.4929
		200	40.020	216.689		

representing the situations at the same time instances. It also contains v and D values, calculated from the differences between the two sets of values according to eqn. (24). Finally, in Fig. 4 the concentration distributions for a peak, resulting from some of these matrixes, are compared. It may be concluded that the convergence of the numerical solutions is quite satisfactory.

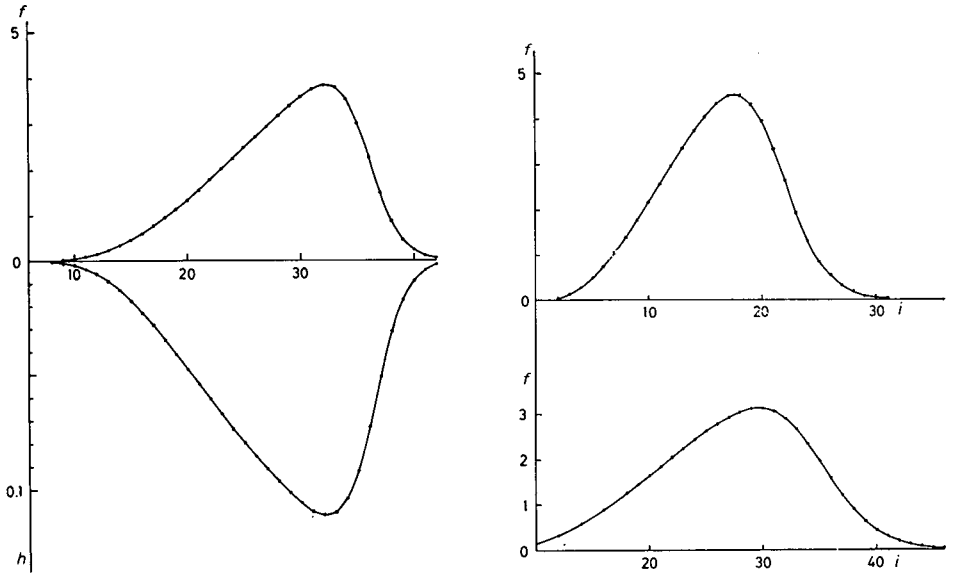

Fig. 1. Concentration distribution in the mobile and stationary phases. Column $j = 200$ of matrix No. 2.

Fig. 2. Concentration distribution in the mobile phase. Columns $j = 100$ and $j = 200$ of matrix No. 14.

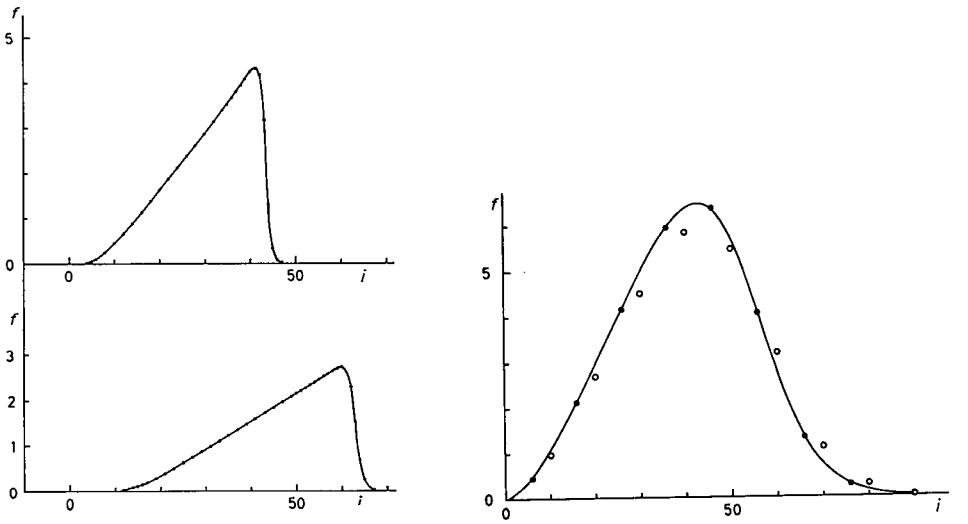

Fig. 3. Concentration distribution in the mobile phase of an overloaded chromatographic column Columns $j = 100$ and $j = 200$ of matrix No. 19.

Fig. 4. Concentration distributions in the mobile phase resulting from calculations with finite differences of varying size. The curve represents column $j = 200$ of matrix 26, filled circles column $j = 100$ of matrix 24 and unfilled circles column $j = 20$ of matrix 22.

Frontal analysis

We will next consider a column fed with a solution of constant concentration. This case is amenable to a straightforward analytical treatment and has been studied by earlier investigators[4,5]. We will indicate here a more direct approach where longitudinal diffusion is also taken into account. We start with eqns. (1) and (2) and investigate their solution for a stationary boundary. The existence of such a boundary is guaranteed by the nonlinearity of the sorption isotherm, which makes the movement of different points of the boundary a function of concentration.

Denoting the velocity of the stationary boundary by ω, we may determine it directly from the mass balance equation:

$$vtV_1 f_0 = \omega t V_1 f_0 + \omega t h_\infty \tag{25}$$

where f_0 and h_∞ refer to feed concentration and equilibrium concentration of f and h, respectively. From (25) and (2) (with $\partial h/\partial t = 0$) we get:

$$\frac{\omega}{v} = \frac{f_0}{f_0 + \frac{h_\infty}{V_1}} = \frac{1}{1 + \frac{1}{V_1}\frac{c}{f_0 + \frac{k_2}{k_1}}} \tag{26}$$

We will next switch to a new coordinate system, which follows the movement of the boundary. Thus we make the transformation:

$$\xi = x - \omega t \tag{27}$$

Eqns. (1) and (2) take the form:

$$\frac{\partial f}{\partial t} = D_1 \frac{\partial^2 f}{\partial \xi^2} - (v - \omega)\frac{\partial f}{\partial \xi} - \frac{1}{V_1}\left(\frac{\partial h}{\partial t} - \omega \frac{\partial h}{\partial \xi}\right) \tag{28}$$

$$\frac{\partial h}{\partial t} - \omega \frac{\partial h}{\partial \xi} = k_1 f(c - h) - k_2 h \tag{29}$$

For a stationary boundary we have:

$$\frac{\partial f}{\partial t} = \frac{\partial h}{\partial t} = 0 \tag{30}$$

Hence:

$$D_1 \frac{\mathrm{d}^2 f}{\mathrm{d}\xi^2} - (v - \omega)\frac{\mathrm{d}f}{\mathrm{d}\xi} + \frac{\omega}{V_1}\frac{\mathrm{d}h}{\mathrm{d}\xi} = 0 \tag{31}$$

$$\omega \frac{\mathrm{d}h}{\mathrm{d}\xi} + k_1 f(c - h) - k_2 h = 0 \tag{32}$$

A first integration of (31) gives:

$$D_1 \frac{\mathrm{d}f}{\mathrm{d}\xi} - (v - \omega) f + \frac{\omega}{V_1} h = K \tag{33}$$

For an originally empty column we have:

$$f = h = \frac{df}{d\xi} = 0 \tag{34}$$

hence:

$$K = 0$$

Thus, in this case the stationary boundary is determined by the following equations:

$$D_1 \frac{df}{d\xi} - (v - \omega) f + \frac{\omega}{V_1} h = 0 \tag{35}$$

$$\omega \frac{dh}{d\xi} + k_1 f (c - h) - k_2 h = 0 \tag{36}$$

These equations may be solved directly for $D_1 = 0$. Then according to (35):

$$f = \frac{\omega}{V_1 (v - \omega)} \cdot h \tag{37}$$

With (37) and (26), the integration of (36) yields:

$$\frac{f}{f_0} = \frac{1}{1 + e^{\frac{k_1 f_0}{\omega} \xi}} \tag{38}$$

The case $D_1 \neq 0$ is more troublesome. However, owing to the small value of D_1, the solution for $D_1 = 0$ is a good first order approximation. It is therefore possible to solve the full equations by iteration, inserting the approximate solution into the non-linear term in eqn. (36). The resulting linear equations may then be solved by standard methods.

In Table II numerical solutions are given for the case $\alpha = 0$ and $\alpha = 0.8$. Choosing $\tau = 1$ sec. this gives $D_1 = 0$ and $D_1 = 4 \cdot 10^{-5}$ cm^2 sec^{-1} respectively. In Table II only the values of A_0 are given. They determine the first moment μ of the boundary. We have:

$$\mu f_1 = \sum_{i=1}^{\infty} i(f_i - f_{i+1}) = \sum_{i=1}^{\infty} i f_i - \sum_{i=1}^{\infty} (i + 1) f_{i+1} + \sum_{i=1}^{\infty} f_{i+1} = \sum_{i=1}^{\infty} f_i = A_0 \tag{39}$$

Here f_1 is the constant concentration in the plateau region. In the mobile phase we have $f_1 = 100$, hence $\mu = A_0/100$. From the data in Table II we see that the velocity of a stationary boundary is constant. It has exactly the value predicted by eqn. (26). In Fig. 5, the boundaries for the two cases are shown in detail. We see that in the case of non-vanishing longitudinal diffusion the boundary is not symmetrical. The effect of diffusion is seen to be rather small, however, and the translational velocity of the boundary remains unaffected.

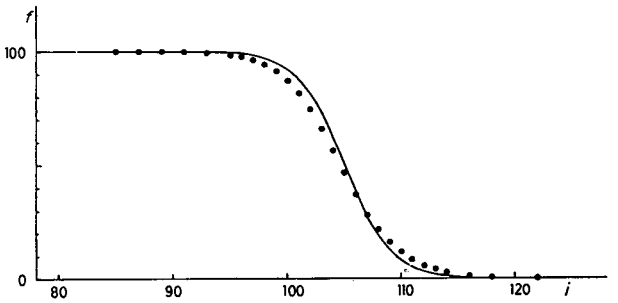

Fig. 5. Concentration profiles in frontal analysis. Columns $j = 200$ of matrixes Nos. 20 (line) and 21 (filled circles).

APPENDIX

Some aspects concerning the errors involved in the application of the finite difference method to chromatography are now considered. First, for the sake of generality, the recursion formulae in partition and sorption chromatography are reformulated on a common basis, and then take the form:

$$f^0_{ij} = f_{ij} + {}^1/_2\,\alpha\,(f_{i-1,\,j} - 2\,f_{ij} + f_{i+1,\,j}) \tag{40}$$

$$f_{i+1,\,j+1} = f^0_{ij} - \beta\delta_{ij} \tag{41}$$

$$h_{i,\,j+1} = h_{ij} + \delta_{ij} \tag{42}$$

Here the term δ_{ij} represents the exchange of solute between the mobile and stationary phases in a cell, and is thus determined by the kinetics of the chromatographic process. The parameter β has the values V_2/V_1 and $1/V_1$ for partition and sorption chromatography respectively. In the case of partition chromatography we get according to eqns. (23), (24) in ref. 2:

$$\beta\delta_{ij} = \eta\left(f^0_{ij} - \frac{1}{\gamma}\,h_{ij}\right) \tag{43}$$

(here h_{ij} is the solute concentration in the stationary phase, but is designated γg_{ij} in ref. 2).

In sorption chromatography with Langmuir kinetics we get according to eqns. (12)–(16) in ref. 1

$$\delta_{ij} = \delta^1_{ij} + \delta^2_{ij} + \ldots + \delta^m_{ij} \tag{44}$$

with

$$\delta^k_{ij} = \frac{\tau}{m}\,[k_1 f^{k-1}_{ij}\,(c - h^{k-1}_{ij}) - k_2 h^{k-1}_{ij}] \qquad k = 1, 2, \ldots, m \tag{45}$$

(here, by comparison to ref. 1, the indices have been changed for convenience).

The object is now to establish the variation with time in the first and second moments of a concentration distribution and compare the results with exact formulae. Such formulae are available in partition chromatography and the treatment will therefore be restricted to this case only, the results also being valid asymptotically

for sorption chromatography. We will consider isolated peaks and, in the formulae below, let all summation limits refer to points on both sides of the peak, in regions of zero concentration. For the second moment at time $j + 1$ we then get:

$$A_{2,\,j+1} = \sum_i i^2 f_{i,j+1} = \sum_i (i+1)^2 f_{i+1,\,j+1} = (1-\eta) \sum_i (i+1)^2 f^0_{ij} + \\ + \eta/\gamma \sum_i (i+1)^2 h_{ij} \quad (46)$$

To evaluate the first term on the right hand side we substitute for f^0_{ij} from eqn. (40) and use the identities:

$$(i+1)^2 = i^2 + 2i + 1$$

$$(i+1)^2 = (i-1)^2 + 4(i-1) + 4$$

Then:

$$A_{2,\,j+1} = (1-\eta)(A_{2\,j} + 2A_{1j} + A_{0j} + \alpha A_{0j}) + \eta/\gamma \sum_i (i+1)^2 h_{ij} \quad (47)$$

Using the same procedure we get for the first moment:

$$A_{1,\,j+1} = (1-\eta)(A_{1\,j} + A_{0j}) + \eta/\gamma \sum_i (i+1) h_{ij} \quad (48)$$

In general these expressions are dependent on the original concentration distributions (f_{i0}, h_{i0}) and hence become exceedingly complicated for high values of j. However, when the reaction rate is so high that equilibrium between the two phases in a cell is established in an equilibration step, this dependence disappears and the equations take simple forms. We may use eqns. (21)–(24) in ref. 2 and, as then:
$m = \infty$ in the expressions for η and ξ, we get
$\eta = \xi$, which implies:

$$f_{i+1,\,j} = \frac{1}{\gamma} h_{ij} \quad (49)$$

Also, as the concentration in a phase is now constant, we use normalized distributions and put:

$$A_{0j} = 1 \quad (50)$$

Inserting the last two equations into (47) and (48) we get:

$$A_{2,\,j+1} = A_{2\,j} + (1-\eta)(2A_{1\,j} + 1 + \alpha) \quad (51)$$

$$A_{1,\,j+1} = A_{1\,j} + 1 - \eta \quad (52)$$

For the variance we get:

$$\mu_{2,\,j+1} = A_{2,\,j+1} - A^2_{1,\,j+1} = \mu_{2\,j} + \alpha(1-\eta) + \eta(1-\eta) \quad (53)$$

We are now in the position to write down expressions for the peak velocity v and spreading coefficient D. In local units they take the form:

$$v = A_{1,\,j+1} - A_{1\,j} = 1 - \eta \tag{54}$$

$$D = {}^1/_2\,(\mu_{2,\,j+1} - \mu_{2\,j}) = {}^1/_2\,\alpha(1-\eta) + {}^1/_2\,\eta(1-\eta) \tag{55}$$

Substituting the values of α and η and comparing the formulae with eqns. (36) and (39) in ref. 6 we find that no error is involved in the expression for v, and that in the expression for D the term representing longitudinal diffusion is exact, while the chromatographic dispersion is subject to the error ${}^1/_2\,\eta\,(1-\eta)$.

This is strictly valid for an infinitely fast equilibration reaction and it is therefore of interest to consider the error at lower reaction rates. Although a general theoretical analysis of this problem is impracticable, some information may be obtained from the numerical data in this paper and in ref. 3. Thus, it appears that for steady state conditions the error in v always is very small and that the error in D generally decreases with decreasing reaction rate. This indicates that ${}^1/_2\,\eta\,(1-\eta)$ represents the upper limit of the error of the chromatographic dispersion. Further the fact that no error is involved in the longitudinal diffusion is of great interest. If pure diffusion is considered, this implied that the finite difference method (Schmidt's formula, *cf.* ref. 7) leads to macroscopically correct results (with respect to μ_2, *cf.* ref. 8). This result may be generalized and, with the help of the formula in ref. 8, it may be shown that the result is correct even when the diffusion coefficient is a linear function of concentration.

ACKNOWLEDGEMENTS

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SUMMARY

The operation of a chromatographic column with a sorption reaction following Langmuir kinetics has been simulated by numerical calculations on a digital computer. The operational conditions of the column are varied within wide limits and the results are related to theoretical considerations. It is shown that in sorption chromatography the conditions of partition chromatography are asymptotically approached and the process may then be described by the exact analytical formulae of linear partition chromatography. The errors in the finite difference method are discussed and evaluated for some special cases.

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DETERMINATION OF THE TRISATURATED GLYCERIDE CONTENT OF FATS BY COLUMN CHROMATOGRAPHY OF THE MERCURIC ACETATE ADDUCTS ON FLORISIL*

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INTRODUCTION

The trisaturated glyceride (GS_3) content of natural fats can be determined by the traditional permanganate oxidation techniques described by HILDITCH[1]. It has been shown by ESHELMAN AND HAMMOND[2] that these methods do not yield reliable results with milk fat. In addition, the procedure is involved and time consuming. In a research project designed to study the trisaturated glyceride content of milk fat (to be published elsewhere) a method has been developed which proved suitable for the rapid determination of the GS_3 content of fats. It is based on the quantitative addition of mercuric acetate to the double bonds of all unsaturated glycerides (GU) followed by separation of the GS_3 and GU by column chromatography on Florisil. The GU can be recovered from the adducts with simultaneous re-establishment of the original structure by elution with an acid-containing solvent.

EXPERIMENTAL

Margarine fat was obtained from commercial samples of margarine by melting, washing with warm water to remove non lipid material, drying under reduced pressure and filtration. The other fats were obtained from commercial sources and used without further treatment.

The trisaturated glyceride content was determined by weighing 700–800 mg of fat into a 50 ml Erlenmeyer flask with ground joint, and adding 3 g of mercuric acetate, 12 ml of methanol and a few boiling chips. The mixture was refluxed for 30 min and the contents of the flask transferred to a 500 ml separatory funnel fitted with a Teflon stopcock which contained 200 ml of water. The flask was rinsed several times with a total volume of 75 ml of chloroform, which was added to the contents of the separatory funnel. The organic solvent layer was removed and the aqueous layer washed 4 times with 50 ml of chloroform. The combined chloroform extracts were evaporated. The chromatographic separation was carried out on a column of Florisil (activated magnesium silicate). The Florisil was deactivated with 14 % by wt. of distilled water. The columns were Pyrex tubes, about 400 × 20 mm in size each fitted with a fritted glass disk and a Teflon stopcock. The elution solvent was a

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mixture of hexane–diethyl ether, 8:2. The columns were charged with 30 g of deactivated Florisil slurried in elution solvent.

The reacted fat, after evaporation of the solvent, was taken up in 10 ml of the elution solvent and introduced into the chromatographic column. The GS_3 were eluted with 500 ml of the hexane–ether mixture and weighed after evaporation of the solvent.

The unsaturated glycerides could be obtained and regenerated from the adducts adsorbed on the Florisil by a second elution with 180 ml of a solvent mixture consisting of 95 % ethanol, chloroform and hydrochloric acid, 9:8:1. The eluent was transferred to a 500 ml separatory funnel, 200 ml of water and 200 ml of petroleum ether added and the organic solvent layer washed several times with water. The organic layer was then neutralized, washed again and evaporated.

The fatty acid compositions of the GS_3 and GU were determined by GLC using a method similar to the one described by DEMAN[3]. The results are expressed in weight % as methyl esters.

RESULTS AND DISCUSSION

The reliability of the outlined procedure is indicated by the fatty acid analysis of the GS_3. In no case was there more than a trace (less than 0.1–0.2 %) of unsaturated fatty acid in the GS_3. Fig. 1 shows the gas chromatograms of a sample of shortening (sample No. 2), and of the GS_3 obtained from it. Recovery trials of added trilaurin and trimyristin to vegetable oils indicated recoveries within ± 0.6 %, when GS_3 was added at levels of 20–40 %. Repeatability was checked by performing 10 replications on a sample of milk fat, which resulted in values ranging from 35.2 to 36.6 % with a standard deviation of the mean of 0.15 %.

The GS_3 contents of two samples of lard (Table I) were 10.3 and 11.1 %. The fatty acid compositions of sample 1, its GS_3 and GU are given in Table II. The values for GS_3 content correspond with figures reported by COLEMAN[4], who found 9.2 and 12.9 mole % in two samples. VANDER WAL[5] found only 0.6 mole % and YOUNGS[6] 8.0 mole %. The GS_3 contains palmitic and stearic acid as major components in approximately equal amounts, and small amounts of lauric and myristic acid. The

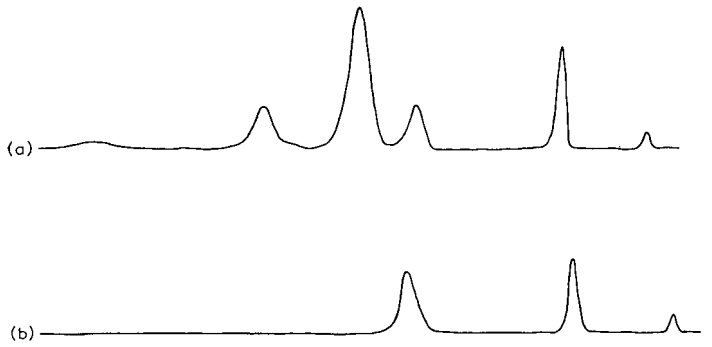

Fig. 1. Gas chromatograms of the fatty acid methyl esters of a sample of shortening (No. 2, Table I) and of the trisaturated glycerides obtained from it by the mercuric acetate method. Peaks from right to left: (a) 14:0, 16:0, 18:0, 18:1, 18:2, and 18:3; (b) 14:0, 16:0, and 18:0.

TABLE I

TRISATURATED GLYCERIDE (GS_3) CONTENT OF SOME FATS AS DETERMINED BY THE MERCURIC ACETATE ADDUCT PROCEDURE

Product	% GS_3
Lard 1	11.1
Lard 2	10.3
Shortening 1	17.1
Shortening 2	11.5
Coconut oil	81.9
Margarine fat 1	4.1
Margarine fat 2	9.0
Margarine fat 3	6.3
Margarine fat 4	5.4
Margarine fat 5	9.3

TABLE II

FATTY ACID COMPOSITION IN WEIGHT % AS METHYL ESTERS OF LARD SAMPLE I AND THE TRISATURATED (GS_3) AND UNSATURATED GLYCERIDES (GU) OBTAINED FROM IT BY THE MERCURIC ACETATE ADDUCT PROCEDURE

Fatty acid	*Whole fat*	*GS_3*	*GU*
12:0	0.1	0.9	0
14:0	1.2	3.6	1.3
16:0	25.1	49.7	23.7
16:1	1.8	0	2.3
18:0	12.1	45.6	10.7
18:1	45.7	trace	49.1
18:2	12.6	0	10.8
18:3	1.5	0	2.0

TABLE III

FATTY ACID COMPOSITION IN WEIGHT % AS METHYL ESTERS OF SHORTENING SAMPLE I AND THE TRISATURATED (GS_3) AND UNSATURATED GLYCERIDES (GU) OBTAINED FROM IT BY THE MERCURIC ACETATE ADDUCT PROCEDURE

Fatty acid	*Whole fat*	*GS_3*	*GU*
12:0	0.1	0.9	0
14:0	6.5	7.8	5.0
16:0	26.3	49.4	20.9
16:1	8.3	1.0	10.5
18:0	13.8	40.8	9.8
18:1	29.7	trace	34.3
18:2	11.2	0	14.1
18:3	4.2	0	5.4

lauric acid was found to be associated with the GS_3. MAGIDMAN *et al.*[7] have reported 0.07 % lauric acid in lard. The stearic acid content of the GS_3 was close to four times higher than in the whole fat, whereas the palmitic acid content was only about twice as high. This indicates that, in lard, palmitic acid seems to combine more readily with unsaturated acids into glycerides than does stearic acid.

The shortening samples analyzed contained 17.1 and 11.5 % GS_3. There seems to be no published information on the content and composition of the GS_3 of shortenings. Table III gives the fatty acid composition of shortening sample No. 1 and its GS_3 and GU. As in the lard there was a relatively greater accumulation of stearic acid than palmitic acid in the GS_3.

Coconut oil had a GS_3 content of 81.9 %. ESHELMAN *et al.*[8] reported 72.2 and 76.2 % in two samples analyzed by the mercaptoacetic acid method. COLLIN AND HILDITCH[9] found 84–86 mole %. The fatty acid composition of coconut oil, its GS_3 and GU is listed in Table IV. It is interesting to note that palmitic acid is present in a higher percentage in the GU than in the GS_3.

TABLE IV

FATTY ACID COMPOSITION IN WEIGHT % AS METHYL ESTERS OF COCONUT OIL AND THE TRISATURATED (GS_3) AND UNSATURATED GLYCERIDES (GU) OBTAINED FROM IT BY THE MERCURIC ACETATE ADDUCT PROCEDURE

Fatty acid	*Whole fat*	*GS_3*	*GU*
6:0	0.8	0.9	0.5
8:0	7.6	8.6	4.6
10:0	6.5	7.1	2.9
12:0	47.3	53.8	23.1
14:0	17.3	19.3	9.5
16:0	8.2	7.2	11.2
16:1	0.1	0	0.5
18:0	2.7	3.0	2.6
18:1	7.2	trace	35.5
18:2	2.3	trace	9.6
18:3	trace	0	trace

Five samples of margarine fat analyzed had GS_3 contents ranging from 4.1 to 9.3 %. The fatty composition of sample No. 1 and its GS_3 and GU is given in Table V.

The examples given above indicate that this method can be applied to determine the GS_3 content of a variety of fats, it is considerably easier to perform and less time consuming than other methods.

TABLE V

FATTY ACID COMPOSITION IN WEIGHT % AS METHYL ESTERS OF MARGARINE FAT SAMPLE I AND THE TRISATURATED (GS_3) AND UNSATURATED GLYCERIDES (GU) OBTAINED FROM IT BY THE MERCURIC ACETATE ADDUCT PROCEDURE

Fatty acid	*Whole fat*	*GS_3*	*GU*
14:0	1.2	16.8	0.4
16:0	12.3	61.0	10.4
18:0	5.9	22.2	5.4
18:1	49.0	trace	51.0
18:2	31.6	0	32.8

SUMMARY

A new method for the determination of the trisaturated glyceride (GS_3) content of fats is described. The method is based on the formation of mercuric acetate adducts of the unsaturated glycerides (GU) of the fat and the subsequent column chromatographic separation of the GS_3 and the mercuric acetate adducts on deactivated Florisil. The GU can be regenerated and eluted with an acid-containing solvent. The fatty acid compositions of the fats, GS_3 and GU of lards, shortenings, coconut oil, and margarine fats were determined.

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QUANTITATIVE DETERMINATION OF α- AND γ-2,4-DINITROPHENYL-ISOMERS OF α,γ-DIAMINOBUTYRIC ACID WITH AN AUTOMATIC AMINO ACID ANALYZER AS A METHOD OF STUDYING $N^{\alpha} \rightleftarrows N^{\gamma}$ MIGRATION IN PEPTIDES OF α,γ-DIAMINOBUTYRIC ACID AND POLYMYXIN M

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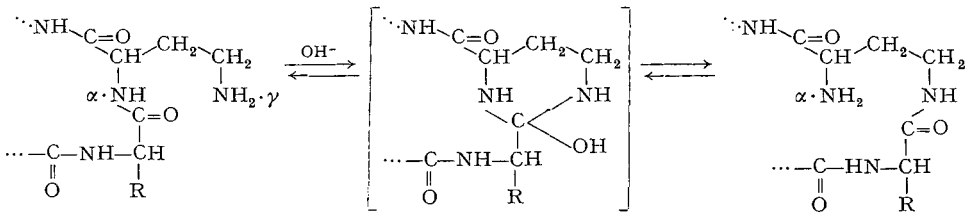
According to the data reported in the literature, salts of antibiotics of the polymyxin group are stable in weak acid and neutral solutions, but are inactivated in acid solutions and in particular in alkaline ones[1–5]. The study of the alkaline inactivation of the antibiotic polymyxin M has shown that in 0.1 *N* NH_4OH at 37° the antibiotic almost completely loses its antibacterial activity in three to five days[6,7]. The inactivation is caused by the change of the secondary structure of the molecule, by a partial racemization of the amino acids forming the molecule of polymyxin M, and finally by intramolecular transfer of the amino acid acyl residue from the α-amino group to the γ-amino group of α,γ-DAB*, which takes place according to the following scheme[8–10].



The possibility of such $N^{\alpha} \rightarrow N^{\gamma}$ and vice versa $N^{\gamma} \rightarrow N^{\alpha}$ migrations occurring was later qualitatively demonstrated on model peptides[11,12]. The problem confronting us was that of the quantitative estimation of the $N^{\alpha} \rightarrow N^{\gamma}$ migration. To solve it, one had to know the composition of the α-peptide of α,γ-DAB and of the γ-peptide formed as a result of $N^{\alpha} \rightarrow N^{\gamma}$ migration and the quantity of γ-peptide and α-peptide formed from it in the case of $N^{\gamma} \rightarrow N^{\alpha}$ migration. The simplest way to achieve this is by marking the free α- or γ-amino group of the α,γ-DAB forming the peptide with, for example, a DNP-residue. Then the quantity of α-peptide in the mixture may be easily determined from the percentage of γ-DNP-DAB, while the quantity of γ-

* Abbreviations: Pel = pelargonic acid; α,γ-DAB = α,γ-diaminobutyric acid; DNP = 2,4-dinitrophenyl-; FDNP = 1-fluoro-2,4-dinitrobenzene; Gly-DAB = glycyl-diaminobutyryl diketopiperazine; DAB-DAB = α,γ-DAB diketopiperazine; Thr = threonine; Leu = leucine.

peptide may be determined from the amount of α-DNP-DAB obtained upon hydrolysis and separation of the DNP-isomers of α,γ-DAB. The main difficulty consists in the separation of the α- and γ-DNP-derivatives of α,γ-DAB. There are only a few, not very effective, ways of separating the α-DNP- and γ-DNP-isomers of α,γ-DAB described in the literature[8,10,13,14]. The most complete separation was achieved by paper electrophoresis at pH 9.1 (ref. 14). However, the analysis takes a long time (17 h) and requires additional correction factors for the adsorption of DNP-derivatives on the paper. In the present study we used an automatic amino acid analyzer for the separation of the α- and γ-DNP-isomers[15,16].

α- and γ-peptides of α,γ-DAB and polymyxin M, after being sustained under conditions of inactivation of the antibiotic (0.1 *N* NH_4OH, 37°, for four days), were subjected to dinitrophenylation in a bicarbonate buffer pH 9.5 according to methods described previously[17]. The completeness of dinitrophenylation was checked by paper electrophoresis in the mixture: 85 % formic acid–glacial acetic acid–water (28:20:52) (300 V, 2–3 h)[18,19]. Then the DNP-peptides, without preliminary refining to prevent losses, were hydrolyzed by 6 *N* HCl for 16 h at 106–108°, and after complete removal of HCl, studied with the "Hitachi" amino acid analyzer, KLA-2 type. The analysis was carried out on a 0.9 × 15 cm column filled with Amberlite CG-120, type III (400 mesh). A 0.30 *N* Na^+-citrate buffer with a pH of 5.28 ± 0.02 was used as eluting buffer. The separation of α-DNP- and γ-DNP-isomers of α,γ-DAB is based on their differences in solubility and basicity. Fig. 1 depicts an elution curve for α- and γ-DNP-DAB, α,γ-DAB and NH_3. The first peak is that of α-DNP-DAB (106 min ± 3 min), the final one (195 min ± 3 min) is the peak of γ-DNP-DAB. The whole analysis takes about 3.5 h.

The clear-cut separation of the pairs α-DNP-DAB and α,γ-DAB, and NH_3 and γ-

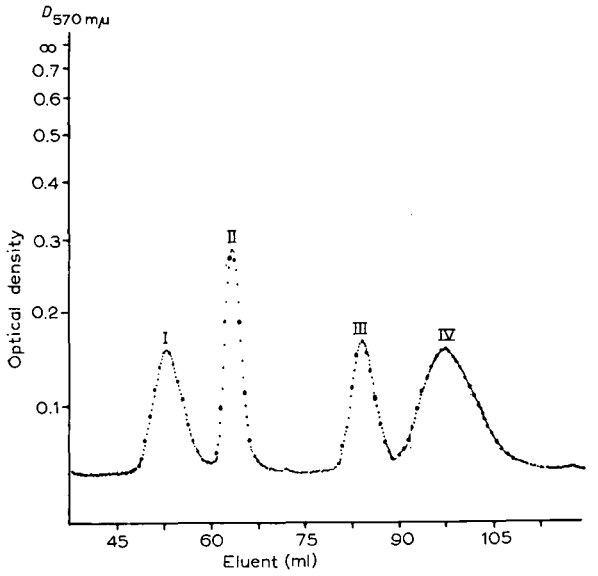


Fig. 1. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.30 *N* Na^+-citrate buffer, pH 5.28 ± 0.02. I = α-DNP-DAB; II = α,γ-DAB; III = NH_3; IV = γ-DNP-DAB.

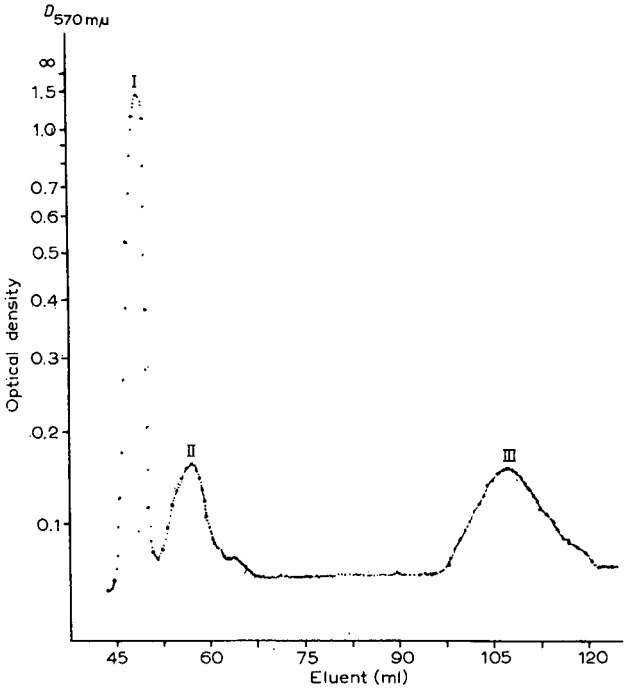


Fig. 2. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.30 *N* Na^{+}-citrate buffer, pH 5.28 ± 0.02. I = Lysine; II = α-DNP-lysine; III = ε-DNP-lysine.

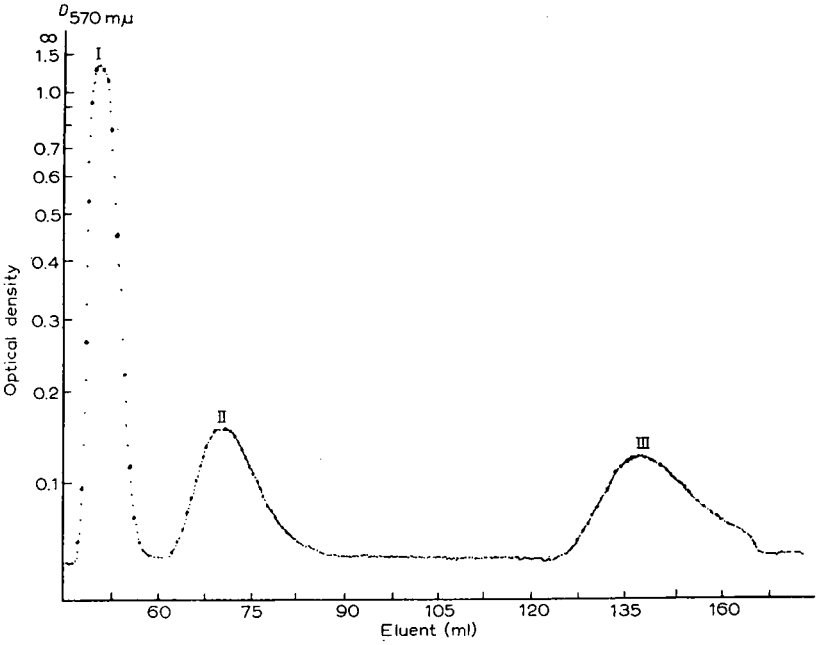


Fig. 3. The separation of the main amino acids and their mono-DNP-derivatives with the KLA-2 "Hitachi" analyzer on a column of 15 cm at a temperature of 50° with 0.7 *N* Na^{+}-citrate buffer, pH 5.28. I = Ornithine; II = ε-DNP-ornithine; III = δ-DNP-ornithine.

TABLE I

THE RESULTS OF THE QUANTITATIVE ANALYSIS OF PEPTIDES AND OF AN INACTIVE POLYMYXIN M

15-cm column; temperature 50°; eluent 0.30 *N* Na^+-citrate buffer; pH 5.28 ± 0.02.
The analysis of the peptides was carried out after their complete dinitrophenylation and acid hydrolysis under standard conditions. The data presented in the table are given as the degree of breakdown of the DNP-amino acids during hydrolysis: α-DNP-DAB is destroyed to an extent of 16.6% (6 *N* HCl, 16 h, 105–108°), γ-DNP-DAB to an extent of 10.5% (according to data in the literature[13] 10–11%). (a) Before keeping in a thermostat in 0.1 *N* NH_4OH; (b) after keeping in a thermostat in 0.1 *N* NH_4OH for 4 days at 37 ± 1°.

No.	*Compound analysed*		*Amino acid residue*						*Quantity of isomer peptide (degree of re-acylation)*
			α-DNP-DAB		*γ-DNP-DAB*		*α,γ-DAB*		
			μmole	%	*μmole*	%	*μmole*	%	
1	α-Pel-DAB		0.95	56.5	0.65	39.3	0.07	4.2	56.5
2	γ-Pel-DAB		1.12	59.4	0.63	33.5	0.13	7.1	33.5
3	α-Gly-DAB	(a)	—	—	1.05	—	—	—	—
		(b)	2.48	76.8	0.39	11.7	0.43	13.1	76.8
4	γ-Gly-DAB	(a)	1.38	92.8	—	—	0.12	8.0	—
		(b)	0.56	81.7	0.07	9.9	0.06	8.5	9.9
5	Inactive DNP-polymyxin M*		0.341	50.2	0.304	44.8	0.034	5.0	50.2
6	Gly-DAB		0.58	26.1	0.19	8.9	1.43	65.0	—
7	DAB-DAB		0.25	14.1	0.68	38.0	0.86	47.9	—

* In estimating we took into consideration quantities of α-DNP- and γ-DNP-DAB and an excess quantity of α,γ-DAB as compared to the active DNP-polymyxin M (see text p. 65).

DNP-DAB ensures a precise identification and a quantitative estimation according to the absorption at 570 mμ. Under these conditions the micromolar absorption coefficient (*G*) is equal to 6.6 for α-DNP-DAB, 12.6 for γ-DNP-DAB, and 13.1 for α,γ-DAB. It should be mentioned that under identical conditions a clear-cut separation of α-DNP-lysine, ε-DNP-lysine and lysine is also obtained (see Fig. 2). The separation of ornithine and its DNP-isomers is satisfactorily carried out in 0.7 *N* Na^+-citrate buffer pH 5.28 (see Fig. 3). Neutral amino acids and their DNP-derivatives as well as FDNB and dinitrophenol are eluted from the column in the first 50–65 min. Table I shows the results of the analysis of four synthetic peptides* and of two diketopiperazines of α,γ-DAB and of an inactive DNP-polymyxin M.

Table I illustrates that re-acylation occurs in α- as well as in γ-peptides of α,γ-DAB. However, $N^{\alpha} \rightarrow N^{\gamma}$ migration goes on more extensively than $N^{\gamma} \rightarrow N^{\alpha}$-migration, *i.e.* a shift towards the greater formation and preservation of the γ-peptide is observed. In other words, under conditions of polymyxin M inactivation an equilibrium mixture is formed with 58% of the γ- and 36% of the α-peptide in the case of α- and γ-Pel-DAB, and 79% of the γ- and 10% of the α-isomer in the case of α- and γ-Gly-DAB. This phenomenon may be explained by a lower basicity of the α-amino group of α,γ-DAB as compared to that of the γ-amino group, as well as by a possible steric hindrance of the attack of the —C(O)NH(γ) bond by an α-amino group. In addition, the degree of re-acylation also depends on the kind of acyl residue. Thus, for γ-Pel-DAB $N^{\gamma} \rightarrow N^{\alpha}$ migration is 33.5%, whereas for γ-Gly-DAB it is only 9.9%.

* The peptides were synthesized by K. PODUSHKA (ČSSR) and by one of our colleagues[12].

It is noteworthy that in hydrolysates of all the peptides and of an inactive DNP-polymyxin M, inactivated in 0.1 *N* NH_4OH and processed by FDNB, it was possible to find from 4.2 to 13.1 % of free α,γ-DAB upon acid hydrolysis. Control hydrolysates of α-DNP-DAB, γ-DNP-DAB and dinitrophenylated α-Gly-DAB did not yield free α,γ-DAB. However, in the hydrolysate of the DNP-peptide of γ-Gly-DAB, 8 % of free α,γ-DAB was discovered. Its presence may be explained either by the formation of an intermediate substance (II) (see the scheme), during dinitrophenylation, or simply by the breakdown of this peptide in the process of hydrolysis.

The intermediate substance II, exposed to FDNB, upon hydrolysis yields neither α- nor γ-DNP-DAB, but only free α,γ-DAB. Therefore, according to the quantity of the latter it is possible to estimate quantitatively in the reaction mixture the amount of the intermediate substance II formed during the period of inactivation.

The method for the quantitative estimation of α- and γ-DNP-isomers of α,γ-DAB suggested in this paper enables the complete amino acid composition of the DNP-derivatives of active and inactive polymyxin M to be determined and, on this basis, the quantitative estimation of the $N^{\alpha} \rightarrow N^{\gamma}$ migration in the antibiotic during its inactivation. It was found that active DNP-polymyxin M has the following proportions: Leu:Thr:α,γ-DAB:γ-DNP-DAB = 1.0:2.7:0.9:4.9, respectively, which coincides with the data on its structure[20,21]. For an inactive polymyxin M the following proportion of amino acids was obtained: Leu:Thr:α,γ-DAB:γ-DNP-DAB:α-DNP-DAB = 1:2.5:1.2:2.3:2.5, respectively. Thus, an inactivated antibiotic, as well as an active one, contains five free amino groups of α,γ-DAB and the $N^{\alpha} \rightarrow N^{\gamma}$ migration taking place in it is 50 % (see Table I). It is also important that in the case of an inactivated DNP-polymyxin M one may observe the formation of a small excess (5 %) of free α,γ-DAB as compared to the active antibiotic. In our opinion it would be wrong to think that only half of the γ-amino groups took part in re-acylation. In this case $N^{\alpha} \rightarrow N^{\gamma}$ migration in these γ-amino groups should have been 100 %. However, the experiment with the model peptide α-Pel-DAB-demonstrated a 56.5 % migration, whereas with α-Gly-DAB it was 76.8 %.

It is believed that under the conditions described all five free amino groups of the antibiotic take part in $N^{\alpha} \rightarrow N^{\gamma}$ migration. The per cent of re-acylation is an arithmetical mean.

Parallel to the change in the secondary structure and in optical activity, caused by the long-term effect of an alkali medium, the appearance of free α-amino groups of α,γ-DAB and of an intermediate substance II in each molecule of polymyxin M causes a sharp change in its microbiological properties. This fact is worthy of further detailed study.

It is of interest that this method also permitted a quantitative study of isomerization of Gly-DAB and DAB-DAB diketopiperazines, described previously[12]. It was shown that under conditions of inactivation of polymyxin M, as a result of ammonolysis, 53 % of α-DAB-pyrrolidone and 12 % of α-aminopyrrolidone are formed from DAB-DAB, whereas $N^{\alpha} \rightarrow N^{\gamma}$ migration does not take place in diketopiperazine. Gly-DAB yields under these conditions 71 % of α-glycyl-pyrrolidone; only 8 % of the initial diketopiperazine remains.

The quantitative study of $N^{\alpha} \rightarrow N^{\gamma}$ migration in different peptides of α,γ-DAB is being continued.

CONCLUSIONS

A rapid and convenient method of separation and quantitative estimation of α- and ω-DNP-derivatives of α,γ-DAB, ornithine and lysine has been suggested.

A quantitative estimation of the $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in four peptides of α,γ-DAB has been carried out.

The extent of migration in inactive polymyxin M has been determined.

It was shown that in α- and γ-DNP-isomers of N-Pel-DAB, under conditions of inactivation of polymyxin M, a unique mixture is formed which contains about 58 % of the γ- and 36 % of the α-isomer; in N-Gly-DAB peptides, under these conditions, the mixture formed has 79 % of the γ- and 10 % of the α-isomer.

SUMMARY

For a quantitative estimation of the value of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in inactive polymyxin M and in peptides of α,γ-diaminobutyric acid (α,γ-DAB) a rapid method of separation and quantitative determination of the α- and γ-2,4-dinitrophenyl-derivatives of α,γ-DAB is suggested. Their breakdown during acid hydrolysis can be followed with an automatic amino acid analyzer (a 15-cm column, Amberlite CG-120; 0.30 *N* Na^+-citrate buffer, pH 5.28, 50°; 3.5 h). This method enables the α- and ω-2,4-dinitrophenyl-isomers of lysine and ornithine to be separated. The method has also been used to study the $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in four peptides of α,γ-DAB and in inactive polymyxin M. It is shown that in the α- and γ-isomers of N-pelargonyl-DAB, under conditions for inactivation of polymyxin M, a unique mixture is formed, containing about 58 % of the γ- and 36 % of the α-isomers; in peptides of N-glycyl-DAB, the mixture formed has 79 % of the γ- and 10 % of the α-isomer. With polymyxin M $N^{\alpha} \rightarrow N^{\gamma}$ migration is 50 %. It is suggested that in polymyxin M all five γ-amino groups take part in $N^{\alpha} \rightarrow N^{\gamma}$ migration. The quantitative aspect of the transformations of diketopiperazines of α,γ-DAB and of glycyl-DAB, occurring under conditions for inactivation of polymyxin, have also been studied.

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SEPARATION OF MALTODEXTRINS BY CHARCOAL CHROMATOGRAPHY*

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INTRODUCTION

The use of charcoal columns for the chromatographic separation of homologous oligosaccharides is a method of great potential in that it is capable of handling relatively large amounts of material fairly rapidly with fair to good resolution. The chromatographic support is inexpensive, and eluting solvents are available in a state of high purity. The method has been shown[1–9] to be capable of resolving starch and cellulose oligosaccharides up through six or more D-glucose units. Both stepwise and gradient elution have been used.

In the present study, we have automated the monitoring of the carbohydrate content of the effluent from charcoal columns by using a continuous recording polarimeter or by analyzing fractions using the Technicon Autoanalyzer. This has enabled us to examine the effect of some experimental variables such as the ratio of sample size to weight of charcoal, column size, steepness of gradient, use of *n*-BuOH** and *tert.*-BuOH as eluants, and pretreatment of the charcoal by various acids. While the handling of charcoal columns is still to a large degree an art, and not exactly reproducible, we usually have been able to separate linear starch oligosaccharides up through G_{11} or G_{12} and occasionally through G_{15}.

From amylase digests of amylopectin, we have also been able to effect clean separations of branched oligosaccharides in the range of 4 to 8 D-glucose units.

Use of *n*-BuOH or *tert.*-BuOH has consistently given superior results in comparison with EtOH. *tert.*-BuOH was selected as being the highest monohydric aliphatic alcohol completely miscible with water. The concentration of *n*- or *tert.*-BuOH required was very much less than that of EtOH for elution of a given oligosaccharide. In our experience with EtOH, it has usually been found that, for each successively higher oligosaccharide, an increase of 5% in EtOH concentration is required. Such increases rather rapidly lead to such high EtOH concentrations that the oligosaccharides become relatively insoluble. On the other hand, with *tert.*-BuOH, the increases were approximately 1% of alcohol for each higher homolog. Still smaller concentrations of *n*-BuOH were required.

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** The following abbreviations are used: G_2, G_3, etc. = maltose, maltotriose, etc.; B_6, etc. = branched hexasaccharide, etc.; EtOH = ethanol; *n*-BuOH, *tert.*-BuOH = normal and tertiary butyl alcohol, respectively.

Deactivation of the charcoal by prior treatment with acid was very effective in improving the resolution and preventing tailing of the peaks. TAYLOR AND WHELAN[7] have emphasized the importance of using acidic conditions (0.01 *N* formic acid in the eluting solvent). In our first trials, 0.01 *N* HCl was equally effective. However, stearic acid pretreatment of the charcoal, as suggested by ALM *et al.*[1] has given superior results when *tert.*-BuOH was used as eluant. With *n*-BuOH as eluant, we have obtained good recovery and superior resolution, even without using the stearic acid treatment. However, a much lower concentration of *n*-BuOH is required in the gradient-developing system, and in some cases, we have resorted to a 3-chamber gradient system.

The ratio of sample size to amount of charcoal (1 %) recommended by WHISTLER AND DURSO[3] has given satisfactory results. However, resolution of the higher compounds has been slightly improved by using a 0.7 % ratio.

In view of the successful extension of the method into the megalosaccharide range, we wish to present our preliminary results, even though much work remains to be done in improving the methods.

EXPERIMENTAL

Carbohydrates

In the initial work, a mixture of chromatographically pure D-glucose and commercial "C-P" maltose was used. The maltose contained appreciable quantities of maltotriose and other oligosaccharides which were frequently resolved. The carbohydrate sample for most of the work has been a digest of amylose ("Superlose", Stein-Hall Mfg. Co.) with crystalline *Aspergillus oryzae* α-amylase (Taka A)[10]. Ten grams of Superlose were suspended in 100 ml of dimethyl sulfoxide and heated to 90° until a clear solution was obtained; water was then slowly added with stirring to a volume of 900 ml; 100 ml of 200 m*M* acetate buffer pH 5.7 were added; 15 units* of *A. oryzae* α-amylase were added to effect the reaction, which was allowed to proceed at 40° (*ca.* 2 h.) until the reducing value indicated 25 % apparent maltose ($\overline{DP} = 8$). The reaction was stopped by adding 200 ml of glacial acetic acid; after a period of 2 h, the acid was neutralized to pH 5.4 with 200 ml of concentrated ammonium hydroxide. The digest was concentrated in a rotary vacuum evaporator to 500 ml. Paper chromatography of such a digest showed linear starch oligosaccharides from G_2 up through at least G_{11}.

Branched oligosaccharides were obtained by the action of crystalline human salivary α-amylase[11] on waxy maize starch. Waxy maize starch (25 g) was suspended in 1.5 l of water; 20 ml of 3 *M* sodium hydroxide was added, and the suspension was warmed in a boiling water bath until solution occurred; it was then cooled and neutralized (pH 6.5); 120 ml of 500 m*M* citrate buffer pH 6.5, and 300 ml of 1 *M* sodium chloride were added; the mixture was then diluted to 3 l. The reaction was effected by 1000 units* of crystalline human salivary α-amylase. The reaction was allowed to proceed at 37° under toluene for 72 h; it was terminated by boiling for 5 min and the reaction mixture was concentrated under vacuum to 200 ml. The bulk of the glucose, G_2 and G_3 were removed by a crude preliminary separation on a large charcoal column without attempting to resolve the individual branched components.

* Units are expressed as micromoles of glucosyl bonds cleaved per minute at pH 5.7 and 40°

Apparatus

The apparatus is indicated schematically in Fig. 1. The sintered glass plate indicated in Fig. 1 can be replaced by glass wool, which is more satisfactory especially with small columns. A solvent gradient was formed by allowing solvent (*e.g.* 10% *tert.*-BuOH) from a reservoir to flow into a large mixing chamber (initially containing pure water). The mixed solvent was conducted into the charcoal column. The effluent passed first through a flow cell in a continuous recording polarimeter (Model 143A,

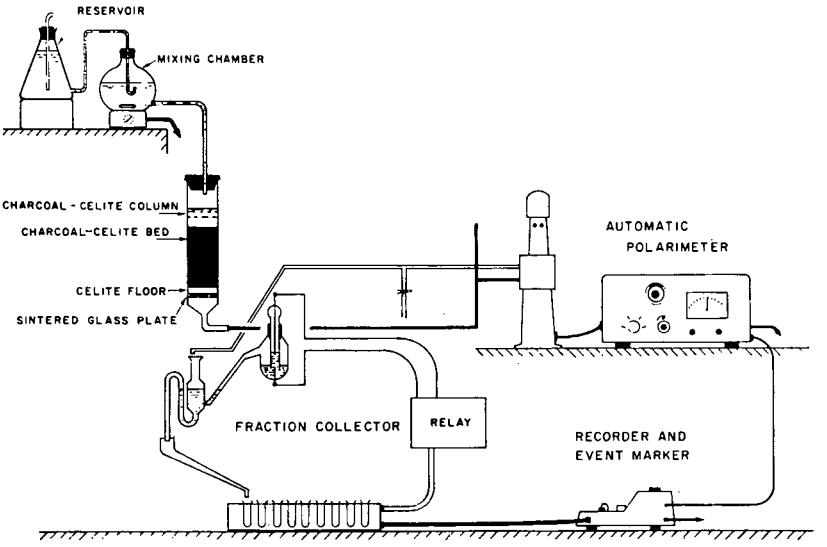


Fig. 1. Apparatus employed in the polarimetric automated charcoal columns.

Bendix Corporation, Cincinnati Division, Cincinnati, Ohio) and thence into a fraction collector. The recorder for the polarimeter was equipped with an event marker so that, at each advance of the fraction collector turntable, a signal was sent to the event marker. In this way, it was possible to obtain a record of the optical rotation of each fraction. The continuous flow cells supplied with the polarimeter were unsatisfactory for mixed solvents owing to the swelling of the gasket material and concomitant bubble collection within the cell. The bubble problem was partially remedied by using an all-glass flow cell and a simple but effective bubble trap and debubbling device. In some runs, the carbohydrate content of each tube was measured by determining the reducing value by the alkaline ferricyanide–cyanide procedure using the Autoanalyzer (Technicon Instruments Corp., Chauncey, N.Y.).

For chromatography at higher temperatures, a jacketed column, similar to that used for high temperature cellulose chromatography[12], was used.

Column preparation and chromatography

For the majority of the studies, an intermediate size column (3.6 × 40 cm) and a mixture of linear dextrins from the *A. oryzae* α-amylase digestion of amylose was used. The column packing was prepared by mixing 100 g of Darco G-60 (Atlas Chemical Industries, Wilmington, Dela.) with 75 g of Celite 560 (Johns-Manville,

Manville, N.J.) which was slurried with 1 *M* HCl and allowed to stand overnight. The slurry was filtered by suction and washed with water until chloride-free. If stearic acid treatment was used, the mixture was suspended in 666 ml of absolute EtOH which contained 16.7 g of stearic acid (2.5 % w/v). After 30 min it was filtered and resuspended in 666 ml of 50 % EtOH (v/v). It was then filtered and washed with about 1 l of water. The treated charcoal was slurried in about 400 ml of water, poured into the column, and washed with 2–5 l of water. A sample of 700 mg of carbohydrate in 100 ml of water was layered onto the top of the column and washed down with three 15 ml portions of water. After the sample and washings were absorbed onto the column, the charcoal was overlaid with about 100 ml of water and the gradient system started. At no time after making the column was it allowed to run dry. The gradient system consisted of two 6 l flasks. The first contained 6 l of 10 % *tert.*-BuOH and the second or mixing chamber 5 l of water. The flow rate was about 2.5 ml/min with a total hydrostatic head of approximately 2 m.

We were also successful in scaling up the method by using six times the amount

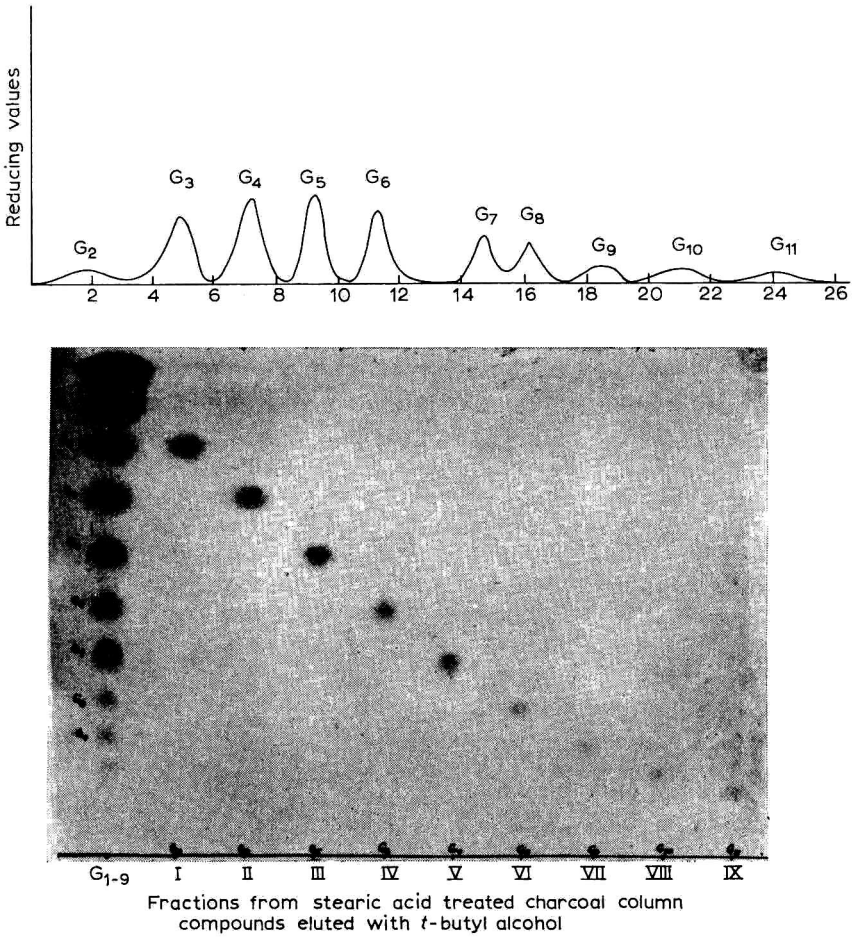


Fig. 2. Elution diagram and chromatographic analysis of linear maltodextrins (G_2–G_{11}) on a small stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution.

of charcoal, Celite, sample, and stearic acid–EtOH solutions. The eluting system consisted of 20 l of 10 % *tert.*-BuOH and 15 l of water, calculated from the gradient elution formula given by ALM *et al.*[1]. The dimensions of the larger columns were 6.5 × 70 cm.

For the optimum separation of the branched dextrins, less stearic acid was required for the charcoal treatment. The stearic acid concentration in the EtOH was reduced from 2.5 % to 1.0 % w/v. The volume of EtOH and the 50 % EtOH wash remained the same. Results similar to those obtained for the small columns were obtained for the branched dextrins when the method was scaled up six times.

RESULTS AND DISCUSSION

Fig. 2 shows the elution diagram and paper chromatographic analysis of the pooled fractions from a typical separation of the linear maltodextrins. Fig. 3 shows

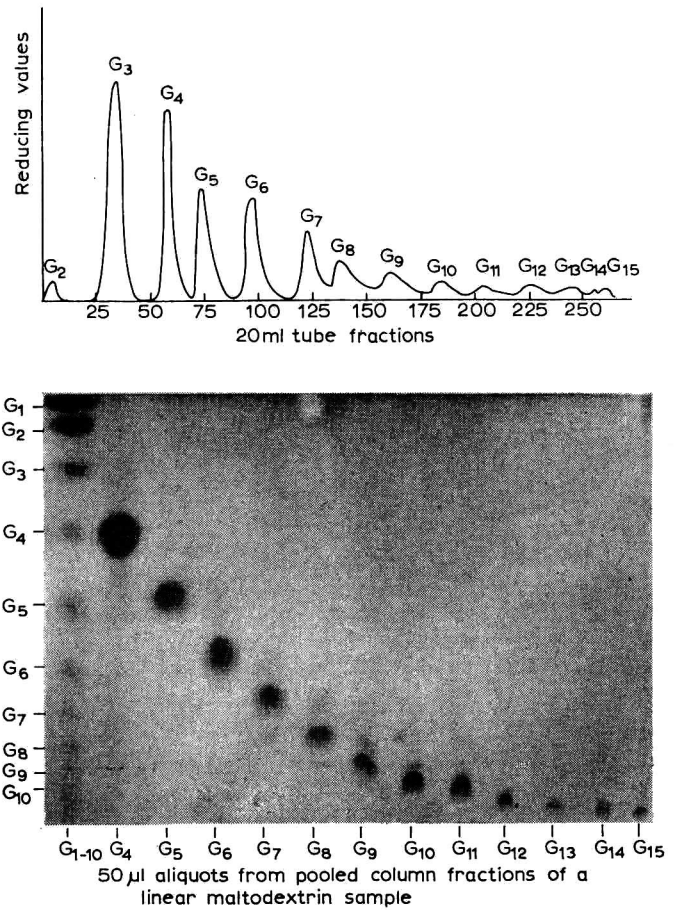


Fig. 3. Elution diagram and chromatographic analysis of linear maltodextrins on a large stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution. Column: 600 g charcoal–450 g celite, treated with 2.5 % stearic acid. Sample: 4.2 g (G_2–G_{15}). Elution gradient: 10 % *tert.*-butyl alcohol (20 l) into H_2O (15 l). Flow rate: *ca.* 4 ml/min.

the elution diagram and chromatographic analysis of the pooled fractions from a large column separation of the linear maltodextrins. For the separation of the linear maltodextrins, the *tert.*-BuOH gradient system with a 2.5 % stearic acid treatment of Darco G-60 charcoal gave the best separation.

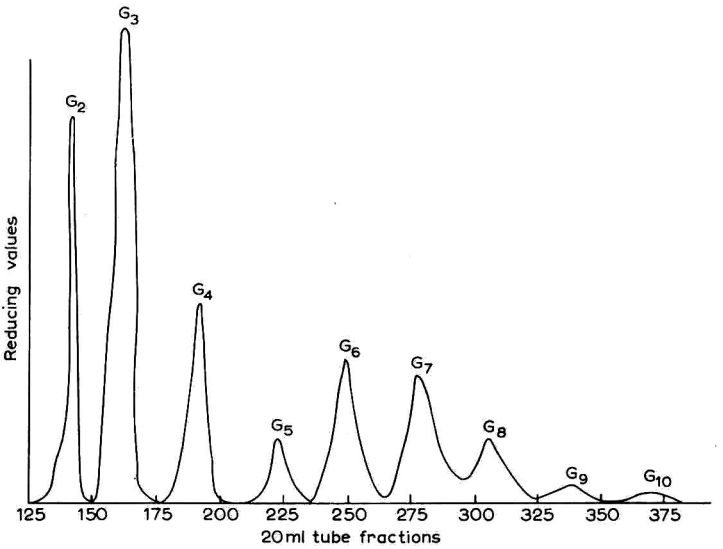


Fig. 4. Elution diagram of linear maltodextrins on a charcoal–celite column with *n*-butyl alcohol gradient elution. Column: 100 g charcoal–75 g celite. Sample: 700 mg maltodextrin (G_2–G_{10}). Elution gradient: 6 l 3 % *n*-butyl alcohol into 5 l water, followed by 6 l 3.5 % *n*-butyl alcohol. Flow rate: *ca.* 2.5 ml/min.

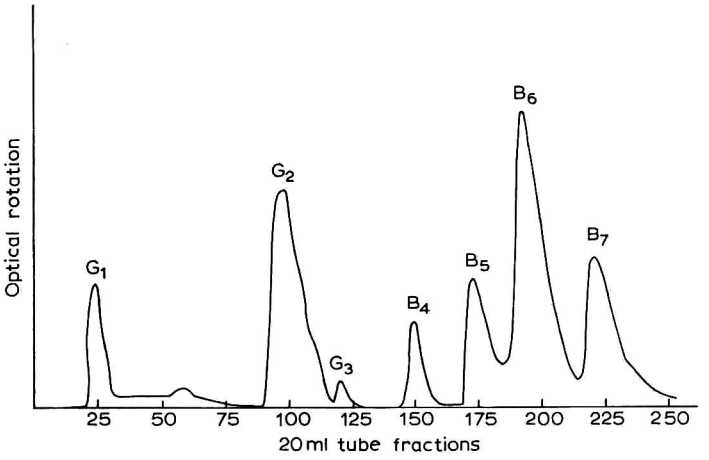
Fig. 4 shows the results of the separation of the linear maltodextrins for the *n*-BuOH gradient system using Darco G 60 charcoal without stearic acid treatment. Although not completely worked out, this system offers some interesting possibilities. The advantages of *n*-BuOH were: better resolution and less tailing, lower alcohol concentrations required, better recovery of oligosaccharides, and the elimination of the stearic acid treatment.

Fig. 5 shows the elution diagram and chromatographic analysis of the separation of the branched maltodextrins on a large 1.0 % stearic acid treated charcoal column. Although there was not a really clean separation of B_6, B_7 and B_8, relatively pure fractions could be obtained by judiciously cutting the peaks.

Pooled fractions were concentrated under reduced pressure, lyophylized, treated twice with anhydrous acetone and once with absolute EtOH, and dried *in vacuo* at 50°. The resulting specimens were white, easily handled powders.

Although the effect of column dimensions and sample size has not been extensively studied, it was found that, for optimum separation, a minimum column length to diameter ratio of 10:1 was required and that the maximum sample size for the stearic acid treated columns was 0.7 % the weight of the charcoal.

In some cases, use of a higher temperature (50°) has improved resolution and recovery, and reduced tailing. Flow rates at higher temperatures are much faster than at room temperature.



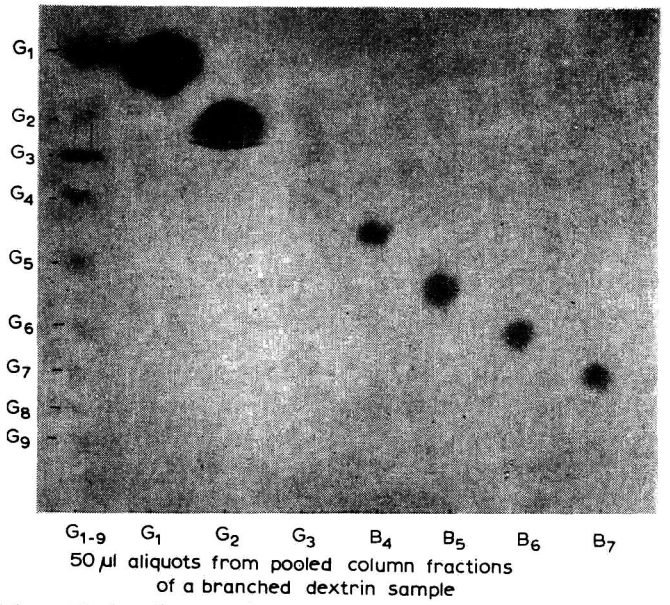


Fig. 5. Elution diagram of branched maltodextrins on a stearic acid treated charcoal–celite column with *tert.*-butyl alcohol gradient elution. Column: 600 g charcoal–460 g Celite, treated with 1.0% stearic acid. Sample: 4.0 g linear and branched maltodextrins. Elution gradient: 10% *tert.*-butyl alcohol (20 l) into H_2O (15 l).

SUMMARY

Gradient elution of charcoal columns using aqueous *tert.*-BuOH has proved very effective for separating individual starch oligosaccharides in the range up to 15 D-glucose units. Effects of sample size, steepness of gradient and pretreatment of charcoal were examined. Carbohydrate in the effluent was monitored by a continuous recording polarimeter or by analyzing fractions with a Technicon Autoanalyzer. Branched, as well as linear, oligosaccharides could be separated.

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THE EFFECT OF GLYCEROL ON THE RATE OF MOVEMENT OF SIMPLE SUGARS ON SILICA GEL AND CELLULOSE THIN LAYERS

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INTRODUCTION

Thin-layer chromatography has been successfully applied to the separation of the sugars, the adsorbents used including aluminium oxide[1], calcium silicate[2], cellulose[3–6], kieselguhr[7–10], magnesium silicate[11] and silica gel[7,12–20]. Many different solvent systems have been used.

Preliminary work using buffered silica gel and cellulose layers with the solvent systems methyl ethyl ketone–glacial acetic acid–methanol (3:1:1)[7,8,17] and ethyl acetate–pyridine–water (60:25:20)[21] respectively yielded satisfactory results with solutions of some sugars in distilled water or isopropanol–water mixtures. Difficulty was encountered, however, when the same methods were applied to pollen extracts preserved in 50 % glycerol solution. Since glycerol is commonly used for the preservation of saccharides in biological fluids, the influence of the presence of glycerol on the rate of movement of some sugars was investigated on buffered silica gel and cellulose layers.

EXPERIMENTAL

Essential details are given in Table I.

Sugars

The following sugars were studied:

Monosaccharides:

aldohexoses	D-galactose, D-glucose, D-mannose
ketohexoses	D-fructose, L-sorbose
aldopentoses	L-arabinose, D-xylose
aldomethylpentoses	L-fucose, D-rhamnose

Disaccharides: cellobiose, lactose, maltose, sucrose

Trisaccharide: raffinose.

Spray reagents

(a) anisaldehyde–sulphuric acid[8]

(b) naphthoresorcinol–phosphoric acid[7,19]

(c) *p*-anisidine–phthalic acid[4].

TABLE I

SUMMARY OF EXPERIMENTAL PROCEDURE

Adsorbent	Silica gel G (Merck) buffered with boric acid	Cellulose MN300 (Macherey, Nagel & Co.)
Thickness	250 μ	250 μ
Activation	Air drying (1 h) 110° for 60 min	Air drying (1h) 105° for 10 min
Solvent system	Methyl ethyl ketone–glacial acetic acid–methanol (3:1:1)	Ethyl acetate–pyridine–water (60:25:20)
Method	Ascending in saturated chamber	Ascending in saturated chamber
Temperature	20–22°	20–22°
Distance	10 cm	10 cm
Load	20 μg (2 μl of 1 % solution)	20 μg (2 μl of 1 % solution)
% Glycerol in water	0, 10, 20, 30, 40 and 50	0, 10, 20, 30, 40 and 50

After spraying, the sugar was located by heating the plate at 100–105° for 20–30 min.

Glycerol can quite easily be distinguished from the sugars by all three spray reagents used, the colour of the glycerol spot being white or pale pink.

RESULTS AND DISCUSSION

Tracings of the results obtained are shown in Figs. 1–4.

Tracings of the movement of glycerol alone are not shown since the presence of the sugar does not affect the position or shape of the glycerol spot. However, the following observations on the behaviour of glycerol alone are pertinent:

(i) The rate of movement of glycerol depends on the concentration, the rate decreasing with increase in concentration.

(ii) The shape of the spot becomes distorted as the glycerol concentration increases. This effect is accentuated on cellulose layers.

The presence of glycerol in the sugar solutions affects both the *rate of movement* and the *shape* of the sugar spots thus making identification of specific sugars extremely difficult. No attempt, therefore, has been made to calculate the R_F values of the sugars.

Rate of movement

The sugars may be considered in two groups: (i) those whose movement is slow compared with that of glycerol and (ii) those whose movement is similar to that of glycerol.

(i) In general, the movement of the more slowly migrating di- and trisaccharides is not greatly affected by the presence of glycerol when chromatographed on buffered silica gel layers although the movement of cellobiose and sucrose (Fig. 3) is retarded in the presence of 40 and 50 % glycerol. The rate of movement of sorbose (Fig. 1) is unaffected by glycerol; other slow moving monosaccharides are retarded only when the glycerol concentration reaches a certain percentage, *e.g.* mannose and arabinose (Fig. 1) in 30 % glycerol and galactose, glucose and fructose (Fig. 1) in 40 % glycerol.

The behaviour of these sugars, in the presence of glycerol, is not identical when chromatographed on cellulose layers. Although there is little effect on the rate of

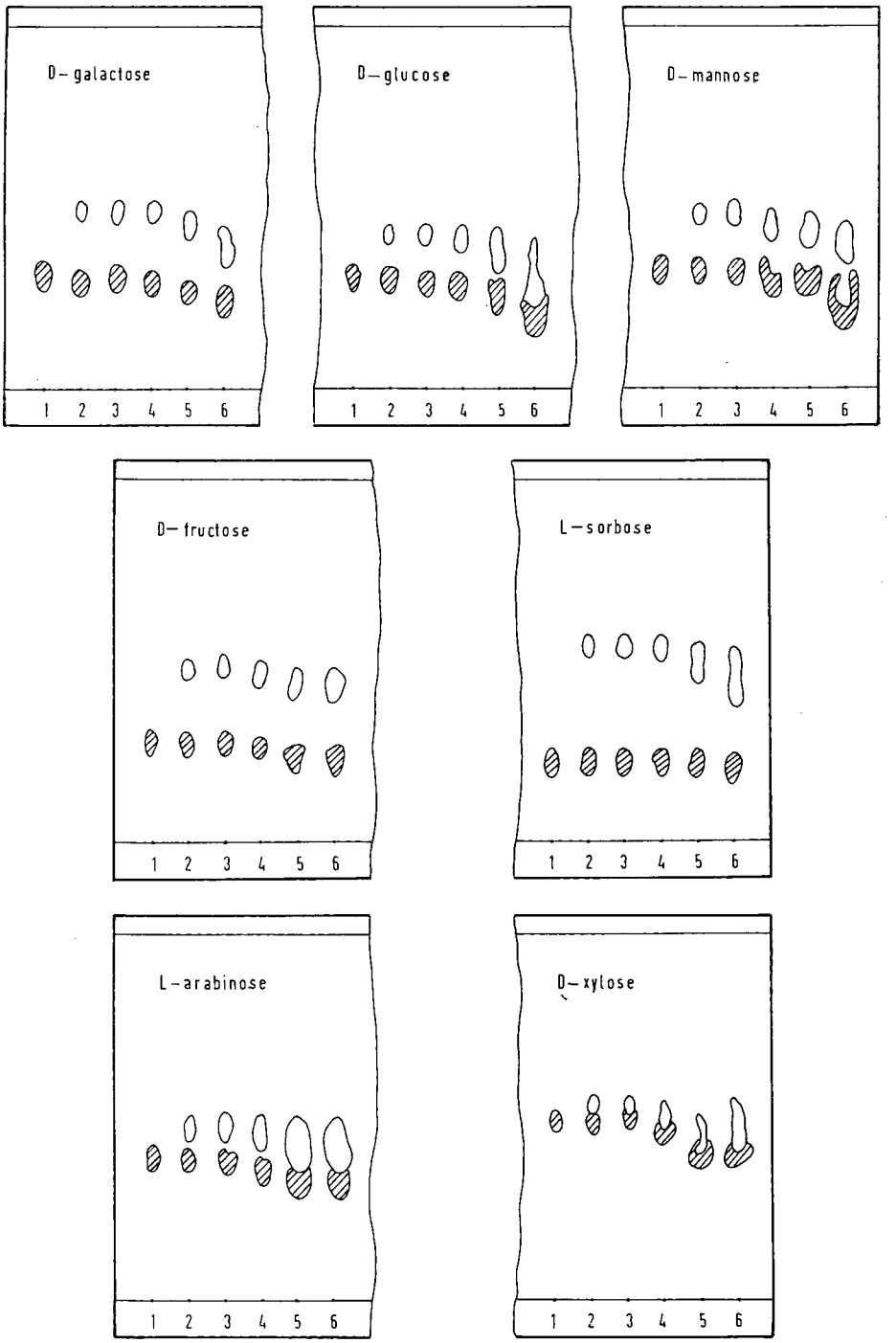

Fig. 1. Tracings of chromatograms of some sugars dissolved in distilled water, 10, 20, 30, 40 and 50% glycerol solution and designated nos. 1–6 respectively on the starting line. Layer: silica gel buffered with boric acid. Hatched areas: sugar; unhatched areas: glycerol.

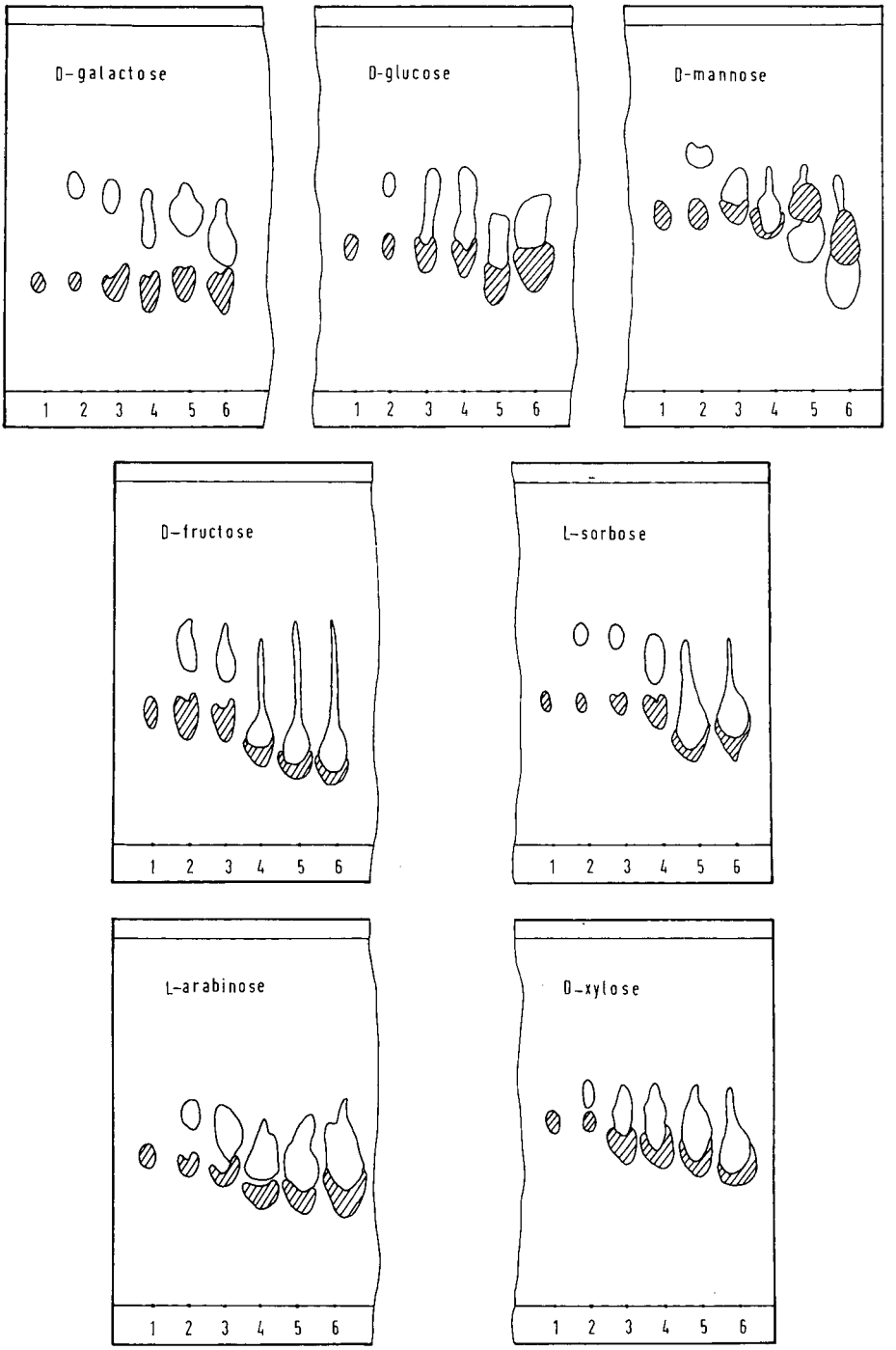

Fig. 2. Tracings of chromatograms of some sugars dissolved in distilled water, 10, 20, 30, 40 and 50% glycerol solution and designated nos. 1–6 respectively on the starting line. Layer: cellulose. Hatched areas: sugar; unhatched areas: glycerol.

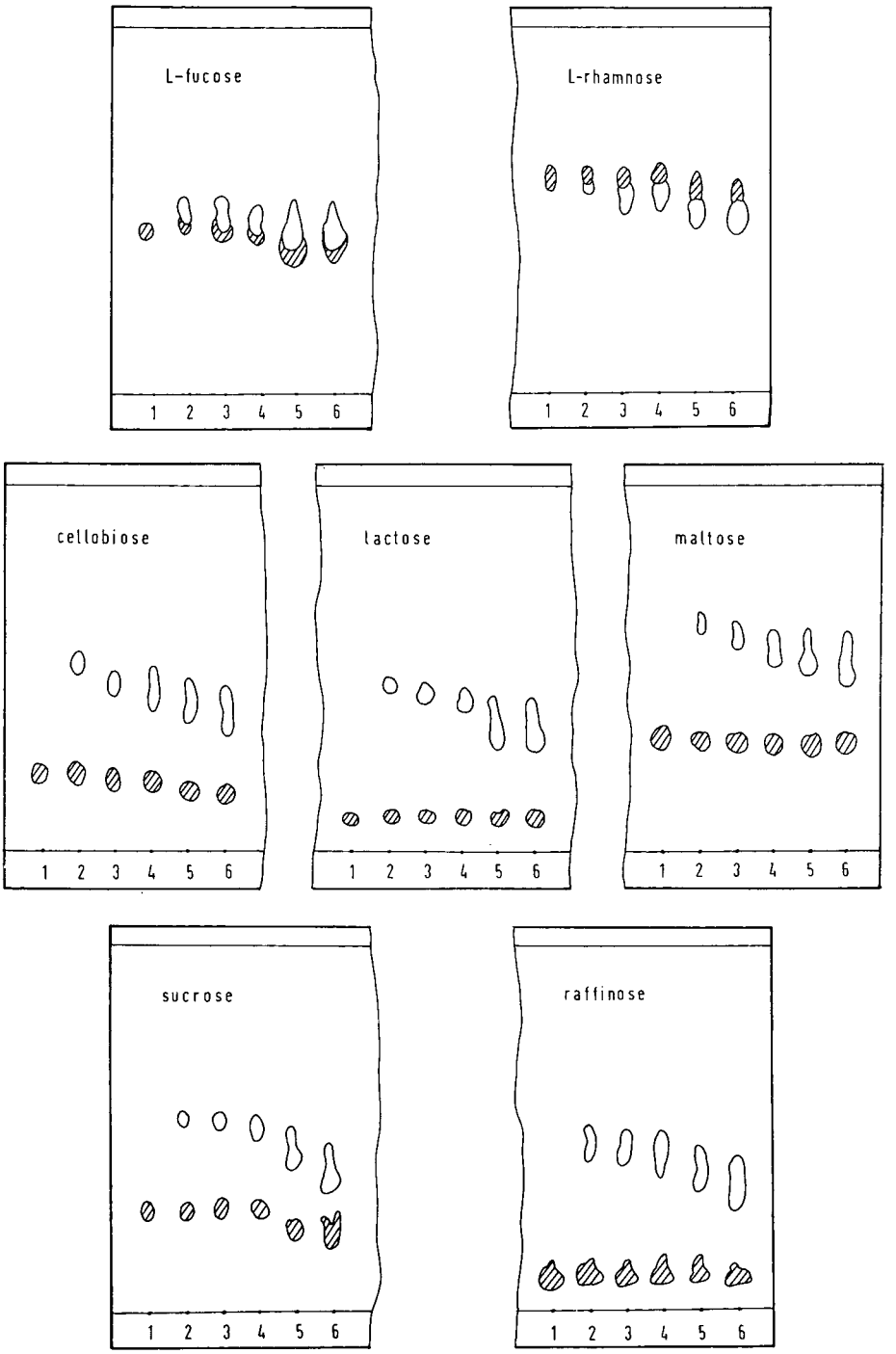

Fig. 3. Tracings of chromatograms of some sugars dissolved in distilled water, 10, 20, 30, 40 and 50% glycerol solution and designated nos. 1–6 respectively on the starting line. Layer: silica gel buffered with boric acid. Hatched areas: sugar; unhatched areas: glycerol.

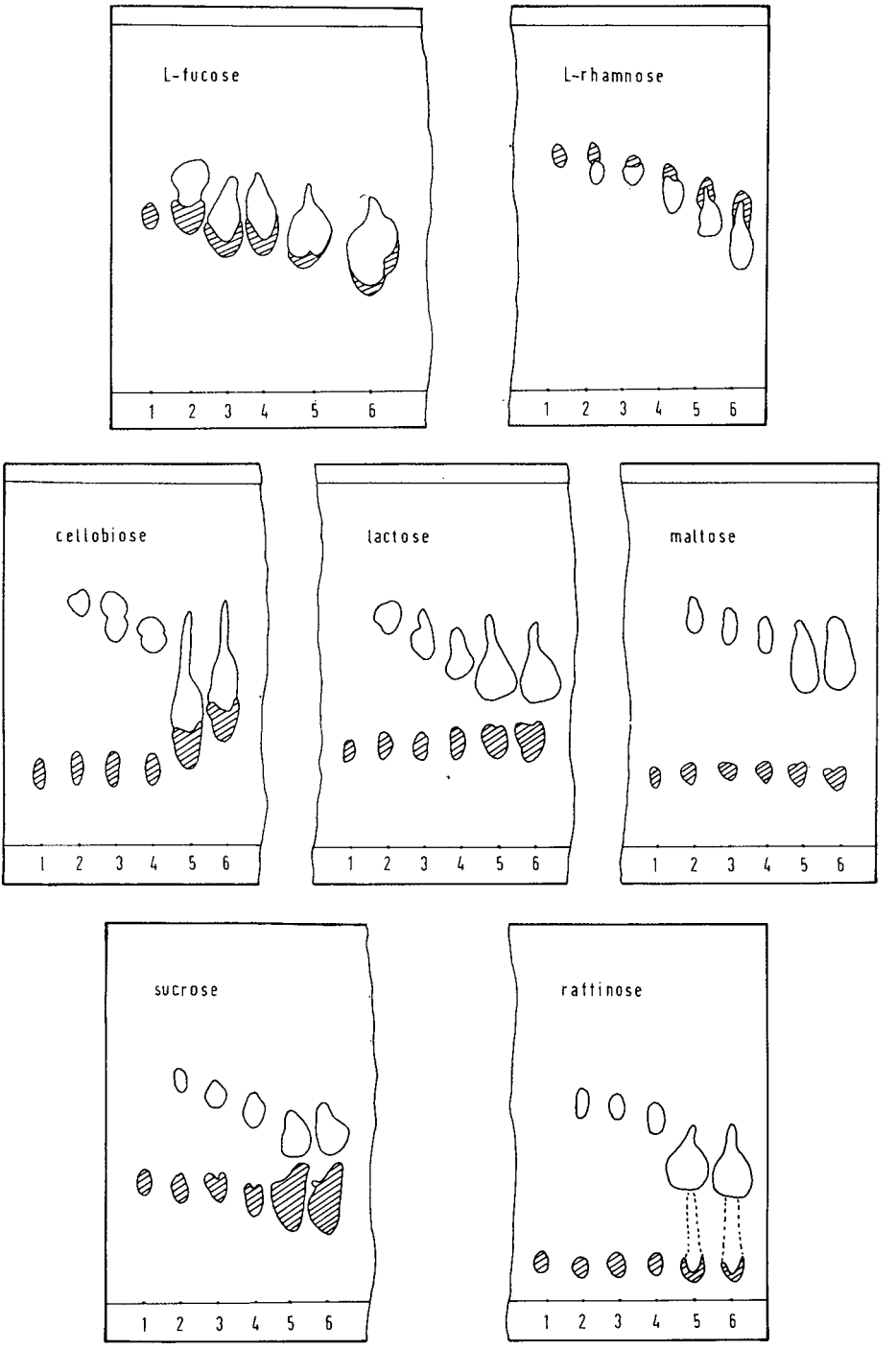

Fig. 4. Tracings of chromatograms of some sugars dissolved in distilled water, 10, 20, 30, 40 and 50% glycerol solution and designated nos. 1–6 respectively on the starting line. Layer: cellulose. Hatched areas: sugar; unhatched areas: glycerol.

movement of raffinose, lactose and maltose the movement of sucrose is retarded when the glycerol concentration reaches 30 % while that of cellobiose is markedly increased in 40 and 50 % glycerol (Fig. 4). It is difficult to compare the *rate of movement* of the monosaccharides chromatographed on cellulose layers (Fig. 2) with that on buffered silica gel (Fig. 1) because of interference in the movement of the sugars resulting from the considerable distortion in the shape of the glycerol spot particularly with the higher concentrations of glycerol. Nevertheless, there is a general tendency for increase in glycerol concentration to retard the movement of the sugar.

(ii) These sugars (xylose, fucose, rhamnose), whose movement is similar to that of glycerol, show a decrease in movement on both buffered silica gel (Figs. 1 and 3) and cellulose (Figs. 2 and 4) irrespective of whether the sugar moves just behind or in front of the glycerol. The effect is more marked on the cellulose layers, where for each sugar a glycerol concentration of 20 % retards the movement, whereas on buffered silica gel retardation is not observed until the glycerol concentration reaches 30 % (xylose, Fig. 1) or 40 % (fucose, rhamnose, Fig. 3).

The cause of this variation in the rate of movement is possibly a direct effect of the increase in the viscosity of the solutions which results in increased drag as the glycerol ascends the layers.

Shape

When chromatographed on buffered silica gel layers those sugars whose rate of movement is slow compared with that of glycerol appear as small, discrete, round to ovoid spots (*e.g.* galactose, fructose, sorbose, Fig. 1; cellobiose, lactose, maltose, Fig. 3). Only at the higher glycerol concentrations in the case of glucose, mannose and arabinose (Fig. 1) and sucrose (Fig. 3) is there a tendency for slight distortion in shape. Raffinose (Fig. 3) in water and all glycerol concentrations exhibits a small peak at the front edge of the spot. Where the movement of the sugar is similar to that of glycerol the shape of the spot is affected more if the sugar lies just behind the glycerol. A marked crescent-shaped spot results (*e.g.* xylose, Fig. 1; fucose, Fig. 3) especially as the concentration of glycerol increases. If the sugar moves just in front of the glycerol (*e.g.* rhamnose, Fig. 3) the shape of the spot is not influenced appreciably.

When chromatographed on cellulose layers the shape of the sugar spot is grossly distorted at glycerol concentrations as low as 10 % (*e.g.* fructose, arabinose, Fig. 2; fucose, Fig. 4). Distortion occurs even when the sugar and glycerol spots are well separated (*e.g.* sucrose and raffinose, Fig. 4).

VOMHOF[5] reported silica gel layers had not been a satisfactory medium for the naturally occurring sugars owing to the poor separations and low capacity of the plates. However, comparison of the spots obtained in the two systems used shows that in the presence of glycerol there is greater distortion in the shape of the spot on the cellulose layers than on buffered silica gel.

To overcome the difficulties encountered in separation of sugars in extracts preserved in 50 % glycerol solution it is desirable to dilute the glycerol concentration to as low a value as possible commensurate with the ability to detect the sugar. On buffered silica gel layers a glycerol concentration of 20 % produces little or no distortion in shape of change in rate of movement compared with the standard sugar in distilled water.

SUMMARY

The movement of some saccharides in the presence of varying concentrations of glycerol has been investigated to account for the marked variation in R_F values obtained for some sugars in pollen extracts preserved in 50 % glycerol. Buffered silica gel and cellulose layers have been compared and, in the presence of glycerol, the former are preferred. It is recommended, commensurate with the ability to detect the quantity of sugar present, that before chromatographing the solution is diluted to contain 10 or 20 % glycerol.

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THE CORRELATION BETWEEN THE STEREOCHEMISTRY OF SOME INDOLE AND OXINDOLE ALKALOIDS AND THEIR BEHAVIOUR ON THIN LAYER CHROMATOGRAMS*

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INTRODUCTION

SHELLARD AND PHILLIPSON[1] have reported the use of thin layer chromatography as an aid to distinguish and identify 12 indole and oxindole alkaloids isolated from species of *Mitragyna*. These alkaloids were first separated on thin layer chromatograms using a scheme described by WALDI, SCHNACKERZ AND MUNTER[2] and then by a series of systems based on the Waldi scheme. Further alkaloids have been isolated from *Mitragyna speciosa* Korth[3,4] and the behaviour of all these alkaloids on thin layer chromatograms has been compared with some indole and oxindole alkaloids of known stereochemistry.

METHODS

Adsorbents: Aluminium oxide G (Merck, 15 % calcium sulphate), 35 g/60 ml distilled water; Silica gel G (Merck), 30 g/60 ml distilled water.

Plate size: 20 × 20 cm.

Layer thickness: 250 μ.

Activation: Air dried for 15 min, heated at 105° for 60 min, stored in a desiccator over silica gel and reheated for 15 min at 105° immediately prior to use.

Solvents: See below; freshly distilled.

Running distance: 10 cm.

Temperature: 22–25°.

Load: Approx. 1 μg of alkaloid.

Detection: Dragendorff's spray reagent.

The thin-layer systems used in the modified Waldi scheme were as follows:

Series A: *untreated plates, neutral solvents*

1. Cyclohexane, silica gel
2. Cyclohexane–chloroform (5:4), silica gel
3. Chloroform, silica gel.
4. Benzene–ethyl acetate (7:2), silica gel

* This work forms part of a thesis submitted by J. D. PHILLIPSON for the degree of Ph.D of the University of London (July 1965).

5. Chloroform–benzene (1:1), alumina
6. Cyclohexane–chloroform (3:7), alumina
7. Ether, silica gel
8. Chloroform, alumina
9. Chloroform–acetone (5:4), silica gel
10. Methanol, silica gel

Series B: untreated plates, alkaline solvents

1. Cyclohexane–diethylamine (9:1), silica gel
2. Cyclohexane–chloroform–diethylamine (5:4:1), silica gel
3. Chloroform–diethylamine (9:1), silica gel
4. Benzene–ethyl acetate–diethylamine (7:2:1), silica gel
5. Chloroform–benzene–diethylamine (1:1:0.001), alumina
6. Cyclohexane–chloroform–diethylamine (3:7:0.005), alumina
7. Ether–diethylamine (9:1), silica gel
8. Chloroform–diethylamine (9:1), alumina
9. Chloroform–acetone–diethylamine (5:4:1), silica gel
10. Methanol–diethylamine (9:1), silica gel

Series C: alkaline plates, neutral solvents

The solvent systems 1–10 were the same as in Series A. Plates were prepared with $N/10$ sodium hydroxide solution instead of distilled water.

RESULTS

System 2, Series B, silica gel/cyclohexane–chloroform–diethylamine (5:4:1) can be used to distinguish between the known indole and oxindole alkaloids isolated from *Mitragyna* species, indole alkaloids having hR_F* values greater than 35 and oxindole alkaloids below 35. System 8, Series A, alumina/chloroform was the best general system for mixtures of indole and oxindole alkaloids. For the separation of the indole alkaloids three systems were found to be most useful:

System 5, Series A, alumina/chloroform–benzene (1:1)
System 7, Series A, silica gel/ether
System 9, Series A, chloroform–acetone (5:4).

The results of the separations obtained are illustrated in Figs. 1–4.

DISCUSSION

Ajmalicine, isolated from *Mitragyna speciosa* Korth (ref. 4), has been compared with similar alkaloids by means of thin layer chromatography. Fig. 1 illustrates the separation of four ajmalicinoid alkaloids using the three solvent systems which were found to be most useful for the separation of the indole alkaloids of *Mitragyna* species. From these chromatograms it can be observed that these alkaloids behaved as two distinct pairs, those with a methoxy substituent in position 11 having a slightly lower hR_F value than the non-aromatic-substituted alkaloid with the same stereo-

* $hR_F = 100 \times R_F$.

chemistry. Thus it would appear that the effect of a methoxy substituent in the indole ring of these alkaloids is to slightly lower the hR_F value on thin layer chromatograms.

The pair of alkaloids with *cis* D/E ring junctions, C-15 H α and C-20 H α, reserpinine (I, R = $-OCH_3$) and tetrahydroalstonine (I, R = —H) have higher

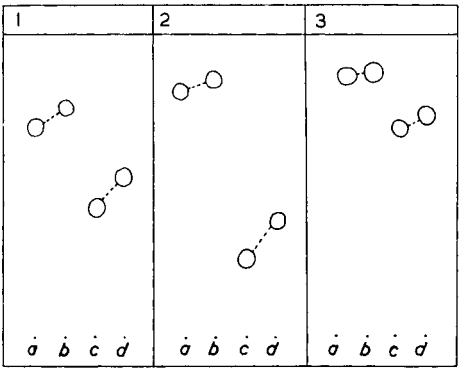
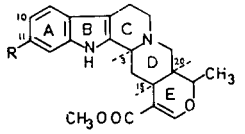


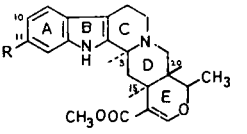
Fig. 1. Thin layer chromatography of some ajmalicinoid alkaloids. a = Reserpinine (I, R = $-OCH_3$); b = tetrahydroalstonine (I, R = —H); c = tetraphylline (II, R = $-OCH_3$); d = ajmalicine (II, R = —H); 1 = chloroform–benzene (1:1), alumina; 2 = ether, silica gel; 3 = chloroform–acetone (5:4), silica gel.

hR_F values than the pair of alkaloids with *trans* D/E ring junctions, C-15 H α and C-20 H β, tetraphylline (II, R = $-OCH_3$) and ajmalicine (II, R = —H). When aricine was run on the same three thin layer systems it always corresponded in hR_F value with reserpinine and it is interesting to note that the stereochemical structure of reserpinine and aricine is identical; aricine differs from reserpinine in that the methoxy substituent is in position 10 instead of position 11. Thus it appears that the hR_F value is little affected by substitution in either position 10 or 11.



I

(I) Reserpinine (R = $-OCH_3$)
Tetrahydroalstonine (R = —H)



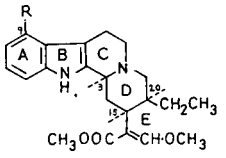
II

(II) Tetraphylline (R = $-OCH_3$)
Ajmalicine (R = —H)

Because of this correlation between hR_F value and the stereochemistry of the D/E ring junction in these ajmalicinoid alkaloids it was decided to investigate the behaviour of some new *Mitragyna* alkaloids in these three systems using mitragynine and corynantheidine (isolated from *Mitragyna speciosa*[4]), corynantheine and dihydrocorynantheine, four alkaloids with a known stereochemistry at C-3, C-15 and C-20[5–10].

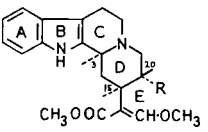
By analyses, equivalent weights, U.V., I.R. and N.M.R. spectra, two new alkaloids from *Mitragyna speciosa* Korth, speciogynine and speciociliatine, have been shown to be isomers of mitragynine (III, R = $-OCH_3$) and a third new alkaloid

paynantheine to be of the mitragynine type with a 9-methoxy substituent but with a vinyl group at C-20, similar to corynantheine (IV, R = $-CH=CH_2$)[4].



III

(III) Mitragynine (R = $-OCH_3$)
Corynantheidine (R = —H)



IV

(IV) Dihydrocorynantheine (R = $-CH_2CH_3$)
Corynantheine (R = $-CH=CH_2$)

The results of the separations of speciogynine and paynantheine with mitragynine, corynantheidine, dihydrocorynantheine and corynantheine on thin layer chromatograms are illustrated in Fig. 2. In these three systems mitragynine (III, R = $-OCH_3$) and corynantheidine (III, R = —H) have almost identical hR_F values but the hR_F value of mitragynine is always slightly lower. This behaviour is similar to that of the 11-methoxy ajmalicinoid alkaloids. Speciogynine has similar hR_F values (slightly lower) to dihydrocorynantheine (IV, R = $-CH_2CH_3$) and different hR_F values (much lower) from mitragynine/corynantheidine thus indicating that the D/E ring junction is *trans* and not *cis*. The I.R. spectra of mitragynine and speciogynine indicate *trans* C/D ring junctions and thus if the stereochemistry at C-17 is identical across the double bond then speciogynine can differ from mitragynine in having a *trans* D/E ring junction[4].

Fig. 2 also illustrates that corynantheine (IV, R = $-CH=CH_2$) and dihydrocorynantheine (IV, R = $-CH_2CH_3$) have similar hR_F values in these three thin layer systems but that of corynantheine is always slightly higher, thus the effect of a vinyl group at C-20 instead of an ethyl group is to slightly increase the hR_F value. Paynantheine runs more closely to speciogynine and corynantheine in these three systems than it does to mitragynine so it would appear that paynantheine resembles these two alkaloids in having a *trans* D/E ring junction.

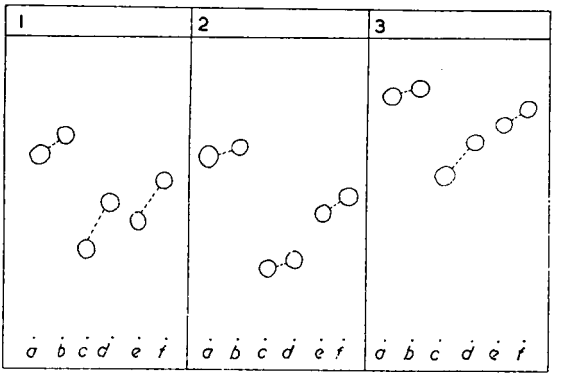
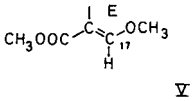


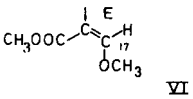
Fig. 2. Thin layer chromatography of some E *seco* indole alkaloids. a = Mitragynine (III, R = $-OCH_3$); b = corynantheidine (III, R = —H); c = speciogynine; d = dihydrocorynantheine (IV, R = $-CH_2CH_3$); e = payantheine; f = corynantheine (IV, R = $-CH=CH_2$); 1 = chloroform–benzene (1:1), alumina; 2 = ether, silica gel; 3 = chloroform–acetone (5:4), silica gel.

The conclusions drawn from these three thin layer systems (Fig. 2) that mitragynine resembles corynantheidine stereochemically and that paynantheine resembles speciogynine stereochemically were further substantiated when these four alkaloids were run on all the systems of the modified Waldi scheme (Fig. 3). The close hR_F values in all these systems between corynantheidine and mitragynine suggest that these two alkaloids have the same stereochemistry at all centres and thus it would appear that the stereochemistry at C-17 is identical for these two alkaloids and not different as proposed, V (mitragynine) and VI (corynantheidine)[9,10].



V

(V) Mitragynine



VI

(VII) Corynantheidine

ZACHARIAS, ROSENSTEIN AND JEFFREY[11] have shown by X-ray crystallography that the C(17)-H in mitragynine is *cis* to the carbomethoxy group (V) and PHILLIPSON[12] as a result of thin layer chromatographic and N.M.R. considerations suggested that the arrangement is the same in corynantheidine. This postulation was independently confirmed by WEISBACH *et al.*[13].

There are four known isomers with a mitragynine-like structure, mitragynine, speciogynine, speciociliatine[4] and mitraciliatine[14]. The relationship between the hR_F values of these four isomers is shown in Fig. 3 and it can be seen that in most systems mitragynine has the highest hR_F, then speciogynine, speciociliatine and the lowest mitraciliatine. The I.R. spectra of mitragynine and speciogynine indicate *trans* C/D ring junctions whilst the I.R. spectra of speciociliatine and mitraciliatine indicate *cis* C/D ring junctions[4,12]. Thus the two alkaloids with *cis* C/D ring junctions have lower hR_F values than the two alkaloids with *trans* C/D ring junctions. If it is assumed that the stereochemistry at C-17 is identical for these four alkaloids then it is likely that these four alkaloids correspond to the four possible stereochemical skeletons for mitragynine, namely *normal, pseudo, allo* and *epiallo*. The behaviour on thin layer chromatograms suggests that since mitragynine has a *cis* D/E ring junction then speciogynine has a *trans* D/E ring junction and the same argument would suggest that speciociliatine has a *cis* D/E ring junction and mitraciliatine a *trans* D/E ring junction. These proposals are summarised in Table I:

Insufficient alkaloids are available for comparison of the stereochemistry of the oxindole alkaloids with their behaviour on thin layer chromatograms but it is

TABLE I

	Configuration of H atom at		
	C-3	*C-15*	*C-20*
Mitragynine (*allo*)	α	α	α
Speciociliatine (*epiallo*)	β	α	α
Speciogynine (*normal*)	α	α	β
Mitraciliatine (*pseudo*)	β	α	β

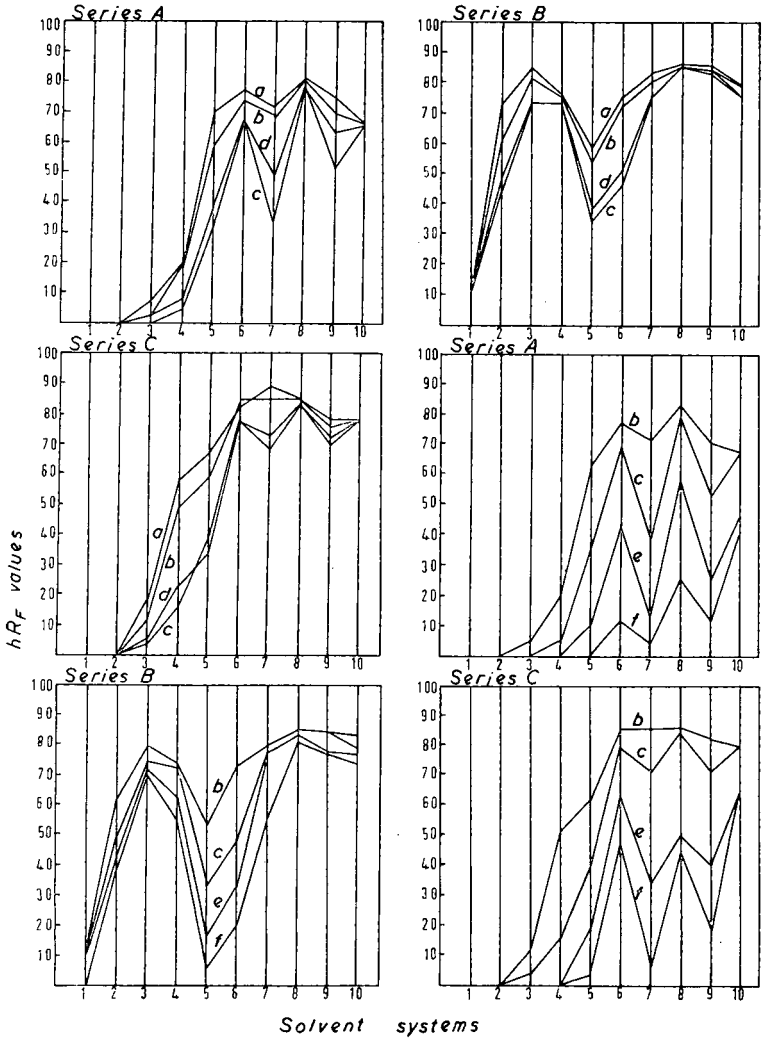


Fig. 3. hR_F values of corynantheidine (a), mitragynine (b), speciogynine (c), paynantheine (d), speciociliatine (e) and mitraciliatine (f) in the modified Waldi scheme.

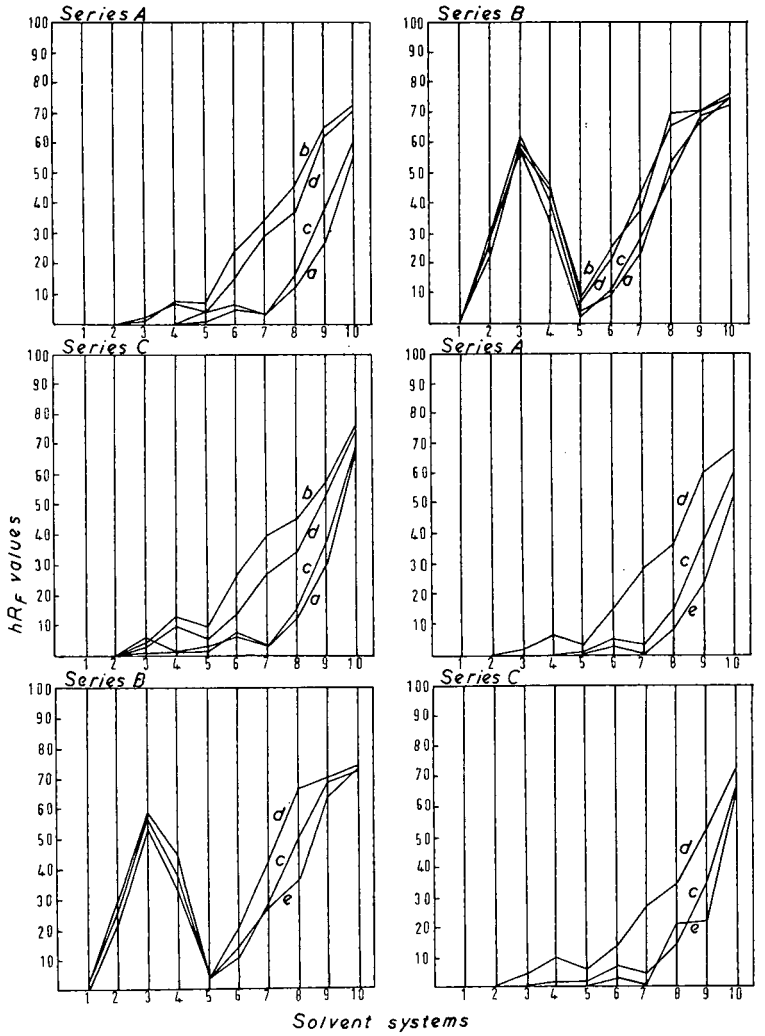
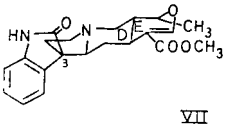


Fig. 4. hR_F values of rhynchophylline (a), isorhynchophylline (b), mitraphylline (c), isomitraphylline (d) and speciophylline (e) in the modified Waldi scheme.

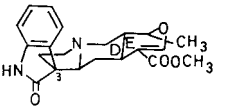
possible to use thin layer chromatography in order to ascertain the stereochemistry at the spiro carbon C-3.

Mitraphylline (VII) and isomitraphylline (VIII) and rhynchophylline (IX) and isorhynchophylline (X) have the same stereochemistry at the D/E ring junction and each pair is isomeric at the spiro carbon C-3[15–17]. Fig. 4 shows that rhynchophylline and mitraphylline have similar hR_F values in most systems of the modified Waldi scheme whilst isorhynchophylline and isomitraphylline have similar hR_F values to each other, the latter pair moving more quickly. Although no explanation can be offered, it is interesting to note that mitraphylline usually has a higher hR_F value than rhynchophylline whilst the reverse is true for the iso alkaloids.



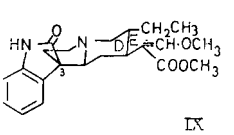
VII

(VII) Mitraphylline



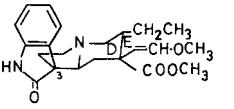
VIII

(VIII) Isomitraphylline



IX

(IX) Rhynchophylline



X

(X) Isorhynchophylline

Speciophylline, a new oxindole alkaloid from *Mitragyna speciosa*, has been characterised by equivalent weight, analysis, U.V., I.R., and N.M.R. spectra as an isomer of mitraphylline. Thin layer chromatography has indicated that speciophylline is different from mitraphylline, isomitraphylline, uncarine A and uncarine B, the four known isomers of mitraphylline. Fig. 4 shows that on thin layer chromatograms speciophylline resembles mitraphylline rather than isomitraphylline; thus it is proposed that the stereochemistry of the spiro carbon at C-3 is the same for speciophylline as for mitraphylline.

ACKNOWLEDGEMENTS

We thank the Smith Kline and French Laboratories, Philadelphia, for gifts of reserpinine, tetrahydroalstonine and tetraphylline and Prof. JANOT, Paris for a gift of corynantheine.

The dihydrocorynantheine was obtained from S.B. Penick and Co. (as the hydrochloride).

SUMMARY

Thin layer chromatography may be used to distinguish between indole alkaloids having *cis* or *trans* C/D and D/E ring junctions, with or without methoxy substituents,

and with vinyl groups instead of ethyl groups at C-20. The behaviour on thin layer chromatograms of some new indole and oxindole alkaloids from *Mitragyna* species has been examined and proposals made for their stereochemical differences.

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SEPARATION OF EPOXY, HYDROXY, HALOHYDROXY AND KETO FATTY ACIDS AND DERIVATIVES BY THIN-LAYER CHROMATOGRAPHY*

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(Received January 18th, 1966)

Separation by thin-layer chromatography (TLC) of oxygenated fatty acid derivatives has been reported by SUBBARAO *et al.*[1,2], MORRIS[3–5], SGOUTAS AND KUMMEROW[6] and KAUFMANN AND KO[7]. The use of glycol complexing agents such as boric acid, sodium borate and sodium arsenite for the separation of *erythro* and *threo* isomers of di- and tetrahydroxy fatty acids was first reported by MORRIS[3]. In later work[4,5], he coupled this elegant technique with the use of silver nitrate as an ethenoid complexing agent[3,8,9] to separate methyl esters of various positional and geometrical isomers of oxygenated fatty acids differing in degree of unsaturation. In this paper, the TLC behaviour of a number of hydroxy, epoxy, halohydroxy and keto fatty acids, esters and alcohols prepared as earlier reported[10–13], using direct (D), reversed-phase (RP) and boric acid-coated (B) silica gel plates is described.

MATERIALS AND METHODS

The various epoxy fatty acids, esters and alcohols used in this study were prepared according to the method of FINDLEY *et al.*[14]. *Erythro* and *threo* acids were prepared by hydroxylation of *cis* unsaturated acids with alkaline potassium permanganate[15] and performic acid[16] and the esters were prepared by the usual procedure with methanol and sulphuric acid. *Threo-* and *erythro*-hydroxyacetoxy compounds were prepared by refluxing *cis-* and *trans*-epoxy compounds with acetic acid while the diacetoxy fatty acids were obtained by treating the dihydroxy acids with acetic anhydride and pyridine. *Erythro-* and *threo*-2,3-dihydroxydocosanoic acids were prepared by the method of ROOMI *et al.*[13]. Halohydroxy compounds were prepared according to the method of KING[17]. 9(10)-Hydroxyoctadecanoic and 13(14)-hydroxydocosanoic acids were obtained by catalytic hydrogenation of *cis*-9,10-epoxystearic and 13,14-epoxydocosanoic acids[11] and the 9(10)-ketostearic acid by chromium trioxide oxidation of the 9(10)-hydroxy acid[18]. Both the 9-hydroxy-10-keto and 10-hydroxy-9-ketostearic acids were prepared by neutral permanganate oxidation of oleic acid by KING's method[19]. Permanganate oxidation of 9,10-stearolic acid[20] gave the 9,10-diketostearic acid. Reduction of esters using lithium aluminium hydride in tetrahydrofuran gave the corresponding alcohols.

The direct TLC procedure in use in this laboratory, employing Desaga equipment, 275-μ layers of silica gel G, and ether–light petroleum for development, has

* Taken in part from the Ph.D. Thesis of M. W. ROOMI submitted to the Osmania University in September, 1965.

been described earlier[1]. For reversed-phase TLC, the dried, coated plate was uniformly impregnated with silicone oil (Dow Corning silicone fluid 200) by allowing a 5 % solution in ether to ascend the plate in a developing chamber[21]. Both for acids and esters, only one solvent system was used for reversed-phase TLC, *viz.* acetonitrile–acetic acid–water (70:10:20, v/v). Impregnation with boric acid (2.8 g/25 g silica gel) was carried out by the method of MORRIS[4], and ether–light petroleum mixtures were used for development.

RESULTS AND DISCUSSION

Epoxy compounds

The separation of epoxy acids, esters and alcohols by direct and reversed-phase TLC is indicated in Table I. By DTLC *cis*-epoxy compounds are separated from their *trans* isomers. The monoepoxy has a higher mobility than the corresponding diepoxy compound. Positional isomers of the same carbon chain length are separable (*cis*-6,7- from *cis*-9,10-), and also epoxy compounds of different chain lengths (18 from 22). Esters move faster than either acids or alcohols. By RPTLC also, separations are possible on the basis both of chain length and the number of epoxy groups in the chain.

TABLE I

SEPARATION OF EPOXY ACIDS, ESTERS AND ALCOHOLS BY TLC

R_F values × 100.
D = direct, RP = reversed-phase.

Compound	*Carbon chain length*	*Acids*		*Esters*		*Alcohols*	
		D[a]	*RP*[b]	*D*[a]	*RP*[b]	*D*[a]	*RP*[b]
cis-6,7-Epoxystearic acid	18	41	76	48	50	—	—
trans-6,7-Epoxystearic acid	18	44	76	54	50	—	—
cis-9,10-Epoxystearic acid	18	45	77	54	51	30	71
trans-9,10-Epoxystearic acid	18	48	77	60	51	34	70
cis,cis-9,10,12,13-Diepoxystearic acid	18	32	96	3	81	—	—
cis-13,14-Epoxydocosanoic acid	22	48	64	72	33	42	63
trans-13,14-Epoxydocosanoic acid	22	58	64	80	33	46	63

[a] Ether in light petroleum (30:70, v/v).
[b] Acetonitrile–acetic acid–water (70:10:20, v/v).

Hydroxy acids, esters and alcohols

(a) *Monohydroxy acids.* By DTLC using 30 % ether in light petroleum, 2-hydroxydocosanoic acid was separated from the 13(14)-hydroxy isomer (Table II). MORRIS[5] showed for hydroxystearic esters that two similar maxima occur for the 12(13)-hydroxy and the 2-hydroxy isomers respectively. In the present instance the 2-hydroxydocosanoic acid moves faster than the 13-hydroxy isomer (R_F × 100, 96 and 85 respectively). 6(7)- and 9(10)-Hydroxyoctadecanoic acids and esters are separable from each other, the 6-hydroxy isomer having a lower R_F value. Close positional isomers (6- and 7-, 9- and 10-hydroxy) can be well separated, confirming

TABLE II

SEPARATION OF MONOHYDROXY ACIDS AND ESTERS BY TLC

R_F values × 100.
D = direct.

Compounds	*Acids*	*Esters*
	D[a]	*D*[b]
6(7)-Hydroxyoctadecanoic acid	62, 57	27, 22
9(10)-Hydroxyoctadecanoic acid	71, 66	36, 30
2-Hydroxydocosanoic acid	96	93
13(14)-Hydroxydocosanoic acid	85	78

[a] Ether–light petroleum (30:70, v/v).
[b] Ether–light petroleum (20:80, v/v).

an earlier report[2]. Separation is also effected according to chain length, *e.g.* C_{18} from C_{22}, the latter having greater mobility.

(b) *Dihydroxy acids, esters and alcohols.* Table III shows the separation of dihydroxy compounds by D-, RP- and BTLC. As with epoxy compounds, RPTLC separates according to carbon chain length and number of hydroxy groups, and is ineffective in separating stereoisomers. DTLC on the other hand shows slight separation of *erythro* and *threo* isomers. Incorporation of boric acid as now well established causes the *threo* isomer to move considerably faster than the *erythro*. Positional dihydroxy isomers (6,7- and 9,10-dihydroxyoctadecanoic acids and 2,3- and 13,14-dihydroxydocosanoic acids) are separable by DTLC and BTLC but not by RPTLC. The behaviour of 2,3-dihydroxydocosanoic acids is anomalous. By analogy with the

TABLE III

SEPARATION OF DI- AND TETRAHYDROXY ACIDS, ESTERS AND ALCOHOLS BY TLC

R_F values × 100.
D = direct, B = boric acid-coated, RP = reversed-phase.

	Acids			*Esters*			*Alcohols*	
	D[a]	*B*[b]	*RP*[c]	*D*[a]	*B*[b]	*RP*[c]	*D*[d]	*B*[d]
threo-6,7-Dihydroxystearic acid	8	42	84	24	55	78	—	—
erythro-6,7-Dihydroxystearic acid	5	30	84	20	29	78	—	—
threo-9,10-Dihydroxystearic acid	14	48	85	33	60	78	—	—
erythro-9,10-Dihydroxystearic acid	10	35	85	29	33	78	—	—
threo,threo-9,10,12,13-Tetrahydroxystearic acid	0	10	94	0	17	87	—	—
erythro,erythro-9,10,12,13-Tetrahydroxystearic acid	0	5	94	0	10	87	—	—
threo-2,3-Dihydroxydocosanoic acid	0	0	—	60	64	—	23	45
erythro-2,3-Dihydroxydocosanoic acid	0	0	—	55	56	—	22	44
threo-13,14-Dihydroxydocosanoic acid	25	61	72	46	84	64	55	53
erythro-13,14-Dihydroxydocosanoic acid	22	48	72	39	43	64	54	47

[a] Ether–light petroleum (50:50, v/v).
[b] Ether–light petroleum (40:60, v/v).
[c] Acetonitrile–acetic acid–water (70:10:20, v/v).
[d] Ether–light petroleum (70:30, v/v).

2-monohydroxy acid, the 2,3-dihydroxy acids were expected to move faster than the corresponding 13,14-isomers. However, both the *erythro-* and *threo*-2,3-dihydroxydocosanoic acids did not move from the starting line even when pure ether or methanol was used as solvent. As expected, however, esters of 2,3-dihydroxydocosanoic acids moved farther than the corresponding 13,14-isomers on DTLC. *Erythro* and *threo* isomers were well separated on a boric acid-coated plate. In this instance the 2,3-dihydroxy esters had a lower mobility than the 13,14-isomer.

The 1,2,3- and 1,13,14-docosanetriols were separable both by DTLC and BTLC. Using BTLC, the *erythro* and *threo* isomers of 1,13,14-docosanetriols were separated from each other but not the isomers of 1,2,3-docosanetriols.

A dihydroxy compound can be separated from a tetrahydroxy compound by all three methods. Ester separations were superior, but the actual resolution of acids recorded could be further improved by increasing the volume of ether in the solvent system.

Some aspects of separation of *erythro-* and *threo*-dihydroxy fatty acids and their esters have been discussed by MORRIS[4]. Similar separation of carbohydrates has been attributed to ease of dehydration to form a five-membered acidic complex[22]. When one or both hydroxy groups were acetylated, TLC separation on boric acid plates no longer occurred (Table IV) probably because of the absence of protons from vicinal hydroxy groups necessary for complex formation.

TABLE IV

SEPARATION OF DIHYDROXY, HYDROXYACETOXY AND DIACETOXY ACIDS BY TLC

R_F values × 100.
D = direct, B = boric acid-coated.

Compound	*D*[a]	*B*[a]
threo-9,10-Dihydroxystearic acid	7	34
erythro-9,10-Dihydroxystearic acid	3	28
threo-9(10)-Hydroxy-10(9)-acetoxystearic acid	35, 26	43, 50
erythro-9(10)-Hydroxy-10(9)-acetoxystearic acid	35, 26	43, 50
threo-9,10-Diacetoxystearic acid	52	74
erythro-9,10-Diacetoxystearic acid	52	74

[a] Ether–light petroleum (30:70, v/v).

The diacetoxy acids moved faster than the others, followed by the hydroxyacetoxy and the dihydroxy acids considerably behind. On opening a 9,10-epoxy ring with acetic acid, the two isomers obtained, *viz.* 9-hydroxy-10-acetoxy and 9-acetoxy-10-hydroxy, are well separated by TLC, the spot intensity suggesting roughly equal proportions of the isomers, as during other ring openings of epoxy fatty acids[11].

Halohydroxy acids and esters

The separation of close positional isomers is also possible for halohydroxy acids (Table V). Separation both of positional isomers, *e.g.* 6,7- and 9,10-chlorohydroxystearic acids and by chain length, *e.g.* 9,10-chlorohydroxystearic from 13,14-chlorohydroxydocosanoic acid is possible. Slight separation of even close positional isomers such as 9-chloro-10-hydroxy and 10-chloro-9-hydroxystearic acids is possible.

TABLE V

SEPARATION OF HALOHYDROXY ACIDS AND ESTERS BY TLC

R_F values × 100.
D = direct.

Compound	*Acids*	*Esters*
	D^a	D^a
C_{18} *series*		
threo-6(7)-Chloro-7(6)-hydroxy	25, 22	29, 25
erythro-6(7)-Chloro-7(6)-hydroxy	25, 22	29, 25
threo-9(10)-Chloro-10(9)-hydroxy	31, 28	35, 31
erythro-9(10)-Chloro-10(9)-hydroxy	32, 28	35, 31
C_{22} *series*		
threo-13(14)-Chloro-14(13)-hydroxy	44	67
erythro-13(14)-Chloro-14(13)-hydroxy	40	67

[a] Ether–light petroleum (20:80, v/v).

Esters move faster than acids. Chloro-, bromo- and iodohydroxy acids are not separated from each other. RPTLC failed to give any of these separations except according to chain length.

Hydroxy and keto acids

From Table VI a diketo acid is seen to move faster than a monoketo acid, which in turn moves faster than a monohydroxy acid. A monoketo acid has an R_F value between those of a diketo and a ketohydroxy acid, and similarly a monohydroxy acid lies between a dihydroxy and ketohydroxy acid. Positional ketohydroxy compounds are not separable. When the crude reaction product of peracid epoxidation of an unsaturated acid is separated by DTLC with 30 % ether–light petroleum, the unreacted unsaturated acid moves with the solvent front and the epoxy, dihydroxy and hydroxyacetoxy acids follow the pattern shown in Table VI.

TABLE VI

SEPARATION OF EPOXY, HYDROXY AND KETO ACIDS BY TLC

R_F values × 100.
D = direct.

Compound	*Acids*
	D^a
cis-9,10-Epoxystearic acid	37
9(10)-Hydroxystearic acid	17, 13
9(10)-Ketostearic acid	37
9,10-Diketostearic acid	53
9-Hydroxy-10-ketostearic acid	21
10-Hydroxy-9-ketostearic acid	21
threo-9,10-Dihydroxystearic acid	3
erythro-9,10-Dihydroxystearic acid	1

[a] Ether–light petroleum (20:80, v/v).

The mechanism of separations obtained with various systems is thoroughly discussed by MORRIS[5]. Some further general conclusions may be drawn from the present experimental results. Compounds of the same carbon chain length carrying different groups like epoxy, mono-, di- and tetrahydroxy, keto, diketo and halohydroxy are separable from each other. Slight changes in the position of these groups in the carbon chain alter the polarity sufficiently to enable TLC separation. As is now well established, the use of boric acid-coated plates enables stereoisomers of di- and tetrahydroxystearic acids to be separated. Compounds of different carbon chain lengths can be separated by DTLC, BTLC and RPTLC, the last mentioned indeed effecting only this type of separation.

SUMMARY

Epoxy, halohydroxy, hydroxy, dihydroxy, keto, diketo and tetrahydroxy fatty acids of C_{18} and C_{22} chain lengths are separable by direct thin-layer chromatography. *Erythro* and *threo* di- and tetrahydroxy fatty acids are better separated on a boric acid-coated silica gel plate. Separation both of positional isomers (6,7 from 9,10 compounds) and of close positional isomers (9-hydroxy-10-acetoxy from 9-acetoxy-10-hydroxy) is possible. All the three systems, *viz.* direct, boric acid-coated and reversed-phase thin-layer chromatography, separate according to chain length The first two systems are generally considerably more useful for oxygenated products.

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF CHENODEOXYCHOLIC ACID AND DEOXYCHOLIC ACID IN HUMAN DUODENAL CONTENTS*

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We previously described a method[1] for the quantitation of free and conjugated cholic acid in bile and duodenal contents by using thin-layer chromatography (TLC). However, the quantitative separation of the dihydroxycholanic acids, chenodeoxycholic acid (CDC) and deoxycholic acid (DC), from a mixture was particularly difficult. We shall show that, despite the contiguity of the spots for CDC and DC on TLC plates, sufficient separation can be obtained to permit the quantitative determination of both of these bile acids.

METHODS

Preparation of plates and samples

Silica gel G and standard equipment for TLC were used. The commercial silica gel G was washed in sulfuric and hydrochloric acids as described previously[1]. Pure CDC and DC were supplied by Dr. H. L. MASON, Section of Biochemistry, Mayo Clinic, and were dissolved in ethanol for use as standards. Samples of duodenal contents were obtained by intubation. A methanol–acetone (1:1, v/v) extract was prepared with 4 volumes of methanol–acetone for each 1 volume of sample. After centrifugation, the supernate was concentrated in a stream of air. An aliquot of the concentrated extract was submitted to alkaline hydrolysis in 2 ml of 2 *N* NaOH for 3 h at 15 p.s.i. in a pressure cooker. After acidification with concentrated HCl the free bile acids were extracted three times with 3-ml portions of ethyl ether. The ether extracts were taken to near-dryness in an airstream and the residue was redissolved in 0.1 to 0.2 ml of 95 % ethanol. Appropriate aliquots of this preparation, usually 5 to 25 μl, were used for TLC as previously described[1].

Solvent systems

Two systems were used: isooctane–ethylene chloride–acetic acid (2:1:1) as modified from the system described by HAMILTON[2] and isooctane–ethyl acetate–acetic acid (5:1:1) as described by ENEROTH[3]. The latter system was used primarily

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because of the slightly better separation that could be obtained. The tanks were equilibrated with the solvents for 1 h prior to chromatography. The solvents were allowed to ascend approximately 18 cm on the 20 cm plates; this required approximately 2 h.

Identification and quantitation of CDC and DC

One column on each plate was sprayed with 15 % phosphomolybdic acid in 95 % ethanol, which stained the bile acids dark blue after heating. The remainder of the plate was carefully sprayed with water until the areas that contained bile acids stood out as light spots against the darker, wet background. The locations of the CDC and DC spots were identified and a fine, interrupted, dividing line was drawn between the CDC and DC areas. Then the plates were dried for a few minutes at 100°. The CDC and DC spots identified by water spraying were scraped off the plates

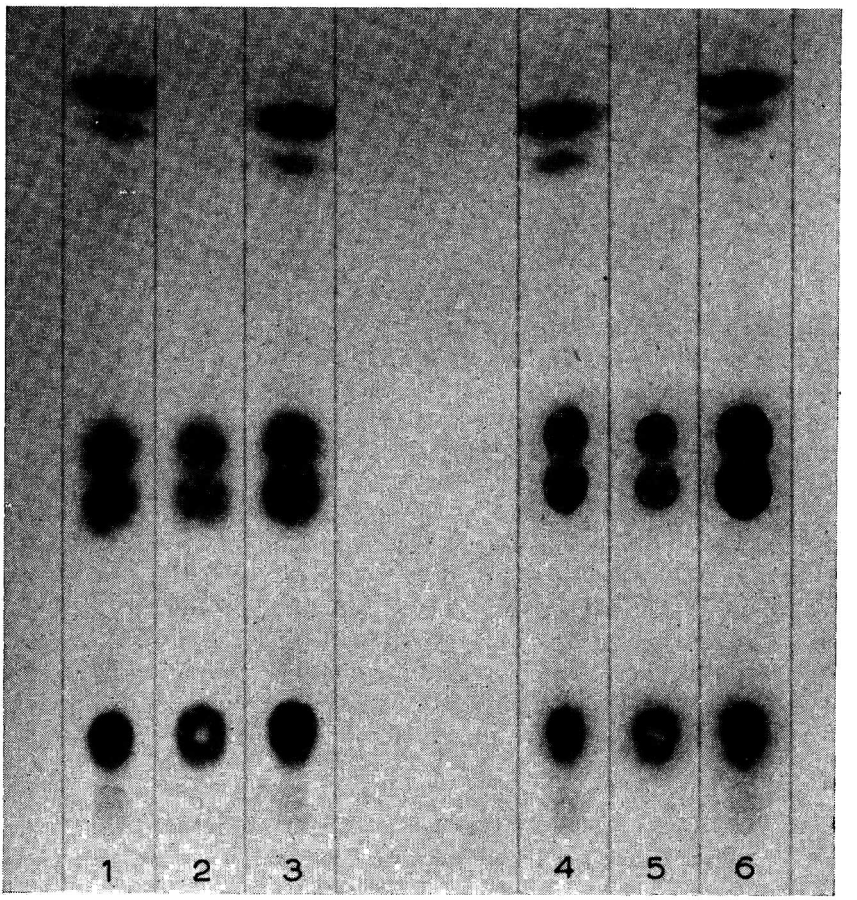

Fig. 1. Thin-layer chromatogram of free bile acids, developed in isooctane–ethyl acetate–acetic acid (5:5:1), from a mixture of standards and from hydrolysates of bile extracts. Columns 1 to 3 were stained immediately after development; columns 4 to 6 were stained after water spraying. Columns 2 and 5 represent the mixture of standards; columns 1 and 4, hydrolyzed bile extract; columns 3 and 6, bile extract plus standard bile acids. Standards on columns 2 and 5 from below upward are cholic acid, chenodeoxycholic acid, and deoxycholic acid.

separately and transferred to test tubes. The amount of bile acid present in each spot was determined spectrophotometrically in 65 % (w/w) sulfuric acid according to the method of ERIKSSON AND SJÖVALL[4] as modified for TLC by GÄNSHIRT and co-workers[5]. Four milliliters of 65 % sulfuric acid was added to the test tube and mixed with a Vortex mixer. After incubation for 60 min at 60° and centrifugation for 60 min at 20° and 3,000 r.p.m., the clear, slightly yellow supernate was poured into 1 × 1 cm cuvettes and read against a blank of 65 % sulfuric acid, in a Beckman DU spectrophotometer. CDC was measured at 380 mμ and DC, at 385 mμ[4]. An equivalent area of silica gel from a blank column of the plate was similarly treated and the optical density of this was subtracted from the sample readings to obtain the net extinctions.

Isotope techniques

Multiple samples of duodenal contents were obtained from persons who had received tracer doses of either ^{14}C-labeled cholic acid alone or ^{14}C-labeled cholic acid and tritiated CDC (cholic acid-carboxyl-^{14}C and randomly tritiated CDC, prepared by the method of WILZBACH, were supplied by Tracerlab, Waltham, Massachusetts). The CDC-^{3}H was purified by TLC. The administration of ^{14}C-labeled cholic acid to humans results in the appearance of DC-^{14}C in bile. Thus, bile extracts contained DC-^{14}C as well as cholic-^{14}C acid and CDC-^{3}H. After identification of CDC and DC on the TLC plates by spraying with water, the area containing each radioactive bile acid was scraped into plastic counting vials and the amount of radioactivity associated with either CDC or DC was assessed by liquid-scintillation spectrophotometry in a Packard Tri-Carb counter, by a modification of the techniques described by SNYDER AND STEPHENS[6] for labeled fatty acids and tripalmitin. A 4 % solution of Cab-O-Sil in a liquid scintillator described by KINARD[7] was used as scintillating solution; the silica gel from the plates was suspended in this solution by vigorous shaking. Tritium and ^{14}C were counted simultaneously. Silica gel exerted no significant quenching effect[6], in contradiction to the report of EKDAHL and associates[8] who found 10 % quenching with unwashed silica. The relative amounts of the two isotopes present in each sample were calculated by the discriminator-ratio method according to KABARA and co-workers[9].

RESULTS

DC and CDC standards each migrated as single spots on chromatograms. Fig. 1 shows the degree of separation that was obtained, both from mixtures of standards and from extracts of bile. After spraying with water, the edge of each spot was more sharply outlined than it was before spraying. On the other hand, the faint halos around the spots suggest that some minor spreading and diffusion of substances does occur.

When anisaldehyde in sulfuric and glacial acetic acids was used as a stain for bile acids[10], CDC and DC appeared as distinctly different colors, both in visible light and in ultraviolet light, and the different colors were sharply separated from each other. This separation remained sharp when the plates were sprayed with water prior to the staining with anisaldehyde and was especially distinct when viewed under ultraviolet light.

To verify the method of quantitation, various amounts of pure CDC and DC

were measured before and after TLC. Fig. 2 demonstrates that the bile acids followed Beer's law within the concentration ranges studied. The slope of the plot was less steep after TLC; however, linearity remained.

Table I summarizes recovery studies of standards chromatographed as pure substances (singly or combined) or as internal standards added to hydrolyzed bile extracts. Recovery of standards averaged 82 % for DC and CDC with similar values

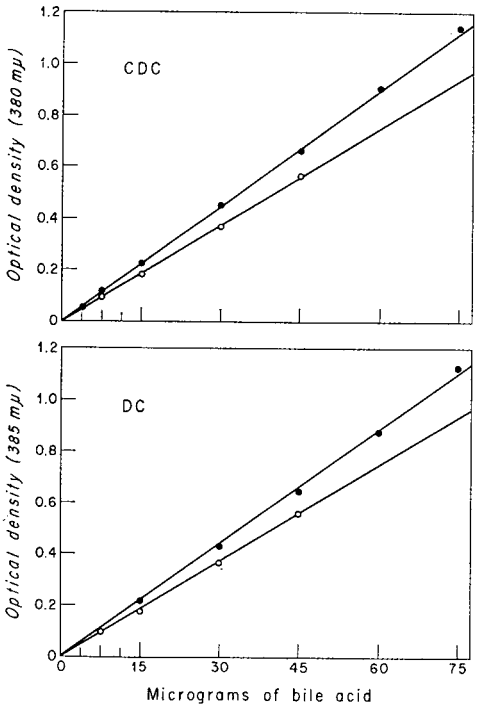

Fig. 2. Standard curves for deoxycholic acid (lower panel) and chenodeoxycholic acid (upper panel) in 4 ml of 65 % sulfuric acid without TLC (solid circles) and following TLC in the presence of silica gel (open circles).

for internal standards. The standard deviations for internal standards were somewhat larger because of the additional variability of the amount of bile acid present in the hydrolyzed extracts.

Studies with varying ratios of CDC and DC in the mixture, ranging from 1:3 to 3:1, resulted in essentially the same percentage recovery for each of the constituents. Only when the amount of one bile acid to be chromatographed was less than 5 μg did the identification by spraying with water become difficult.

CDC and DC were determined in duplicate in 68 samples of hydrolyzed bile extracts; the variation between duplicates is summarized in Table I.

The use of bile acids labeled with ^{14}C and ^{3}H permitted precise evaluation of cross contamination of the bile acids during TLC. After the chromatographic separation of CDC and DC, the total radioactivity of both spots was determined. The contamination of one spot with ^{14}C or ^{3}H counts from the adjacent spot was expressed as a percentage of the total ^{14}C or ^{3}H count associated with the bile acid of the adja-

TABLE I

RESULTS OF RECOVERY AND REPRODUCIBILITY STUDIES

System	*Chenodeoxycholic acid (CDC)*		*Deoxycholic acid (DC)*	
	No. of samples	*Mean ± S.D. (%)*	*No. of samples*	*Mean ± S.D. (%)*
Recovery of pure standards*	26	82.1 ± 4.2	23	82.7 ± 4.8
Recovery of internal standards*	32	82.2 ± 7.9	18	84.8 ± 7.0
Variation between duplicate determinations**	68	2.1 (0–7.1)***	68	2.6 (0–10.5)***

* In each case, 15 μg of CDC or DC was added.
** Calculated as the deviation of the mean from the lower value in duplicate runs.
*** Range of values.

cent area (Table II). Cross contamination was least when the absolute counts for ^{14}C and ^{3}H were in the same order of magnitude. When the disparity between radioactivity in the two bile acids became great, the calculated value for cross contamination tended to be greater for one of the bile acids.

TABLE II

STUDIES OF CROSS CONTAMINATION*

	No. of samples	*Mean ± S.D. (%)*
Contamination of CDC by DC (1 isotope only)	10	8.5 ± 2.9
Contamination of CDC by DC (2 isotopes)	40	7.9 ± 5.2
Contamination of DC by CDC (2 isotopes)	40	9.4 ± 4.6

* Contamination is expressed as percentage of counts found to be associated with the area of CDC when DC was labeled, or vice versa, following separation by TLC.

DISCUSSION

In an excellent review, SJÖVALL[11] discussed the different approaches used for the separation and quantitation of bile acids. GÄNSHIRT and co-workers[5], who first used TLC for the quantitative determination of bile acids, could not quantitate CDC and DC from a mixture because of inadequate chromatographic separation of these two bile acids. Although more suitable solvent systems have been developed in recent years[2,3] to separate these two bile acids, the difference in the R_F values on thin-layer chromatograms remained small.

FROSCH AND WAGENER[12] bypassed this difficulty by scraping off CDC and DC as a combined spot and using a specific color reaction[13,14] for each of the acids. Thus, they were able to use TLC to quantitate each bile acid in the presence of the other from mixtures of pure bile acids and also from duodenal contents[15]. However, a number of factors make it desirable to separate the bile acids before quantitation is carried out. These include the lack of absolute specificity of the color reagents[11] and the modification of the absorption spectra by the presence of silica gel which makes it impossible to measure these colors at their peak optical densities.

In a previous report[1] we noted that commercial silica gel G contains impurities which interfere with the quantitation of bile acids in 65 % sulfuric acid. This proved to be particularly true for CDC and DC. Only when the silica gel was treated by a specific washing procedure before being made into plates could we obtain reproducible results and linear extinctions in the concentration ranges studied. The recovery of standards is quite constant and compares favorably with the results obtained previously[1] for taurocholic, glycocholic, and cholic acids.

The reasons for the consistent 15 to 18 % loss of internal standard are not fully understood. Migration losses during chromatography do not appear to be a significant factor because the same magnitude of loss was noted when standard bile acids were spotted on a TLC plate and the spotted areas were transferred directly to test tubes without chromatography. Studies of the supernate obtained after centrifugation of a mixture of silica and 65 % sulfuric acid revealed the presence of a Tyndall effect and fluorescent properties in this fraction. The Tyndall effect suggests that a colloidal suspension of silica particles remains in the supernate. The Tyndall effect and the fluorescence could interfere with the spectrophotometric determination of the bile acids, but, ordinarily, the silica blank would be expected to correct for this. The addition of more silica gel to the supernate after the color reaction of the bile acids in sulfuric acid had reached its end point did not result in further decreased recoveries. When standard bile acid was pipetted into 65 % sulfuric acid, the characteristic color allowed to develop, and then more silica added and the mixture centrifuged, there was no additional loss of optical density in the supernate. Incomplete elution of the bile acids from the silica gel may be a reason for the losses. However, since these losses are linear in the ranges studied (Fig. 2), they are of no significance as far as the reliability of the method is concerned. It should be emphasized that the recoveries and the low optical densities of the silica blank were consistent only when the duration of the centrifugation of the sulfuric acid suspension was extended to 60 min at 2,700 to 3,000 r.p.m.

Water was used by GÄNSHIRT and co-workers[5] as a spray reagent to locate the different bile acids on the plates. Compared to other means of identification, water has a distinct advantage in that the same spot can subsequently be used for quantitation. It was not possible to locate and identify the CDC and DC spots accurately by staining adjacent strips or columns because of the inevitable small fluctuations in the R_F values between different columns even on the same plate. The detection of as little as 5 μg of bile acid by water spraying is unique for free acids. Larger amounts of conjugated acids are necessary for detection by this technique.

The anisaldehyde stain[10] and the studies with labeled CDC and DC support the feasibility of the approach described. As a consequence of virtually eliminating interfering substances from the silica, the presence of silica became less of a problem and measurements could be carried out at the peak of the absorption spectra. In 68 samples of duodenal contents the ratio between CDC and DC was usually close to 1, with a range of 1:2 to 3:1. In no instance did this range present a problem as far as identification on the plates was concerned.

FROSCH AND WAGENER[15] used the specific color reactions for CDC and DC for the quantitation of the dihydroxycholanic acid conjugates as well as for the free CDC and DC. At present, no solvent systems are known for separation of either the glycine or the taurine conjugates of CDC and DC by TLC. Attempts were made to

quantitate the conjugated dihydroxycholanic acids by a two-step chromatographic procedure. The conjugates were isolated from bile extracts by TLC in suitable systems and identified as previously described[1]. The combined taurine-conjugate spot and the combined glycine-conjugate spot were scraped into separate test tubes and submitted to hydrolysis in the presence of the silica gel. Each hydrolysate was chromatographed again as described above and an attempt was made to quantitate the resulting free bile acids separately. Reproducible results could not be obtained and the recoveries of standards did not exceed 65 %. This is most likely due to the very small amounts (10 to 50 μg) of conjugates subjected to hydrolysis. It has been pointed out that[11,16], when very small amounts of bile acids are hydrolyzed in glass tubes, a significant amount of bile acid material sticks to the glass or may even form silicate complexes. A loss of this type may be enhanced in the presence of silica. When large amounts of conjugated bile acids were hydrolyzed, recoveries of standards after hydrolysis and TLC were only a few per cent lower than after TLC alone. For this reason, whenever possible we used aliquots of bile extract containing at least 1 mg of conjugated bile acid material for hydrolysis.

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SUMMARY

A method is described for quantitating chenodeoxycholic acid and deoxycholic acid in a mixture after separating them by thin-layer chromatography. The procedure involves the preliminary purification of the silica and the identification of the bile acids on thin-layer plates by water spraying. The feasibility of the method and the reproducibility of the results have been demonstrated by special staining techniques and radiotracer studies. The method was applied to human duodenal contents.

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THE RAPID RESOLUTION OF NATURALLY OCCURRING AMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

The use of unidimensional chromatography is seldom sufficient to resolve complex mixtures of naturally occurring amino acids. Even with two-dimensional chromatography it has not hitherto been possible to separate all the common amino acids satisfactorily on one chromatogram.

Over the past few years much work has been carried out on the application of thin-layer chromatography to the separation of amino acids[1–7]. However, despite the superiority of this technique over paper chromatography, great difficulty has been encountered in separating adequately all the natural amino acids on a single chromatogram. In particular, the pair of amino acids leucine/isoleucine have rarely been resolved under such conditions. Nevertheless, by the use of a series of different solvent pairs and chromatograms, it is possible, by successive partial separations, to analyse a complex mixture. This technique was employed by FAHMY, NIEDERWIESER, PATAKI AND BRENNER[4], and also by VON ARX AND NEHER[7]. The properties of filter paper and cellulose powder resemble each other in that chromatograms run on these two solid phases may be developed by partition chromatography. Solvent systems which are capable of separating amino acids on paper are also effective for their separation on thin layers of cellulose, the latter separation being usually superior because of the inherent advantages of thin-layer chromatography.

Some success has previously been achieved in separating the amino acids leucine and isoleucine by paper chromatography[8]. Because of this, and the fact that TEICHERT *et al.*[5], WOLLENWEBER[6], and VON ARX AND NEHER[7] have shown that thin layers of cellulose may be applied to the separation of amino acids, cellulose powder was chosen as the adsorbent in the present work. This paper describes the unambiguous separation of 23 amino acids which are commonly present in proteins. A brief preliminary report of the work has already appeared[9].

EXPERIMENTAL

Materials and equipment

Equipment. Throughout this work Shandon** equipment was used, the layer-spreading equipment being particularly useful because it enables layers of adsorbent slurry to be spread on to glass plates of different thickness at the same time.

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** Shandon Scientific Co., 65 Pound Lane, London, NW 10.

Adsorbent powder. The thin layers used in the present investigation were prepared from Cellulose Powder MN 300 manufactured by Macherey, Nagel & Co.*

Standard solutions. Standard amino acid solutions were prepared as suggested by SMITH[10]. Sufficient of each amino acid was accurately weighed out to produce a 0.05 *M* solution in 25 ml of aqueous propan-2-ol (10 %, v/v). The quantities of amino acids were dissolved in 10–15 ml of the propan-2-ol solution with the addition, if necessary, of the minimum quantity of hydrochloric acid to dissolve those amino acids insoluble in water. The final volumes were then made up to 25 ml.

Ninhydrin–collidine chromogenic reagent. This reagent was similar to that used by BRENNER AND NIEDERWIESER[3]. It consisted of ninhydrin (0.3 g), glacial acetic acid (20 ml), and collidine (2,4,6-trimethylpyridine) (5 ml) in sufficient ethanol to produce a final volume of 100 ml.

Solvents for chromatographic development. All solvents used in this work were of Analar grade**, with the exception of *tert.*-butanol, which was GPR grade**.

PREPARATION OF CELLULOSE THIN LAYERS

TRUTER[11] states that slurries of cellulose may be obtained by dispersing cellulose powder (1 g) in acetone (6 ml). However, thin layers produced in this way were found to be thicker than those produced from water slurries of cellulose powder, and they also adhered to glass less firmly. They had the further serious disadvantage that when samples, in aqueous solution, were applied to the layer, fissure occurred around the circumference of the sample spot during drying thus isolating the spot from the rest of the layer.

The most satisfactory thin layers of cellulose powder were produced in a manner similar to that employed by VON ARX AND NEHER[7]. A quantity of cellulose powder (15 g) of MN 300 was slurried with a mixture containing 70 ml of water and 10 ml of ethanol. From this quantity of slurry five 20 × 20 cm glass plates were coated with layers of slurry each 300 μ in thickness. When the surfaces of the thin layers became matt (this occurred after about 20 min), the plates were transferred to a chromorack and stored overnight at room temperature.

During this time the cellulose thin layers came to equilibrium with atmospheric moisture at room temperature. This is important, since cellulose layers as used, constituted partition chromatographic systems, the water adsorbed on the layers acting as the liquid phase on a solid cellulose support. RANDERATH[12] correlates paper chromatography and cellulose thin-layer chromatography with partition systems.

The final thickness of a cellulose thin layer (when dry) is much less than the initial thickness of the layer, when water is used to form the slurry. Layers initially spread to a depth of 300 μ produce cellulose layers only 100 μ thick when dry.

DEVELOPMENT OF TWO-DIMENSIONAL CHROMATOGRAMS

Sample spots were so positioned on thin layers that development of the first dimension was always "along the grain" of the layer, thus maintaining a standard procedure (see Fig. 1). Amino acid solutions, 1 μl or less, were placed on the thin

* Macherey, Nagel & Co., agents Camlab (Glass) Ltd., Cambridge.
** Hopkin & Williams Ltd., Freshwater Road, Chadwell Heath, Essex.

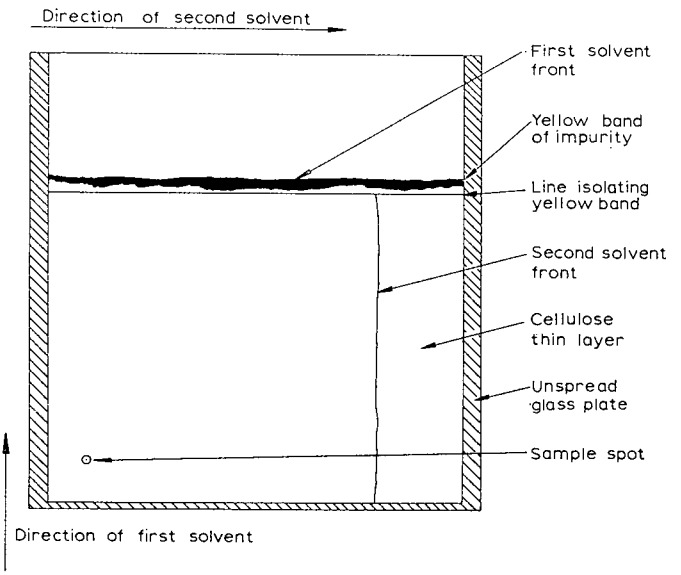

Fig. 1. Two-dimensional thin-layer chromatography on cellulose thin layers.

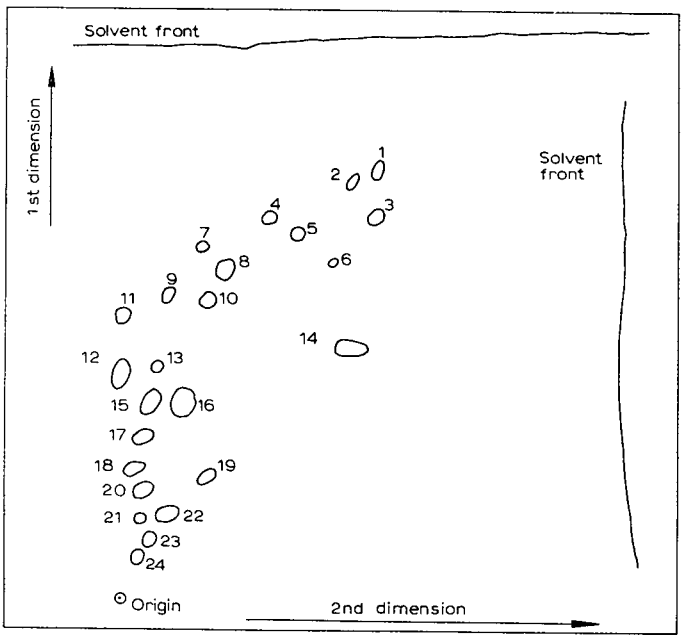

Fig. 2. Amino acid separations on thin layers of cellulose powder. 1 = leucine; 2 = isoleucine; 3 = phenylalanine; 4 = valine; 5 = methionine; 6 = tryptophan; 7 = 2-amino-*n*-butyric acid; 8 = tyrosine; 9 = α-alanine; 10 = proline; 11 = glutamic acid; 12 = aspartic acid; 13 = hydroxyproline; 14 = threonine; 15 = glycine; 16 = serine; 17 = glutamine; 18 = arginine; 19 = taurine; 20 = lysine; 21 = ornithine; 22 = histidine; 23 = cysteic acid; 24 = cysteine. Solvents: 1st dimension: propan-2-ol–formic acid–water (40:2:10, v/v); 2nd dimension: *tert.*-butanol–methyl ethyl ketone–0.88 NH_3–water (50:30:10:10, v/v).

layer, using 1-μl capillary tubes, at a point 1.5 cm from the two edges of the layer in the bottom left-hand corner of the plate. Each spot was dried in a stream of warm air after application to the layer. The solvent for development in the first dimension consisted of propan-2-ol–formic acid–water (40:2:10, v/v)[7]. The thin layer was placed into a tank immediately after the solvent had been added, so that the tank atmosphere was not saturated with solvent vapour prior to development. This was found to be important with both solvent systems if good separation of the amino acids was to be obtained. Ascending development of the chromatogram was allowed to continue at room temperature until the solvent front had travelled a distance of 12–13 cm above the origin (initial sample spot position). This took place over a period of approx. 3 h. The solvent was blown off the layer with warm air until no odour of formic acid could be detected. The latter process required 3–4 min.

It was found that an undulating yellow-coloured band, caused by impurities, was formed along the solvent front. This yellow band held back the solvent front, during development in the second dimension, along the portion of the thin layer which was covered by the band. However, it was possible to isolate the yellow band from the portion of the layer carrying amino acids by breaking the layer, with a scribe, along a line parallel to the edge of the glass plate, just below the yellow band, as illustrated in Fig. 1.

The second solvent, a modification of one of the solvents devised by BOISSONNAS[8], consisted of *tert.*-butanol–methyl ethyl ketone–ammonia (0.88)–water (50:30:10:10, v/v). Once again the plate was developed by the ascending technique until the solvent front was 12–13 cm above the origin, which occurred in this case over a period of about 2.5 h. Excess solvent was removed from the layer by warm air until no odour of solvent could be detected.

DETECTION OF AMINO ACIDS ON THIN-LAYER CHROMATOGRAMS

After two-dimensional development, the amino acids on the chromatograms were detected by means of the ninhydrin–collidine chromogenic reagent. The reagent was sprayed on to the thin layers, which were then held in a stream of warm air. Heating was continued until coloured spots, denoting the positions of amino acids, appeared. As each coloured spot appeared its centre was marked to facilitate calculation of the R_F values.

PAPER CHROMATOGRAPHY

Although this paper is mainly devoted to work carried out by thin-layer chromatography, it is of interest to mention that the solvent systems used for the development of two-dimensional thin-layer chromatograms were also successfully applied to amino acid separations by paper chromatography. Using 10-in. sq. of Whatman No. 1 paper, conventional two-dimensional ascending chromatography was carried out with the same solvent systems as used for thin-layer chromatography. The times required were 8 h for the development of the first dimension and 5 h for the second dimension. Good separations of the amino acid pairs leucine/isoleucine, valine/methionine, and glycine/serine were produced, but the overall separation was inferior to that resulting from thin-layer chromatography.

TABLE I

R_F AND $R_{Leucine}$ VALUES OF AMINO ACIDS SEPARATED BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

Amino acid		*Colour with ninhydrin–collidine chromogenic reagent*	*First dimension IFW*[a]		*Second dimension TMAW*[b]	
			$R_F \times 100$	$R_{Leucine} \times 100$	$R_F \times 100$	$R_{Leucine} \times 100$
α-Alanine	(Ala)	Violet	55	71	10	19
2-Amino-*n*-butyric acid	(Abut)	Violet	64	82	16	32
Aspartic acid	(Asp)	Green	41	61	0	0
Arginine	(Arg)	Violet	24	31	3	6
Cystine group						
Cystine	$(CyS)_2$	Grey	0	0	0	0
Cysteine	(CySH)	Violet	8	10	3	6
Cysteic acid	$(CySO_3H)$	Blue	11	14	5	11
Glutamic acid	(Glu)	Violet	52	67	1	2
Glutamine	$(Glu(NH_2))$	Violet	29	38	4	9
Glycine	(Gly)	Brown	36	46	7	13
Histidine	(His)	Grey/Brown	15	20	9	18
Hydroxyproline	(Hyp)	Yellow	42	54	7	15
Isoleucine	(Ile)	Violet	76	97	45	90
Leucine	(Leu)	Violet	78	100	52	100
Lysine	(Lys)	Violet	20	25	4	9
Methionine	(Met)	Violet	66	85	36	68
Ornithine	(Orn)	Violet	15	20	4	9
Phenylalanine	(Phe)	Brown	69	89	51	99
Proline	(Pro)	Yellow	54	70	17	33
Serine	(Ser)	Violet	36	46	13	25
Taurine	(Taur)	Violet	22	28	17	33
Threonine	(Thr)	Violet	45	59	45	88
Tryptophan	(Trp)	Violet	60	78	42	81
Tyrosine	(Tyr)	Brown	60	78	21	41
Valine	(Val)	Violet	69	89	30	58

[a] IFW = propan-2-ol–formic acid–water (40:2:10, v/v).
[b] TMAW = *tert.*-butanol–methyl ethyl ketone–ammonia (0.88)–water (50:30:10:10, v/v).

RESULTS

The colours of the amino acids produced by the ninhydrin–collidine chromogenic reagent and their R_F and $R_{Leucine}$ values in both dimensions are given in Table I. The coloured spot arising from the presence of threonine appeared only slowly, as did those for other amino acids when present in small amounts (less than 1 μg). The identification of several amino acids, *e.g.* proline, histidine, was made easier by the fact that they produced characteristically coloured spots.

The quality of any separation cannot be judged purely from a knowledge of the R_F values of the spots, because it is the actual size of the spots which determines the true effective separation of neighbouring spots. Fig. 2 shows the actual sizes of the spots and the effective separations produced by thin-layer chromatography.

DISCUSSION

The results of the presents work show that twenty-three naturally occurring amino acids have been unambiguously resolved on a single thin-layer chromatogram. It has not been possible in the past to achieve this. Even pairs of amino acids, which have previously been troublesome to resolve when present at the same time, such as leucine/isoleucine, valine/methionine, and glycine/serine are among the twenty-three amino acids separated.

The necessity for the tank atmosphere to be unsaturated with respect to solvent vapour before development of cellulose thin-layer chromatograms was stated by VON ARX AND NEHER[7] and has been verified by the present work. Indeed prior saturation of the tank atmosphere resulted in inferior separations of the amino acids being produced.

The thin-layer technique which has been described has been used satisfactorily for the analysis of acid-hydrolysed peptides and desalted urine samples.

SUMMARY

Using a two-dimensional thin-layer chromatographic technique, with thin layers of cellulose powder, twenty-three naturally occurring amino acids have been separated unambiguously on a single chromatogram. The separation takes only six hours and includes a resolution of the amino acids leucine and isoleucine.

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THIN-LAYER ELECTROPHORESIS*

PART III. STUDIES ON THE DISSIPATION OF HEAT

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Previous studies have indicated that a load of 0.021 W/cm^2 is tolerable for electrophoresis with thin-layer plates of cellulose[2] or kieselguhr G[3] for periods of up to 15 min. A lower load is recommended for longer electrophoretic runs[2,3]. Despite a build up of heat, the above loads may be exceeded, for example, the electrophoresis of food dyes on kieselguhr G has been found to be satisfactory for 2 h at an initial load of 0.026 W/cm^2 and for 15 min at the greater initial load of 0.041 W/cm^2 (ref. 3). However, these increased loads bring about a quicker breakdown in the electrophoretic process and, unless steps are taken to dissipate the heat that is necessarily produced by the passage of current, there is a rather severe limit to the load that can be used.

It is true that the heat produced can be diminished by diluting the electrolyte thus lowering the current passing, but a common practice, developed initially for paper electrophoresis, is to place the thin-layer plate on a cooling plate through which water is circulated[4–6]. Where extra cooling is required, passage of brine through the cooling plate has been recommended[6], and prior passage of the cooling water through a refrigerated thermostat has also been employed[7].

With the trend for thin-layer electrophoresis to be used for fingerprinting studies on amino acids and peptides, there is the tendency for the thin-layer electrophoretic process to be carried out at higher voltages. The availability of suitable methods of dissipating excessive heat is, therefore, a matter of prime importance, and it is for this reason that the present investigation on the dissipation of heat during thin-layer electrophoresis has been carried out.

EXPERIMENTAL

The thin layers (250 μ thick) were prepared from a slurry of 30 g of the appropriate support material in 60 ml water with a Shandon "Unoplan" Leveller and Spreader and dried at 105°.

The electrolyte solution was 0.05 *M* aqueous borax (pH 9.2).

The Shandon Constant Voltage Power Pack was used for the experiments in conjunction with a specially constructed horizontal electrophoresis tank made of Perspex and provided with a specially thin bottom (1.5 mm thickness) to the bridge compartment to facilitate the conduction of heat.

* For Part II of this series, see ref. 1.

For the experiments in which cooling was employed the electrophoresis tank rested on a prototype 4 × 6 (15 A module) couple "Frigistor" thermoelectric stage cooler (20 × 12.5 cm) through which tap water was circulated. When in operation the cooler was connected to a "Frigistor" 1503 D.C. Power Supply of maximum output 15 A and 3 V.

A few experiments were conducted with the glass plate supporting the thin-layer resting directly on the "Frigistor" stage cooler by using an electrophoresis tank from which the bottom of the bridge compartment had been removed. On these occasions the stage of the cooler was electrically insulated by painting with a solution of polystyrene in chloroform.

The thin-layer plates were saturated with electrolyte by capillary action in the manner previously described[3] and electrophoresis conducted in the usual way at different constant voltages and with different degrees of cooling by the "Frigistor" cooler.

RESULTS AND DISCUSSION

The dissipation of heat during electrophoresis using thin-layer material as support has been studied at initial loads within the range 0.03 to 0.18 W/cm^2. Using 0.05 *M* borax as electrolyte and thin layers of kieselguhr G of 250 μ thick, these loads correspond to applied voltages ranging from 500 to 1000 V for a 20 cm long plate, that is, 25 to 50 V/cm. Inspection of Table I shows that the loads and conditions employed are among the most severe of those previously reported in the literature.

TABLE I

SUMMARY OF LOADS AND CONDITIONS EMPLOYED (OR RECOMMENDED) FOR THIN-LAYER ELECTROPHORESIS

Initial load (*W/cm^2*)	*Voltage* (*V/cm*)	*Current* (*mA/cm*)	*Duration*	*Substrate*	*Method of cooling*	*Ref.*
0.014	23	0.63	1 h	Silica gel G	None	8
0.022	22	0.98	1 h	Silica gel G	None	8
0.0125–0.0333	10–13.7	1.25–2.5	1.5–2 h	Plaster of Paris	None	9
0.021	—	—	15 min	Cellulose	None	2
0.073*	49	1.5	1 h	Silica gel G	Water	5
—	46	—	5 min	Kieselguhr	Water	4
—	45	—	2 min	Kieselguhr	None	4
0.076	47.5	1.6	40 min	Silica gel H	Refrigerator at −1°	7
—	20	—	1.5 h	Silica gel G	None	10
—	20	—	1.5 h	Kieselguhr	None	10
0.026	20	1.30	2 h	Kieselguhr G	None	3
0.041	25	1.65	15 min	Kieselguhr G	None	3
0.120**	40	3.0	30 min	Kieselguhr G	Water at 6.5°	11
0.088	40	2.2	28 min	Kieselguhr G	"Frigistor"	Present work
0.160	50	3.2	7 min	Kieselguhr G	"Frigistor"	Present work

* This figure relates to separations quoted by RITSCHARD[5] but this author also claims that water provided sufficient cooling for loads within the range 0.071–0.200 W/cm^2.

** Using Desaga Thin-Layer Electrophoresis Tank with Shandon Constant Voltage Power Pack.

Experiments with bridge of electrophoresis tank resting on "Frigistor" stage cooler

As stated above, the procedure adopted for the electrophoresis was that normally employed, except for the use of the "Frigistor" stage cooler for exercising different degrees of cooling. Typical results obtained in these circumstances are summarised in Table II for thin layers of kieselguhr G using 0.05 *M* borax as electrolyte. Similar trends were also observed for thin layers of silica gel G and alumina G.

It is clear from Table II that use of the "Frigistor" stage cooler appreciably extends the length of time during which electrophoresis can be successfully carried out at the loads studied. At the lower applied voltages, moderate cooling output by the "Frigistor" stage cooler is adequate for dissipating the heat produced. However, at the higher applied voltages, a cooling output approaching the maximum by the cooler serves only to extend for a short time the period for which electrophoresis can be carried out with 5 cm wide plates before the electrophoresis current rises to the maximum of 25 mA permitted by the Shandon Constant Voltage Power Pack.

It is, of course, possible that the cooler is potentially capable of controlling the heat produced at higher loads. However, since a likely application of the present

TABLE II

EFFECT OF VARIOUS DEGREES OF COOLING BY "FRIGISTOR" UNIT IN DISSIPATING HEAT PRODUCED DURING ELECTROPHORESIS AT CONSTANT VOLTAGE

Support: kieselguhr G (250 μ thick); electrolyte: 0.05 *M* borax; plate dimension: 20 × 5 cm.

Voltage (*V*)	*Initial load* (W/cm^2)	*Load after 60 min unless otherwise stated* (W/cm^2)	*"Frigistor" setting* (*A*)	*Temperature change on surface of a glass plate resting on the bridge compartment* (°C)*
500	0.031	0.030	13	4–6
500	0.035	0.042	10	4–8
500	0.037	0.048	5	8–12
500	0.042	0.120**	—	20–30
600	0.038	0.044	13	2–7
600	0.050	0.114 (90 min)	10	4–12
600	0.055	0.138** (75 min)	5	8–15
600	0.060	0.144** (20 min)	—	20–27
700	0.056	0.081	13	4–10
700	0.070	0.172** (30 min)	10	3–10
700	0.077	0.172** (15 min)	5	7–14
700	0.084	0.172** (7 min)	—	21–24
800	0.088	0.208** (28 min)	13	2–7***
800	0.096	0.200** (15 min)	10	4–12
800	0.108	0.196** (5 min)	5	9–10
800	0.116	0.200** (4 min)	—	20–22
1000	0.160	0.260** (7 min)	13	1–2
1000	0.180	0.250** (1.5 min)	—	20–21

* Recorded by covering the thermometer bulb with wet filter paper and resting on the glass plate.

** These runs were terminated because the current carried by the 5 cm wide plates approached the measuring capacity of the power pack.

*** Temperature of thin layer at end of experiment was 17°. This was recorded by covering thermometer bulb with wet filter paper and resting on the thin layer.

work is to two-dimensional electrophoretic/chromatographic studies using 20 × 20 cm plates, a power pack with a current output of at least 100 mA will be required, particularly where electrolytes of the conducting power of 0.05 *M* borax are used. An output of this order approaches those of power packs used by RITSCHARD[5] and STEGEMANN AND LERCH[7].

Experiment with the thin-layer plate resting directly on "Frigistor" stage cooler

When the bridge compartment of the electrophoresis tank rests on the "Frigistor" stage cooler there are several heat insulating layers between the thin layer and the cooling surface of the "Frigistor" itself. The effect of two of these insulating layers, namely, an air layer and the layer of Perspex forming the bottom of the bridge of the electrophoresis tank, is removed by carrying out electrophoresis with the thin-layer plate resting directly on the "Frigistor" stage cooler. This has the effect of greatly improving the cooling performance during electrophoresis as is shown by the typical results quoted in Table III for experiments using applied voltages of 700 and 800 V.

TABLE III

COOLING PERFORMANCE OF "FRIGISTOR" UNIT ILLUSTRATING EFFECT OF REMOVING BOTTOM OF BRIDGE COMPARTMENT OF ELECTROPHORESIS TANK

Support: kieselguhr G (250 μ thick); electrolyte: 0.05 *M* borax; output of "Frigistor": 10 A; plate dimension: 20 × 5 cm.

Voltage (V)	*Load (W/cm^2)*			
	Bottom of bridge compartment in position		*Bottom of bridge compartment removed*	
	Initial	*After time stated*	*Initial*	*After time stated*
700	0.070	0.172* (30 min)	0.070	0.161 (150 min)
				0.154 (180 min)
800	0.096	0.200* (15 min)	0.088	0.200* (75 min)

* These runs were terminated because the current carried by the 5 cm wide plates approached the maximum measuring capacity of the power pack.

CONCLUSION

The "Frigistor" stage cooler is able to usefully extend the length of time for which electrophoresis on thin layers at applied voltages of 40 to 50 V/cm can be carried out. The results indicate that a power pack of high current measuring capacity is desirable together with a "Frigistor" stage cooler of greater cooling capacity than that used in the present investigation.

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SUMMARY

The application of the "Frigistor" thermoelectric cooling device as a means of dissipating the heat produced during thin-layer electrophoresis has been studied at applied voltages within the range 25 to 50 V/cm for thin layers of kieselguhr G, silica gel G, and alumina G and using 0.05 *M* borax as electrolyte. The detailed results for kieselguhr G are presented and discussed and clearly indicate that the "Frigistor" stage cooler is able to usefully extend the length of time for which electrophoresis under the stated conditions can be carried out.

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THE USE OF DIPHENYLAMINE–ANILINE–PHOSPHORIC ACID REAGENT IN THE DETECTION AND DIFFERENTIATION OF MONOSACCHARIDES AND THEIR DERIVATIVES ON PAPER CHROMATOGRAMS

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INTRODUCTION

Diphenylamine–aniline–phosphoric acid reagent*** which is used for the detection of mono- and oligo-saccharides on paper, displays a wide range of colors with carbohydrates of different structures. This reagent has been used to advantage for distinguishing between various sugars, and some interrelationships between structure and the color produced have been described[1,2].

The aim of the present paper is to draw attention to another useful application of this reagent in the detection and differentiation of some monosaccharide derivatives. The reagent, as used successfully for several years in our laboratory, is a modification of the original reagent of BUCHAN AND SAVAGE[3], developed independently by VÍTEK[4,5].

EXPERIMENTAL

Materials

Most of the sugars and sugar derivatives used were prepared in our laboratory or were gifts from the laboratories acknowledged below. Some of the free sugars were commercial preparations.

Chromatograms

In the majority of cases, the sugars and their derivatives were applied to papers (Whatman No. 3) in 1 % aqueous or ethanolic solutions, in quantities of 10–50 μg, and, if not otherwise stated, were developed by downward irrigation in *n*-butanol–acetic acid–water (10:1:3). The dipping technique of detection was used as a rule, the chromatograms being dried at room temperature and finally heated at 95–100° in a thermostatic oven until the background was faintly gray. For the detection of acid-labile non-reducing derivatives, the time of heating depended on the stability of the hydrolysable bond and, if necessary, was prolonged until distinct spots appeared. In addition to the time of heating, the shades of the colors given in the tables depend more or less on the quantities of sugars applied; in this respect the most marked

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*** Abbreviation: DAPA reagent.

TABLE I

COLOR REACTIONS ON PAPER CHROMATOGRAMS OF SOME COMMON SUGARS WITH THE DIPHENYLAMINE–ANILINE–PHOSPHORIC ACID REAGENT IN VÍTEK'S MODIFICATION

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
(*a*) *Aldopentoses*		
Arabinose	0.22	green-gray
Ribose	0.31	green-gray
Xylose	0.28	green-gray
(*b*) *Ketopentoses*		
Ribulose	0.32	orange-yellow
Xylulose	0.32	green-yellow
(*c*) *Aldohexoses*		
Altrose	0.15	blue-gray
Galactose	0.14	blue-gray
Glucose	0.14	blue-gray
Mannose	0.15	blue-gray
(*d*) *Ketohexoses*		
Fructose	0.16	brown-red*
Sorbose	0.18	yellow-brown**
Tagatose	0.16	brown-red
(*e*) *Disaccharides*		
Cellobiose	0.58***	blue-gray
Lactose	0.48***	blue-gray
Maltose	0.60***	bright blue
Sucrose	0.81***	green-brown
(*f*) *Uronic acids*		
Galacturonic acid	0.05	blackish brown
Glucuronic acid	0.07	blackish brown

* During the heating the color changes from orange-yellow to brown-red.
** During the heating the color changes from yellow-green to yellow-brown.
*** R_{Glc}.

variations of colors were observed with ketoses (Table I). When exposed to the atmosphere of the laboratory, the colors of the spots, as well the background, gradually change, the background acquiring a more blue or blue-green shade. In some cases the spots of otherwise undetectable sugars appear (higher concentrations of sugar alcohols) or those giving a weak reaction (1-thioaldoses) are intensified.

Reagent

Diphenylamine (0.15 g) is dissolved in about 20–25 ml of ethyl acetate from a total volume of 100 ml. Then 0.8 ml of aniline, the remainder of the ethyl acetate and 11 ml of 80 % (w/v) phosphoric acid are added. During the addition of phosphoric acid a voluminous precipitate of amine phosphates is formed, which disappears on further addition of the solvent, when gently shaken. Phosphoric acid must be added after the dissolution of the bases is complete, otherwise an insoluble precipitate can be formed.

When stored in the dark at 0–5° the reagent is stable for 2–3 weeks. After prolonged periods of storage changes in the color shades produced were observed.

RESULTS

Types of compounds examined

According to their reactivity or stability toward the DAPA reagent under the conditions given above, four principal groups of carbohydrates have been tested: *viz.* (1) free sugars, (2) sugar derivatives with acid-labile blocking groups, (3) sugar derivatives with acid-stable blocking groups and (4) sugar alcohols. While the compounds of the second group give, as a rule, the same color reactions as the corresponding free sugars, the colors produced by the derivatives of the third group may differ markedly from those of the parent sugars, due to the different chromogen formed by the action of the reagent. Free 1-thioaldoses, 1-selenoaldoses and 2-amino-2-deoxyaldoses give but non-characteristic weak yellow spots, while sugar alcohols do not react under the given standard conditions.

Owing to the slightly different composition of the reagent used in this work the colors differ, in some cases, from colors given by the reagents reported in previous papers. For comparison Table I shows colors given by some more important common sugars. The results summarized below are divided according to the type of carbohydrate compounds tested.

(*a*) *Derivatives without a secondary hydroxyl group adjacent to the carbonyl group or with an acid-stable blocked one (Table II)*

Absence or acid-stable blocking of the secondary hydroxyl group in the neighborhood of the carbonyl group results in a typical color change with DAPA reagent

TABLE II

DERIVATIVES WITHOUT A SECONDARY HYDROXYL GROUP ADJACENT TO THE CARBONYL GROUP OR WITH AN ACID-STABLE BLOCKED ONE, ALL GIVING VARIOUS SHADES OF RED-VIOLET WITH DAPA REAGENT

System: *n*-butanol–acetic acid–water (10:1:3), if not otherwise stated.

Sugar	R_F
(*a*) *Deoxy-sugars and derivatives*	
2-Deoxy-D-*erythro*-pentose (2-deoxy-D-ribose)	0.42
2-Deoxy-D-*lyxo*-hexose (2-deoxy-D-galactose)	0.33
2-Deoxy-D-*arabino*-hexose (2-deoxy-D-glucose)	0.34
2,6-Dideoxy-3-O-methyl-D-*ribo*-hexose (cymarose)	0.83
2^G-Deoxysucrose	0.14
2-Deoxy-D-ribohexonic (2-deoxygluconic) acid lactone	0.60
2-Deoxy-D-*lyxo*-hexose-1-phosphate	0.25*
2-Deoxy-D-*arabino*-hexose-6-phosphate	0.23*
(*b*) *Glycals*	
D-Galactal	0.54
D-Glucal	0.61
(*c*) *Ethers and α-D-glycopyranosyl derivative*	
2-O-*p*-Tolyl-D-altrose	0.85
2-O-Methyl-D-glucose	0.26
3-O-Methyl-D-fructose	0.34
3-O-α-D-Glucopyranosyl-D-fructose (turanose)	0.05

* System: *n*-propanol–ammonia–water (6:1:3).

to various gradations of violet or red-violet. Reactions of 2-deoxyaldoses, as well as glycals, are especially sensitive and the color predominates also in acid-labile oligosaccharides containing a 2-deoxyaldose moiety in addition to other monosaccharide units, as in 2^{G}-deoxysucrose or 2^{Gal}-deoxyraffinose[6]. A red-violet color is also given by 2-O-methyl-D-glucose and 3-O-methyl-D-fructose and even turanose which is 3-O-α-D-glucopyranosyl-D-fructose. The rule does not apply when the secondary hydroxyl group adjacent to the carbonyl function is replaced by a more polar group, *e.g.* an amino or sulfhydryl group in 2-amino-2-deoxyaldoses or 2-thioaldoses. Similarly, further acid-stable substitution on remaining free hydroxyl groups as in 2,4,6-tri-O-methyl-D-glucopyranose (see Table III) brings about the loss of this typical reaction.

(*b*) *Aldohexoses in which a hydroxyl group not adjacent to the carbonyl group is missing or is acid-stably blocked, or aldohexoses which besides the secondary acid-stably blocked hydroxyl group adjacent to the carbonyl function have additional acid-stably blocked hydroxyl groups* (*Table III*).

For the few compounds of this type available no definite regularity in the color reaction could be established. It seems likely that in aldohexoses the absence (or stable blocking) of the single hydroxyl group at C-3 or C-4 results in a change of the originally blue-gray color of the parent hexose to yellow or green. The blue-gray color given by the higher methylated glucoses is remarkable when compared with the red-violet given by 2-O-methyl-D-glucose.

TABLE III

ALDOHEXOSES IN WHICH A HYDROXYL GROUP NOT ADJACENT TO THE CARBONYL GROUP IS MISSING OR IS ACID-STABLY BLOCKED, OR ALDOHEXOSES WHICH BESIDES THE SECONDARY ACID-STABLY BLOCKED HYDROXYL GROUP ADJACENT TO THE CARBONYL FUNCTION HAVE ADDITIONAL ACID-STABLY BLOCKED HYDROXYL GROUPS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
3-Deoxy-D-*ribo*-hexose (3-deoxy-D-glucose)	0.26	dull yellow
4-Deoxy-D-*xylo*-hexose (4-deoxy-D-glucose, 4-deoxy-D-galactose)	0.25	dull yellow
4-O-Methyl-D-galactose	0.31	green-gray
2,4,6-Tri-O-methyl-D-glucopyranose	0.67	blue-gray
2,3,4,6-Tetra-O-methyl-D-glucopyranose	0.82	blue-gray
1,2-O-Isopropylidene-5,6-O-carbonyl-α-D-glucofuranose	0.80	blackish brown

(*c*) *Simple aliphatic, alicyclic and aromatic glycosides* (*Table IV*)

Most of the aliphatic glycosides are easily detectable in amounts of about 200 μg and produce the same color as the parent sugar within 1 to 10 min. The time necessary for splitting off the aglycone and hence for the color development is in good agreement with the general rules holding for the stability of the glycosidic linkage and can be used as a means of differentiation between glycosides of various structures. From the compounds examined the alkyl β-D-fructofuranosides are the most labile ones, whereas some of the methyl α-D-aldohexopyranosides show a considerable stability; methyl α-D-glucopyranoside fails to react at all.

The sensitivity limits lay at 100 μg for most aliphatic and at about 200 μg for

TABLE IV

SIMPLE ALIPHATIC AND AROMATIC GLYCOSIDES

System: *n*-butanol–acetic acid–water (10:1:3).

Glycoside	R_F	*Minimum time necessary for developing color (min)*	*Color*
(*a*) *Aliphatic glycosides*			
Methyl β-D-arabinopyranoside	0.34	< 5	gray-green
Methyl α-D-altropyranoside	0.32	> 5	blue-gray
Methyl α-D-galactopyranoside	0.22	< 10	blue-gray
Methyl β-D-galactopyranoside	0.21	< 10	blue-gray
Ethyl α-D-galactopyranoside	0.35	< 10	blue-gray
n-Propyl β-D-glucopyranoside	0.48	< 10	blue-gray
n-Butyl β-D-glucopyranoside	0.58	< 10	blue-gray
Methyl α-D-glucopyranoside	—	—	nil
Methyl β-D-glucopyranoside	0.24	5	blue-gray
Methyl 3-keto-β-D-glucopyranoside	0.25	> 5	blue-gray
Isopropyl β-D-glucopyranoside	0.47	< 5	blue-gray
Isobutyl β-D-glucopyranoside	0.61	< 5	blue-gray
tert.-Butyl β-D-glucopyranoside	0.51	< 5	blue-gray
Cyclohexyl β-D-glucopyranoside	0.59	< 5	blue-gray
Methyl α-D-mannopyranoside	0.35	10	blue-gray
Methyl β-D-fructofuranoside	0.35	< 1	brown-red
Ethyl β-D-fructofuranoside	0.45	< 1	brown-red
n-Propyl β-D-fructofuranoside	0.56	< 1	brown-red
n-Butyl β-D-fructofuranoside	0.65	< 1	brown-red
Amyl β-D-fructofuranoside	0.71	< 1	brown-red
Benzyl β-D-fructofuranoside	0.66	< 1	brown-red
(*b*) *Aromatic glycosides*			
o-Hydroxyphenyl β-D-glucopyranoside	0.40	> 10	faint yellow
m-Hydroxyphenyl β-D-glucopyranoside	0.30	> 10	faint yellow
p-Hydroxyphenyl β-D-glucopyranoside (arbutin)	0.24	> 10	yellow
Phenyl β-cellobioside	0.31	> 10	yellow-brown

aromatic glycosides. The weak spots of aromatic glycosides appear after an average of 10 min heating and have non-characteristic shades of dull yellow.

(*d*) *Glycosans (Table V)*

Being internal β-D-glycosides, glycosans react readily, developing, as a rule, the color of the parent sugar.

(*e*) *Alkylidene, arylidene and acyl derivatives (Table VI)*

The various acid-labile compounds listed in Table VI react smoothly. Increase in molecular weight caused by the various blocking groups attached, leads to a corresponding decrease in sensitivity, but, as a rule, not to a perceptible color change.

(*f*) *Thio and selenosugars (Table VII)*

1-Thioaldoses and 1-selenoaldoses give after prolonged heating non-characteristic light spots on a dark background. The use of the reagent for this class of sugars

TABLE V

GLYCOSANS, ANHYDROSUGARS, AND THEIR DERIVATIVES

System: *n*-butanol–acetic acid–water (10:1:3).

Glycosan	R_F	*Color*
1,6-Anhydro-β-D-glucopyranose	0.40	blue-gray
1,6-Anhydro-4-deoxy-β-D-altropyranose	0.47	yellow
1,6-Anhydro-4-deoxy-β-D-glucopyranose	0.53	yellow
1,6-Anhydro-2-O-*p*-tolyl-β-D-altropyranose	—	nil
1,6-Anhydro-2-O-*p*-tolylsulphonyl-β-D-altropyranose	—	nil
1,6:3,4-Dianhydro-β-D-altropyranose	0.62	blue-gray
1,6:3,4-Dianhydro-β-D-galactopyranose	0.71	blue-gray
1,6:2,3-Dianhydro-β-D-gulopyranose	0.75	green
1,6:2,3-Dianhydro-β-D-mannopyranose	0.62	blue-gray

TABLE VI

ALKYLIDENE, ARYLIDENE, AND ACETYL DERIVATIVES OF SUGARS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar derivative	R_F	*Color*
1,2:3,4-Di-O-isopropylidene-L-arabinopyranose	0.98	green-gray
1,2-O-Isopropylidene-α-D-glucofuranose	0.70	blue-gray
1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose	0.97	blue-gray
1,2-O-Isopropylidene-5,6-anhydro-α-D-glucofuranose	0.78	green-gray
Methyl 2-deoxy-4,6-O-benzylidene-α-D-*arabino*-hexopyranoside	0.90	red-violet
1,2-O-Isopropylidene-3-deoxy-α-D-*ribo*-hexofuranose	0.75	yellow
6-O-Acetyl-D-galactose	0.29	blue-gray
6-O-Acetyl-D-glucose	0.29	blue-gray
1,6-Anhydro-3-O-acetyl-β-D-glucopyranose	0.96	blue-gray
3,4,6-Tri-O-acetyl-D-galactal	0.98	red-violet
Hepta-O-acetyl-2G-deoxysucrose	0.96	red-violet

TABLE VII

THIOSUGARS AND SELENOSUGARS

System: *n*-butanol–acetic acid–water (10:1:3).

Sugar	R_F	*Color*
1-Thio-D-glucose	0.25	nil*
6-Thio-D-glucose	0.35	green-gray
1,6-Dithio-D-glucose	0.62	nil*
1-Seleno-D-glucose	0.08	nil*
2-Thio-D-altrose	0.33	yellow-green

* Pale yellowish spots on deep gray or blue-gray background on prolonged heating.

is of no particular advantage. On the other hand, reducing thiosugars with secondary —SH groups (other than on the anomeric C-atom), as far as examined, give well-developed spots differing in shade from the corresponding —OH compounds.

(*g*) *Amino sugars*

Of the amino sugars only 2-amino-2-deoxy-aldohexoses were tested. They only produce non-typical dull yellow spots. DAPA reagent is not suitable for detection of this type of sugar.

(*i*) *Sugar alcohols*

In the usual concentrations sugar alcohols do not react with DAPA reagent. At higher concentrations (more than 500 μg) light spots appear after prolonged heating on the gray or blue-gray background which, on prolonged exposure to the atmosphere at room temperature, turn bright blue, acquiring a shade similar to that given by maltose.

DAPA reagent has been used with success for the detection and differentiation of sugar derivatives in plant extracts and in mixtures of enzymic transglycosylation products. Furthermore it has been proved valuable in following the course of reaction in synthetic carbohydrate chemistry. For example, the two different ethers (at C-2 or C-3) which arise, as a rule, by splitting 2,3-anhydro rings in aldohexose epoxy sugars by the action of alkali alcoholates or phenolates, can clearly be distinguished on paper, by the red-violet and yellow-green colors given by the respective ethers.

DISCUSSION

In this paper two advantages of practical application of DAPA reagent are demonstrated; they are (1) the specific color reactions given by the reagent with different types of reducing sugars and (2) the detection of various non-reducing sugar derivatives which are hydrolysed by the action of the reagent to the free sugars.

Due to the limited number of sugar compounds of a particular structure that were at our disposal, it was not possible to present a detailed study on the interrelationships between the structure and the corresponding color produced by the reagent. Nevertheless, a few groups of sugars of similar structure could be shown to give characteristic and more or less identical colors: (a) aldohexoses, blue-gray; (b) aldopentoses, green-gray; (c) hexuronic acids, dark brown; (d) derivatives with no secondary hydroxyl group adjacent to the carbonyl group, or with an acid-stable blocked one, red-violet.

On the other hand there are groups of sugars of very similar structure, *e.g.* ketoses like fructose and sorbose, or ribulose and xylulose which show clear-cut differences in color and thus bear evidence to the complexity of the color reaction. Another example is represented by the reactions given by 2-O-methyl-D-glucose and the higher methylated glucose derivatives. While 2-O-methyl-D-glucose follows the general rule found for derivatives summarized under (d) and gives a red-violet color, 2,4,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucopyranose give, contrary to all expectations, the blue-gray color of free glucose.

These rather contradictory results make it difficult to suggest a mechanism for the chromogen formation involved. Apparently, the simple theory which ascribes

the color production to the preliminary conversion of the sugar by acid to furfural, or a derivative, which then reacts with the reagent to give a colored compound[7] cannot give a sufficient explanation for the variety of cases described. The practical aspects of the reaction should, however, stimulate its further investigation.

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The R_F values of fructofuranosides given in Table IV are taken from the Diploma Thesis by Mr. J. VOLC[8] whose cooperation in this part of the work is gratefully acknowledged.

SUMMARY

The diphenylamine–aniline–phosphoric acid reagent, as modified by VÍTEK, has been used for the detection of various monosaccharides and their derivatives. Under the defined conditions, the reagent gives specific color reactions and can be used (1) to distinguish between sugars of different structure and (2) to detect various non-reducing sugar derivatives with acid-labile blocking groups.

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MOBILITIES OF AMINO ACIDS AND PEPTIDES IN PAPER ELECTROPHORESIS

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INTRODUCTION

The relative zone mobility u_r of an ion X is defined as the ratio of its zone mobility to the zone mobility of a standard ion (we have used butylammonium as the standard cation and picrate as the standard anion[1]). In a previous communication[1] we showed that u_r is given by the equation:

$$u_r = u\rho/u_{(std)}\rho_{(std)} \quad (1)$$

where u and $u_{(std)}$ are the mobilities of X and of the standard ion in free solution, and ρ and $\rho_{(std)}$ are the adsorptive factors of X and of the standard ion. The adsorptive factor measures the retardation of an ion by reversible adsorption on to the cellulose fibres of the paper and for most small ions can be ignored (*i.e.* $\rho = 1$). However, for ions having large flat hydrophobic faces, adsorptive effects become appreciable and $\rho < 1$.

The possibility of calculating u_r depends on the ability to calculate $u/u_{(std)}$ and $\rho/\rho_{(std)}$.

Our previous communication[1] dealt with the calculation of the first term, when the shape of the ion under consideration was approximately spherical or ellipsoidal. In the present paper we extend the calculations to several amino acids and peptides, some of them of irregular shape, measured against butylammonium as the standard cation. The second term $\rho/\rho_{(std)}$ has been evaluated experimentally; the instances where it may safely be assumed equal to unity are discussed below.

CALCULATION OF $u/u_{(std)}$

The amino acids and peptides listed in Table I were run against butylammonium as standard ion in hydrochloric acid at pH 1.51 $\pm$ 0.04 (measured with a glass electrode). The experimental procedure has already been described[1]. Since all the ions under investigation are monovalent, they will suffer about the same proportional retardation from interionic effects in a solution of given ionic strength, so that approximately:

$$u^+/u^+_{(std)} = u_0{}^+/u_0{}^+_{(std)} \quad (2)$$

where $u_0{}^+$ and $u_0{}^+_{(std)}$ are cation mobilities in infinitely dilute solution.

The mobility of butylammonium ion at infinite dilution in water at 25° is $3.88 \cdot 10^{-4}$ $cm^2 \cdot sec^{-1} \cdot V^{-1}$ (ref. 2). The limiting mobilities (in $cm^2 \cdot sec^{-1} \cdot V^{-1}$) of the

$$\underset{\text{(I)}}{NH_3^+\text{–CHR–}CO_2H} \rightleftarrows \underset{\text{(II)}}{NH_3^+\text{–CHR–}CO_2^-} \rightleftarrows \underset{\text{(III)}}{NH_2\text{–CHR–}CO_2^-}$$

cations (I) of the amino acids (II) in water at 25° may be calculated using a modified STOKES equation[2,3]:

$$u_0^+ = (1.602 \times 10^{-12} z)/5\pi r_w^+ \eta (f/f_0) \tag{3}$$

where z is the ionic charge, r_w^+ is the Van der Waals radius of the cation (in Å), η the viscosity of the solvent (in poises), and (f/f_0) is the "frictional ratio" ($\geqslant 1$), which measures the increased resistance to the passage of the ion if its shape is not spherical[4].

(a) Calculation of charge z

The effective charge (z) of the cation (I) depends on the extent of conversion of the zwitterion (II) and hence on the hydrogen ion activity a_{H+} of the solution:

$$z = [I]/([I] + [II]) = a_{H+}/(a_{H+} + K_1') \tag{4}$$

where K'_1 is the apparent dissociation constant for the reaction: I $\rightleftarrows$ II + H^+, and $-\log a_{H+}$ = pH is measured potentiometrically using a glass electrode. The apparent dissociation constant K_1' (= — antilog pK_1') is related to the thermodynamic dissociation constant K_1 (= — antilog pK_1) by the equation:

$$pK_1' = pK_1 + 0.5\sqrt{w}/(1 + Qa\sqrt{w}) - K_R w \tag{5}$$

where w is the ionic strength of the solution, $Q(\cong 0.33)$ is a constant, a is the collision diameter of the cation, and K_R is a salting-in term[5]. For our present calculations we have, following NEUBERGER[6], assumed $Qa \approx 1$, and have set $K_R = 0.32$ for the amino acids (Nos. 1–16 of Table I), 0.4 for glycylglycine, and 0.6 for diglycylglycine. These values of K_R seem reasonable for the α-amino acids (Nos. 1–12) and peptides[5,6], but may be too low for the aminobenzoic acids (Nos. 14–16).

The values of z calculated from K_1 values listed by ROBINSON AND STOKES[7] are given in Table I. These values have a possible error of about ± 5 % because of variations in the pH of the hydrochloric acid solutions and because of the various approximations in the calculations using Eqns. (4) and (5).

(b) Calculation of frictional ratio (f/f_0)

The frictional ratio (f/f_0) of the cations derived from simple amino acids such as glycine and alanine, which have approximately ellipsoidal shapes, can be obtained by the application of PERRIN's equations[4]. However, the shapes of most of the amino acids are too irregular for these equations to be applicable. Consequently, the frictional ratios were obtained from the limiting diffusion coefficients $D_0^{\pm}$ of the amino

TABLE I

RELATIVE ZONE MOBILITIES (u_r^+) OF AMINO ACIDS AND PEPTIDES AT pH 1.5

No.	*Compound*	*z*	$r_w^{\pm}/r_w^+$	u_r^+ *(calc.)*	u_r^+ *(obs.)*
1	Leucine	0.890	1.01	0.65	0.67
2	Norleucine	0.890	1.01	0.65	0.68
3	Valine	0.880	1.01	0.69	0.68
4	Norvaline	0.898	1.01	0.70	0.65
5	Threonine	0.823	1.01	0.66	0.67
6	Serine	0.853	1.01	0.76	0.70
7	Proline	0.772	1.01	0.69	0.66
8	Alanine	0.895	1.02	0.82	0.83
9	Asparagine	0.810	1.01	0.68	0.68
10	Phenylalanine	0.720	1.01	0.51	0.53
11	Tryptophan	0.904	1.01	0.48	0.49
12	Glycine	0.895	1.02	0.96	0.97
13	β-Alanine	0.994	1.10	1.02	1.02
14	*o*-Aminobenzoic acid	0.831	1.01	0.61	0.19
15	*m*-Aminobenzoic acid	0.982	1.10	0.68	0.65
16	*p*-Aminobenzoic acid	0.910	1.10	0.69	0.66
17	Glycylglycine	0.892	1.10	0.86	0.82
18	Diglycylglycine	0.985	1.07	0.70	0.72

acid zwitterions (II). It has been shown that a modified STOKE's equation applies to the diffusion in water of these and other neutral organic molecules[7]:

$$D_0^{\pm} = kT/5\pi r_w^{\pm}\eta(f/f_0) \tag{6}$$

$$(f/f_0) = kT/5\pi D_0^{\pm} r_w^{\pm}\eta \tag{6a}$$

where k is Boltzmann's constant, T the absolute temperature, and $r_w^{\pm}$ the Van der Waals radius of the zwitterion. Equation (6) has been found to hold fairly well for glycine and alanine (which are sufficiently ellipsoidal for (f/f_0) to be obtainable from PERRIN's equations)[8], and hence may be assumed to apply to the more irregular amino acids which deviate widely from ellipsoidal shapes. Values of $D_0^{\pm}$ for the amino acids and peptides of Table I are available from the careful work of LONGSWORTH[9]. LONGSWORTH's experimental values are for low solute concentrations, rather than for infinite dilution, but will differ by only 1–2 % from values for the latter condition.

(c) Calculation of the ionic radius r_w^+

The Van der Waals volume V_w of organic ions may be obtained by the addition of the volumes of the constituent atoms or groups, which have already been listed[1,2,10,11]. The Van der Waals radius r_w is thus given by the relation: $V_w = 4\pi r_w^3/3$. However, in aqueous solution it was sometimes found necessary to assume an increase in volume because of hydration, in order to compute correct ionic mobilities by the use of Eqn. (3). Thus it was found that the volume of the ionized carboxylate group (29.5 Å^3) had to be increased by about 40 Å^3 to account satisfactorily for the mobilities of ions containing this group[2]. On the other hand, it was not found necessary to increase the Van der Waals volume (11.5 Å^3) of the $-NH_3^+$ group to account satisfactorily for the mobilities of substituted ammonium ions in water[2,3], in

spite of the fact that other types of evidence[12] indicate this group also to be hydrated. It would seem that with substituted ammonium ions the effect of hydration is counterbalanced by other effects, such as loosening of the surrounding water structure[13], insofar as mobilities are concerned.

The Van der Waals volumes calculated in this way prove satisfactory in explaining the mobilities of monofunctional and some simple polyfunctional compounds[2,3]. However, there is some evidence that hydration increases when several polar groups are near each other in an ion[2], perhaps through cooperative effects in binding water molecules. Thus the cations of glycine (I; R = H) and alanine (I; R = CH_3) were found to have mobilities considerably less than calculated from Eqn. (3)[1]. The experimental mobilities may be brought into line with calculated values if it is assumed that the Van der Waals volumes of these cations are increased by about 30 Å^3.

The work described in the present paper with the more extended series of amino acids of Table I corroborates this finding. The relative mobilities of these cations were obtained from Eqn. (7), obtained by combining Eqns. (3) and (6):

$$u_0^+ = 1.602 \times 10^{-12} z r_w^{\pm} D_0^{\pm} / k r_w^+ T \qquad (7)$$

For water solutions at 25° this becomes:

$$u_0^+ = 38.9 z D_0^{\pm} r_w^{\pm} / r_w^+ \qquad (8)$$

D being measured in cm$^2 \cdot$sec^{-1}. When butylammonium ($u_0 = 3.88 \times 10^{-4}$ cm$^2 \cdot$ sec$^{-1} \cdot$V^{-1} at 25°) is used as the standard ion:

$$u_0^+ / u_{0(\mathrm{std})}^+ = 1.00 \times 10^5 z D_0^{\pm} r_w^{\pm} / r_w^+ \qquad (9)$$

The mobility ratio calculated from Eqn. (9) is for aqueous solutions at 25°. However, the relative mobilities of organic ions having radii larger than about 3 Å are not affected by temperature[2]. In the present work temperatures were about 30°, but were not controlled closely.

(d) Determination of $\rho/\rho_{(\mathrm{std})}$

The adsorptive factor ($\rho_{(\mathrm{std})}$) of the butylammonium ion is known to be 1.00[1]. The same adsorptive factors would be anticipated for the cations of all the amino acids and peptides listed in Table I, except perhaps phenylalanine, tryptophan and the aminobenzoic acids, which contain aromatic rings. The adsorptive factors were determined by the method described earlier[1], and were found to be approximately 1.00 for all the amino acids (including phenylalanine) in aqueous hydrochloric acid (pH 1.5) on Whatman No. 1 paper, with the exception only of *o*-aminobenzoic acid ($\rho = 0.86$), *m*-aminobenzoic acid ($\rho = 0.82$), *p*-aminobenzoic acid ($\rho = 0.82$), and tryptophan ($\rho = 0.80$). The different values obtained for phenylalanine and the aminobenzoic acids illustrate the fact that while the adsorptive factor is in general governed by the area of flat hydrophobic surface in the molecule, other more specific factors are also involved[2].

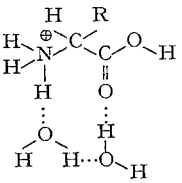
COMPARISON OF CALCULATED AND OBSERVED RELATIVE ZONE MOBILITIES

Combining Eqns. (1), (2) and (9) gives:

$$u_r^+ = 1.00 \times 10^5 \rho z D_0^{\pm} r_w^{\pm} / r_w^+ \qquad (10)$$

when butylammonium is the standard ion. If no hydration of the amino acid cation (I) is assumed, $r_w^{\pm}/r_w^+$ varies from 1.18 for the smallest ion (glycine) to 1.07 for the largest (tryptophan), because of the increase in volume on ionization of unhydrated $-CO_2H$ to hydrated CO_2^-*. The use of such values led to systematically high values of u_r^+, the error being greater for the small ions than for the large ions. However, when it was assumed that V_w^+ of the cations was increased by 33 Å^3 by hydration, and the consequent values of $r_w^{\pm}/r_w^+$ given in Table I were obtained, the calculated values of u_r^+ were found to be in reasonably good agreement with the observed values**. Better agreement could not be expected, considering the various approximations in the calculations, and the precision of only about 3–4 % in the observed values.

The unexpected hydration of the cations (I) may be a consequence of a cooperative effect of the ammonium and carboxyl groups in binding water molecules:



In that case the effect should drop off with separation of the groups. Some support for this idea comes from the cations after No. 12 in Table I. An increase of 10 Å^3 in the V_w^+ for the β-amino acids (Nos. 13 and 14), and of zero for the remaining acids and peptides (Nos. 15–18) has been assumed in arriving at the calculated values of u_r^+ in the table. It is evident that these values are in rough agreement with observed values, except in the case of *o*-aminobenzoic acid. We cannot explain the anomalously low mobility of this cation, which merits further investigation.

It is realized that in this treatment a naive picture of the conduction process is used: the decrease in mobility is attributed solely to an increased volume of migrating ion because of hydration. In fact the retardation of ions in aqueous solutions is due to a number of causes[16], of which this is only one. However, this treatment is justified by its operational usefulness. It suggests that the mobilities of ammonium ions having aldehyde, keto or nitro groups near the $-NH_3^+$ group may be less than those having these groups more distant from the $-NH_3^+$ group.

Attempts were made to analyse the relative zone mobilities of amino acid

* The question of the relative sizes of amino acid cations, anions and zwitterions seems first to have been considered by SVENSSON[14], who, however, lacked any experimental evidence on the subject.

** In a previous treatment[15] relating u^+ and $D_0^{\pm}$, it was assumed that $r^{\pm}{}_w \sim r_w^+$. It is apparent, from inspection of $r_w^{\pm}/r_w^+$ values in Table I, that this assumption is in rough agreement with the results in the present paper.

anions (III) in aqueous piperidine at pH 11.4, using picrate as the standard anion. Qualitatively, the results appeared to indicate hydration of the anions (III) considerably in excess of the hydration assumed for simple carboxylate ions. However, the mobilities of α-amino acids in this and in other alkaline media varied in an erratic manner, for reasons not yet understood. The attempt to clarify this behaviour is being continued.

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SUMMARY

The relative zone mobilities of several protonated amino acids and two peptides have been calculated from the diffusion coefficients of the zwitterionic forms of the compounds. Good agreement with observed values was obtained if it was assumed that the Van der Waals volume of protonated α-amino acids is increased by 33 Å^3 by hydration.

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CHROMATOGRAPHIC ANALYSIS OF PHOTOSENSITIZING DYES

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The studies reported here were undertaken with the initial objective of establishing the purity of several dyes which had been used in our investigations concerning their photochemical properties. Questions as to the purity of several dyes arose because in several instances our inability to demonstrate the photosensitizing properties of a dye was thought to be due to the presence of sensitizing impurities in dye samples used by other workers. In addition the determination of the spectroscopic properties of dyes, a field of investigation greatly stimulated by the development of laser systems, necessitates the use of pure dyestuffs.

There are several reviews[1–3] dealing with methods of dye purification in general, and with the chromatographic assay of dye purity in particular. For paper chromatography of thiazine dyes an excellent system was developed by TAYLOR[4,5]. It quickly became apparent, however, that the solvent systems generally used are extremely slow, and did not always give good resolution. In developing the solvent system reported here, our aim was to achieve a system which would give good separation of constituents in relatively short time periods, and one which could be applied to a wide variety of dye systems.

EXPERIMENTAL

The dyes used in this investigation are listed in Table I. Thionolin was prepared and purified by a method in the literature[6] and further purified by extraction of an acid solution with chloroform. Formamide (grade: "for vitamin assay"), *n*- and *tert.*-butanol, and acetone were purchased from Fischer as certified reagent grades. Most of the dyes were dissolved in 10% of the final volume absolute ethyl alcohol, and diluted to final volume with water; the final concentrations of the dyes were 1–4 mg per ml solution; 0.01–0.20 mg of dye (0.01–0.10 ml of solution) were applied to paper using a hypodermic syringe under a stream of air to minimize spreading of the spot. Thionolin and methylene violet were dissolved in methanol.

Solvents for development

Three solvent systems were used as described below:

A. *tert.*-Butanol–acetone–0.2 *M* HCl (aq.) (35:35:30; parts by volume). The apparent pH of this mixture as measured with a glass electrode is 1.9.

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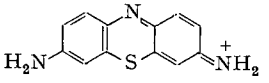
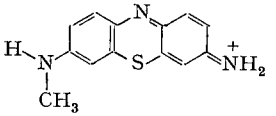
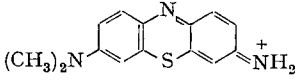
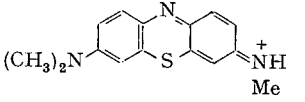
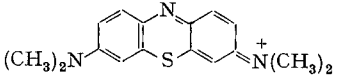
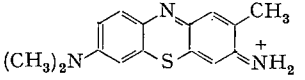
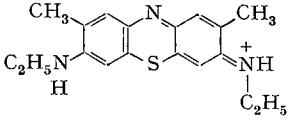
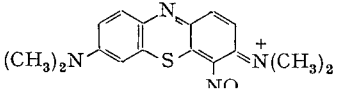
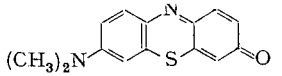
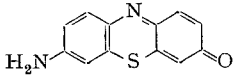
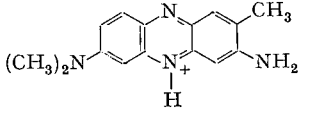
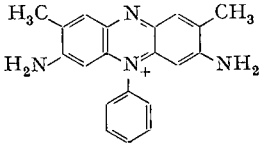
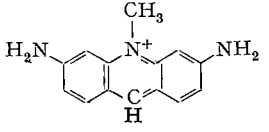
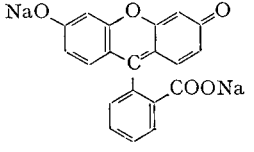
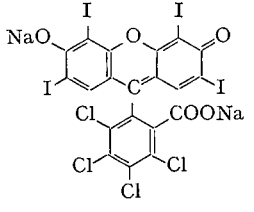
TABLE I

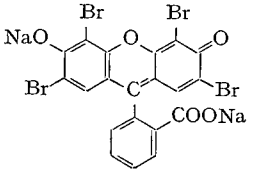
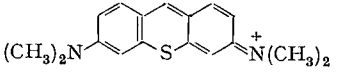
DYES USED FOR CHROMATOGRAPHY

Dye	*Structural formula*[7]	*Color Index No.*	*Certific'n No. (C) or lot No. (L)*	*Stated dye content (%)*	*Supplier*
Thiazines					
Thionine		5200	NT 20 (C)	85	National Aniline
Azure C		—	NAc 5 (C)	—	National Aniline
Azure A		—	NAz 16 (C)	93	National Aniline
Azure B		52010	NAb (C)	69	National Aniline
Methylene blue		52015	741220 (L)	88	Fisher Scientific
Toluidine blue O		52040	NU 12 (C)	76	National Aniline
New methylene blue N		52030	14642 (L)	—	National Aniline
Methylene green		52020	1623 p (L)	—	National Aniline

Methylene violet		—	NLv 8 (C)	70	National Aniline
Thionolin		—	—	—	Synthesized by authors according to ref. 6
Azines					
Neutral red		50040	N 129 (C)	83	Fisher Scientific
Safranine O		50240	NS 22 (C)	85	National Aniline
Acridines					
Acriflavine		46000	14435 (L)	—	National Aniline
Xanthenes					
Uranine		45350	731575 (L)	—	Fisher Scientific
Rose bengal		45440	724158 (L)	80	Fisher Scientific

(*continued on p. 134*)

TABLE I (*continued*)

Dye	*Structural formula*[7]	*Color Index No.*	*Certific'n No. (C) or lot No. (L)*	*Stated dye content (%)*	*Supplier*
Eosin Y		45380	732702 (L)	87	Fisher Scientific
Thiopyronines					
Thiopyronine		—	—	—	E. Merck AG.

B. *tert.*-Butanol–acetone–0.2 *M* NH_3 (aq.)–0.2 *M* HCl (aq.) (35:35:21:9). The apparent pH of this mixture is pH 9.0. This pH was chosen because it was found to prevent "ghosting" of components in the case of the alkaline thiazine dyes.

C. *tert.*-Butanol–*n*-butanol–0.2 *M* NH_3 (aq.) (40:30:30), with an apparent pH of 10.6.

Chromatographic development was carried out on wedge-shaped strips[7] of Whatman 3MM paper, 4 cm wide and 45 cm long, in a Chromatobox (Warner-Chilcott Laboratories). Chromatography was carried out in the dark, and the chambers were sealed with parafilm in order to minimize evaporation of the volatile solvent components.

In order to keep conditions constant, a standard developing time of eighteen hours was adopted for solvents A and B. With solvent C forty hours were necessary. At the end of these time periods the solvent front had reached to within two cm of the end of the paper.

RESULTS AND DISCUSSION

The results of the chromatographic analysis of thiazine dyes are listed in Table II. The value α is the absorbance at the absorption maximum in ml per mg of dye. If the different fractions in a dye sample do not differ greatly in molar extinction coefficient, the values of α can be taken as the relative molar proportions of the components of the mixture. Thus one can calculate that the methylene blue sample analyzed is contaminated with 2.3 % Azure A and 8.1 % Azure B. The reproducibility of the R_F values and of α are illustrated for the analysis obtained in the case of methylene blue, where the standard deviation of ten determinations is listed. For those fractions whose spectra showed a definite shoulder in the absorption maximum this wavelength is listed, together with the relative absorbance at this wavelength compared to that at the absorption maximum. These values frequently aid in the identification of the various fractions. It should be noted that the spectral data given were obtained by eluting the fractions into 50 % (v/v) aqueous formamide (in order to get quantitative elution) and that these spectra therefore differ from those obtained in acetate or in methanol: the shoulders in the absorption spectra are less pronounced. In the eighth column of the table are listed what we believe to be the identity of the various fractions obtained. These are based on absorption maxima, R_F values and the comparison of the spectra in acetate buffer or methanol with spectra reported in the literature.

Excellent separation of components was achieved both at alkaline and acid pH. None of the samples tested was pure, some containing as much as sixty per cent of contaminant. Our results are in overall agreement with those reported for thiazine dyes by TAYLOR[4,5] who used circular chromatography, and developed chromatograms using solvents based on cyclohexanone. Using this author's solvents in a closed system such as ours produced poor results: his method depends on the continued evaporation of the volatile solvent from the edge of the strip. It is therefore unsuited to the identification of dye components by their R_F values, since the values so obtained are all relative to the fastest-moving component.

Our identification of the various fractions occurring in those commercial samples of thiazine dyes which we analyzed, in general coincide with those assigned by TAYLOR[4,5]. Several other authors[3,8–10] obtain slightly different proportions of

J. Chromatog., 24 (1966) 131–140

TABLE II

CHROMATOGRAPHIC SEPARATION OF THIAZINE DYES

Dye	*Amount (mg)*	*Developing solvent A (pH 1.9)*						*Developing solvent B (pH 9.0)*				
		R_F	λ_{max} ($m\mu$)	λ_{sh} ($m\mu$)	$\frac{OD\lambda_m}{OD\lambda_{sh}}$	α	*Identif.*	R_F	λ_{max} ($m\mu$)	λ_{sh} ($m\mu$)	$\frac{OD\lambda_{max}}{OD\lambda_{sh}}$	α
Thionine	0.10	0.55	538	564	0.87	4.5	—	decomp.				
		0.50	602			190	Thionine	0.64	602	570	0.56	146.0
Azure C	0.01	0.47	610			36.0	Azure C	0.57	609			18.0
		0.45	627			50.0	Azure A	0.55	628			35.0
		0.33	647			36.0	Azure B	0.41	646			31.0
		0.21	661	620	0.60	15.0	Methylene blue	0.29	662	619	0.58	12.0
Azure A	0.02	0.45	618			48.0	—	0.58	616			23.5
		0.40	632			97.0	—	0.53	632			84.0
		0.31	643			27.0	—	0.43	643			23.5
		0.23	659	619	0.61	14.0	Methylene blue	0.30	660	617	0.65	11.0
Azure B	0.10	0.64	604	574	0.75	0.8	N-Me-thionolin					
		0.59	618	579	0.65	1.6	N,N-Di-Me-thionolin	0.78	—			—
		0.45	629			17.5	Azure A	0.58	629			15.5
		0.38	648			65.0	Azure B	0.52	647			55.0
		0.31	664	619	0.45	85.0	Methylene blue	0.44	664	620	0.49	72.0
Methylene blue	0.10	0.41 ± 0.01	629			5.4 ± 0.1	Azure A	0.55	629			4.3
		0.35 ± 0.01	648			19.1 ± 1.4	Azure B	0.49	647			15.8
		0.31 ± 0.01	665	620	0.50	213.0 ± 4.2	Methylene blue	0.45	663	619	0.45	203.0

Methylene violet	0.10	0.90	596	431	0.90	4.0	Thionol	0.84	614	583	0.67	127.0
		0.65	606	574	0.70	35.0	N-Me-thionolin	0.70	597			3.3
		0.58	619	581	0.61	101.0	N,N-Di-Me-thionolin	0.59	603			2.6
		0.44	603			4.6	Thionine	0.52	625			2.3
		0.38	629			3.0	Azure A	0.44	646			4.5
		0.32	647			5.5	Azure B	0.33	663	619	0.57	4.5
		0.26	662	620	0.55	6.9	Methylene blue					
Thionolin	0.10	0.69	592	560	0.84	128.0	Thionolin	0.79	593	560	0.86	122.0
Toluidine blue O	0.02	0.45	616			13.5	—	0.57	617			8.2
		0.43	632			47.0	—	0.53	632			41.5
		0.36	638			54.5	—	0.48	638			42.0
New methylene blue N	0.02	0.63	639			15.0	—	0.82	639			4.5
		0.60	633			92.0	New methylene blue N	0.80	635			12.5
								0.69	633			63.0
Methylene green	0.10	0.57	644			2.9	—	decomp.				—
		0.54	656	623	0.81	79.0	Methylene green	decomp.				—
		0.33	649			5.7	Azure B	0.44	648			4.9
		0.30	664	617	0.45	41.8	Methylene blue	0.38	663	619	0.48	33.3

TABLE III

CHROMATOGRAPHIC SEPARATION OF VARIOUS DYES

Dye	*Amount (mg)*	*Development*		R_F	λ_{max} ($m\mu$)	α	*Fluorescence of band*	*Development*		R_F	λ_{max} ($m\mu$)	α
		Solvent	*Time (h)*					*Solvent*	*Time (h)*			
Rose bengal	0.20	B	18	0.84	555	65.0	strong red	C	40	0.59	555	67.0
				(0.71	532	4.9	slight red			0.53	532	6.1
				0.64)	526	2.6	yellow			0.48	524	2.3
				0.45	518	0.4	very slight blue			0.41	517	0.4
										0.33	—	<0.1
Uranine	0.20						slight pink	C	40	0.52	505	0.3
							green			0.38	496	143.5
							pink			0.24	500	0.5
Eosin Y	0.20						yellow	C	40	0.51	524	106.0
							yellow-green			0.44	517	11.5
Acriflavine	0.10	A	18	0.66	—	<0.5	pink	B	18	0.75	—	<0.1
				0.59	460	6.7	orange-yellow			0.70	460	7.6
				0.56	450	60.0	yellow			0.66	450	61.0
				0.47	458	68.0	green-yellow			0.56	457	65.0
				0	—	—	reddish			—	—	—
Neutral red	0.10	A	18	0.50	526	1.8	red	B	18			
				0.47	540	85.0	dark red			0.702	540	
Safranine	0.10	A	18	0.85	525	17.3	red					
				0.78	528	81.0	red					
Thiopyronine	0.04	A	18	0.382	566	198.0	orange-red	B	18	sample decomposed		

contaminant, and, in some cases incorrectly designate the fast (pink) component in thionine as thionolin[8]. This unknown component is a weak base which can readily be extracted into chloroform from an aqueous solution at pH 9. The absorption spectra of the major component in the sample labelled "Azure A" in formamide, or in pH 5 acetate buffer do not resemble those of Azure A (absorption max. in formamide at 627–629 mμ).

By the use of the alkaline solvent ("solvent B") one obtains lower values for total absorbance (α) than in the acid system A. This is probably due to decomposition of several of the thiazine components at this pH. The more acid solvent is therefore to be preferred for the chromatographic separation of thiazines. Thionol, the fastest-moving component in methylene violet (solvent A) is resolved better in solvent system B.

The necessity for using pure samples in evaluating the photochemical reactivity of a dye is well illustrated by our observations on methylene green. On chromatography this dye sample was shown to contain at least forty per cent of contaminants, of which methylene blue is by far the major constituent. The ability of these fractions to sensitize the photopolymerization of calcium acrylate[11] is vastly different: when the components were used at equal absorptivity, whereas the methylene blue fraction caused polymer formation within seconds, the methylene green fraction caused only slight polymerization after six minutes. The decreased reactivity of the methylene green as compared with methylene blue can be ascribed to the presence of a nitro group[12]. Methylene green has been reported to be a good sensitizer for the photodynamic inactivation of viruses[13]. On the basis of our findings we would suspect that the dye sample used by this author was heavily contaminated with methylene blue, which was probably responsible for the photodynamic effect.

Table III gives results obtained for the chromatographic analysis of various photosensitizing dyes other than thiazines. Good separation was achieved with neutral red at acid pH, and for the acridine dye, acriflavine, at both acid and alkaline pH. Safranine O gave very poor separation in solvent system A. Increasing the polarity and the acidity of the resolving solvent may somewhat improve the resolution obtained. In the case of xanthene dyes, the alkaline butanol–acetone solvent (solvent B) gave poor resolution of the secondary bands, which were quite broad, although these were well-differentiated from the main component. Replacement of acetone with *n*-butanol and increasing the apparent pH of the mixture resulted in better separation of the contaminants, but the time needed to achieve resolution is twice as long. Our results agree with those obtained by others[14–16] using different solvent systems, in that eosin Y, for instance, produced two zones; the minor component is probably 2,4,5-tribromofluorescin[15]. Since the authors cited do not quote R_F values, it is difficult to decide whether the separations achieved are comparable.

None of the solvent systems used could usefully be applied to the analysis of triphenylmethane dyes: the polarity of the solvents is such that these dyes move with the solvent front. Reverse-phase chromatography, although it is a cumbersome technique seems best suited for the analysis of these dyes[17].

SUMMARY

Simple one-phase solvent systems were developed which are useful for the

chromatography of a variety of water-soluble acid and alkaline dyes. Using these solvents the purity of several commercial preparations of thiazine, azine, acridine, and xanthene dyes was assayed, and it was shown that such preparations can contain up to sixty percent of isomeric contaminants. These findings point to the need for purifying dye samples before their use in determining absolute quantum yields of photoreduction or photoxidation, spectral changes in absorption due to dye-binding, and spectroscopic properties.

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SEPARATION OF SOME INORGANIC IONS BY REVERSED-PHASE PARTITION CHROMATOGRAPHY ON THIN LAYERS

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The widespread application of thin-layer chromatography to the separation of small quantities of organic material is well known and the technique has found wide acceptance by virtue of its speed, selectivity and convenience. By contrast, separation of inorganic mixtures on thin layers has received relatively little attention and the investigations that have been carried out have been primarily concerned with separations on layers of conventional adsorbants such as kieselguhr, silica gel and cellulose[1]. Reversed-phase partition chromatography in which an organic extractant is retained as stationary phase on an inert carrier, has provided good separation of inorganic mixtures by column and paper chromatographic techniques[2], and retention of extractants on thin layers of carriers spread on glass plates is also possible[3,4]. We have already reported rapid separation of certain rare earth mixtures by thin-layer reversed-phase partition chromatography on a polymeric layer retaining di-(2-ethylhexyl) hydrogen phosphate[5], but in this particular case emphasis was placed on speed, rather than resolution and a relatively coarse grade of carrier was used. Subsequently, separation of a very wide range of inorganic ions has been achieved on a fine grade of carrier, employing a variety of stationary and mobile phases, and using solvent extraction data to provide a guide to the conditions required. As examples, we report in this paper a number of separations carried out with three different extractants and using two different carriers, silica gel and powdered polyvinyl chloride.

EXPERIMENTAL

Preparation of thin layers

Two carrier materials were used for these experiments, silica gel supplied commercially for thin-layer chromatography (Whatman SG 41) and granulated polyvinyl chloride (Corvic D 55/3, Imperial Chemical Industries Ltd.) A poly(vinyl chloride–vinyl acetate) copolymer (Corvic R 51/83) has been used previously as carrier for reversed-phase partition chromatography in columns[6] or sintered sheets[7] and even on thin layers[5], but the particle size of this solid was much coarser than that usually employed for thin-layer chromatography, and the layers, although robust gave relatively poor resolution. Consequently a number of different polymers were assessed as carriers and Corvic D 55/3 was found to be the most satisfactory of those tried.

Neither the silica gel nor the Corvic was sufficiently free from inorganic impurities to permit their use for chromatography without chemical purification and the silica gel needed particularly thorough treatment. After passing through a 300-mesh sieve, both carriers were repeatedly washed, first with concentrated hydrochloric acid until the acid remained clear, and then with demineralised water. When tests with colour-forming reagents indicated that inorganic impurities were still present these impurities were usually removed by washing with a solution of ethylenediaminetetraacetic acid in deionised water.

Thin layers retaining an organic extractant were made by first spreading a layer of Corvic on a glass plate and subsequently allowing a solution of the extractant in a volatile organic solvent to run over it. The plate was used after evaporation of the solvent.

Standard commercial equipment was used to prepare the thin layers, but a special spreader, constructed of polymeric materials was made to reduce trace element contamination of the thin layers, and to permit the Corvic to be added to the plates in a slurry of dilute acid if required. In practice, however, thin layers could be satisfactorily prepared from a slurry of the carrier in water, with the result that use of a polymeric spreader was not essential, particularly when radiotracer techniques were used to locate the position of the migrating solutes.

After the slurry had been spread over the plates and the water on the surface allowed to evaporate, the plates were heated in an oven. The silica gel layers were heated at 110° for 30 min but the Corvic was heated at a rather lower temperature to avoid the risk of the particles sintering which caused uneven fronts, and 30 min at 70° proved satisfactory.

The stationary phase was added to the carrier by dissolving it in diethyl ether or some other volatile solvent and feeding the resulting solution to the horizontal plate by means of a paper wick. This method of allowing the solution to run over the layer was chosen in preference to dipping the plate in a solution of the reagent to avoid damaging the fragile thin layers of silica which became detached from the glass backing plates rather readily. Corvic layers were very much more robust and could be dipped in a variety of organic solutions without damage, but they were prepared in exactly the same way as the silica layers to enable direct comparison to be made. Whenever possible mobile and stationary phases were saturated with the other before use and extractants purified by standard techniques.

Ascending, descending and horizontal methods of development were tried and in all cases satisfactory results were obtained with Corvic layers. However, silica showed least tendency to come away from the glass backing plates during horizontal development and therefore this technique was always used for silica layers.

Layers were spread on standard plates 20 cm long and 20, 10 or 5 cm wide; development was usually allowed to proceed for about 10 cm.

Both radioactive and inactive solutes were spotted on to the plates and their final positions located, either by spraying the plates with a colour-forming reagent such as 8-hydroxyquinoline or diphenylthiocarbazone, or by removing portions of the thin layer and counting the radioactivity. γ-Ray spectroscopy was used whenever possible to identify the active nuclides when mixtures of radioactive elements were separated by thin-layer chromatography.

RESULTS AND DISCUSSION

An initial series of experiments was conducted to find a thickness of layer which gave satisfactory surfaces and good reversed-phase partition chromatograms for the elution of inorganic solutes. The procedure finally adopted was to move the spreader slowly over the plates with the blade set at a height of 0.25 mm above the glass backing plate.

The quantity of extractant on the thin layer was found to influence both the R_F value of the solute and the speed of elution. Increases in reagent loading of the carrier in reversed-phase partition chromatography have been shown to reduce the R_F value of inorganic ions migrating on paper[8] and in columns[9], and this effect was also found for partition chromatography on thin layers with solutes migrating more slowly on the more heavily loaded layers.

The rate of development was also dependent upon the reagent loading of the carrier with the most heavily loaded layers requiring the largest development time. Under the experimental conditions required for the separations reported below, the usual time necessary for the mobile phase to traverse 10 cm of the loaded thin layer was 40–50 minutes.

At high reagent loadings, particularly when di-(2-ethylhexyl) hydrogen phosphate was retained on silica, the extractant tended to strip from the carrier and was pushed ahead of the advancing aqueous phase. A suitable reagent loading of the carrier which gave adequate capacity without showing any tendency to strip was finally obtained by adding the extractant in an organic solvent as a 10 % solution. The uniformity of the distribution of the stationary phase on the thin layer was assessed by placing several spots of the same radioactive element across the bottom of a number of plates and after development locating the new positions of the elements by removing and counting 0.5 cm lengths of each track of the thin layer. Positions of maximum activity were never found to vary by more than one 0.5 cm fraction on the same plate, irrespective of the point across the bottom of the plate at which the spot started and usually maximum activity occurred in the same fraction, thus indicating that the extractant was evenly spread over the plate. Similar values of R_F were obtained for the same elements eluted under similar conditions on plates of the same batch, but appreciable variations were sometimes found from batch to batch and it was customary to elute at least one standard, together with the mixture on a thin layer.

Generally, slightly sharper eluted spots were obtained on layers of silica gel than on Corvic, and small initial spots of solute were more easily formed on silica, but the robust polymer layer was easier to handle and was always preferred when conditions permitted.

Three different extractants were employed as stationary phases for these experiments, and all were liquids which had previously proved satisfactory for reversed-phase partition chromatography in column operation; they were the cation exchanger di-(2-ethylhexyl) hydrogen phosphate (HDEHP)[6,10–14], the anion exchanger tri-iso-octylamine (TIOA)[15] and the neutral phosphorus ester tri-*n*-butyl phosphate (TBP)[16–18].

In all cases aqueous acid was used as mobile phase and gave straight fronts when advancing over the stationary phase.

By choice of extractant and mobile phase a large number of different separations have been carried out on thin layers, but for brevity we give below only one or two typical examples of separations achieved with each extractant.

Tri-n-butyl phosphate

The use of TBP for liquid–liquid extraction has been extensively investigated and the extraction of about fifty elements from aqueous hydrochloric acid by 100 % TBP has been reported[19]. A number of elements of the first transition series were spotted onto corvic and silica layers which had been contacted with 10 % TBP in diethyl ether and developed with hydrochloric acid of different strengths. The results are shown in Fig. 1 as a plot of R_F against the normality of the developing acid and

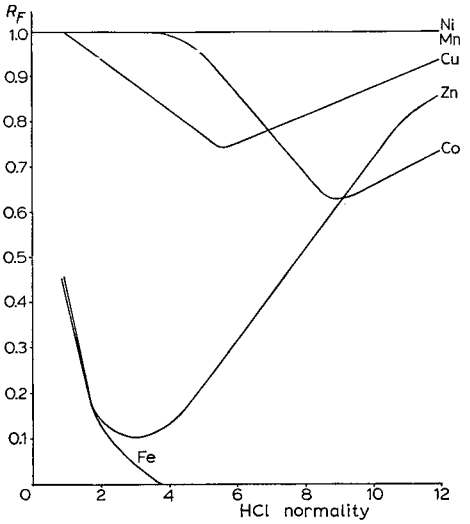

Fig. 1. Plot of R_F value against hydrochloric acid concentration for the migration of a number of elements over layers of Corvic retaining TBP.

are of the form expected from liquid–liquid extraction data, assuming that distribution ratios for the solvent extraction system are the same function of aqueous acid concentration as is the parameter $(1/R_F - 1)$ for the partition chromatographic system. Thus nickel and manganese which show relatively low extraction by 100 % TBP from hydrochloric acid move with the solvent front at all acidities, while iron, which exhibits a high distribution ratio when extracted from a range of strongly acid aqueous solutions by 100 % TBP stays essentially at the starting point when eluted with acids stronger than about 4 M. As an example of the separations that can be achieved on thin layers retaining TBP, chromatograms obtained for the elution of a number of elements with hydrochloric acid individually and as a mixture are given in Fig. 2. The spots were well defined permitting essentially complete disengagement of the different elements by this thin-layer technique.

Di-(2-ethylhexyl) hydrogen phosphate

HDEHP has been extensively used for reversed-phase partition chromatography of the rare earths because of the good separation factors that can be obtained

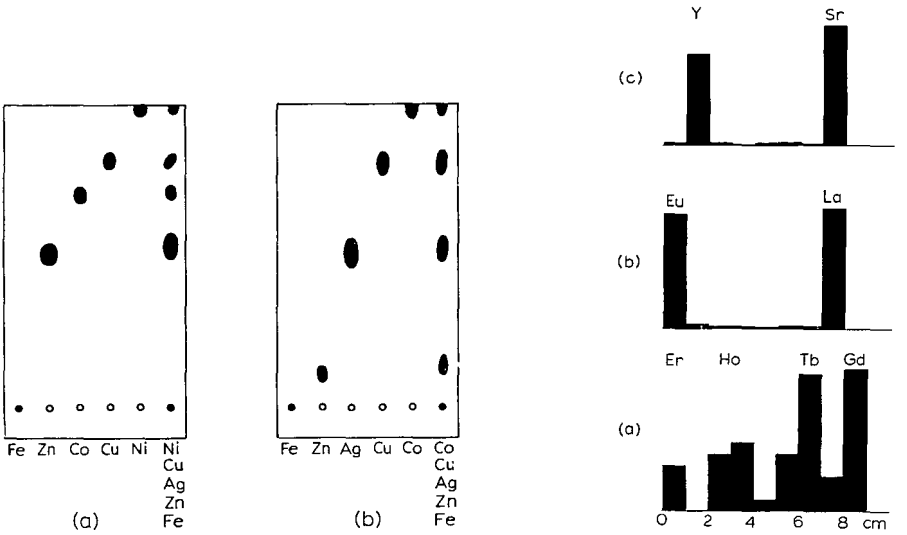

Fig. 2. Separation of a number of elements on TBP Corvic layers by elution with hydrochloric acid. Acid strength: (a) 8 *N*, (b) 4 *N*.

Fig. 3. Histograms of separations carried out on HDEHP–silica layers with hydrochloric acid as eluant. Acid strength: (a) 1.75 *N*, (b) 0.3 *N*, (c) 1.5 *N*.

for adjacent elements with this extractant[20]. The rare earths are normally eluted with aqueous acid and their mobilities are found to decrease with increasing atomic number. We have already reported the rapid separation of a number of rare earths on layers of a coarse grade of Corvic retaining HDEHP[5]; for the experiments reported here the reagent was added either to silica gel, or to a finer grade of Corvic with particles of the size more usually employed for thin-layer chromatography. No attempt was made to obtain a plot of the zone migration parameter, $R_M \log (1/R_F - 1)$ against the hydrogen ion concentration of the aqueous phase as this has already been reported for the thin-layer system[5], and instead a number of separations were attempted by eluting elements and mixtures on Corvic and silica layers retaining HDEHP as the stationary phase. An example is given in Fig. 3. Development was carried out at room temperature and not at the elevated temperatures used for some column separations. Again elements showing the highest distribution ratios in the respective liquid–liquid systems gave the lowest R_F values, and separation factors were of the order expected from column chromatography although accurate separation factors could not be calculated from histograms of the type shown in Fig. 3.

Tri-iso-octylamine

The third extractant used as stationary phase was the liquid anion exchanger TIOA. The use of high molecular weight amines for the extraction of inorganic ions has received considerable attention[21] and distribution data for the extraction of more than sixty elements from hydrochloric acid into TIOA has been reported[22]. Experience with TIOA retained on a carrier as stationary phase for column chromatography indicated that the amine might also be of use for reversed-phase partition chromatography on thin layers.

The behaviour of TIOA retained on Corvic D 55/3 for thin-layer chromatography was similar to that retained on Corvic R 51/83 for column chromatography which has been described in greater detail elsewhere[15] and will not be re-considered here. Examples of separations obtained on stationary phases of TIOA are shown in Fig. 4 and again the elements which migrate furthest are those which show lowest extraction in the comparable liquid–liquid system.

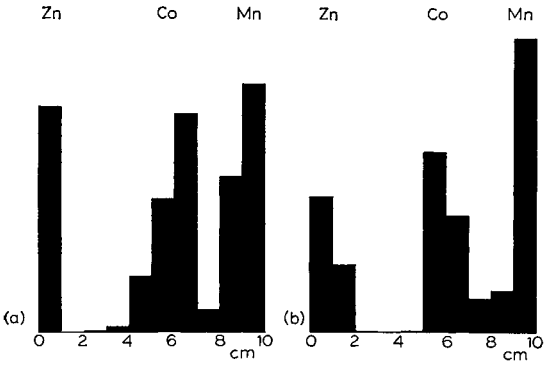

Fig. 4. Histograms of separations carried out with a stationary TIOA phase. (a) carrier: Corvic; eluant: 4 *N* hydrochloric acid; (b) carrier: silica; eluant: 4 *N* hydrochloric acid.

CONCLUSION

Reversed-phase partition chromatography employing a stationary phase of organic extractant retained on a thin layer of carrier can provide separation of a wide variety of inorganic ions, selectivity for the technique being achieved by careful choice of extractant and eluting phase.

Provided that rapid mass-transfer between the two phases is possible and the usual precautions are taken to minimise spot size good resolution can be attained.

Powdered polyvinyl chloride can be used as an alternative to the more conventional carriers used for thin-layer chromatography as it provides very robust thin layers, without requiring the addition of any binder.

SUMMARY

Separation of mixtures of inorganic ions has been obtained by elution with aqueous acid on a stationary phase of an organic extractant retained on a thin layer of a carrier. Di-(2-ethylhexyl) hydrogen phosphate, tri-iso-octylamine and tri-*n*-butyl phosphate were used as extractant and silica gel and powdered polyvinyl chloride employed as carriers.

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ION EXCHANGE IN FUSED SALTS

II. DISTRIBUTION COEFFICIENTS OF SOME ALKALI METAL IONS ON ZIRCONIUM PHOSPHATE IN FUSED NITRATES

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In a previous paper[1] the chromatographic behaviour of various metal cations dissolved in fused salts, on glass fiber paper impregnated with synthetic inorganic ion exchange materials, has been reported.

These preliminary results, in spite of their qualitative nature, showed that there is a marked analogy between the adsorption of a given metal ion and its solubility in fused salts; and that metal ions are adsorbed by ion exchange, although other mechanisms such as oxide precipitation on the exchanger materials, are possible.

To extend the knowledge on ion exchange in fused salts and to confirm the differences predicted[1] between the properties of ion exchangers in aqueous solutions and in ionic melts more quantitative data were required.

In the present communication the distribution coefficients of lithium, sodium, potassium and cesium ions, on zirconium phosphate (ZP), in fused nitrates, are reported and discussed.

Owing to the well known high solubility of alkali metal oxides in fused nitrates, the distribution coefficients of these metal cations should not be affected by oxide precipitation on the ion exchanger.

The lack of knowledge on the behaviour of ZP in ionic fused media, also made it necessary to determine the rate of ion exchange, the extent of ZP hydrolysis and the degree of condensation of $=HPO_4$ to P—O—P groups, under the operating conditions.

EXPERIMENTAL

Preparation of the solvents

$LiNO_3$, $NaNO_3$, and KNO_3 (Erba RP) vacuum dried at 100° for 24 h, were weighed and mixed in the proportions shown in Table I, then vacuum dried again at 110°.

ZP salt forms

ZP in the sodium form (ZPNa) and in the potassium form (ZPK) were prepared from the H^+ form of ZP (ZP-1, Bio Rad Laboratories, grain size >100 mesh) according to a procedure described elsewhere[2]. After dehydration at 110° materials containing 5.2 mequiv. Na^+/g ZPNa (corresponding to 5.9 mequiv. Na^+/g anhydrous ZPH) and 4.7 mequiv. K^+/g ZPK (corresponding to 5.8 mequiv. K^+/g anhydrous ZPH) were obtained.

TABLE I

DISTRIBUTION COEFFICIENTS FOR LITHIUM, SODIUM AND POTASSIUM IONS ON ZPK AND ZPNa IN FUSED NITRATES, AT 160°

Exchanger	*Initial molar composition of melts* (%)		*Mequiv. Me⁺ per g of melt (final composition)*		*Mequiv. Me⁺ per g of anhydrous ZPH*		K_d[a]		*Selectivity coefficients*	
ZPK	$LiNO_3$	43	Li^+	4.8	Li^+	4.1	K_d^{Li}	0.85	K_K^{Li}	3.7
	KNO_3	57	K^+	6.6	K^+	1.5	K_d^K	0.23		
ZPNa	$LiNO_3$	43	Li^+	4.8	Li^+	5.1	K_d^{Li}	1.06	K_K^{Li}	15
	KNO_3	57	Na^+	0.11	Na^+	0.15	K_d^K	0.069		
			K^+	6.5	K^+	0.45				
ZPK	$LiNO_3$	30	Li^+	3.3	Li^+	3.0	K_d^{Li}	0.91	K_K^{Li}	3.6
	$NaNO_3$	23	Na^+	2.6	Na^+	1.2	K_d^{Na}	0.46	K_{Na}^{Li}	2.2
	KNO_3	47	K^+	5.4	K^+	1.4	K_d^K	0.26		
ZPNa	$LiNO_3$	30	Li^+	3.3	Li^+	3.7	K_d^{Li}	1.12	K_K^{Li}	15
	$NaNO_3$	23	Na^+	2.7	Na^+	1.6	K_d^{Na}	0.59	K_K^{Na}	7.9
	KNO_3	47	K^+	5.3	K^+	0.40	K_d^K	0.075	K_{Na}^{Li}	1.9

[a] K_d = mequiv. Me^+ per g of anhydrous ZPH/mequiv. Me^+ per g of melt.

ZP in the cesium form (ZPCs) was prepared by equilibrating ZP-1 with a Cs^+ solution, labelled with ^{137}Cs, at about pH 8. The pH value was kept roughly constant by operating in the presence of anion exchangers in OH^- form (Dowex 1, 20–50 mesh). After 4 days, ZPCs was mechanically separated from the anionic resin and analyzed for the Cs^+ content (~ 4 mequiv. Cs^+ per g ZPCs weighed after dehydration at 110°).

Determination of distribution coefficients

The experiments were performed at 160 ± 2°, in a dry nitrogen atmosphere, in an oven (Bicasa) provided with an internal mechanical stirrer. 1.130 g of ZPNa or 1.234 g of ZPK (weighed after drying at 110° and corresponding to 1 g of anhydrous ZPH*) was left for 48 h at the operating temperature (160°), then added to 50 g of alkali nitrates melt, contained in a pyrex cylinder, provided with a sintered glass disk (G4) at the bottom to separate ZP from the melt after equilibration by vacuum filtration. After filtering and cooling, known amounts of 2 *N* HCl solution were passed through ZP until complete elution of all alkali cations was achieved.

Analytical procedures

The concentrations of lithium, sodium and potassium ions were determined by an E.E.L. flame photometer. Potassium concentrations were also determined gravimetrically by precipitating with tetraphenylboron[3].

Cesium was determined radiometrically.

Nitrate ion concentration was determined by VOGEL's[4] method, after reducing NO_3^- to ammonia with Devarda's alloy.

ZP hydrolysis and P—O—P formation were determined according to the techniques described elsewhere[5].

* For this equivalence, see ref. 2.

RESULTS AND DISCUSSION

ZP hydrolysis

It is well known that zirconium phosphate hydrolyzes in aqueous solutions[6]. Under our experimental conditions, it was found that hydrolysis occurs only to a very small extent (< 1 %) in fused alkali nitrates.

Pyrophosphate formation

On heating ZP-1 above 160–180° a gradual condensation of $=HPO_4$ to P—O—P groups takes place and the ion exchange rate correspondingly decreases considerably[5]. Since ZP in salt form, prepared as described above, contain a high percentage of the hydrogen form, all the experiments were performed at 160°. After 70 h at this temperature in a fused $LiNO_3$–KNO_3 eutectic, formation of P—O—P groups was found to be negligible (< 1 %).

Rate of ion exchange

Fig. 1 shows the equivalent ionic fractions of potassium and lithium found in 1 g of ZPK, left in contact with 50 g of $LiNO_3$–KNO_3 eutectic, plotted *vs.* time of equilibration. The experiments were performed with and without stirring. It can be seen that stirring does not appreciably influence the rate of ion exchange. Owing to the high ionic concentration of fused salts, the rate-determining step is probably controlled by particle diffusion, therefore it does not depend on stirring. All the following experiments were therefore performed without stirring. Fig. 1 also shows that, when considering the exchange between potassium and lithium, the equilibrium is achieved in about 10–12 h.

Since a decrease in the rate of exchange between potassium and ions with greater ionic radii is to be expected, the time for all the ion exchange experiments was not less than 70 h. It must be pointed out that the presence of lithium in all the ionic media employed facilitates ion exchange, even when it should be very slow or hindered, considering a two-way traffic mechanism[7].

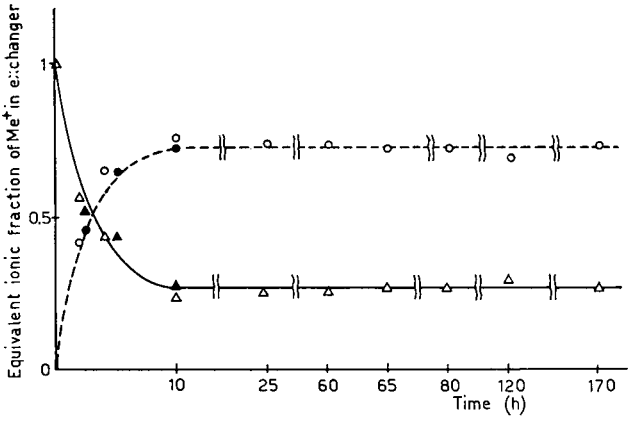

Fig. 1. Equivalent ionic fraction of K^+ (solid line) and Li^+ (dashed line) in zirconium phosphate (initially in potassium form) as a function of time of equilibration. (● ▲) stirred solution; (○ △) unstirred solution.

Distribution coefficients

Many difficulties arise when the distribution coefficients of alkali metal ions on ZP are determined. As pointed out above, the salt forms of ZP contain a remarkable number of P–OH groups which condense to P–O–P groups at temperatures higher than 160°, and thus decrease the ion exchange rate. Therefore it was not possible to study fused systems, such as $LiNO_3$–$NaNO_3$ or $NaNO_3$–KNO_3, owing to their relatively high melting points.

Furthermore, some of the melt, even after careful filtration, is found to adhere to the walls of the pyrex container and to the surface of the exchanger. For this reason alkali metal ions of the melt are mixed, during the elution, with the exchangeable ions of the exchanger. Since the alkali ions of the melt are present as nitrates and the melt molar composition is known (see Table I), the proper corrections can be made by determining the nitrate concentration in the eluate.

It was also observed that the distribution coefficients of alkali metal ions depend greatly on the initial salt form of the exchanger.

Table I shows that, after 90 h of equilibration, 1.5 and 0.45 mequiv. K^+ per g of anhydrous ZPH are found using ZPK and ZPNa, respectively. Such a difference could be due to incomplete equilibrium, but, since the amount of K^+ present in the exchanger does not decrease much from 12 to 100 h of equilibration (see Fig. 1), it was concluded that about 1 mequiv. of K^+ ion is exchanged at a very low rate or even encaged in the exchanger.

Therefore, when ZPK is used, the values of the distribution and selectivity coefficients are invalidated by the excess of potassium remaining in the exchanger even after 90 h of equilibration.

Increase of the ionic crystal radius of the initial exchangeable ion increases this effect and could be connected with the shrinking of the exchanger produced by dehydration at 160°. It was found *e.g.*, that by adding 4 g of ZPCs to 40 g of a melt composed by 38.4 g of $LiNO_3$–KNO_3 eutectic and 1.6 g $CsNO_3$, less than 0.1 mequiv. Cs^+/g ZPCs was exchanged, while addition of 4 g of ZPK to 40 g of a melt composed by 35.3 g of $LiNO_3$–KNO_3 eutectic and 4.7 g $CsNO_3$, resulted in less than 0.1 mequiv. K^+/g ZPK being exchanged by Cs^+ ions (after 100 h of equilibration). In this case, owing to the large difference between the results obtained with ZPCs and ZPK, it is not possible to draw any conclusion on the selectivity of ZP for Cs^+ ion dissolved in molten alkali nitrates.

On the other hand, Table I shows that when ZPNa is left in contact with $LiNO_3$–KNO_3 eutectic, only 0.15 mequiv. Na^+ per g of anhydrous ZPH are found in the exchanger, part of this being due to the distribution of Na^+ between the exchanger and the melt. Therefore when ZPNa is employed, the small amount of Na^+ ion eventually not exchanged (<0.15 mequiv. Na^+ per g of anhydrous ZPH) will not affect appreciably the distribution of alkali metal ions on zirconium phosphate.*

It should be ponted out that the K_{Na}^{Li} value obtained using ZPK is in good agreement with that determined on ZPNa. Therefore the distribution coefficients of Li^+ and Na^+ ions are altered by the amount of K^+ ion not exchanged whilst the coefficient K_{Na}^{Li} is not.

* It should be expected that an even smaller amount of unexchanged Li^+ will remain in the exchanger initially in the lithium form, but it was not possible to test this point since the conversion of ZPH to ZPLi, by the method previously reported[2], appears to be unsatisfactory, owing to the poor solubility of lithium phosphate in water.

From the values of the selectivity coefficients found when the exchanger was initially in the sodium form, the following order of selectivity in molten alkali nitrates is derived: $Li > Na \gg K$.

By comparing the ion exchange behavior of ZP in nitrate melts and in aqueous solution, the most striking feature is that on decreasing the crystal radii of alkali metal ion, the selectivity of the ZP increases in fused media while it decreases in aqueous solutions. This confirms what was already found in a previous paper[1] from a qualitative point of view, and is also in good agreement with the results obtained by LIQUORNIK AND MARCUS[8] with a Linde Molecular Sieve 4 A in fused nitrates.

ACKNOWLEDGEMENT

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SUMMARY

Some experiments on the ion exchange properties of zirconium phosphate in fused alkali nitrates, at 160°, are reported. The results obtained show that the ion exchange equilibrium is reached after about 10–12 hours and that condensation and hydrolysis of the exchanger are negligible. Values of distribution coefficients for some alkali metal ions are reported. The selectivity decreases with increasing the ionic crystal radius in fused media and this is found to be reversed relative to aqueous solutions.

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TRENNUNG DER SELTENEN ERDEN DURCH FLÜSSIGEN IONENAUSTAUSCH

V. DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG VON VIELKOMPONENTEN-GEMISCHEN DER SELTENEN ERDEN

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In einer früheren Mitteilung[1] wurde gezeigt, dass es möglich ist, drei oder vier benachbarte Elemente der Seltenen Erden durch Dünnschichtchromatographie an mit Di-(2-äthylhexyl)-phosphorsäure (HDEHP) imprägniertem Kieselgel zu trennen. Mit der beschriebenen Methode war es nicht möglich, eine grössere Anzahl von Seltenerdkomponenten nebeneinander nachzuweisen. Deshalb wurde versucht, durch Erhöhung der Kapazität der Dünnschicht, Veränderung der Schichtdicke und zweidimensionale Elution eine Erweiterung der Methode zu erreichen. Wenn nicht anders vermerkt, entsprechen die experimentellen Einzelheiten unserer früheren Mitteilung.

ABHÄNGIGKEIT DER R_F-WERTE VON DER HNO_3-KONZENTRATION IM ELUTIONSMITTEL

In Tabelle I sind die R_F-Werte bei verschiedener HNO_3-Konzentration zusammengestellt. Fig. 1 zeigt die Abhängigkeit der R_M-Werte ($R_M = \lg (1/R_F - 1)$) von der Salpetersäurekonzentration.

TABELLE I

R_F-WERTE DER SELTENEN ERDEN IN ABHÄNGIGKEIT VON DER HNO_3-KONZENTRATION DES ELUTIONSMITTELS

Dünnschicht: 30 g Kieselgel D + 6 ml HDEHP in 40 ml *n*-Butanol. Schichtdicke: 500 μ.

	HNO_3-Molarität								
	0.2	*0.3*	*0.5*	*0.75*	*1.0*	*1.5*	*2.0*	*3.0*	*4.0*
La	0.26	0.51	0.81						
Ce	0.14	0.34	0.70						
Pr	0.11	0.29	0.62						
Nd	0.10	0.24	0.57	0.78					
Sm		0.06	0.22	0.44	0.67				
Eu			0.11	0.29	0.46	0.73			
Gd			0.06	0.20	0.36	0.62	0.79		
Tb				0.06	0.10	0.28	0.46	0.74	0.86
Dy					0.04	0.11	0.26	0.55	0.74
Ho						0.06.	0.12	0.38	0.60
Y								0.26	0.44
Er								0.20	0.41

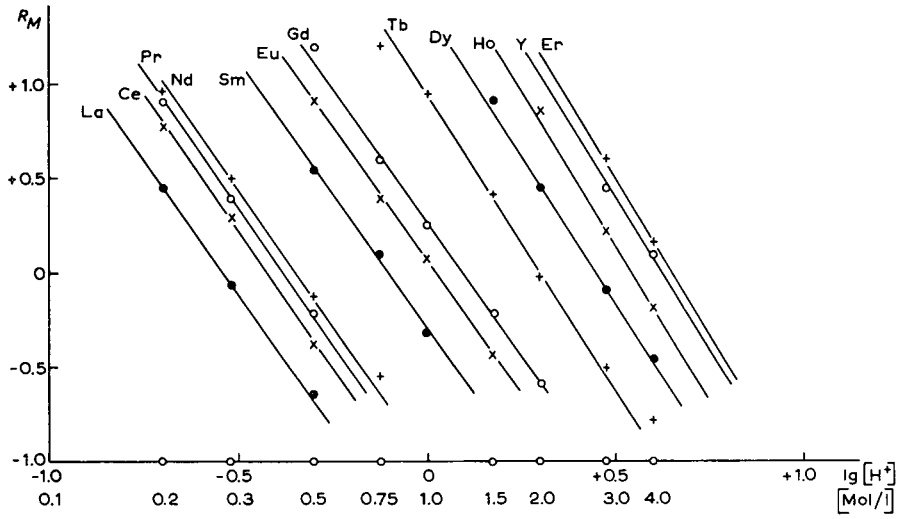

Fig. 1. Abhängigkeit der R_M-Werte verschiedener Seltener Erden von der Salpetersäurekonzentration.

Wie bei der papierchromatographischen Trennung der Seltenen Erden an HDEHP-Papier[2] haben die Geraden in Fig. 1 eine Neigung von nahezu —3, wodurch der gleiche Austauschmechanismus bewiesen ist.

Zwischen dem R_F- bzw. R_M-Wert der Papier- oder Dünnschichtchromatographie und dem Verteilungskoeffizient VK der Extraktion bzw. des flüssigen Ionenaustausches besteht nach CERRAI UND TESTA[2] folgender Zusammenhang:

$$\lg VK = \lg \left(\frac{1}{R_F} - 1\right) + \text{const.}$$

oder:

$$\lg VK = R_M + \text{const.} \qquad VK = K \cdot 10^{R_M}$$

Da der Trennfaktor β_Z^{Z+1} der Quotient der unter gleichen Bedingungen bestimmten Verteilungskoeffizienten ist, kann man für den Trennfaktor zwischen benachbarten Elementen schreiben:

$$\beta_Z^{Z+1} = \frac{VK_{Z+1}}{VK_Z} = 10^{R_{M(Z+1)} - R_{M(Z)}}$$

wobei Z die Ordnungszahl bedeutet.

Tabelle II enthält die so berechneten Trennfaktoren (Spalte 3), denen die durch Extraktion mit HDEHP und Chromatographie an HDEHP-Säulen erhaltenen Werte[3] gegenübergestellt sind.

ABHÄNGIGKEIT DER R_F-WERTE UND TRENNFAKTOREN VON DER HDEHP-KONZENTRATION

Mit der Änderung der HDEHP-Konzentration in der Imprägnierungslösung ändern sich sowohl die R_F-Werte als auch die Trennfaktoren, wie aus Tabelle III hervorgeht.

TABELLE II

TRENNFAKTOREN BENACHBARTER SELTENER ERDEN

Seltenerd-Paar	*HDEHP-Extraktion aus wässriger HCl*	*Säulenchromatographie an HDEHP-behandeltem Träger. Elution mit wässriger HCl*	*Dünnschichtchromatographie an HDEHP-behandeltem Kieselgel. Elution mit wässriger HNO_3*
Ce–La	2.4	2.4	2.1
Pr–Ce	2.8	1.5	1.5
Nd–Pr	1.7	1.4	1.3
Sm–Nd	5.0	4.4	4.5
Eu–Sm	2.2	2.0	2.0
Gd–Eu	1.6	1.4	1.6
Tb–Gd	3.2	3.3	4.2
Dy–Tb	2.0	1.9	2.3
Ho–Dy	2.1	1.9	2.0
Y–Ho			1.8
Er–Y			1.2
Er–Ho	2.1	2.7	2.2
Durchschnittlicher Trennfaktor benachbarter Elemente	2.2	2.1	2.1

TABELLE III

R_F- UND R_M-WERTE SOWIE TRENNFAKTOREN VON DYSPROSIUM, HOLMIUM UND ERBIUM IN ABHÄNGIGKEIT VON DER HDEHP-BELADUNG DES KIESELGELS

Material zur Herstellung der Dünnschicht: 30 g Kieselgel D + 46 ml Lösung (30·a ml HDEHP + (46 — 30·a) ml n-Butanol). Schichtdicke: 500 μ. Elutionsmittel: 4 N HNO_3.

Beladung a ml HDEHP/ g Kieselgel	*Dy*		*Ho*		*Er*		β_{Dy}^{Ho}	β_{Ho}^{Er}
	R_F	R_M	R_F	R_M	R_F	R_M		
0.1	0.84	—0.72	0.75	—0.48	0.59	—0.16	1.7	2.1
0.2	0.72	—0.41	0.57	—0.12	0.38	0.21	1.9	2.1
0.3	0.61	—0.21	0.45	0.09	0.27	0.43	2.0	2.2
0.4	0.55	—0.09	0.37	0.23	0.20	0.60	2.1	2.3

TABELLE IV

R_F- UND R_M-WERTE SOWIE TRENNFAKTOREN VON SAMARIUM, EUROPIUM UND GADOLINIUM IN ABHÄNGIGKEIT VON DER SCHICHTDICKE

Dünnschicht: 30 g Kieselgel D + 46 ml Lösung (6 ml HDEHP + 40 ml n-Butanol). Elutionsmittel 0.75 N HCl.

Schichtdicke (mm)	*Sm*		*Eu*		*Gd*		β_{Sm}^{Eu}	β_{Eu}^{Gd}
	R_F	R_M	R_F	R_M	R_F	R_M		
0.25	0.39	0.19	0.24	0.50	0.16	0.72	2.0	1.7
0.50	0.42	0.14	0.27	0.43	0.19	0.63	2.0	1.6
0.75	0.45	0.09	0.30	0.37	0.21	0.57	1.9	1.6
1.00	0.48	0.04	0.33	0.31	0.24	0.50	1.9	1.5

Es erscheint folglich günstig, bei möglichst hoher HDEHP-Beladung des Kieselgels zu arbeiten. Dem wirkt jedoch die Zunahme der Elutionszeit entgegen. Die Elution der Yttererden mit 4 N HNO_3 dauert bei Verwendung einer Schicht von 0.4 ml HDEHP/g Kieselgel 6 Std. anstatt 4 Std., wenn nur bei halber Beladung gearbeitet wird. Ausserdem ist es unmöglich, mehr als 0.5 ml HDEHP pro Gramm Kieselgel zu verwenden, da so hergestellte Schichten uneben sind und feucht bleiben.

ABHÄNGIGKEIT DER R_F-WERTE UND TRENNFAKTOREN VON DER SCHICHTDICKE

Aus Mischungen von 30 g Kieselgel D mit 6 ml HDEHP in 40 ml *n*-Butanol wurden mit einem verstellbaren Streichgerät Schichten von 250, 500, 750 und 1000 μ Dicke hergestellt. Bei Elution mit 0.75 N HCl wurden die in Tabelle IV und Fig. 2 dargestellten Ergebnisse erhalten.

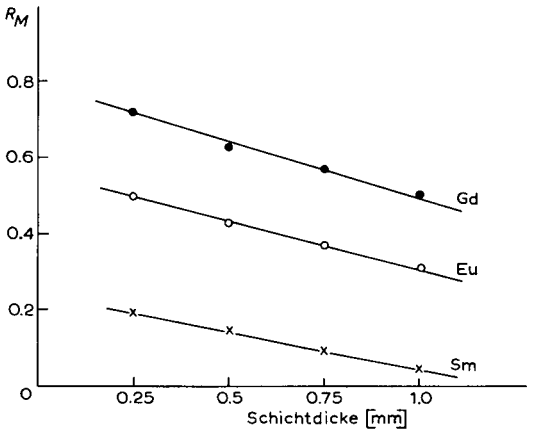

Fig. 2. Abhängigkeit der R_M-Werte von der Dicke der mit Di-(2-äthylhexyl)phosphorsäure behandelten Kieselgel-Schicht.

Mit steigender Schichtdicke nehmen die Trennfaktoren etwas ab. Dies ist erklärlich, da der Austausch im wesentlichen auf die Oberfläche der dünnen Schicht beschränkt ist.

TRENNUNG EINES VIELKOMPONENTEN-GEMISCHES DURCH EINDIMENSIONALE DÜNNSCHICHTCHROMATOGRAPHIE

Nach den vorhergehenden Untersuchungen ist festzustellen, dass man die Trennung der Seltenen Erden an möglichst dünnen Schichten mit hoher HDEHP-Beladung durchführen sollte. Da zu dünne Schichten (250 μ) nicht sehr bequem handhabbar sind, wurden die folgenden Untersuchungen mit Kieselgel-Schichten von 500 μ und einer HDEHP-Beladung von 12 ml HDEHP + 34 ml *n*-Butanol auf 30 g Kieselgel durchgeführt.

Die Fig. 3 und 4 zeigen die Ergebnisse der Trennungen von je 5 Elementen der Cerit- bzw. Yttererden mittels 0.50 N HCl bzw. 4 N HNO_3. Da kurz hinter der Lösungsmittelfront alle Verunreinigungen angereichert sind, die weniger als die

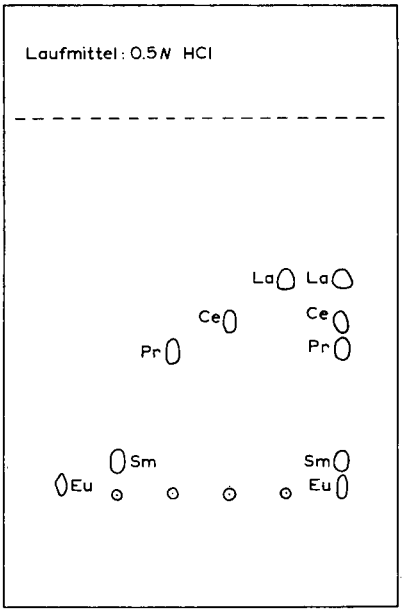

Fig. 3. Trennung der Ceriterden.

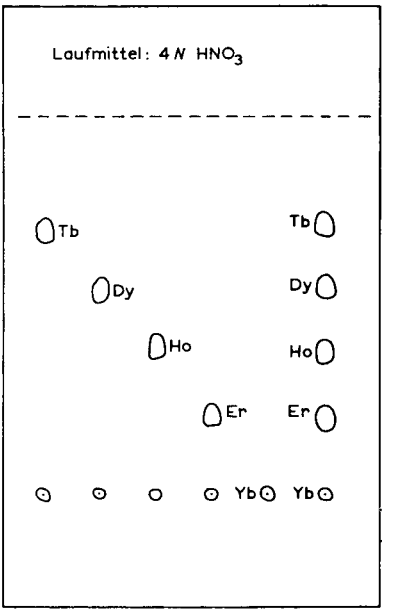

Fig. 4. Trennung der Yttererden.

Seltenen Erden von HDEHP zurückgehalten werden, müssen bei der Elution stets solche Bedingungen gewählt werden, dass die maximalen R_F-Werte bei 0.80 liegen. Nur dann sind die Flecke nach Besprühen mit Oxin im U.V. Licht exakt lokalisierbar.

Es ist ersichtlich, dass maximal 5 Elemente auf einem Chromatogramm zu erkennen sind.

TRENNUNG EINES VIELKOMPONENTEN-GEMISCHES DURCH ZWEIDIMENSIONALE DÜNNSCHICHTCHROMATOGRAPHIE

Um eine Trennung eines Mehrkomponenten-Gemisches zu erzielen, wurde eine Reihe von Versuchen mit der üblichen Elution in zwei Laufrichtungen durchgeführt. Nachdem zunächst mit 0.50 N HNO_3 eine Elution der Ceriterden erfolgte, wurden die Platten im Trockenschrank bei 70° getrocknet und anschliessend senkrecht zur ursprünglichen Richtung mit 4 N HNO_3 behandelt. Nach Besprühen mit Oxinlösung liessen sich im U.V.-Licht nur die Yttererden erkennen. Ihre R_F-Werte stimmten mit denen in Fig. 4 überein. Die Flecke der Ceriterden an der Lösungsmittelfront sind aus den oben angeführten Gründen nicht zu erkennen. Um diese Schwierigkeiten zu überwinden, wurde in folgender Weise gearbeitet (Fig. 5): Die Substanzmischung (je 15 μg der zu trennenden Seltenen Erden) wird sowohl im Punkt A als auch im Punkt B aufgetragen. Nun wird zunächst mit 0.50 N HNO_3 bis zur Lösungsmittelfront F_1 eluiert. Dabei werden die Ceriterden aufgetrennt, die Yttererden verbleiben am Start. Nach dem Trocknen der Platte wird diese um 90° gedreht und mit 4 N HNO_3 vom Punkt B bis zur Lösungsmittelfront F_2 eluiert.

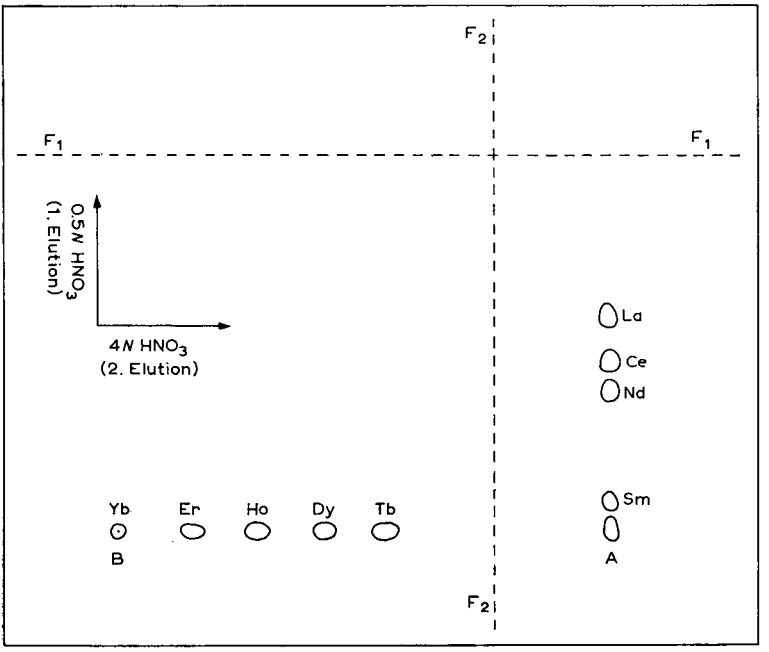

Fig. 5. Trennung der Seltenen Erden durch zweidimensionale Elution.

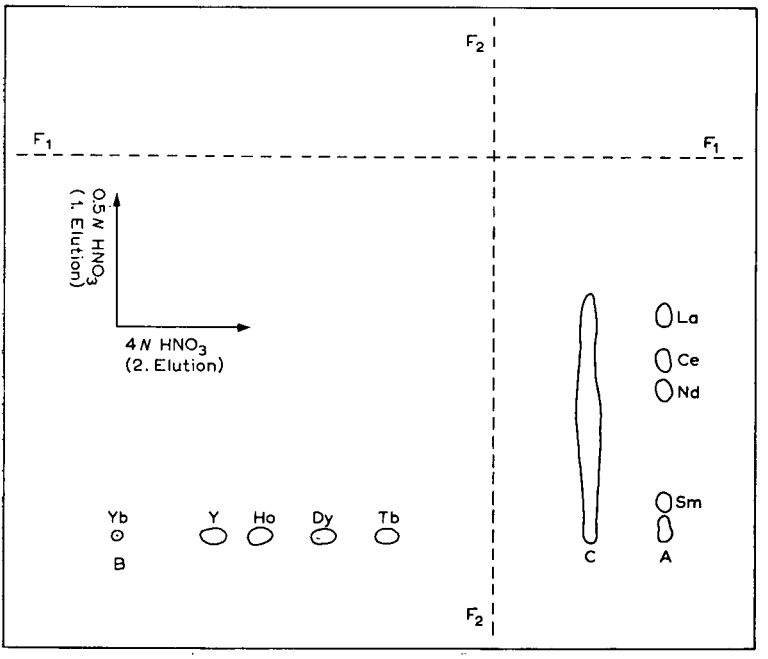

Fig. 6. Trennung der Seltenen Erden durch zweidimensionale Elution bei grossen Konzentrationsunterschieden von Cerit- und Yttererden.

Dabei werden die an Punkt B befindlichen Yttererden aufgetrennt. Die Ceriterden vom Punkt B sind nicht mehr nachweisbar.

Auch wenn die Konzentration der Ceriterden im Vergleich zu der der Yttererden grösser ist, kann man eine Trennung durchführen (Fig. 6). Bei B und C enthält die aufgetragene Probelösung je 7 μg von 5 Yttererden und je 35 μg von 4 Ceriterden. Bei A ist eine auf das Fünffache verdünnte Probelösung aufgegeben worden. Während in der ursprünglichen Lösung die Yttererden gut aufgetrennt wurden, die Ceriterden dagegen nicht, können nach der Verdünnung auch die Ceriterden deutlich getrennt werden.

TRENNUNG DER SELTENEN ERDEN AUS TECHNISCHEN PRODUKTEN

Die Trennung der Seltenen Erden wurde an technischem Ceritcarbonat (Fa. Auer-Remy, Hamburg) und an Seltenen Erden, die durch Tributylphosphat-Extraktion aus Kola-Apatit gewonnen wurden[4], überprüft. Im Folgenden wird die Trennung der Seltenen Erden aus Kola-Apatit beschrieben.

600 mg des erhaltenen Rohoxid-Gemisches werden in HCl gelöst. Vorhandenes Cer(IV) wurde mit H_2O_2 reduziert, die Lösung zur Trockne eingedampft und 10 ml einer 0.1 *N* salzsauren Lösung hergestellt. 0.005 ml dieser Lösung dienten zur Trennung der Yttererden (B). Für die Trennung der Ceriterden wurden die Lösung fünffach verdünnt (Punkt A). Wie Fig. 7 zeigt, sind die Elemente Lanthan, Cer, Yttrium sowie die Summe von Praseodym und Neodym nachweisbar. Die übrigen Seltenen Erden sind wegen ihres geringen Anteils in der Mischung nicht zu erkennen.

Sie sind zu weniger als 10 % gegenüber der Hauptkomponente vorhanden.

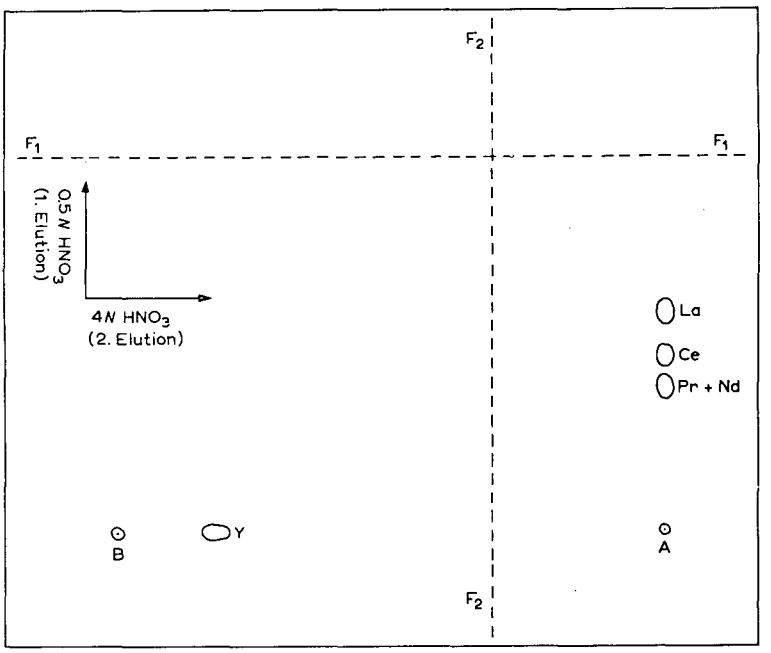

Fig. 7. Trennung der Seltenen Erden des Kola-Apatit.

ZUSAMMENFASSUNG

Es werden die Bedingungen für die Trennung eines Vielkomponenten-Gemisches der Seltenen Erden beschrieben. Die Trennfaktoren benachbarter Elemente wachsen mit steigender Beladung des Silikagel mit Di-2-äthylhexyl-phosphorsäure und abnehmender Schichtdicke. Durch zweidimensionale Elution ist eine Trennung von nahezu allen Seltenerd-Elementen möglich.

SUMMARY

The conditions for separation of a multi-component mixture of rare earths by thin-layer chromatography are described. The separation factor of adjacent elements is enhanced by increasing the loading of the silica gel with di-(2-ethylhexyl) phosphoric acid and decreasing the thickness of the layer. By two-dimensional elution the separation of nearly all rare earth elements is possible.

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Notes

Gaschromatographische Trennung der theoretisch möglichen bindungs- und konfigurationsisomeren *n*-Hexensäure-(1)-methylester

Für das systematische Studium von mit einer Doppelbindungsisomerisierung verbundenen Reaktionen ungesättigter Carbonsäuren bzw. Carbonsäurederivaten war es notwendig, ein gaschromatographisches Analysenverfahren zur Erfassung aller theoretisch möglichen Bindungs- und Konfigurationsisomeren einer höhermolekularen ungesättigten Carbonsäure auszuarbeiten. Bereits von der Capronsäure leiten sich sieben bindungs- bzw. konfigurationsisomere *n*-Hexensäuren-(1) ab, so dass schon hier mit erheblichen Schwierigkeiten bei der Ausarbeitung eines einwandfreien quantitativ auswertbaren gaschromatographischen Analysenverfahren gerechnet werden musste. Von MABROUK und Mitarb.[1] wurde kürzlich über die gaschromatographische Analyse der drei bindungsisomeren *trans-n*-Hexensäuren-(1) berichtet. Die gleichzeitige Bestimmung aller theoretisch möglichen bindungs- und konfigurationsisomeren *n*-Hexensäuren-(1)-isomeren war bisher noch nicht möglich.

Wir stellten die als Testsubstanzen benötigten einzelnen *n*-Hexensäure-(1)-isomeren entweder in reiner Form oder als *cis–trans*-Isomerengemische nach den in der Literatur beschriebenen Verfahren und mit der in der Tabelle I angegebenen Reinheit bzw. Zusammensetzung dar.

Reine *trans-n*-Hexen-(2)-säure-(1)[2,3] und reine *trans-n*-Hexen-(3)-säure-(1)[4] konnte, wie von LINSTEAD und Mitarb. gefunden wurde, durch Doebner-Kondensation von *n*-Butyraldehyd mit Malonsäure in Pyridin/Piperidin bzw. in Triäthanolamin erhalten werden.

TABELLE I

DIE SYNTHETISIERTEN *n*-HEXEN-(*x*)-SÄUREN-(1) UND IHRE GASCHROMATOGRAPHISCH* ERMITTELTE REINHEIT

		Gehalt in %							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
n-Hexen-(5)-säure-(1)	(A)	94.8	3.7	1.5	—	—	—	—	—
trans-n-Hexen-(4)-säure-(1)	(B)	—	79.5	20.5	—	—	—	—	—
cis-n-Hexen-(4)-säure-(1)	(C)	—	79.5	20.5	—	—	—	—	—
trans-n-Hexen-(3)-säure-(1)	(D)	—	—	—	95.8	0.4	2.5	—	1.3
cis-n-Hexen-(3)-säure-(1)	(E)**	—	—	—	82.0	14.4	2.3	—	1.0
trans-n-Hexen-(2)-säure-(1)	(F)	—	—	—	Spur	—	97.9	2.1	—
cis-n-Hexen-(2)-säure-(1)	(G)	—	—	—	0.4	—	13.7	78.2	7.7
n-Hexansäure-(1)	(H)	—	—	—	—	—	—	—	100.0

* In Form der Methylester.

** *cis–trans*-Isomerengemisch erhalten durch Konfigurationsisomerisierung von reinem *trans-n*-Hexen-(3)-säure-(1)-methylester mit N_2O_4 nach N. A. KHAN[6,7].

Die *cis*-*n*-Hexen-(2)-säure-(1) konnte durch Halbhydrierung von *n*-Hexin-(2)-säure-(1) neben *trans*-*n*-Hexen-(2)-säure-(1) und *n*-Hexansäure-(1) mit Hilfe von Lindlar-Kontakt erhalten werden (vergleiche z.B. Lit. 5). Der völlig quantitative Umsatz der Hexinsäure zur Hexensäure verursachte neben der teilweisen Weiterhydrierung zur Hexansäure auch die Bildung einer grösseren Menge der konfigurationsisomeren *trans*-*n*-Hexen-(2)-säure-(1).

Aus der *trans*-*n*-Hexen-(3)-säure-(1) wurde ein *cis*–*trans*-Isomerengemisch der *n*-Hexen-(3)-säure-(1) durch reine Konfigurationsisomerisierung mit Hilfe von NO_2 dargestellt[6,7], so dass damit auch die Retentionszeit des *cis*-*n*-Hexen-(3)-säure-(1)-methylesters festgestellt werden konnte.

Über eine Malonestersynthese mit Crotylbromid wurde ein *cis*–*trans*-Isomerengemisch der *n*-Hexen-(4)-säure-(1) synthetisiert (vergleiche z.B. Lit. 8). MABROUK und Mitarb.[1] berichten, dass sie bei der gleichen Synthese ein Gemisch von 75 % *trans*-*n*-Hexen-(4)-säure-(1) und 25 % *trans*-*n*-Hexen-(3)-säure-(1) erhalten hätten. Die *n*-Hexen-(3)-säure-(1) kann aber, wie auch die Versuche in der vorliegenden Arbeit zeigen, bei der Malonestersynthese mit Crotylbromid nicht entstehen. Dagegen muss hier immer *cis*-*n*-Hexen-(4)-säure-(1) gebildet werden, da eine schnell verlaufende Allylumlagerung die Bildung beider konfigurationsisomeren *n*-Hexen-(4)-säuren-(1) sowie der 3-Methyl-penten-(1)-säure-(5) verursacht. Bei der von MABROUK und Mitarb.[1] als Nebenprodukt der Malonestersynthese mit Crotylbromid gefundenen *trans*-*n*-Hexen-(3)-säure-(1) handelt es sich deshalb wahrscheinlich um die *cis*-*n*-Hexen-(4)-säure-(1).

Die *n*-Hexen-(5)-säure-(1) schliesslich wurde durch Überführen von 5-Brom-*n*-penten-(1) mit Magnesium in die Grignardverbindung und Umsetzung dieser mit trockenem CO_2 in der Kälte hergestellt[9].

Da es aussichtsreicher erschien, ein Analysenverfahren für die Methylester der *n*-Hexensäuren ausarbeiten zu können, überführten wir die *n*-Hexensäuren mit BF_3–Methanol nach METCALFE UND SCHMITZ[10] quantitativ in die Methylester und setzten diese in die Gaschromatographie ein. Eine Stellungs- oder *cis*–*trans*-Isomerisierung tritt bei dem Veresterungsverfahren mit BF_3–Methanol nicht ein.

Olefinisomerengemische werden am vorteilhaftesten unter Verwendung von stationären Phasen, die Silbersalze gelöst enthalten, gaschromatographisch getrennt (vergleiche z.B. Lit. 11). Solche Kolonnen dürfen jedoch im allgemeinen nur bis etwa 80° erhitzt werden. Bei höhersiedenden Verbindungen können sie demnach nicht verwendet werden. Allerdings sind silbernitrathaltige β,β'-Oxidipropionitril- oder 1,2,3-Tris-(2′-cyanoäthoxy)-propan-Phasen, wie wir fanden, ohne Schwierigkeiten bis ca. 100° zu verwenden, so dass sie für die zwischen 150° und 160° siedenden Methylester der *n*-Hexensäuren eben noch eingesetzt werden konnten. Zahlreiche Versuche zeigten aber, dass auch bei Verwendung dieser Phasen keine vollständige Trennung aller *n*-Hexensäuremethylester erreicht werden konnte: Die Retentionszeiten von *trans*-*n*-Hexen-(2)-säure-(1)- und *n*-Hexen-(5)-säure-(1)-methylester lagen so nahe beieinander, dass eine auch nur halbquantitative Analyse dieser beiden Verbindungen nicht möglich war.

Eine befriedigende Lösung des Problems gelang uns schliesslich durch den Einsatz einer 400 m langen und 0.5 mm weiten mit N,N-Bis-(2-cyanoäthyl)-formamid belegten Kapillarkolonne. Mit Hilfe dieser sehr wirksamen Trennsäule war es möglich, alle sieben theoretischen *n*-Hexensäure-(1)-methylester zusammen mit dem *n*-Hexan-

säure-(1)-methylester einwandfrei quantitativ zu analysieren. Die Methylester der *cis*-(3)- und *cis*-(4)-*n*-Hexensäure werden dabei zwar nicht vollständig voneinander getrennt, doch ist ihre quantitative Analyse nebeneinander ohne Schwierigkeiten möglich.

TABELLE II

QUANTITATIVE GASCHROMATOGRAPHISCHE ANALYSE EINES TESTGEMISCHES VON *n*-HEXENSÄURE-(1)-METHYLESTERN UND *n*-HEXANSÄURE-(1)-METHYLESTER

	Retentionszeit (Min.)	*Einwaage* (%)	*Analyse* (%)	*Abweichung* (%)
cis-n-Hexen-(2)-säure-(1)-methylester	102	15.8	15.8	±0.0
trans-n-Hexen-(4)-säure-(1)-methylester	116	18.2	18.3	+0.1
n-Hexen-(5)-säure-(1)-methylester	119	15.7	16.4	+0.7
trans-n-Hexen-(3)-säure-(1)-methylester	122	16.5	16.1	−0.4
cis-n-Hexen-(4)-säure-(1)-methylester	127	4.7	5.1	+0.4
trans-n-Hexen-(2)-säure-(1)-methylester	134	17.0	15.5	−1.5*
n-Hexansäure-(1)-methylester	98	11.8	12.8	+1.0*

* Die grösste Abweichung vom Sollwert zeigt bei einem Isomerengemisch ganz allgemein immer die Komponente mit der kürzesten Retentionszeit, die in zu hoher Konzentration und die Komponente mit der längsten Retentionszeit, die in zu geringer Konzentration als der Einwaage entspricht, gefunden wird.

Die quantitative Auswertung des Gaschromatogramms eines Testgemisches ohne *cis-n*-Hexen-(3)-säure-(1)-methylester zeigt die Tabelle II, das Gaschromatogramm selbst die Fig. 1. Die Einwaage des Testgemisches erfolgte unter Berücksichtigung der Isomerenanteile, die in den synthetisierten Hexensäureestern enthalten waren. Bei der Berechnung der Reinheit der einzelnen Ester wurde angenommen, dass die Flächen unter den Kurvenzügen den prozentualen Anteilen der einzelnen Komponenten genau entsprechen. Die Richtigkeit dieser Annahme wurde durch das Ergebnis der Analyse des Testgemisches dann auch bestätigt.

Die Fig. 2 zeigt ein Gaschromatogramm, das auch den *cis-n*-Hexen-(3)-säure-(1)-methylester enthält.

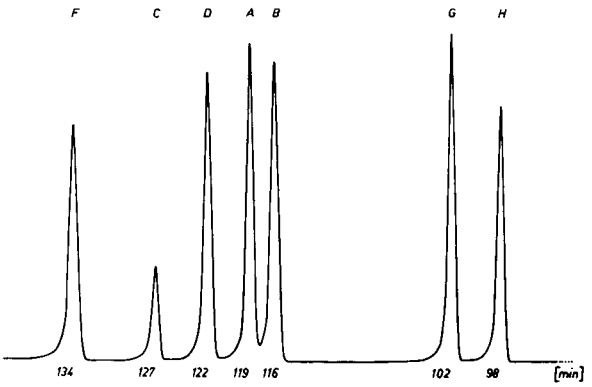

Fig. 1. Gaschromatogramm des Testgemisches der *n*-Hexensäure-(1)-methylester und *n*-Hexensäure-(1)-methylester (Bezeichnung vgl. Tabelle I).

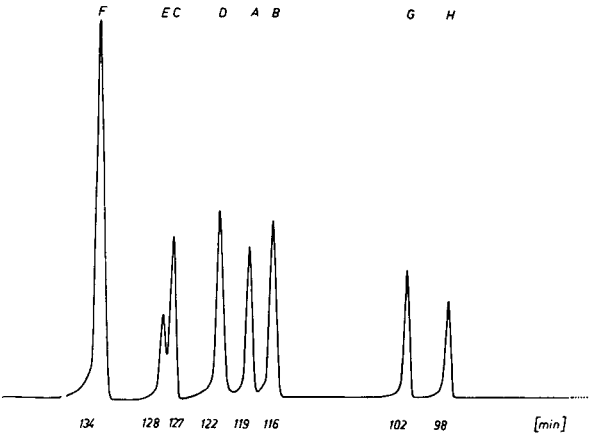

Fig. 2. Gaschromatogramm eines Gemisches aller *n*-Hexensäure-(1)-methylester und des *n*-Hexansäure-(1)-methylesters (Bezeichnung vgl. Tabelle I).

Die gaschromatographischen Arbeitsbedingungen waren:

Gerät: RSCo 60–10 (Research Specialties Co., jetzt Warner Chilcott Laboratories, Richmond, Calif.).

Kapillarkolonne: 400 m lang, V_2A-Stahl; 0.5 mm Innendurchmesser.

Stationäre Phase: N,N-bis-(2-cyanoäthyl)-formamid.

Trägergas: Helium; Durchflussmenge: 1.2 c.c./Min.

Detektor: FID.

Einspritzmenge: 0.05 µl. Probenteilung: 1:160.

Temperaturen: Kolonne 80°; Detektor 80°; Verdampfer 240°.

Schreiber: Honeywell-Brown 5-mV-Kompensations-Schreiber.

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Direkte gaschromatographische Bestimmung der einfachern Alkoholisomeren bis zu *n*-Pentanol in verdünnten, wässerigen Lösungen

Das in Wasser gut beständige 1-Nonylphenoxypropandiol-2,3 (Nonylphenol-Glyzerinäther, NPGA) eignet sich als stationäre Phase für die Trennung von Alkoholen und Ketonen[1]. Wie systematische Untersuchungen[2] zeigten, lassen sich mit Chromosorb A—als Trägermaterial für Glyzerinäther—verbesserte Trennungen von Substanzgemischen in verdünnten, wässerigen Lösungen erzielen. Da die erwähnte stationäre Phase bis zu einer Temperatur von 100° verwendbar ist, untersuchten wir die Auflösefähigkeiten von NPGA–Chromosorb A-Kolonnen am Beispiel der Trennung von einfachen Alkoholen (die 16 möglichen Isomeren bis und mit 5 C-Atomen) unter Anwendung eines Temperaturprogrammes und eines empfindlichen Flammenionisationsdetektors (zur "Elimination" des Wassers).

Im Folgenden soll über die Möglichkeiten dieser Trennsäule berichtet werden.

Apparatives

Gaschromatograph: Beckman GC4 mit zwei Säulen, Doppelflammenionisationsdetektor und Temperaturprogrammiereinheit.

Kolonnen: 12 ft. Stahlsäulen, 3/16 in. innerer Durchmesser, 10 % NPGA und 0.2 % Alkaterge T auf Chromosorb A, 60/80 mesh (Johns Manville). Die Kolonnen wurden vor Gebrauch 24 Std. bei 140° ausgeheizt.

Trägergas: Stickstoff *ca.* 17 ml pro Min.

Testlösungen: Für die Herstellung der Testgemische wurden analysenreine Alkohole (Merck, Fluka) verwendet.

Resultate

Dimensionen der Trennsäulen: Die Trennsäulen wurden für die Analyse von Probemengen in der Grössenordnung von 10–15 μl ausgelegt. (Diese Mengen lassen sich—im Hinblick auf spätere quantitative Bestimmungen — ohne weiteres genau reproduzieren, z.B. mit dem Beckman liquid sampler.) Als geeignet erwies sich ein Säulendurchmesser von 3/16 in. mit Trägermaterial von 60/80 mesh Korngrösse. 1/8 in. Kolonnen ergaben durchwegs schlechtere Trennfaktoren, 1/4 in. Kolonnen erforderten zu lange Analysenzeiten.

Temperaturprogramm: Für die vorliegende Untersuchung wurde folgendes Temperaturprogramm gewählt: 6 Min isotherm bei 70°, anschliessend aufheizen bis auf 100° mit 1°/Min. Dadurch ergibt sich eine Retentionszeit für *n*-Pentanol von *ca.* 45 Min.

Nach dieser Zeit ist die Säule sofort wieder verwendbar, da das Wasser bereits eluiert ist (Retentionszeit des Wassers ist etwas grösser als diejenige von 2-Methylbutanol-2). In Fig. 1 ist ein mit diesem Temperaturprogramm ermitteltes Chromatogramm dargestellt (Alkoholgehalt je $1^0/_{00}$ in Wasser).

Auflösefaktoren: Gemische mit (gewichtsmässig) gleichen Teilen der Alkohole mit 1 bis 5 C-Atomen wurden analysiert (Einspritzmenge je 15 μl). Entsprechend der Alkoholkonzentration wurde das Detektor-Signal so verstärkt, dass sich am Schreiber ein Ausschlag pro Substanz von durchschnittlich 50 % registrieren liess. Aus den erhaltenen Chromatogrammen ergaben sich die in Tabelle I angeführten Trennfaktoren (vgl. auch Fig. 1).

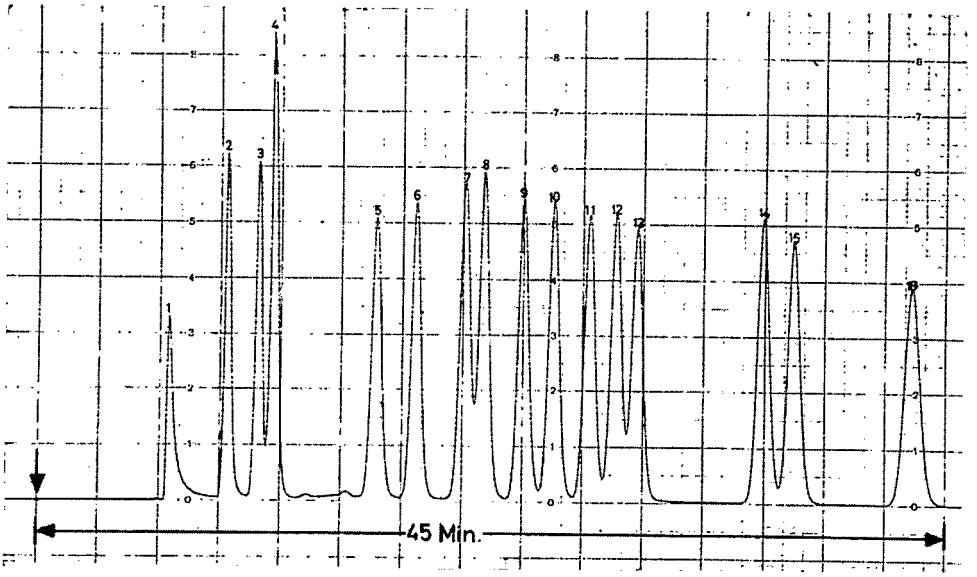

Fig. 1. Direkte Trennung der 16 Alkoholisomeren bis zu *n*-Pentanol in Wasser. Konzentration: je 1‰. 1 = Methanol; 2 = Äthanol; 3 = Propanol-2; 4 = 2-Methylpropanol-2; 5 = *n*-Propanol; 6 = Butanol-2; 7 = 2-Methylbutanol-2; 8 = 2-Methylpropanol-1; 9 = 2,2-Dimethylpropanol-1; 10 = 3-Methylbutanol-2; 11 = Pentanol-3; 12 = *n*-Butanol; 13 = Pentanol-2; 14 = 2-Methylbutanol-1; 15 = 3-Methylbutanol-1; 16 = *n*-Pentanol.

Diskussion

Wie aus Fig. 1 hervorgeht, lassen sich auf NPGA–Chromosorb A-Säulen alle 16 möglichen einfachen Alkohol-Isomeren mit 1 bis 5 C-Atomen recht gut auftrennen. Der niedrigste Trennfaktor lässt sich zwischen 2-Methylbutanol-2 und 2-Methylpropanol-1 mit *ca.* 0.6 beobachten (vgl. Tabelle I). Alle andern untersuchten Alkohole werden besser aufgetrennt. Die Auflösefähigkeit der Säulen wird offensichtlich

TABELLE I

TRENNFAKTOREN SCHWIERIGER ZU TRENNENDER ALKOHOLPAARE IN DIVERSEN VERDÜNNUNGEN

Substanzpaare	*Konzentration des einzelnen Alkohols*					
	Ohne Wasser	*In Wasser je*				
		5‰	1‰	0.5‰	0.1‰	0.05‰
Äthanol Propanol-2	0.99	1.0	0.99	0.99	0.99	0.92
Propanol-2 2-Methylpropanol-2	0.81	0.90	0.90	0.85	0.82	0.78
2-Methylbutanol-2 2-Methylpropanol-1	0.48	0.73	0.73	0.69	0.66	0.66
Pentanol-3 Butanol-1	0.71	0.93	0.93	0.92	0.90	0.85
Butanol-1 Pentanol-2	0.78	0.77	0.77	0.77	0.77	0.72
2-Methylbutanol-1 3-Methylbutanol-1	0.92	0.95	0.95	0.94	0.93	0.89

durch zwei Faktoren beeinflusst, einerseits durch das Wasser und andrerseits durch die Menge der einzelnen Substanzen. Letzteres manifestiert sich vor allem bei den wasserfreien Alkoholgemischen durch niedrigere Trennfaktoren. Der Grund dazu dürfte in einer Überlastung der Säulen zu suchen sein.

Eine Verschlechterung der Trennfähigkeit infolge des Wassergehaltes tritt erst bei sehr starken Verdünnungen, 0.1 $^0/_{00}$, andeutungsweise zutage. Störend wirkt sie sich bei noch niedrigeren Konzentrationen im Bereiche von 0.05 $^0/_{00}$ aus (vgl. Tabelle I).

Die Dauer einer Analyse liegt mit dem vorgeschlagenen Temperaturprogramm unter einer Stunde. *n*-Pentanol erscheint nach *ca.* 45 Min. Zudem ist die Trennsäule sofort wieder betriebsbereit, da das Wasser schon nach dem 2-Methylpropanol-2 eluiert wird. Allerdings wird der Wasser-peak durch den Flammenionisationsdetektor nur durch eine geringfügige Nullpunktverschiebung — und nur bei hoher Empfindlichkeit — angezeigt. Die vorgeschlagene Säule eignet sich somit für rasche, direkte Analysen wässeriger Alkohollösungen bis hinunter zu Konzentrationen von 0.1 $^0/_{00}$.

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J. Chromatog., 24 (1966) 165–167

A simple reproducible technique for sample introduction in analyses of volatile fatty acids by gas chromatography

The determination of the composition of volatile fatty acid mixtures by gas–liquid chromatography in association with automatic titration was first described by JAMES AND MARTIN[1]. Their technique has the advantage over more elaborate detection systems in that absolute values may be estimated easily and with greater precision. Usually relative ratios only of acids are obtained with other types of detectors. To judge from the literature, *cf.* for example SMITH[2], if actual concentrations are desired correction factors must be applied to the calculations.

A difficulty in the JAMES AND MARTIN technique is to obtain a satisfactory method for sample injection which gives a quantitative and smooth delivery of the ethereal solution. The technique now described is satisfactory for this purpose.

A pellet of sintered glass is immersed in the ethereal solution of volatile fatty acids prepared as described by MCINNES[3] and then placed inside the inlet end of the column well within the heated vapour jacket. The amount of acids introduced to the column depends on pellet size and time of immersion. With the equipment in the Fats Research Division D.S.I.R., New Zealand, satisfactory graphs were obtained using

ethereal solutions containing 50 to 150 μM/ml of a mixture of fatty acids comprising mainly acetic with lesser amounts of propionic and butyric, together with traces of iso- and *n*-valeric acids. The pellet (5 × 10 × 30 mm) was immersed for 2 min.

The column head and carrier gas inlet of the chromatograph was modified as shown in Fig. 1 to allow easy introduction of the sample.

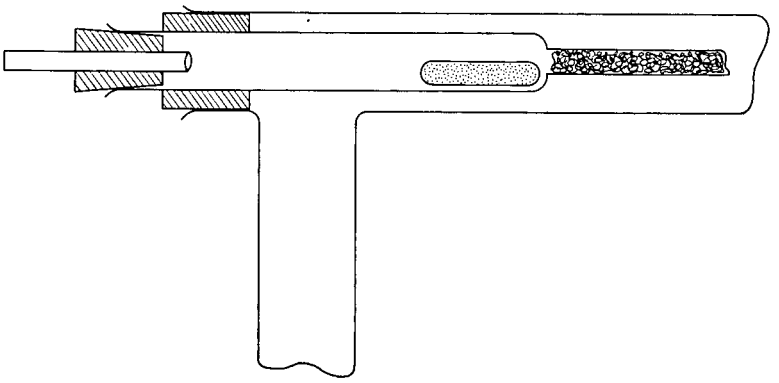

Fig. 1. Modified inlet of gas chromatograph column showing sintered glass pellet in position.

The carrier gas was preheated by passing it through a copper coil to improve the delivery of the sample to the column and prevent possible losses of volatile fatty acids by condensation. The coil was heated by wrapping it several times around the aluminium jacket of a heating mantle set at 220 V.

Comparison of the values of total volatile acids as estimated by titration of ethereal solutions with those calculated from GLC results showed a recovery of 99 ± 3 %.

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Analyse des acides aminés par chromatographie

I. Considérations générales sur l'intégration des pics. Une méthode rapide d'intégration manuelle

Les perfectionnements récents des divers types d'analyseurs automatiques d'acides aminés, dérivés de celui de MOORE ET STEIN, permettent actuellement d'obtenir plusieurs chromatogrammes en 24 h : jusqu'à 8 selon certains constructeurs*. L'intégration prend alors d'avantage de temps que le travail analytique proprement dit. C'est donc sur ce point que l'on peut désormais attendre des améliorations. La méthode préconisée par SPACKMAN, MOORE ET STEIN[1] est certainement la plus employée. Elle demande environ une heure par chromatogramme. Il existe des intégrateurs automatiques** qui donnent d'excellents résultats. Un appareil semi-automatique moins onéreux a été décrit récemment[2].

Méthode générale d'intégration

La surface en unités de densité optique d'un pic enregistré en transmission peut être obtenue en additionnant les densités optiques de points régulièrement espacés sur l'axe des temps (Fig. 1) :

$$S = c \sum_{i=1}^{i=n} H_i \qquad \text{(a)}$$

H_i est la densité optique du point d'abscisse "i".

SPACKMAN, MOORE ET STEIN[1] ont remarqué qu'il existe, dans le cas de pics symétriques, un rapport constant entre l'aire réelle du pic et celle du triangle de même hauteur et de même largeur à la moitié de l'ordonnée du maximum (Fig. 1) :

$$S = k \cdot H \cdot L \qquad \text{(b)}$$

où H est l'ordonnée du maximum et L la largeur du pic à mi-hauteur.

Ce résultat est en accord avec les études théoriques de VERMEULEN ET HIESTER[3] qui ont montré que, d'une manière générale, on peut admettre que les profils d'élution obtenus avec des échangeurs d'ions sont des courbes de Gauss, ou en diffèrent très peu.

Dans ces conditions, pour mesurer l'aire d'un pic, il suffit d'en déterminer deux paramètres convenablement choisis, leur produit est proportionnel à la surface du pic, le coefficient de proportionnalité ne dépendant que du choix des paramètres.

A priori rien n'oblige à prendre, comme l'ont fait SPACKMAN *et al.*, la largeur à mi-hauteur comme second paramètre. Nous avons calculé, en adoptant la hauteur comme paramètre fixe, les valeurs du coefficient k dans la formule (b), en fonction de la fraction x de la hauteur totale à laquelle le paramètre de largeur L_x est mesuré. Le Tableau I groupe les résultats obtenus pour soixante pics, pris au hasard dans dix chromatogrammes.

Ces calculs montrent que la précision reste bonne si l'on mesure le paramètre

* Technicon Instruments Corporation, Chauncey, New York.
** Infotronics, Houston, Texas.

TABLEAU I

VALEUR DES COEFFICIENTS D'INTÉGRATION EN FONCTION DE L'ORDONNÉE À LAQUELLE EST MESURÉ LE PARAMÈTRE DE LARGEUR

Ordonnée où est mesuré le paramètre de largeur (H)	k^*	k'^{**}	*Ecart maximum pour k (%)*	*Observations (%)*
0.5	1.045 (1.064)	1.00 (1.00)	2.5	55 valeurs sur 60 à ± 1.43
0.6	1.21 (1.239)	1.16 (1.16)	4.4	58 valeurs sur 60 à ± 2.48
0.7	1.44 (1.483)	1.38 (1.39)	5.2	55 valeurs sur 60 à ± 3.47
0.75	1.60 (1.651)	1.53 (1.55)	5.3	56 valeurs sur 60 à ± 3.44
0.8	1.82 (1.876)	1.74 (1.76)	5.3	55 valeurs sur 60 à ± 3.84
0.9	2.63 (2.730)	2.52 (2.56)	8.2	55 valeurs sur 60 à ± 5.70

* k Est le coefficient d'intégration à appliquer pour connaître l'aire réelle; entre parenthèses on a mis le coefficient correspondant calculé pour la courbe de Gauss.

** k' Est le coefficient à appliquer pour connaître l'aire du triangle construit sur la hauteur et la largeur à demi-hauteur du pic; entre parenthèses: le même coefficient calculé pour la courbe de Gauss.

de largeur jusqu'aux huit dixièmes de la hauteur. Aux neuf dixièmes l'écart est encore inférieur à 6% dans neuf cas sur dix.

Dans la plupart des cas, il n'y a aucun avantage à mesurer le paramètre de largeur à une hauteur différente de $H/2$, sauf pour vérifier éventuellement un résultat; c'est cependant la méthode la plus rapide et parfois la seule applicable pour intégrer des pics qui se chevauchent. Il suffit en effet, dans ce cas, de se placer à une ordonnée telle que la contribution de la coloration due au second amino-acide soit négligeable, pour mesurer le paramètre de largeur.

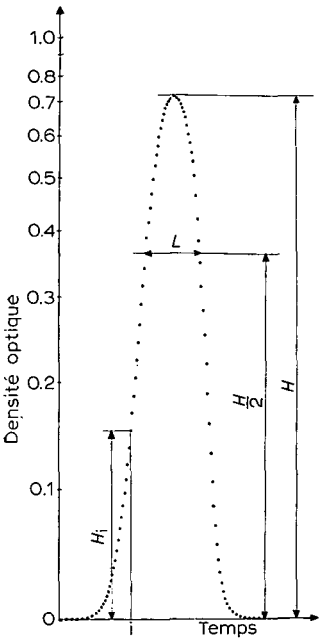

Fig. 1. Intégration d'un pic enregistré en transmission.

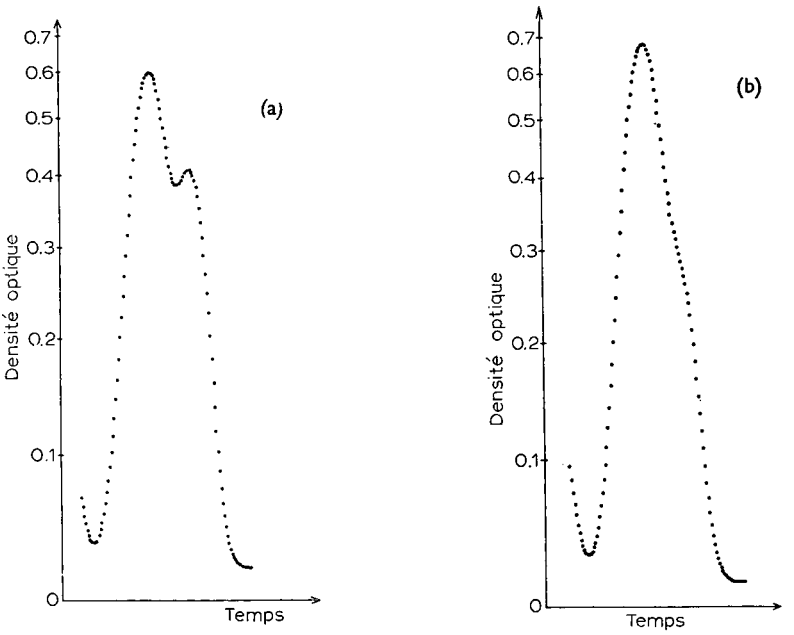

Fig. 2a et b. Deux exemples de séparations incomplètes où l'intégration reste possible.

Ceci suppose bien entendu que le profil de l'élution d'un acide aminé ne soit pas affecté par le chevauchement d'une autre substance. Il en est bien ainsi car nous avons constaté, en révélant simultanément des chromatogrammes par la ninhydrine et par un réactif spécifique des guanidines, que la forme du pic d'un acide aminé était indépendante de la présence d'une guanidine ninhydrine négative éluée simultanément[4].

L'application de la méthode est illustrée par les exemples ci-dessous: dans les deux cas le pic principal est celui de la sérine, le pic secondaire ou l'épaulement correspond à la glutamine.

Dans le premier cas, il n'est pas possible de mesurer $L_{1/2}$ (Fig. 2a).

L'intégration à toutes les ordonnées où elle est possible donne les valeurs suivantes pour la sérine:

$S_{0.9} = 17.53$ $S_{0.75} = 17.86$ S_x est l'aire calculée en mesurant
$S_{0.8} = 17.70$ $S_{0.7} = 18.49$ la largeur à l'ordonnée $x \cdot H$.

Les trois premières valeurs sont concordantes et leur moyenne peut être prise pour une bonne évaluation de l'aire du pic principal.

Dans le second cas (Fig. 2b) où le pic présente un épaulement on calcule les valeurs:

$S_{0.9} = 20.11$ $S_{0.7} = 20.97$
$S_{0.8} = 20.45$ $S_{0.6} = 21.72$
$S_{0.75} = 20.64$ $S_{0.5} = 22.93$

Le fait de trouver une valeur commune à partir d'une certaine ordonnée permet d'affirmer que celle-ci est une bonne estimation de l'aire du pic principal. On peut évaluer l'aire correspondant au second acide aminé par le même procédé d'intégration, s'il est applicable, ou en retranchant la valeur calculée pour le pic principal de celle obtenue par sommation des densités optiques pour l'ensemble des deux acides aminés.

Nous n'avons pas vérifié si les valeurs que nous avons calculées sont valables pour des colonnes ou des systèmes d'élution différents de ceux que nous utilisons. Vu le faible écart des valeurs expérimentales et des valeurs théoriques calculées à artir de la courbe de Gauss (Tableau I) nous pensons qu'il en est probablement ainsi.

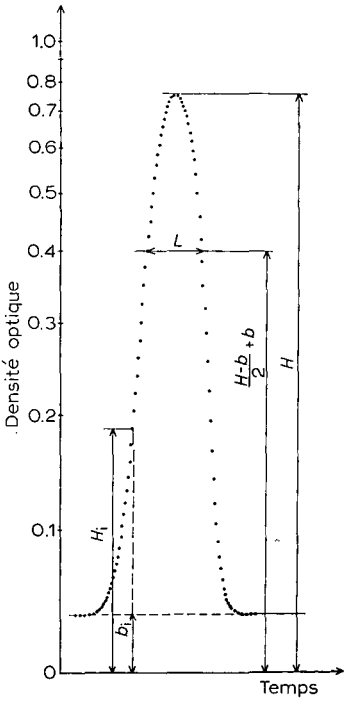

Fig. 3. Intégration d'un pic lorsque la ligne de base n'est pas confondue avec le zéro de l'enregistreur.

Intégration rapide

Lorsque la ligne de base du chromatogramme n'est pas confondue avec le zéro du papier d'enregistrement (Fig. 3), il faut en tenir compte pour l'intégration. Les formules (a) et (b) deviennent respectivement:

$$S = c \sum_{i=1}^{i=n} (H_i - b_i) \qquad (a')$$

et

$$S = k(H - b)L \qquad (b')$$

où b_i est l'ordonnée de la ligne de base interpolée à l'abscisse "i" et b est l'ordonnée de la ligne de base interpolée à l'abscisse du maximum.

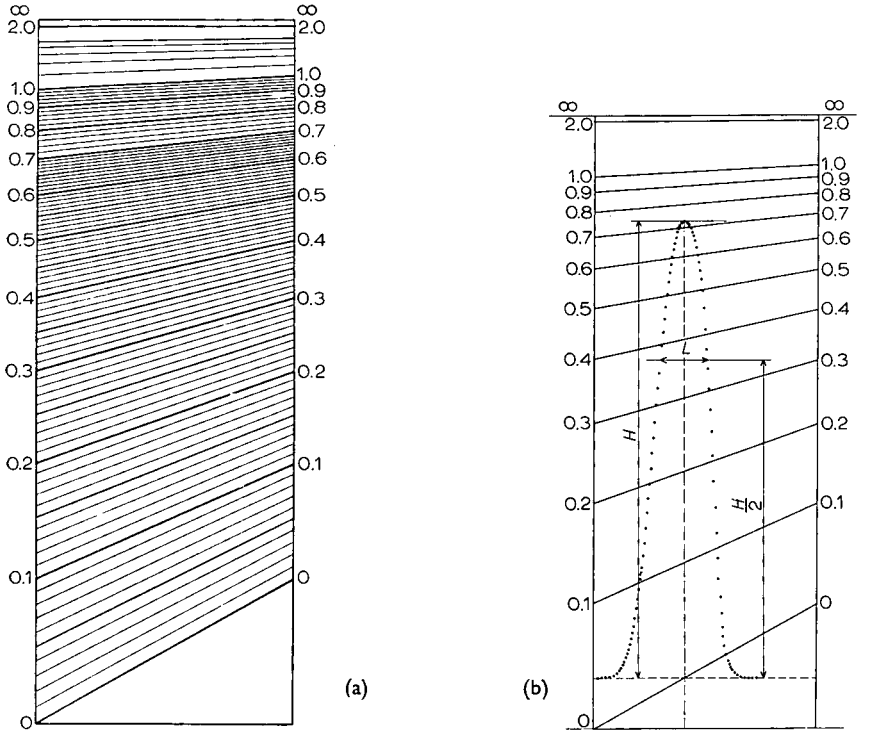

Fig. 4. (a) Échelle en densité optique de module variable. (b) Intégration au moyen de l'échelle de module variable.

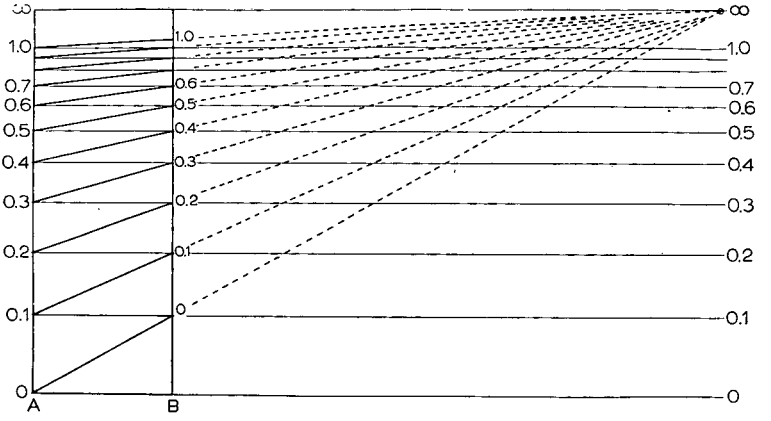

Fig. 5. Construction géométrique de l'échelle.

Nous utilisons pour ceci une échelle (Fig. 4a) transparente; en la plaçant sur l'enregistrement de telle sorte que les lignes de densité infinie soient superposées et que la droite marquée zéro coupe la ligne de base à l'abscisse du maximum, on lit directement la densité optique réelle (*H—b*) de ce point. (Fig. 4b). On repère ensuite sur le papier le point correspondant à la demi-hauteur et après avoir retiré l'échelle on mesure sur l'horizontale passant par ce point la largeur du pic *L*, par la méthode des points de SPACKMAN *et al.*[1].

On peut facilement réaliser une telle échelle en traçant le faisceau des droites s'appuyant sur l'échelle des ordonnées et passant par un point de la droite de densité infinie (Fig. 5). La même figure montre que l'on peut également joindre deux à deux les points situés sur deux parallèles, A et B, à l'axe des densités optiques et différant entre eux d'une quantité constante. Par exemple, on aura une échelle utilisable pour des lignes de base comprises entre 0 et 0.1 en joignant le point 0 de A au point 0.1 de B, le point 0.1 de A au point 0.2 de B, etc.

La pente de la droite de densité optique nulle dépend de l'espacement des deux droites A et B ou de l'éloignement du point de concours à l'axe des ordonnées dans la première construction. Il importe, pour ne pas diminuer la précision, que cette droite ne soit pas trop inclinée sur l'axe des abscisses; d'autre part, il ne faut pas que l'échelle devienne trop encombrante, celle que nous utilisons, pour des lignes de base comprises de 0 à 0.1 de densité optique, a 10 cm de large pour 28 cm de haut.

La précision obtenue sur la mesure de la hauteur réelle et le repérage de la demi-hauteur du pic est au moins égale à celle de la méthode classique car elle substitue une coïncidence optique, facile à réaliser, à une interpolation non linéaire.

Cette méthode rapide nous permet d'intégrer un chromatogramme complet en une demi-heure.

L'utilisation d'une machine à calculer imprimante (DIEHL-transmatic) nous a permis de réduire le temps d'intégration à 20 min.

Toutes nos mesures ont été faites sur des chromatogrammes obtenus en 22 h sur auto-analyseur Technicon par Mlle MAILLE.

Les calculs sont de Mme S. DELHAYE.

Station Centrale de Physiologie Végétale, C.N.R.A., Versailles (France)

JACQUES TEMPÉ

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Reçu le 16 février 1966

Gas-chromatographic analysis of tri-*n*-butyl phosphate

A gas-chromatographic method was found to be applicable for the quantitative analysis of dilute mixtures of tri-*n*-butyl phosphate (TBP), containing water and other impurities.

Normally, indirect methods[1] are used to determine the TBP content in mixtures. Mono- and di-*n*-butyl phosphates (MBP, DBP) are determined by potentiometric titration[2] or by paper chromatography[3]. Butyl alcohol is measured by its reaction with dichromate in sulphuric acid[4]. For water, either Karl-Fisher reagent is used or the weight loss is determined after evaporation under vacuum[5]. These methods are inconvenient and time consuming because each component has to be analyzed separately. The method described below (which is a programmed temperature gas-chromatographic analysis) is simple and rapid.

While this work was in preparation, BERLIN *et al.*[6] published results of isothermal gas-chromatographic analyses of several classes of organophosphorus compounds.

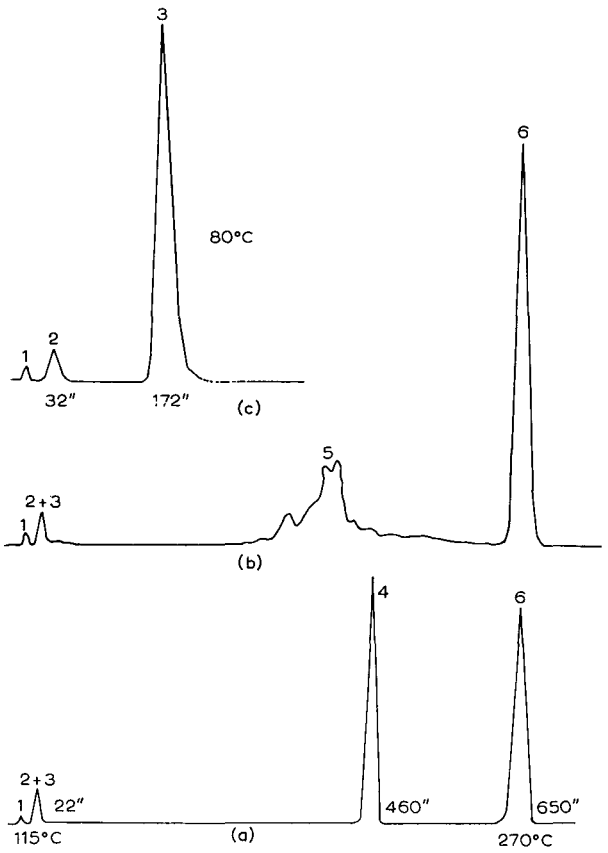


Fig. 1. Chromatographic analyses of TBP–diluent mixtures. (a) 60% v/v TBP + 40% v/v dodecane, saturated with water, 1.5 μl; (b) TBP + kerosene, saturated with water, 3 μl; (c) butyl alcohol + water, 1 μl. 1 = air; 2 = water; 3 = butanol; 4 = dodecane; 5 = kerosene; 6 = tri-*n*-butyl phosphate.

They found that TBP gave a clean, well defined peak with little or no decomposition. The conditions under which they worked differ from those described here.

Experimental

The gas chromatograph used was an F & M Scientific Corp., Model 810, with a dual thermal conductivity detector. The output signal was recorded by a 1 mV Honeywell Electronik-15 recorder, which was equipped with a Disc Chart Integrator, Model 201-B (Disc Instruments Inc.).

A column (2 m length, $^1/_4$ in. O.D.) was filled with a packing material containing 20% by wt. Apiezon-L on 60–80 mesh Diaport-S (both supplied by F & M, Scientific Corp.). The flow rate was 50 ml of helium per min, as measured by a soap flowmeter. The injector port was maintained at 250° and the detector at 265° (175 mA). The liquid samples (0.5–3.0 μl) were injected with a 5 μl Hamilton microsyringe. The column temperature was programmed from 115° (1 min) to 270° (4 min) at the rate of 20° per min.

All materials were used without further purification. TBP of different degrees of purity was obtained from J. T. Baker Chemical Co., Merck, Eastman-Kodak and Prolabo; together with TBP technical MBP and DBP were supplied by Bios Laboratories Inc., and *n*-butanol A.R. by British Drug Houses, Ltd.

Results and discussion

Typical analyses of synthetic mixtures of TBP, dodecane (or kerosene) and butyl alcohol are presented in Figs. 1a and b. A retention time of 22 sec was found

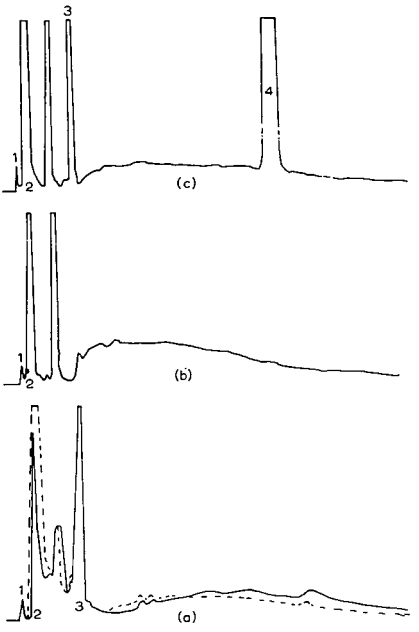


Fig. 2. Thermal decomposition of MBP and DBP. (a) DBP (1 μl), injection port at 240°, and DBP (1 μl), injection port at 290° (dashed lines); (b) MBP (1 μl); (c) 1.5 μl MBP + 1.5 μl DBP + 7 μl TBP. 1 = air; 2 = butanol; 3 = amyl alcohol; 4 = TBP.

for butanol and water, 650 sec for TBP and 460 sec for dodecane. In standard analyses where only the concentrations of TBP and the diluent are of interest, the peaks of butanol and water may remain unresolved. If the water content has to be determined, the programming should be started early, beginning at 80° (other conditions as above). This results in complete resolution of the peaks (Fig. 1c).

Thermal decomposition and very low vapour pressures do not permit the appearance of MBP and DBP peaks. Degradation products like butanol, dibutyl ether, butenes[7] and probably high-boiling hydrocarbons, are presented in Figs. 2a–c. At present the identification of all the peaks, obtained by thermal decomposition, is not quite certain. The problem is complicated by the fact that it is very difficult to obtain MBP and DBP of a high degree of purity[8]. Thermal decomposition strongly depends on the temperature of the injection port (Fig. 2a). This presents a possibility of controlling the pyrolysis by temperature programming of the injection port or by on-column injection.

The diffused and overlapping peaks of high-boiling hydrocarbons have probably little practical value for analytical purposes, but give an indication on TBP purity.

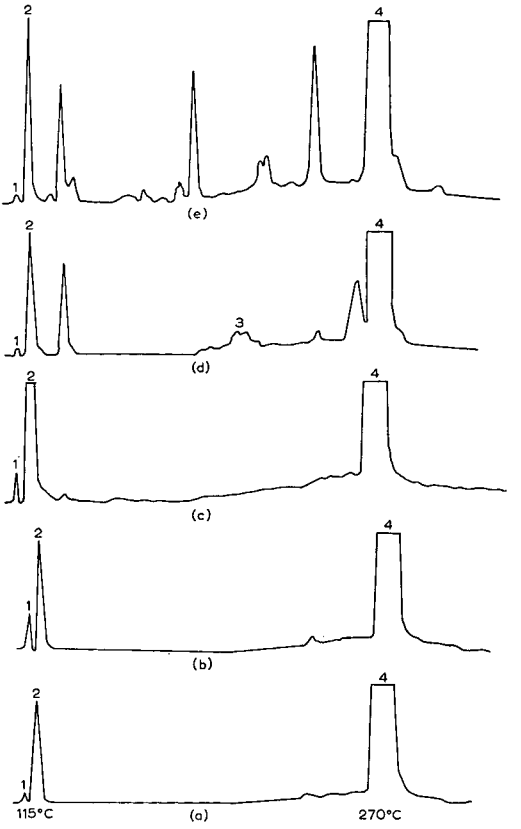
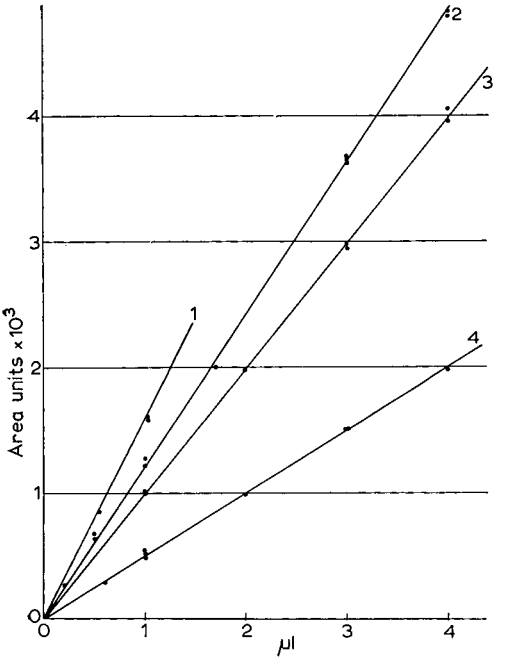


Fig. 3. Chromatograms of TBP (3 μl) obtained from several sources. (a) Baker, lot 4-170; (b) Eastman-Kodak, lot 2957; (c) Merck lot 6835, T 61823; (d) "technical"; (e) Prolabo, lot N-28726. 1 = air; 2 = butanol (+ water); 3 = kerosene; 4 = tri-*n*-butyl phosphate.

The relative purity of TBP obtained from several sources and analysed under identical conditions is presented in Figs. 3a–e.

Calibration curves (Fig. 4) for TBP, dodecane, butanol and water were prepared from synthetic mixtures. The straight lines were obtained for 0–4 μl samples. In order to present all the curves in one figure, arbitrary units of area were chosen.

Our results for solubility of TBP in water (0.39 g TBP/l at 22°) were found to be in agreement with the data compiled by McKAY AND HEALY[9] (0.39 g TBP/l at 25°, obtained by BURGER AND FORSMANN, 0.41 g TBP/l by ALCOCK and co-workers) and with those of HIGGINS, BALDWIN AND SOLDANO[10] (0.414 g TBP/l). The solubility of water in TBP–dodecane mixtures is presented in Fig. 5 and compared with data from the literature. The small discrepancies can be explained by temperature effects and by the nature of the diluents.



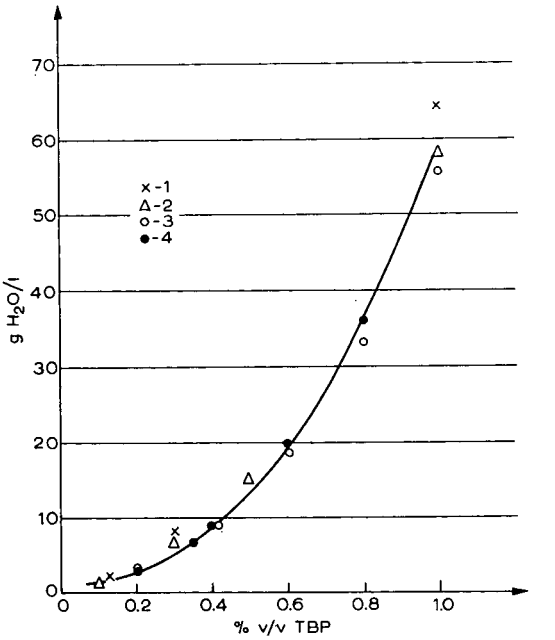


Fig. 4. Calibration curves for (1) butanol, (2) water, (3) TBP, and (4) dodecane.

Fig. 5. Solubility of water in TBP–dodecane mixtures. (1) and (2) data compiled by McKAY AND HEALY[8]; (3) ROZEN's data[12]; (4) this work.

The Apiezon-L column gives good results for analyses of TBP–diluent mixtures (TBP can be diluted not only by hydrocarbons). In addition, a TBP purification procedure[11] can be rapidly controlled by the relative indication of impurities present.

Acknowledgement

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J. Chromatog., 24 (1966) 175–179

Gas-chromatographic characterization of the electrophoretically separated fractions of acid mucopolysaccharides

Acid mucopolysaccharides can be separated by elution of columns of cellulose[1] (after precipitation with cetylpyridinium chloride), Dowex-1[2] or DEAE-Sephadex[3] with magnesium chloride or sodium chloride solutions of increasing concentration. Relatively large (about 10 mg) quantities of mucopolysaccharides are required for these methods.

Very small (5–10 μg) samples of mucopolysaccharides can be analyzed by means of cellulose acetate electrophoresis[4,5], but it has been difficult to characterize the fractions chemically.

Materials and methods

Materials. D-Glucosamine and D-galactosamine, homogenous in paper chromatography, were obtained from Mann Research Laboratories Inc., New York, N.Y., D(+)-glucose, analytical reagent grade, from B.D.H., Poole, Great Britain; galactose, *puriss.* from E. Merck A.G., Darmstadt, Germany; D(+)-glucuronic acid lactone, *puriss.* from Fluka AG, Buchs, S.G., Switzerland; hexamethyldisilazane, *purum* 98 %, and trimethylchlorosilane, *puriss.* 99 %, from Fluka AG, Buchs, S.G., Switzerland; pyridine, reagent grade, redistilled, anhydrous, J. T. Baker Chem. Co., Phillipsburg, N.J.; cetylpyridinium chloride, Recip AB, Stockholm, Sweden; oxoid electrophoretic strips, The Oxoid Division, OXO Ltd., London; Dowex-50, J. T. Baker Chem. Co., Phillipsburg, N.J.; and alcian blue, Gurr Ltd., London.

Iduronic acid was prepared from chondroitin sulphate B and was a gift from K. VON BERLEPSCH, F. Hoffman-La Roche Ltd., Basel, Switzerland.

Samples of keratosulphate and heparitin sulphate were obtained from Dr. M. B. MATHEWS, University of Chicago, Chicago, Ill., and from Prof. K. MEYER, Columbia University College of Physicians and Surgeons, New York, N.Y.

Extraction of mucopolysaccharides. The acid mucopolysaccharides were liber-

ated from acetone-defatted bovine skin by hydrolyzing with papain[2]. Trichloroacetic acid (10 % w/v) was added to precipitate the proteins and nucleic acids, and it was removed from the supernatant with ether. The acid mucopolysaccharides were precipitated at 4° with 4 volumes of ethanol which contained 0.5 % of sodium acetate and the precipitate dissolved in water. The sample was further purified by a precipitation with an excess cetylpyridinium chloride (CPC). The CPC-complexes were dissolved in 3 *N* $MgCl_2$ and the acid mucopolysaccharides reprecipitated with ethanol.

Electrophoretic fractionation. Electrophoresis of mucopolysaccharides was performed on Oxoid cellulose acetate sheets, using a barbiturate buffer, pH 8.6[4], voltage gradient 15 V/cm, and a running time of 25 min. A small strip was cut off the sheet and stained with alcian blue (1 % solution w/v in 25 % acetic acid). The bands were then cut off the unstained parts of the sheets and eluted with water.

Gas chromatography. The carbohydrate components were analyzed as their trimethylsilyl derivates[6–7]. For the determination of hexosamines the mucopolysaccharide fractions were hydrolyzed in 1.5 *N* HCl in sealed tubes at 103° for 17 h. The separation of hexosamines from other carbohydrates was accomplished by using columns of Dowex-50[8]. Uronic acids and neutral sugars were analyzed after hydrolysis of the fractions in 1 *N* HCl at 100° for 3 h. The amino sugars were removed from the hydrolyzates with Dowex-50.

The hydrolyzates were evaporated to dryness, pyridine (0.175 ml), hexamethyldisilazane (0.05 ml) and trimethylchlorosilane (0.015 ml) were added successively. The mixture was shaken and allowed to stand for 30 min at room temperature and the excess of reagents were evaporated off in a stream of nitrogen. The trimethylsilyl ethers were extracted with 5 ml of *n*-hexane and concentrated to the desired volume.

A Barber Colman M-10 chromatograph equipped with a flame ionization detector was employed. The column conditions were as follows: 6 ft. × 3 mm, 1 % SE-30 on 100–140 mesh siliconized Gas-chrom P (Applied Science Laboratories Inc.). Temperature 140°. Carrier gas was nitrogen with a flow rate of 30 c.c./min, pressure 0.5 atm.

Results and discussion

The mucopolysaccharides of the bovine skin were electrophoretically separated nto three fractions (Fig. 1). The electrophoretic mobility of fraction 1 corresponded

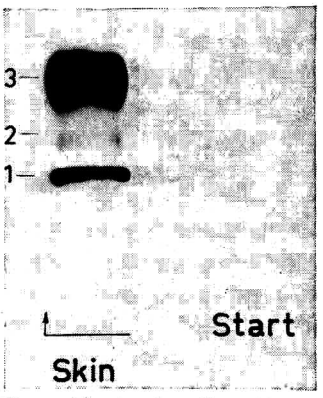

Fig. 1. Electrophoretic pattern of acid mucopolysaccharides of bovine skin. Sample size 10 μg.

to hyaluronic acid which had been isolated from human umbilical cord. Fraction 2 moved in the same manner as the reference samples of heparitin sulphate or keratosulphate and fraction 3 as chondroitin sulphate B. The gas chromatographic analyses showed that galactosamine and a small amount of glucosamine were present in fraction 3 and glucosamine only in fractions 1 and 2 (Fig. 2). The determination of uronic acids and neutral sugars showed that iduronic acid was present in fraction 3, galactose in fraction 2, and glucuronic acid in fraction 1 and also in fraction 3 as a minor component (Fig. 3). When pure cellulose acetate sheets were eluted with water, some glucose was observed in the eluate and it was present also in all the mucopolysaccharide fractions. The quantity of sugars was large enough for identification in samples pooled from 4–8 electrophoretic sheets (total mucopolysaccharides 40–100 μg). After hydrolysis for 3 h small unidentified peaks, possibly impurities, were observed in the gas chromatographic patterns of fractions 1 and 3.

On the basis of this semiquantitative determination of the carbohydrate components, fraction 1 was hyaluronic acid, fraction 2 keratosulphate, and fraction 3 contained chondroitin sulphates, mainly chondroitin sulphate B.

The electrophoresis of mucopolysaccharides on cellulose acetate sheet is a rapid method for the fractionation of small mucopolysaccharide samples. The electrophoretic mobility of keratosulphate and heparitin sulphate is the same at pH 8.6,

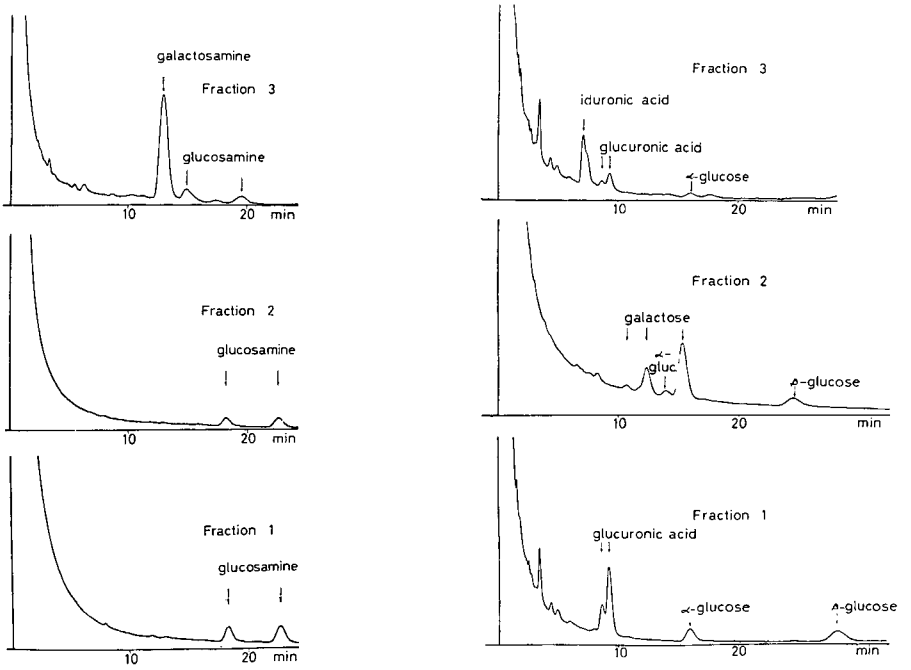

Fig. 2. Gas chromatography of amino sugars of electrophoretically separated mucopolysaccharide fractions. Fraction numbers 1–3 refer to those in Fig. 1. Sample obtained by eluting 4 electrophoresis strips, corresponding to 40 μg total mucopolysaccharide.

Fig. 3. Gas chromatography of uronic acids and neutral sugars of electrophoretically separated mucopolysaccharide fractions. Fraction numbers 1–3 refer to those in Fig. 1. Sample obtained by eluting 8 electrophoresis strips, corresponding to 80 μg total mucopolysaccharide.

but heparitin sulphate and keratosulphate can be differentiated by analysis of the carbohydrate components.

The disadvantage of this method, however, is that chondroitin sulphates A, B and C cannot be separated from each other; though iduronic acid of chondroitin sulphate B and glucuronic acid of chondroitin sulphate A and C can be differentiated by gas chromatography. The quantitative determination of the uronic acids by this method is under investigation.

This procedure is useful for the qualitative and semiquantitative analysis of the tissue polysaccharides, when the individual electrophoretic fractions are available in amounts of 5–50 μg.

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Quantitative determination of griseofulvin by gas–liquid chromatography

Several methods have been described for the determination of griseofulvin in many types of mixtures. The use of a spectrophotometric method[1] is relatively non-specific and therefore an indirect method[2], based on the conversion of griseofulvin to isogriseofulvin, was devised for quantitating griseofulvin in the presence of structurally similar contaminants. There is a spectrophotofluorometric assay[3,4] which is rather more specific and has been used extensively for detecting and estimating griseofulvin in biological fluids. In this assay, however, aspirin, salicylic acid and quinine are likely to interfere[5]. Recently a liquid–solid chromatographic method[6], although the procedure is tedious and time consuming, has been reported for the direct analysis of griseofulvin in complex fermenter broths.

In this study, a new gas chromatographic method was successfully developed for

the rapid, accurate, and direct determination of griseofulvin in pharmaceutical preparations.

Experimental

Apparatus and materials. A Shimadzu Model GC-1B gas chromatograph equipped with a differential hydrogen flame ionization detection system was used for this study. Packings were 1.5 % SE-30 (methyl silicone; General Electric Co.) on acid-washed and silanized Chromosorb W, 80- to 100-mesh (Johns-Manville Co.), and 1.5 % QF-1 (fluorinated alkyl silicone; Dow Corning Corp.) on acid-washed and silanized Anakrom, 80- to 100-mesh (Analabs Inc.), both prepared by the solution-coating technique[7]. A 150 cm (75 cm × 2) × 4 mm I.D. U-shaped stainless steel column was packed in a vertical position by tapping and was preconditioned overnight at 260° before use. Operating conditions were as follows: column and injection port temperature, 230°; detector temperature, 240°; nitrogen as carrier gas at 17.5 ml per min (2 kg per sq.cm) at inlet. Samples of 1 to 2 μl were injected with a 10.0 μl Hamilton syringe.

Calibration curve. A series of synthetic mixtures was prepared for injection by accurately adding 1 to 8 mg of pure griseofulvin, λ_{max}^{EtOH} 291 mμ ($E_{1cm}^{1\%}$ 708) to 1 ml of a solution containing 2 mg per ml diphenyl phthalate (internal standard) in acetone. At the fixed sensitivity and range of the instrument, approximately 1 μl of each mixture was injected into the chromatograph. The peak areas were determined by planimeter and/or by triangulation. By plotting the weight ratios against the peak area ratios of griseofulvin to diphenyl phthalate, a straight line passing through origin was obtained for the calibration curve.

Sample preparation. A quantity of suspension or a finely pulverized sample equivalent to 3 to 15 mg of griseofulvin was extracted twice with 20 ml of ether (for a suspension) or of acetone (for a powder) by shaking vigorously for 2 min. The extract was filtered through filter paper which was then washed with 10 ml of solvent. The filtrate was evaporated to dryness on a hot plate and the residue was dissolved in exactly 2 ml of a 2 mg/ml acetone solution of diphenyl phthalate.

Approximately 1 to 2 μl of solution was injected, the ratio of the peak areas again determined, and the amount of griseofulvin was calculated by comparison with the calibration curve.

TABLE I

RETENTION DATA FOR GRISEOFULVINS

Conditions the same as for Fig. 1.

	Relative retention times	
	1.5 % SE-30	*1.5 % QF-1*
Diphenyl phthalate (internal standard)	1.00*	1.00**
Griseofulvin	1.84	3.25
Isogriseofulvin	2.40	5.15

* Retention time: 5.5 min.
** Retention time: 5.2 min.

Results and discussion

The gas chromatography of griseofulvins without extensive pretreatment was successful on thin film columns of the silicone polymer type. A mixture of griseofulvin, isogriseofulvin, and diphenyl phthalate as the internal standard could be eluted with a satisfactory resolution factor (Table I) both on a 1.5 % SE-30 or on a 1.5 % QF-1 column. A typical chromatogram is reproduced in Fig. 1, in which each peak corresponds to 2 to 5 μg of the samples.

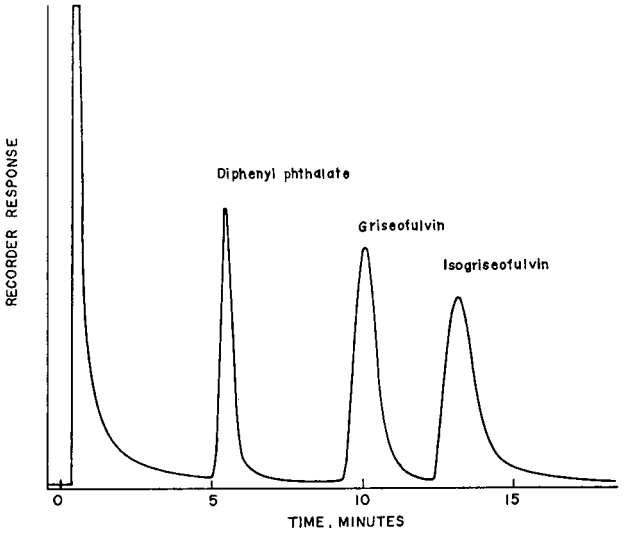

Fig. 1. Chromatogram of a mixture of diphenyl phthalate, griseofulvin, and isogriseofulvin. Conditions: 150 × 0.4 cm I.D. stainless steel column packed with 1.5 % SE-30 on 80- to 100-mesh Chromosorb W; 230°; 17.5 ml per min (2 kg per sq. cm) nitrogen; hydrogen flame ionization detection system.

Lack of thermal degradation of griseofulvin was evidenced by the fact that a single reproducible and almost symmetrical peak was obtained and also by the fact that the effluent collected at the outlet of the column presented the same ultraviolet and infrared spectra (Fig. 2) as the starting material.

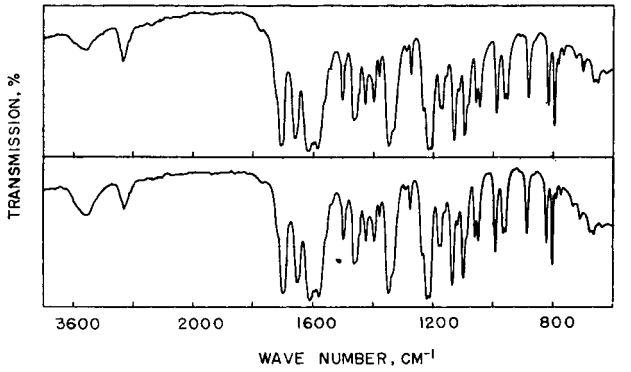

Fig. 2. Infrared spectra of griseofulvin before (lower curve) and after (upper curve) chromatography in a KBr disc.

Interfering peaks were not found in the pharmaceutical preparations examined for griseofulvin. Six synthetic pulverized mixtures were prepared of griseofulvin in milk sugar to study the recovery of a sample subjected to this proposed procedure. Recovery values determined on a SE-30 column are shown in Table II and the average overall recovery of 3.29 to 14.03 mg of griseofulvin added to 0.5 g of milk sugar was 98.9 % with a standard deviation of ± 3.1 % based on peak area measurement.

TABLE II

RECOVERY OF GRISEOFULVIN ADDED TO MILK SUGAR*

Added (*mg*)	*Found* (*mg*)	*Recovery* (%)
3.29	3.34	101.5
4.77	4.81	100.8
7.15	6.77	94.8
8.33	8.55	102.6
12.64	12.24	96.8
14.03	13.60	96.9
Average		98.9
Standard deviation		±3.1

* In separate experiments the precision was found to be ± 0.9 %.

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Gas chromatography of halogenated carbamates

III. *meta*-Fluorosulfonylphenyl derivatives

The recent availability of a new reagent, *meta*-fluorosulfonylphenyl isocyanate[1]*, has stimulated our interest in its facile conversion to substituted carbamates via the standard isocyanate reaction[2,3] with phenolic or alcohol derivatives. Aromatic fluorinated plant growth regulators and intermediates have been the subject of studies by FINGER and his associates[4], who extended their investigations to N-fluorophenyl carbamates. Isomeric mono-fluorophenyl N-methyl carbamates were included in work concerning the anticholinesterase activity and toxicity of substituted phenyl N-methyl carbamates[5]. MCFARLAND AND HOWARD have recently reported preparations of a variety of urethanes from benzenesulfonyl isocyanate[6].

This paper cites the investigation of the gas chromatographic behavior of urethanes prepared from *meta*-fluorosulfonylphenyl isocyanate.

Experimental

The N-*meta*-fluorosulfonylphenyl carbamates were synthesized via the reaction of the isocyanate with the respective phenol or alcohol in a small amount of pyridine and were recrystallized from petroleum ether (35–60° fraction). The melting points were determined on a Fisher–Johns melting point apparatus (Fisher Scientific, Silver Spring, Md., U.S.A.). Gas chromatography was carried out on an F & M model 1609 instrument equipped with a flame ionization detector. The column employed was 6 ft. by 0.25 in. glass coil packed with 4 % QF-1 on 80–100 mesh HMDS-pretreated Chromosorb W (Applied Science Laboratories, Inc., State College, Pa., U.S.A.). Specific operating conditions are given in the footnotes to Table I.

Results and discussion

The chromatographic data are reported in Table I. The carbamates were observed to undergo degradation with the release of the appropriate phenol. A second peak common to all samples was suspected to be *meta*-fluorosulfonylaniline. The elution values are cited relative to this latter component. Isopropanol and cyclohexanol were not detected owing to their rapid elution under the experimental conditions. Interestingly enough, the cyclohexyl derivative produced a large component peak at twice the elution value of the reference peak suggesting perhaps that degradation is not complete for this derivative. The order of elution for the phenols produced via thermal cleavage is essentially the same for those released from N-trichloroacetyl carbamates in earlier studies[7]. The number of theoretical plates for the *meta*-fluorosulfoxyl aniline peak was 680.

A study of other N-haloaryl carbamate derivatives is presently underway.

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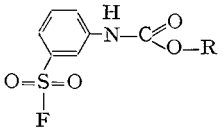
* Aldrich Chemical Company, Inc., Milwaukee 10, Wisc., U.S.A.

TABLE I

GAS CHROMATOGRAPHY OF N-*meta*-FLUOROSULFONYLPHENYL CARBAMATES



R	*Mol.wt.*	*M.p.* (°C)	*Relative elution*[a]
Isopropyl	261	82– 83.5	[b]
Phenyl	295	76– 78	0.041
Cyclohexyl	301	64– 66	[c]
o-Chlorophenyl	329	137–139	0.041
m-Chlorophenyl	329	111–113	0.61
2,3-Dichlorophenyl	363	133–135	0.16
2,5-Dichlorophenyl	363	88– 90	0.17
p-Chlorophenyl	329	124–126	0.18
2,6-Dichlorophenyl	363	114–116	0.19
3,5-Dichlorophenyl	363	110–112	0.35
3,4-Dichlorophenyl	363	98–100	0.48

[a] Elution of phenols released during chromatographic analysis relative to a secondary product (*m*-fluorosulfonyl aniline), present in all samples and eluting at 2.9 min. Column 4% QF-1 on 80–100 mesh HMDS pretreated Chromosorb W, 6 ft. by 0.25 in. glass coil. Operating conditions: column 140°; injection port 70 V; detector 200°; range 1000; nitrogen carrier 91 ml/min; hydrogen 77 ml/min; air 300 ml/min; flame ionization detector.

[b] Eluted in solvent peak.

[c] A major component appeared at 2.0 relative to the *m*-fluorosulfonyl aniline peak, suggestive of intact carbamate.

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Reaction gas chromatography

I. Catalytic aromatization of alicyclic and heterocyclic compounds

In contrast to the wide popularity of gas chromatography as a method for separating the products of chemical reactions, the potentialities of the technique as a tool for simultaneously carrying out reactions and analyzing the products formed have not as yet been fully appreciated.

KOKES *et al.*[1] were the first to apply this idea to the identification of hydrocarbons by catalytic cracking in a reactor attached to the inlet of a gas chromatograph. The term "Reaction Gas Chromatography" for the technique was coined by DRAWERT *et al.*[2,3] and its application illustrated through the conversion of alcohols to olefins employing a reactor packed with H_3PO_4–Sterchamol. Pyrolysis in conjunction with gas chromatography was proposed by JANAK[4] and has been successfully employed by a number of investigators to the analysis of non-volatile organic compounds. ZLATKIS *et al.* developed a method for the examination of amino acids, employing two reactors, one for oxidation of the acids and the other for cracking the aldehydes obtained[5]. Hydrogenolytic gas chromatography developed by BEROZA[6–8] for the determination of C-skeletons of organic compounds has also been used for the estimation of double bonds and certain functional groups[9].

Dehydrogenation reactions often serve as important steps in the structure elucidation of organic compounds. Conventional procedures require relatively large samples, the size of which may, however, be appreciably reduced by means of gas chromatographic analysis of the reaction products[10]. Reaction gas chromatography employing a suitable dehydrogenating catalyst permits such investigations on a micro and even submicro scale. OKAMOTO AND ONAKA[11] suggested an approach of this type when reporting the dehydrogenation of a few monoterpene compounds. Since the technique appeared to be of wider usefulness its applicability to different types of ring compounds was explored. The present investigation reports results obtained with several alicyclic and heterocyclic substances, from which a large number of compounds of pharmaceutical interest are derived.

Experimental

Apparatus

Gas chromatography. A Burrell Kromo-Tog K-2 equipped with a thermal conductivity detector and flash vaporiser unit was used. Column: a glass tube, length 225 cm and I.D. 6 mm. Packing: 20% Carbowax 20 M deposited on alkali-washed firebrick[12]. Carrier gas: helium, 75 ml per min.

Reactor. The glass tube of the accessory flash vaporiser assembly served as reactor. The unit may readily be constructed as shown in Fig. 1. The design may be suitably modified to meet the requirements of gas chromatographs equipped with different types of injectors. The reactor tube was packed with one g of catalyst. The tapered end A of the reactor was inserted into the gas chromatograph while end B, closed with a silicone seal, served as the injection port. By manipulating valves C and D the carrier gas could be made to flow directly through the column or *via* the reactor.

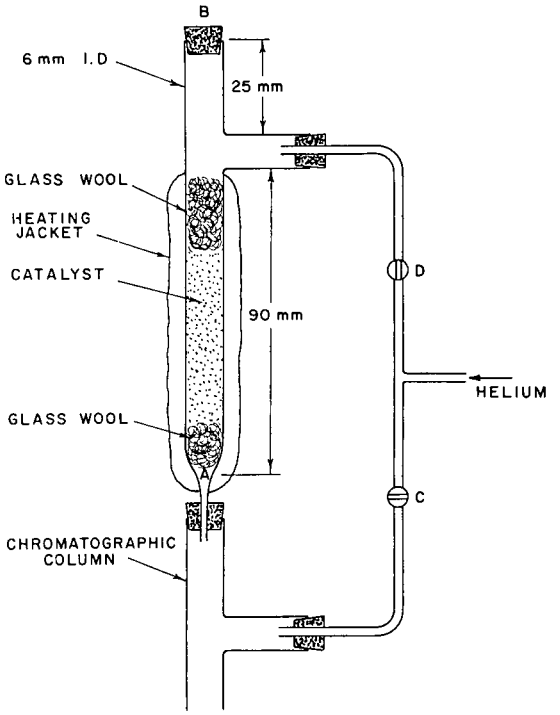

Fig. 1. Reactor for catalytic aromatization.

Catalysts. Two catalysts were employed in this study:

(1) 5 % platinum on alumina, initially obtained from Koch-Light Laboratories, Colnbrook, England, but subsequently prepared by impregnating neutral alumina (5 g) (Woelm) with an aqueous solution of chloroplatinic acid (500 mg), drying at 105° and heating in a slow stream of hydrogen while the temperature was gradually raised to 250°.

(2) 5 % platinum on alkali-washed firebrick[12] prepared in accordance by the same procedure.

Procedure

Aromatization occurred following injection of the samples (0.5–1.0 μl) into the reactor maintained at 280°. Usually dehydrogenation took place almost instantly as the vaporized material swept over the hot catalyst. In some cases, however, only slight aromatization occurred and the residence times had to be increased to realize more effective conversions. This was achieved by means of valves C and D. The carrier gas was allowed to flow directly through the column by closing D and opening C. The sample was then injected into the reactor where it vaporised and was kept under pressure by the carrier gas in the chromatographic column. After the required time for reaction had elapsed valve D was opened, C was closed and the recorder switched on simultaneously. The products of reactions were identified by comparison of their retention times with those of pure reference standards and the yields were estimated by measurement of the corresponding peak areas.

Results and discussion

Choice of reaction conditions

Catalytic dehydrogenation reactions carried out by conventional techniques generally require several hours for completion. Vapour phase reactions require less time but recycling of products is often still necessary. On the other hand reaction gas chromatography, employing the reactor assembly described, permitted very fast and efficient conversions, the short residence time of the sample in the catalyst chamber being compensated by a high catalyst/sample ratio. Minimum temperatures required for dehydrogenation were found to be dependent on the nature of the compounds. For most of the substances investigated the reaction proceeded smoothly at 280°. Helium (flow rate 75 ml/min) provided an inert atmosphere and furthermore accelerated the reaction by sweeping liberated hydrogen immediately off the sites of reaction. This also minimized the occurrence of undesirable side reactions.

Choice of catalyst

Several metals *viz.* platinum, palladium, nickel, rhodium etc., have been used as dehydrogenating catalysts. Platinum and palladium, in particular, are very widely employed. Since palladium exhibits a greater tendency to produce side reactions[13], platinum catalysts were used throughout this study. The nature of the support proved to be important. Thus a 10 % Pd on charcoal catalyst strongly adsorbed many of the compounds and their dehydrogenation products. Tetralin and decalin and their dehydrogenation products, for example, could not be recovered under the experimental conditions described. Similar observations have been reported for a Pt on charcoal catalyst[11]. Employing a 5 % Pt–alumina catalyst, however, both decalin and tetralin were quantitatively converted to naphthalene (Table I). This catalyst proved satisfactory for other alicyclic compounds as well. It was, however, generally unsuitable for analysis of nitrogeneous heterocyclic compounds which adsorbed strongly at the acidic sites of the support[14]. Platinum on alkali-washed firebrick was found to be satisfactory for these products although conversions were low (Table I).

Alicyclic compounds

The platinum–alumina catalyst proved to be more efficient than the platinum–firebrick catalyst for the aromatization of alicyclic compounds (Table I). The ease with which cyclohexene changed into benzene showed that presence of a double bond in the ring facilitates the reaction. Aromatization of cyclohexanol and cyclohexylamine probably proceeds *via* cyclohexene as an intermediate formed at active sites on the alumina[14,15]. Both these compounds gave poor yields on platinum–firebrick. On this catalyst cyclohexylamine remained practically unchanged while cyclohexanol yielded cyclohexene as the main product of reaction. The dehydrogenation of 2-methylcyclohexanone and 3-methylcyclohexanone also appears to be initiated by acidic sites on alumina. In addition to toluene (35 %), methylcyclohexane (54 %) was also obtained. Platinum–firebrick failed to aromatize these ketones to any significant extent and only small quantities of toluene were recovered. Conversions of six membered alicyclic ketones to alcohols and phenols reported to take place during catalytic dehydrogenations[16–19] were not observed with these two ketones under the present experimental conditions.

TABLE I

AROMATIZATION OF ALICYCLIC AND HETEROCYCLIC COMPOUNDS

Reactor temperature: 280°.

Compound	*Aromatic product*	*% Yield of aromatic product**	
		5% Pt on alumina	*5% Pt on alkali-washed firebrick*
Alicyclic compounds			
Hydrocarbons			
Cyclohexane	Benzene	100	2, **36**
Cyclohexene	Benzene	100	83, **93**
Methylcyclohexane	Toluene	77	nil, **7**
Decalin	Naphthalene	100	trace
	Tetralin	nil	10
Tetralin	Naphthalene	100	15
Alcohol			
Cyclohexanol	Benzene	100	6, **8**
Ketones			
2-Methylcyclohexanone	Toluene	65	trace, **4**
3-Methylcyclohexanone	Toluene	46	nil, **2**
Amine			
Cyclohexylamine	Benzene	100	trace
Heterocyclic compounds			
N-Methylpyrrolidine	N-Methylpyrrole	81	trace, **20**
Piperidine	Pyridine	—	32, **71**
N-Methylpiperidine	Pyridine	—	trace, **15**
N-Ethylpiperidine	Pyridine	—	3, **18**
Piperazine	Pyrazine	—	18

* Values in bold face denote yield of aromatic compound when sample was held in reactor for two minutes, all other figures give the yields on direct passage through the reactor.

Heterocyclic compounds

Five nitrogeneous heterocyclic compounds were used in this study (see Table I). Alumina was generally unsuitable as catalyst support for aromatizing these substances. Only N-methylpyrrolidine, a tertiary base, was effectively converted to N-methylpyrrole. The compounds were dehydrogenated on platinum–firebrick when moderate yields of aromatic products were obtained by keeping the sample in the reactor for two minutes. The aromatization of N-alkylpiperidines proceeded *via* elimination of the alkyl groups leading to the formation of pyridine. As expected N-methylpyrrolidine yielded N-methylpyrrole[20].

Scope of the technique

The technique described holds promise for wide application in the field of organic and pharmaceutical chemistry. It has already been used successfully in the author's laboratory for the determination of the C-skeleton of sesquiterpene hydrocarbons[21] and provides a novel approach to the identification and structure elucidation, on a micro scale, of compounds containing aromatizable rings. Many alkaloids possess five and six membered heterocyclic rings. Several piperidine derivatives are

used as narcotics and their detection is of forensic importance. Aromatization of cyclohexyl rings is of special interest in investigations on terpenoids. Applications of the technique to several classes of compounds of pharmaceutical interest are under study and will form the subject of forthcoming publications.

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The pH-dependent elution of malonaldehyde during gel filtration on Sephadex G-10

Malonaldehyde (MA) is formed from the oxidation of polyunsaturated fatty acid esters and squalene[1,2]. MA reacts with proteins[3] and other food constituents[4]. Crystalline MA has been described[5], but it is not a practical source because of its instability. Thus, it is usually obtained by hydrolyzing its bis-(diethylacetal). With MA obtained in this fashion, an unusual elution behavior was noted in an attempt to fractionate on Sephadex G-10 columns.

Experimental

A $4 \cdot 10^{-4}$ *M* solution of distilled MA bis-(diethylacetal) (Kay-Fries) was hydrolyzed by acid[6]. One ml portions of the hydrolyzate were introduced onto a Sephadex G-10 (Pharmacia) column (2 × 105 cm) and eluted with citrate-phosphate buffers[7] containing 0.1 *M* NaCl at pH values, ranging from 2.6 to 7. In each case, about 300 fractions (1 ml) were collected at a flow rate of 1 ml/min (Spinco Accu-flo pump) at room temperature. One ml of TBA reagent (0.02 *M* 2-thiobarbituric acid in 90% acetic acid) had been added to every other test tube in the fraction collector. After 24 h at room temperature, the pink color (MA–TBA reaction product) was measured at 532 mμ. The alternate fractions without added TBA reagent were examined for U.V. absorption spectra, using a Cary 15 spectrophotometer. The void volume (V_0) of the column was determined with Blue Dextran 2000 (Pharmacia).

Results and discussion

The elution volume (V_e) of MA was pH-dependent between pH 3 and 7 (Table I): there were no further changes below pH 3 and above 7. In Fig. 1, the elution patterns of MA at pH 7 (left) and at pH 3 (right) are illustrated. Absorbances at 267 and 245 mμ coincided with those at 532 mμ, after TBA reaction. The U.V. absorption spectra of MA is pH dependent[6]: above pH 7, the absorption maximum is at 267 mμ; below pH 3, the maximum is at 245 mμ. The MA peak in the chromatogram (Fig. 1) becomes broader as the pH of the buffer decreases. The V_0 of the column remained the same at all pH values.

The chromatographic behavior of MA differs from what might be expected from the molecular sieve properties of Sephadex. At least two different mechanisms[8] could be involved; namely, charge effects of the few ionized groups present in the gels and adsorption of solutes to the gel matrix. The former effects are easily distinguished from the latter in that they should be normalized by the presence of salt.

TABLE I

FRACTIONATION OF MA BY SEPHADEX G-10 COLUMN AT DIFFERENT pH

	pH				
	3	*4*	*5*	*6*	*7*
V_e (ml)	278	258	233	223	221
K_d	1.17	1.03	0.85	0.78	0.76

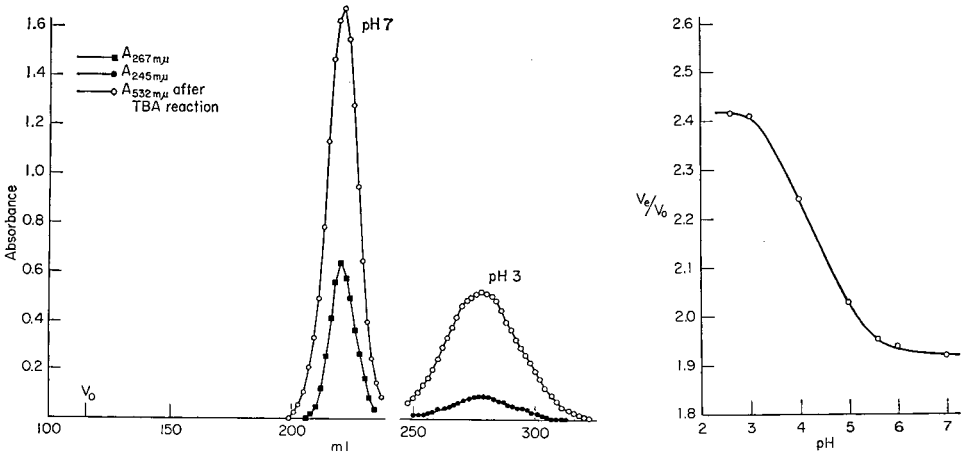
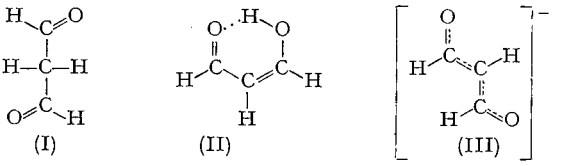


Fig. 1. The chromatographic elution pattern of malonaldehyde (MA) on Sephadex G-10 column at different pH. Column: 2 × 105 cm; buffer: citrate–phosphate buffer, containing 0.1 *M* NaCl; flow rate: 1 ml/min at room temperature; sample: 1 ml $4 \cdot 10^{-4}$ *M* MA bis-(diethyl acetal) hydrolyzate.

Fig. 2. Effect of pH on the V_e/V_0 ratio. Calculation of the ratio, see Table I.

Since the buffers used contain NaCl (0.1 *M*), the charge effects should be eliminated. The distribution coefficient (K_d) of MA changed gradually from 0.76 to 1.17, with the shift in pH of the eluting buffers from 7 to 3 (Table 1). The K_d values higher than unity below pH 4 clearly indicate adsorption of MA to the gel matrix. MA occurs mainly as its enolic tautomer in aqueous solution and dissociation of the enolic MA (pK_a, 4.65) starts at pH 2.8 and is complete at pH 6.5[4,6]. Below pH 2.8, MA (I) is considered to occur as the intra-molecular hydrogen bonded species, the chelated MA (II), while above pH 6.5, as its enolate anion (III)[4,6]. The above finding suggests that the chelated MA has a very high tendency to be adsorbed to the gel matrix. Aromatic and heterocyclic compounds are known to have a greater tendency to be adsorbed than have other water-soluble solutes[8].



A plot of V_e/V_0 against pH is shown in Fig. 2. The shape of the curve is parallel with that of U.V. absorbance[4] of MA against pH and with that of MA recovery by distillation[9] against pH. All three criteria thus indicate that MA occurs as different configurations at different pH levels; only the chelated MA in acidic solutions is volatile[5,9].

The Sephadex gel elution characteristics can be applied to the identification of MA in the presence of other TBA-reactive substances, none of which show the same pH-dependence[2]. Although MA is the principal one, several such substances

occur in oxidized fatty materials[1,2]. The column techniques also provide a method to obtain MA in a given buffer solution at any pH, free from other substances. The original hydrolyzate of MA acetal contains ethanol, and may also contain polymeric MA[2] and other partial hydrolysis products in addition to MA.

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An adjustable column end-piece for gel filtration

The end-piece is constructed from two concentric acrylic tubes (Fig. 1). The inner tube has an expanded end which can be drawn up against the outer tube by a screw thread and nut. A cuff cut from silicone rubber tubing is placed between the ends of the acrylic tubes so that tightening the screw increases its diameter. The expanded end of the inner tube is funnelled to a sharp edge, but is also recessed to take a disc of porous polyethylene which is cemented in with acrylic cement. A silicone tube is pressed into the narrow end of the funnel to convey the column fluid.

Two end-pieces are inserted into a glass cane of slightly larger internal diameter, and tightened till the cuffs expand to fit snugly to the glass. A column of variable bed length can be obtained, and shrinkage can be taken up between experimental runs. The dead space volume of the end-pieces can be made very small, and their porous faces are little smaller than the column cross section. The need for precision bore column tubing is obviated though the diameters of the end-piece and column tube should not be so disparate that fractions with very high or low densities gravitate into the space between them. This risk can be reduced by pressing a little column gel into this space when the end-pieces are finally adjusted.

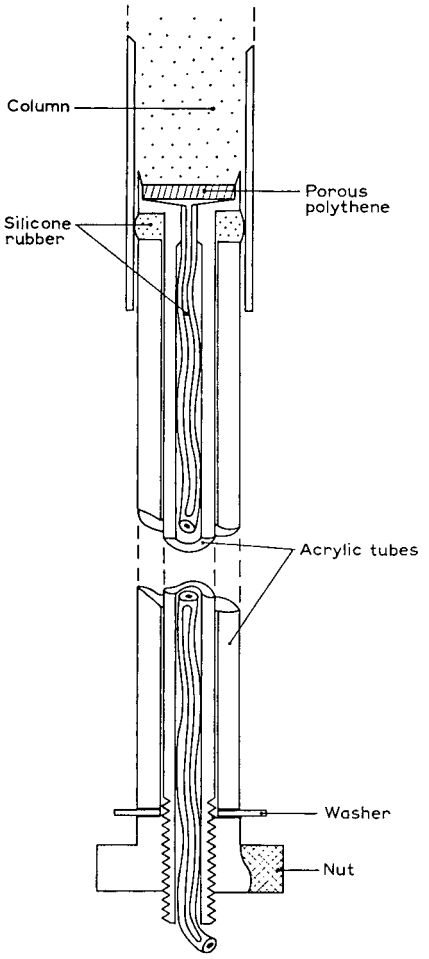

Fig. 1. Column end-piece in longitudinal section.

The end-pieces proved water tight for up to six weeks, but could still be released readily from the glass. Sephadex G-200 grains were completely retained by porous polythene of 80/4 grade, $^1/_8$ in. thick*, at flows of up to 8 ml per hour.

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* Made by Vyon Porous Plastics Ltd., Dagenham Dock, Essex, England.

Separation of transfer-ribonucleic acid from deoxyribonucleic acid by gel filtration

In the work on the development of granulation tissue we were faced with the problem of obtaining information on all the nucleic acids simultaneously. We wished to avoid the preliminary centrifugation of the homogenate and proceed with the phenol extraction[1] with the minimum of delay in order to avoid degradation of the nucleic acids. When HMW-RNA's* were precipitated from the aqueous phase with 3 *M* sodium acetate, DNA and t-RNA remained in the supernatant. It is possible to separate DNA from t-RNA by Sephadex gel filtration.

A Sephadex (G-200, bead form) column (3 cm × 55 cm) was prepared according to manufacturer's (Pharmacia AB, Uppsala, Sweden) instructions and equilibrated with 0.1 *M* sodium acetate buffer, pH 5.1. The void volume (V_0), inner volume (V_i) and distribution coefficients (K_d) were determined according to FLODIN[2].

The mixture of DNA and t-RNA in 3 *M* sodium acetate solution was desalted by ethanol precipitation (final concentration 70 %, v/v), dissolved into 0.1 *M* sodium acetate buffer, pH 5.1, and allowed to drain into the column, which was eluted with the same buffer. The U.V.-absorption in the eluate was followed with Uvicord ultraviolet absorptiometer (LKB Produkter Aktiebolag, Stockholm, Sweden) connected to a recorder. The flow rate was 30 ml/h and 5-ml fractions were collected.

A typical chromatogram is seen in Fig. 1. The first fraction has K_d-value 0, the second 0.57. Sometimes a third fraction is observed with K_d-value 0.9–1.1.

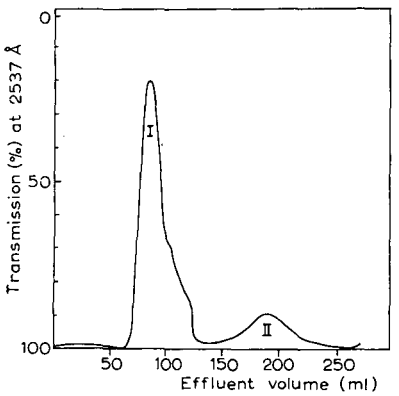

Fig. 1. Separation of DNA (fraction I) and t-RNA (fraction II) on Sephadex G-200 column. The details and the identification of the peaks are described in the text.

Nucleic acid fractions were precipitated with ethanol, washed and dried. The occasional third fraction was not precipitable with ethanol but it was soluble in ether. The base compositions of the fractions were determined after alkaline[3] and acid hydrolysis[4], the sedimentation coefficients with an analytical ultracentrifuge (Spinco Model E, schlieren optics) and the amino acid acceptor capacity was determined according to ZACHAU[5] using amino acid-activating enzymes from rat liver and ^{3}H-lysine (The Radiochemical Centre, Amersham, England).

* Abbreviations: HMW-RNA = high-molecular-weight ribonucleic acid; t-RNA = transfer-ribonucleic acid.

Fraction I was diphenylamine-positive, fraction II negative. No nucleotides were released from fraction I with alkali, but AMP, CMP, UMP, GMP and 5-ribosyluracil monophosphates were released from fraction II. The acid-released bases from fraction I were adenine, thymine, cytosine and guanine. The $S^{\circ}_{20,w}$ of fraction II was 4.6, but the sedimentation coefficient of fraction I was concentration-dependent. The sedimentation velocities agreed with those of SHOOTER AND BUTLER[6] on DNA. The amino acid acceptor-activity of fraction I was only 1.5 % when compared with fraction II.

Separation was complete if the sample volume was less than 10 % of the inner volume of the gel. Amounts up to 15 mg of nucleic acids could be separated satisfactorily.

Recovery of the U.V.-absorbing material from the column ranged from 90 to 96 %. No separation was achieved on Sephadex G-75, which is in agreement with the data by BOSCH *et al.*[7]. Phenol, if still present, is also separated from nucleic acids in Sephadex G-200 column[8].

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Decrease of tailing of chromatographic peaks

It is often found that whereas excellent chromatographic separations can be achieved with small quantities of substances, separation deteriorates badly if the load is increased. The usual reason for this phenomenon of overloading is that the partition coefficient of the substance between the mobile and stationary phases changes with its concentration. This is because the presence of other molecules of the same substance affects the partition of any given molecule between the two phases.

An unusually clear-cut case is the partition chromatography of substances with charged molecules on ion exchange resins, because here the partition coefficients depend largely on two factors. The first is the charge on the solute molecule, itself often determined by the pH of the medium. The second is the competition for the sites of the resin that the molecules of solute suffer. Hence the commonest cause of tailing of a peak on a resin is that the competition suffered by each molecule of the substance being chromatographed is appreciably higher in the peak, because of the presence of other charged molecules of the same substances, than at the edges where only the ions of the eluent solution are present. The peak therefore catches up its leading edge, and leaves a tail behind. This is particularly likely when a multivalent substance is being chromatographed with univalent ions in the eluent, because the effective competition may be appreciably raised in the peak, even though the total concentration of ions is not. The use of an eluent strong in both buffering power and concentration of competing ions usually minimizes such effects.

An exception to this was found on attempting to chromatograph 2-hydroxymethyl-3-hydroxypyridine on a sulphonated polystyrene resin. Very bad tailing of the peaks was observed when the crude product of hydroxymethylating 3-hydroxypyridine by the method of URBANSKI[1] was chromatographed in sodium acetate buffer. The pH of the effluent fractions was measured and found to be constant within 0.02 unit. The load was quite low and the concentration of competing sodium ion 0.5 N, so the usual explanations were inapplicable. It was therefore argued that other molecules of the solute might be more effective competitors than sodium ions. They might possess a greater affinity for the resin phase than that due to ionic attraction, because their aromatic rings could also interact with those of the resin. Chromatography was therefore tried in a buffer of pyridine acetate, in the hope that pyridinium ions would compete more equally with the solute ions. This greatly diminished the tailing as shown in Fig. 1.

Experimental and results

Chromatography. Fig. 1a shows the chromatography of the mixture of products in the buffer of 0.1 M acetic acid and 0.5 M sodium acetate. The skew shape of the peaks is clear. Fig. 1b shows chromatography in a buffer of 0.6 M acetic acid and 0.6 M pyridine. The peaks are much sharper. Since this solution had a pH of 5.0, and the pK's of acetic acid and pyridine are 4.7 and 5.3, this solution is expected to have concentration of 0.2 M of the neutral molecules of pyridine and acetic acid, and concentrations of 0.4 M of pyridinium and acetate ions. Even though the pH was lower than in Fig. 1a (5.0 as opposed to 5.4), which should give the solutes a greater positive charge and so retard them further, and the concentration of competing cations is lower (0.4 M as opposed to 0.5 M) also favouring retardation, the peak is

much further forward. This confirms the idea that pyridinium ions are competing with the solutes much more favourably than sodium ions. Fig. 1c shows the result of modifying the sodium acetate buffer to one (0.6 *M* acetic acid and 1.2 *M* sodium acetate) with a concentration of sodium ions high enough to elute the peaks as far forward as in the pyridine–acetic buffer. It is interesting that the peak is still wider in the sodium-ion solution, even though the competition at this sodium-ion concentration must be as high as in pyridine–acetic solution since the peak is as far forward.

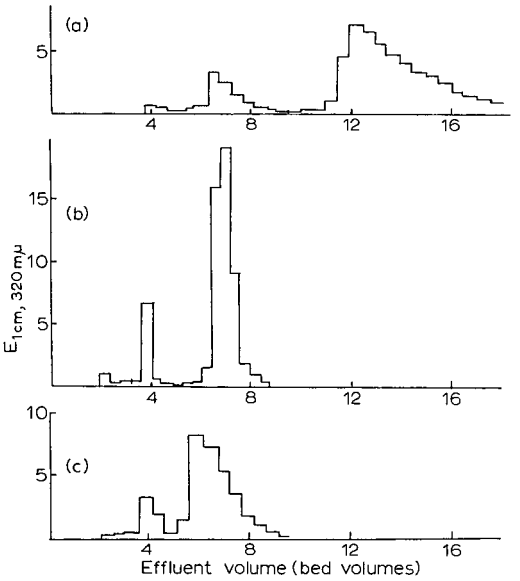

Fig. 1. Chromatograms of pyridine derivatives. A sample of 50 mg of the hydrochlorides of the products of hydroxymethylating 3-hydroxypyridine was applied to the column of 26.2 cm × 0.825 sq. cm sulphonated polystyrene beads of 8% cross linking of under 200 mesh (Zeo-Karb 225, SRC 16), which had been equilibrated, and was eluted at 30 ml cm^{-2} h^{-1}, with one of the following solutions: (*a*) 0.1 *M* acetic acid, 0.5 *M* sodium acetate; (*b*) 0.6 *M* acetic acid, 0.6 *M* pyridine (pH 5.0); (*c*) 0.6 *M* acetic acid, 1.2 *M* sodium acetate (pH 5.0).

Evidently the competition exerted by pyridinium ions is of a different kind. Although the same load was applied to each column the peak was over twice as high in the pyridine–acetic solution. In this solution the column exhibited a resolution of 10 plates per cm calculated by the method of STACK-DUNNE (cited by DIXON[2]). This calculation cannot strictly be applied to skew peaks, but a rough calculation gives about 2 plates/cm for Fig. 1c.

Identification of the substances. On two occasions equal amounts of the two peaks were obtained on treating 3-hydroxypyridine with formaldehyde by URBANSKI's method[1]. A solution of the mixed hydrochlorides (5 g in 100 ml) was run through a column of 20 cm × 3 cm diameter of polystyrene beads, 100–200 mesh, containing quaternary ammonium groups (De-Acidite FF, SRA 71) in the free base form, and displaced with 0.05 *N* HCl at a flow rate of 1.5 ml cm^{-2} min^{-1} (*cf.* PARTRIDGE AND BRIMLEY[3]). Fractions were collected and analysed by paper electrophoresis of 10 μl of each in a volatile buffer at pH 4.4 for 10 min at 80 V/cm in the system of GRAY AND HARTLEY[4]. The hydroxypyridines were detected by the blue fluores-

cence that samples of over 50 μg showed on the dried paper. On the basis of the electrophoretic results the fractions were pooled, acidified with hydrochloric acid, taken to dryness on a rotary evaporator and the hydrochlorides recrystallized by the method of URBANSKI[1] by adding acetone to an aqueous solution. The first material displaced corresponded to the second peak in Fig. 1. On titration of 100 mg of the hydrochloride with 0.1 *N* NaOH it showed p*K*'s of 4.8 and 9.0 and an equivalent weight of 164 ($C_6H_8ClNO_2$ 161.5). This is consistent with its being 2-hydroxymethyl-3-hydroxypyridine, as is the fact that it was the major product in the preparation whose chromatography is shown in Fig. 1, and this was URBANSKI's[1] product. This is supported by the fact that crystallization by his method of the fractions that showed both compounds yielded this same product. The last fractions, containing the substance corresponding with the first peak in Fig. 1, gave a hydrochloride which on titration exhibited p*K*'s of 4.5 and 8.5 and an equivalent weight of 196, and on analysis showed C 44.6% H 5.0% ($C_7H_{10}ClNO_3$ 191.5, C 43.8%, H 5.2%). This is therefore consistent with its being a di-(hydroxymethyl)-3-hydroxypyridine. WILLIAMS[5] identifies it by n.m.r. as 2,6-di-(hydroxymethyl)-3-hydroxypyridine, and confirms that the main compound is 2-hydroxymethyl-3-hydroxypyridine. STEMPEL AND BUZZI[6] and HEINERT AND MARTELL[7] also noted the production of 2,6-di-(hydroxymethyl)-3-hydroxypyridine in URBANSKI's[1] method.

Discussion

Ionic forms of the solutes. The p*K*'s found suggest that these compounds have similar acid-base properties to the 3-hydroxypyridines studies by METZLER AND SNELL[8]. The p*K*'s of 4–5 are therefore probably due to ionization of both the phenolic hydroxyl and the nitrogen-bound proton, with the former predominating. The compounds therefore possessed significant fractions of their molecules in each of the three forms of cation, zwitterion and uncharged molecule under the chromatographic conditions used.

Non-ionic interactions. Although it is clearly unnecessary to call attention to the possibility of specificity in competition for adsorbing sites in fields like enzymology, this possibility may be overlooked with ion exchange resins. Many factors, other than purely ionic ones, will determine the partition of solutes between resin gel and aqueous phases. Unfortunately a custom has grown up of classifying ion-exchange chromatography as a different technique from partition chromatography rather than an example of it. This classification underemphasizes the role of non-ionic interactions in separations on ion-exchange resins. Many of these are important. Thus MOORE AND STEIN[9] pointed out that their separation of amino acids on a sulphonic resin depended on non-ionic as well as ionic interactions. The elution order of glycine, alanine, valine and leucine suggests hydrophobic bonding with the resin phase. They noted that addition of propanol to the eluent selectively accelerated the less polar amino acids. KRESSMAN AND KITCHENER[10] had already pointed out the increased affinity alkyl groups gave ammonium ions for sulphonic resins. SAMSONOV and co-workers (*e.g.* references 11–12) have studied the auxiliary bonds in detail, especially from proton–solute exchange and have measured entropy and enthalpy contributions. DMITRYENKO AND HALE[13] have shown co-operative effects in proton-chlorotetracycline exchange, another departure from ideal behaviour which might be eliminated by using exchange with a more similar substance.

LEDERER AND OSSICINI[14] have clarified the role of salting out in determining the rate at which many ions chromatograph on ion-exchange papers.

DIXON[15] observed change of the partition coefficient of a peptide between a sulphonated polystyrene and an aqueous solution when urea was added to the solution, with the coefficient being altered in favour of the solution. The present example shows that a change in structure of the competing ion may be important, although the nature of the resin phase is also thereby changed.

The tailing of peaks of hydroxypyridine derivatives was greatly decreased when pyridinium replaced sodium cations in competing with these solutes for the sulphonate groups of a resin. This is attributed to a high affinity of pyridinium ions and their derivatives for the resin due to non-ionic interactions. When sodium was the competing cation it would have been impossible to maintain a constant competition as the solute concentration varied.

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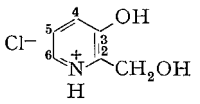
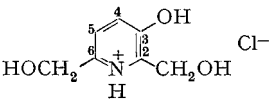
Structure elucidation of mono-(hydroxymethyl)- and di-(hydroxymethyl)-3-hydroxypyridines*

In the previous article, the isolation of mono-(hydroxymethyl)- and di-(hydroxymethyl)-3-hydroxypyridines is described. The plausible structures I and II, respectively, indicated by analysis, equivalent weight and pK values, are established in detail by the n.m.r. spectra of the products as outlined in this accompanying note.



(I)



(II)

The structure of the monosubstituted product is available from its n.m.r. spectra in trifluoroacetic acid and deuterium oxide solutions. In the latter solvent all active hydrogens (OH, NH) are replaced by deuterium; three resonances due to aromatic protons appear in the spectrum, centred at $\delta = 8.31$ p.p.m. (quartet, with splittings of 5 c/s and 2 c/s), $\delta = 8.00$ p.p.m. (perturbed quartet with splittings of 2 c/s and 9 c/s) and $\delta = 7.83$ p.p.m. (perturbed quartet with splittings of ~ 5 c/s and ~ 9 c/s). These resonances are assigned to H-6, H-4 and H-5 respectively. In particular, a large splitting of each of these resonances established that all three protons undergo an *ortho*-interaction. Therefore the hydroxymethyl group is located at C-2 (see I). The methylene protons of the hydroxymethyl group resonate as a sharp singlet at $\delta = 5.40$ p.p.m. in trifluoroacetic acid solution.

The structure of the disubstituted product follows from its n.m.r. spectrum in trifluoroacetic acid solution. Two 2-proton resonances occur as singlets at $\delta = 5.32$ p.p.m. and $\delta = 5.38$ p.p.m. and indicate the presence of two hydroxymethyl substituents. In confirmation, only two proton resonances occur (as an AB system with $J =$ 9 c/s) in the aromatic region at $\delta = 7.89$ p.p.m. and $\delta = 8.25$ p.p.m.** The magnitude of the coupling constant (9 c/s) establishes that the second hydroxymethyl substituent is at C-6 and not at C-4 (see II), since $J_{2,3}$ (or $J_{5,6}$) values are smaller ($\sim$5–6 c/s) in pyridines than the corresponding $J_{3,4}$ (or $J_{4,5}$) values ($\sim$8–9 c/s); the characteristic difference in the J values is probably caused by the electronegativity of the nitrogen atom[1].

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** The pair of lines centered at $\delta = 7.89$ p.p.m. is broadened slightly, presumably due to allylic coupling between H-5 and the methylene protons of the C-6 hydroxymethyl group (see II).

Destaining apparatus for disc electrophoresis gels by current at right angles to gel axis*

In the original apparatus supplied by Canalco** for destaining of disc electrophoretic gel preparations, according to the method of ORNSTEIN[1] and DAVIS[2], the gels are arranged with their long axes vertical, in a glass tube through which current is passed lengthwise. This has been criticized on several grounds: destaining takes hours; it requires that very low current and refrigeration be used to avoid loss of enzyme activity due to heating; diffusion and bowing of the bands occur, however, slightly. To overcome these objections MATSON[3] and SCHRAUWEN[4] have described apparatus whereby the current passes at right angles to the long axis through the column. Their techniques do present very sharply defined bands, even the finest; the current density is not critical and permits a combination of low amperage and short destaining time. The present communication concerns a simple and inexpensive device which may be obtained commercially***, whereby as many as 12 columns of any length up to 4 in. may be accommodated, but which may be used, if desired, for single runs.

This device is made of a plexiglas cylinder, 5 in. high, 4 in. internal diameter, with walls 0.5 in. thick. Twelve holes are bored in this cylinder from top to bottom, their centers being 30° apart and 0.25 in. diameter. The top of this hole is smoothly widened to 7/16 in. to permit easy sliding of the column into place. Opposite each hole, slits 0.25 in. wide and 4.5 in. long are cut through into the plexiglas, from the internal to the external surface of the cylinder. Rims of tygon tubing are made of 0.25 in. external diameter tubing around the cylinder, one just below and the other just above the row of slits, and fixed in place by fitting the ends over a short glass rod. Strips of stainless steel, 0.25 in. wide and 6 in. long, are bent over the tygon rims. These are held firmly by a strip of stainless steel at the upper end and another rim of tygon tubing at the lower end. To the upper fixation strip a lead wire is attached and covered with GE Clearseal plastic. A cover of plexiglas is cut from a plate of convenient thickness and a hole 0.5 in. in diameter is cut in the center. This accommodates a carbon electrode removed from a dry cell battery. This electrode is soldered to a lead wire and the connection covered with GE Clearseal plastic.

To use this device a glass rod of 0.25 in. diameter and 0.5 in. long is placed in the lower end of each hole which is to accommodate a gel column; into all other holes a glass rod of 0.25 in. diameter and 5 in. long is inserted. The gel columns are then slid into the prepared holes, and a length of 0.25 in. glass rod of length suitable to complete the closure of the slit opening is inserted above the gel. In this way all current must pass through the gels. The cylinder may have previously been permanently attached at the bottom to a circular plate of plexiglas by GE Clearseal plastic, or may now merely be set on a solid rubber pad, and the whole placed in a suitable beaker, evaporating dish or the like, and the container and central well of the cylinder filled to within 0.25 in. of the top with 7 % acetic acid. The cover is placed over the cylinder, wires connected to power, and the current, of some 20 mA, allowed to run 20–30 min. If the destaining fluid is continuously run through charcoal the process of destaining may be readily observed and stopped when complete.

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** Canalco, 4935 Cordell Ave., Bethesda, Md. 20014.
*** Plastronic Industries, 35 Alexander Ave., Freeport, N.Y.

Gels, such as 12 % polyacrylamide, swell with this method so that they are not readily removed from their positions, but others are very simply removed by inserting a fine probe beneath the bottom glass rod and lifting. The gels come out easily, without tear or deformation. Even the finest bands are distinct, sharp and flat.

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Use of "lipophilic" Sephadex in peptide synthesis

In the synthesis of peptides the elimination of reaction by-products from the desired peptide derivative is often a cumbersome task. It occurred to us that in cases where the by-products are soluble in organic solvents filtration through methylated Sephadex[1] might be a useful procedure.

As an example we have chosen the widely used "*p*-nitrophenyl ester method" of peptide synthesis[2,3]. One of the advantages of this method is the ease with which it may be ascertained whether or not the main by-product, the yellow *p*-nitrophenol, has been removed. In the early steps of the elongation of a peptide chain, however, the removal of the nitrophenol may be quite time-consuming. It is usually accomplished by repeated extraction of the solution of the reaction mixture in ethyl acetate or ether with aqueous bicarbonate or ammonia. Often some half a dozen extractions are necessary, during which troublesome emulsions may form. Even then traces of nitrophenol are left and for the removal of these filtration through aluminium oxide has been recommended[4].

We have synthesized two peptides, L-leucyl-L-leucine and L-leucyl-L-valine, by the *p*-nitrophenyl ester method, with filtration of the reaction mixtures containing *p*-nitrophenol and benzyloxycarbonyl-L-leucyl-L-leucine methyl ester or benzyloxycarbonyl-L-leucyl-L-valine methyl ester, respectively, through a column of methylated Sephadex. In each case complete separation of the *p*-nitrophenol from the protected dipeptide was achieved. Fig. 1 shows this for the derivative of L-leucyl-L-leucine. The picture was practically identical for benzyloxycarbonyl-L-leucyl-L-valine methyl ester. The latter was isolated in crystalline form. As far as we know it has only been described as a syrup earlier[5].

It is obvious that filtration through lipophilic Sephadex may also find application in analytical work on peptides when derivatives soluble in organic solvents are formed.

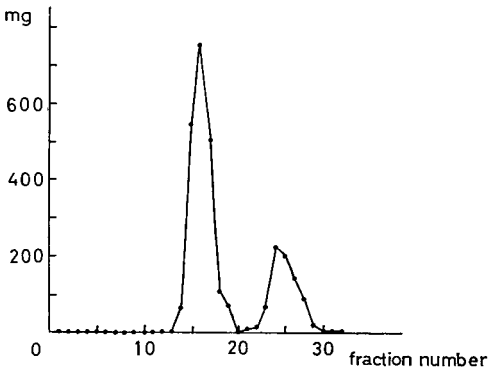

Fig. 1. Filtration of reaction mixture containing *p*-nitrophenol and benzyloxycarbonyl-L-leucyl-L-leucine methyl ester through methylated Sephadex G-25, fine (about 36% –OMe). Column: 4.2 × 43 cm. Solvent: CH_3OH–$CHCl_3$ (1:4). Flow rate: 5 ml/min. Fraction volume: 25 ml. (●—●) = solvent-free weight of fraction. Fractions 22–28 yellow, the others colourless. For details see text.

Experimental

L-Leucine methyl ester hydrochloride was prepared according to HILL *et al.*[6]. L-Valine methyl ester hydrochloride was obtained from Fluka A.G., Buchs, Switzerland. Paper chromatography in the system of WALEY AND WATSON[7] showed that both materials were contaminated with the corresponding free amino acids. For purification the esters were set free from the hydrochlorides by treatment with ice-cold 50% w/v aqueous K_2CO_3 and extracted into ether[8,9]. The ether solutions were dried over anhydrous $MgSO_4$ and filtered. Dry HCl was passed through the solutions whereupon the ester hydrochlorides precipitated in a state of high purity. They were reprecipitated with ether from methanol and dried *in vacuo*.

Benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester was obtained from Fluka A.G. It was recrystallized once from ethanol[3].

The synthesis of benzyloxycarbonyl-L-leucyl-L-leucine methyl ester closely followed the description of the synthesis of benzyloxycarbonyl-L-leucyl-glycine ethyl ester given by BODANSZKY AND DU VIGNEAUD[3]: 6 mmoles of L-leucine methyl ester hydrochloride and 5 mmoles of benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester were dissolved in 5 ml of chloroform and 0.5 mmoles of AcOH[4] and 6.25 mmoles of triethylamine were added to the solution. A precipitate formed but dissolved again. After 24 h at room temperature, 50 ml of ether containing 0.1 ml of concentrated hydrochloric acid were added to the clear, yellow solution. A heavy precipitate formed. After a few hours at +4° this was filtered off and discarded. The ether and chloroform were removed *in vacuo* and the yellow residue taken up in a small volume (*ca.* 5 ml) of MeOH–$CHCl_3$ (1:4). The solution was allowed to sink into a column (4.2 × 43 cm) of methylated Sephadex G-25, fine (*ca.* 36% —OMe), which had been equilibrated with the methanol–chloroform (1:4) solvent. The column was developed with the same solvent. Fractions of 25 ml each were collected at a flow rate of 5 min per fraction. An 0.5 ml aliquot of each fraction was pipetted into a weighed tube and the solvent evaporated in a current of air at 80°. The tubes were reweighed, the difference in weight permitting the calculation of the weight of the solvent-free material in each fraction. The results are evident from Fig. 1. Fractions 22–28 were yellow,

the others colourless. Fractions 14–19 were combined and taken to dryness *in vacuo*. The residue solidified. It had a melting point of about 80°. Crystallization from ether–petroleum ether (b.p. 30–60°) failed to elevate the m.p. significantly. Recrystallization from methanol–water[5], however, gave a material melting sharply at 99.5°. NYMAN AND HERBST[10] found 97–98°, SMITH *et al.*[5] 97.5–98.5°. Yield: 3.2 mmoles.

The synthesis of benzyloxycarbonyl-L-leucyl-L-valine methyl ester followed the same pattern. The residue from evaporation of the solvent from the methylated Sephadex column did, however, not solidify but was a colourless syrup. It was taken up in ethyl acetate and washed consecutively with 0.2 *M* $NaHCO_3$, 0.2 *M* HCl and water. After drying over $MgSO_4$ the ethyl acetate was removed *in vacuo*. The residue, which was still a syrup, was taken up in ether. On the addition of petroleum ether an oily precipitate formed, which crystallized when kept in the cold with vigorous scratching. The m.p. was 70–71° and this did not change on recrystallization of the material from methanol–water. Analysis of the recrystallized material, yield 2.7 mmoles (Firma Analytika, Sollentuna, Sweden) showed: C 63.3%, N 7.3%, calculated for $C_{20}H_{30}O_5N_2$, mol.wt. 378.46: C 63.4% N 7.4%. The isolation of L-leucyl-L-leucine and L-leucine-L-valine from the respective protected peptides followed the directions of SMITH *et al.*[5] and was uneventful. The m.p. was 103.5° for benzyloxycarbonyl-L-leucyl-L-leucine (reported 98–101°[5]) and 113° for benzyloxycarbonyl-L-leucyl-L-valine (reported 108–109°[5]).

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Separation of protected peptides on methylated Sephadex

In a previous paper we have described the preparation and use of methylated Sephadex in the separation of some lipids[1]. Because of the difficulties sometimes encountered in the purification of reaction products in peptide synthesis it appeared of interest to study the behaviour of protected peptides on columns of methylated Sephadex. Some results of this study have been reported[2].

Experimental

Columns of methylated Sephadex G-25 or G-50 were prepared as described previously[1]. Usually 25 g of methylated Sephadex G-25 or 12.5 g of methylated Sephadex G-50 were used for each column. The column diameter was usually kept at 2 cm whereas the column height varied with the solvent used[1].

Protected peptides were generously supplied by Dr. W. RITTEL, Ciba A.G., Basel, Switzerland. The compounds (usually 0.2–0.8 mg) were applied to the top of the column in 0.5–1.0 ml of the same solvent as that used for the preparation and elution of the column. When the sample had entered the gel, solvent was added and the elution performed with a flow rate of 0.5–1.0 ml/min. Fractions of 2–3 ml were collected. The solvents were evaporated and the protected peptide located by a ninhydrin reaction[3] after hydrolysis[4] or by thin-layer chromatography using the chlorine/*o*-tolidine reaction[5]. In all analyses about 0.01 μC of cholesterol-4-^{14}C was added as an internal standard to the sample. Radioactivity was determined by counting an aliquot of each fraction in a gas-flow counter, and the elution volumes of the protected peptides were calculated relative to that of the labeled cholesterol.

Results and discussion

As discussed in a previous paper[1], separations of lipid soluble compounds on methylated Sephadex are determined by several factors. Most important of these appear to be: (a) partition between a stationary gel–solvent phase and the mobile phase; and (b) gel filtration (molecular sieving). Furthermore, the methylated Sephadex contains negatively charged, fixed ions (about 10 μequiv./g in methylated Sephadex G-25, as determined by titration), which may influence the elution rate of compounds with basic and acidic groups (this effect is also seen with regular Sephadex in water[6]). Table I shows the relative elution volumes of some of the protected peptides on methylated Sephadex G-25 and G-50 using chloroform–methanol, 1:1 (v/v), and ethanol–water, 96.5:3.5 (v/v), as the solvents. The results indicate that the effects mentioned may all be of importance in determining the elution volumes of the protected peptides. With the solvents tested gel filtration seems to be most pronounced in chloroform–methanol, 1:1. A plot of relative elution volume in this solvent *versus* log molecular weight is shown in Fig. 1. Two peptides with a free carboxyl group are eluted earlier than peptides of similar molecular weight with protected carboxyl groups. Peptides containing histidine or histidine and nitroarginine are eluted later than peptides of similar molecular weight not having these residues. Occasionally difficulties with tailing of these compounds have been encountered which might indicate adsorption.

With ethanol–water, 96.5:3.5, gel filtration seems to be of minor importance in determining the elution volume. Thus the small peptides a–c (Table I) have elution

TABLE I

ELUTION VOLUMES RELATIVE TO THAT OF CHOLESTEROL OF PROTECTED PEPTIDES ON METHYLATED SEPHADEX G-25 AND G-50 IN TWO SOLVENT SYSTEMS

*Protected peptide**	*Approx. mol. wt.*	*Relative elution volume*			
		G-25		*G-50*	
		Chloroform–methanol (1:1)	*Ethanol–water (96.5:3.5)*	*Chloroform–methanol (1:1)*	*Ethanol–water (96.5:3.5)*
(a) Z·Gly-Gly·OMe	280	1.06	1.55	1.00	1.22
(b) Z·Gly-Gly-Gly·OMe	337	0.97	1.51	0.98	—
(c) Z·Gly-Gly-Gly-Gly·OMe	394	0.93	1.53	1.00	—
(d) Z·Val-Lys(BOC)·OH	479	0.68	0.72	0.78	—
(e) Z·Lys(BOC)-Val-Tyr-Pro·O*t*Bu	795	0.69	0.98	0.86	0.96
(f) H·Lys(BOC)-Val-Tyr-Pro·O*t*Bu	661	0.79	1.00	1.00	1.00
(g) Z·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu	894	0.66	0.93	0.83	0.94
(h) H·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu	760	0.71	0.97	—	0.94
(i) H·Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	436	0.80	1.04	1.00	1.00
(j) Z·Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	669	0.74	0.90	0.89	0.97
(k) Z·Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	782	0.72	—	—	—
(l) Z·Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	761	0.71	0.91	1.00	0.95
(m) Z·His-Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	1032	0.71	0.84	0.86	0.92
(n) H·His-Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	898	0.72	0.78	0.83	0.89
(o) Z·Val-Tyr-Val-His-Pro-Phe·OMe	908	0.76	1.18	1.07	1.06
(p) Z·Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	1109	0.77	—	0.84	—
(q) H·Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	975	0.85	—	0.88	—
(r) Z·Asp(OBzl)-Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	1314	0.74	0.72	0.78	—
(s) Z·Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu	1636	0.60	—	—	—
(t) Z·Glu(O*t*Bu)-Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu	1821	0.50	—	—	—

* Abbreviations: Z = benzyloxycarbonyl; BOC = *tert.*-butyloxycarbonyl; OMe = methyl ester; OBzl = benzyl ester; O*t*Bu = *tert.*-butyl ester; *t*Bu = *tert.*-butyl ether.

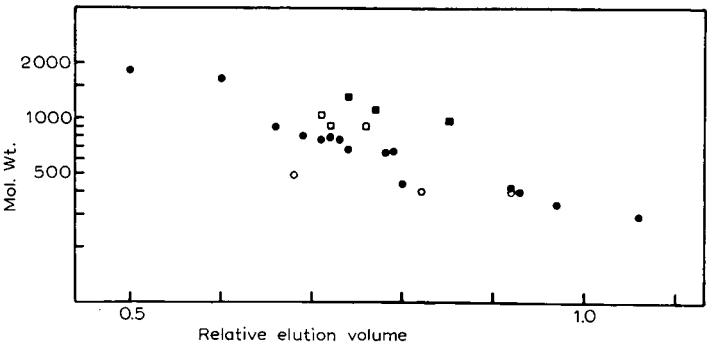

Fig. 1. Plot of relative elution volume *versus* molecular weight. (●) Protected peptides not having histidyl and nitroarginyl residues or a free carboxyl group; (○) peptides with a free carboxyl group; (□) peptides with a histidyl residue; (■) peptides with a histidyl and a nitroarginyl residue. Column: 20 mm. Solvent: chloroform–methanol (1:1); flow rate: 1.0 ml/min.

volumes which are larger than the total column volume (see ref. 1). Partial exclusion from the inner phase seems to occur with peptides above mol. wt. 1000.

With methylated Sephadex G-50 the elution volumes of the peptides studied are closer to that of the reference cholesterol than with methylated Sephadex G-25. This may be due to the higher porosity of this gel giving less separation by gel filtration of the low molecular weight compounds studied.

The results indicate that molecular weight, polarity and charged groups influence the behaviour of protected peptides on columns of methylated Sephadex. In spite of the difficulties involved in predicting the elution volume of a protected peptide it appears, however, that chromatography on methylated Sephadex may be of potential value for the separation and purification of these compounds. Examples of chromatograms are shown in Fig. 2. The method is non-destructive, and since high flow rates can be obtained the separations can be carried out rapidly. Suitable solvent systems may be chosen to increase the difference in polarity between the gel–solvent phase and the mobile phase thereby increasing differences in partition coefficients. It is also possible to use methylated Sephadex of higher or lower polarity by controlling the degree of methylation (the present material had a mean of about 2.3 methoxyl groups per glucose unit[1]).

When the separation factors are small it has been found advantageous to use recycling chromatography[7] permitting the separation of compounds with a separation factor of 1.02–1.03.

Chromatography on methylated Sephadex has been used on a gram scale for removal of *p*-nitrophenol from reaction mixtures in peptide syntheses with the *p*-nitrophenyl ester method[9]. Analysis of microgram amounts can be carried out in capillary columns. The separation shown in Fig. 3 was obtained with a column of methylated Sephadex G-25, superfine, packed in chloroform–methanol–heptane, 1:1:2, in a teflon tubing 1750 × 1.5 mm. The sample was injected in 10 μl of solvent[8]. Material in the effluent was detected with a modified flame ionization detector[10] kindly lent to us by Dr. E. HAAHTI.

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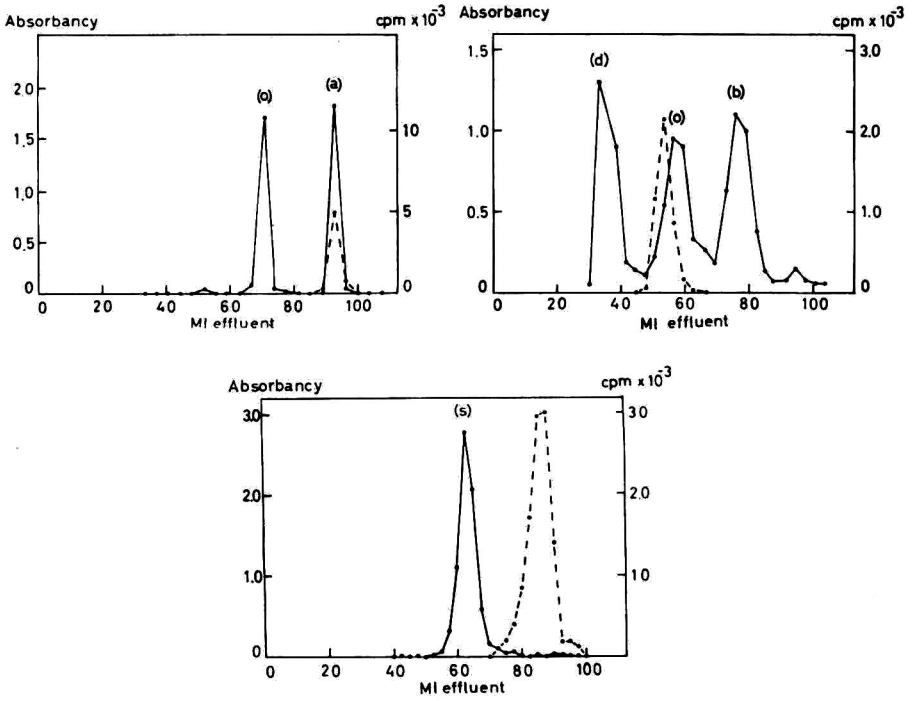

Fig. 2. Upper left curve: Separation of Z·Val-Tyr-Val-His-Pro-Phe·OMe (0.46 mg; o), Z·Gly-Gly·OMe (0.44 mg; a), and cholesterol-4-^{14}C (trace amount: broken line). Column: 25 g methylated Sephadex G-25, 1580 × 8 mm. Solvent: chloroform–methanol (1:1); flow rate: 0.37 ml/min. Fractions of 3.7 ml collected. Upper right curve: Separation of Z·Val-Lys(BOC)·OH (0.27 mg; d), Z·Val-Tyr-Val-His-Pro-Phe·OMe (0.26 mg; o), Z·Gly-Gly-Gly·OMe (0.26 mg; b), and cholesterol-4-^{14}C (trace amount: broken line). Column: 20 g methylated Sephadex G-25, 230 × 20 mm. Solvent: ethanol–water (96.5:3.5); flow rate: 0.8 ml/min. Fractions of 3.0 ml collected. Lower curve: Separation of Z·Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu (0.49 mg; s), and cholesterol-4-^{14}C (trace amount: broken line). Column: 12.5 g methylated Sephadex G-50, 390 × 20 mm. Solvent: chloroform–methanol (4:1); flow rate 0.5 ml/min. Fractions of 2.5 ml collected.

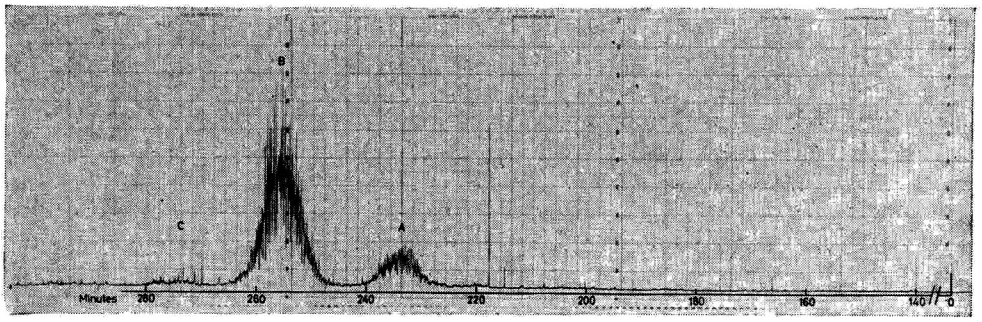

Fig. 3. Separation of Z·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu (about 10 μg; A) and Z·Lys(BOC)-Val-Tyr-Pro·O*t*Bu (about 30 μg; B). The peak eluted at C is an impurity. Column: methylated Sephadex G-25, superfine, 1750 × 1.5 mm. Solvent: chloroform–methanol–heptane (1:1:2), flow rate: 0.57 ml/h. The effluent was monitored with a flame ionization detector[10].

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Separation of vitamins K_2 on capillary columns of methylated Sephadex

In the course of studies of the potential uses of methylated Sephadex for the separation of lipid soluble compounds[1] it was considered of interest to study the separations that could be obtained with a series of isoprenologue compounds. A series of vitamins K_2 were chosen since the effluent could be easily monitored by measurement of the U.V. absorption.

Experimental

Vitamins $K_{2(10)}$–$K_{2(40)}$ were generously supplied by Drs. O. WISS AND U. GLOOR, Hoffmann-la Roche, Basel, Switzerland.

Sephadex G-25 fine and superfine (kindly supplied by Dr. B. GELOTTE, Pharmacia, Uppsala, Sweden) were methylated as described previously[1]. Columns having a diameter of 2 cm were prepared with about 25 g of methylated Sephadex G-25, fine[1]. The samples (0.2–1 mg) were applied to the columns in 0.5–1 ml solvent. Capillary columns were prepared in teflon tubing (outer and inner diameters 2.3 and 1.5 mm, respectively) in the following way:

A small piece of glass wool and a 2 cm piece of stainless steel capillary tube (O.D. $^1/_{16}$ in., I.D. 0.25 mm, cut to a tip in the distal end) were inserted into the distal end of a teflon tubing about 2 m in length. The tubing was filled with the solvent to be used for the chromatography. The proximal end was connected with a stainless steel tubing (O.D. $^1/_{16}$ in., I.D. 0.6 mm, length 5 cm) silver soldered to a stainless steel cylindrical reservoir (O.D. 30 mm, length 100 mm) which contained a slurry of methylated Sephadex G-25, superfine, in the same solvent. The upper end of the

cylindrical reservoir was connected to a nitrogen tank and a pressure of about 1–2 kp/cm² was applied. The slurry passed slowly through the capillary into the teflon tubing. Clogging was prevented by vibrating the reservoir with a Vibro-graver (Burgess Vibrocrafters Inc., Grayslake, Ill.). When the teflon tubing was completely filled with the gel the pressure was released and the tubing was disconnected from the reservoir. An injection port (see Fig. 1) was attached to the proximal end of the teflon column and connected to another cylindrical reservoir (38 × 300 mm) which contained the solvent to be used. Pressure, 1–3 kp/cm² (depending on the solvent used and the length of the column), was applied to the system.

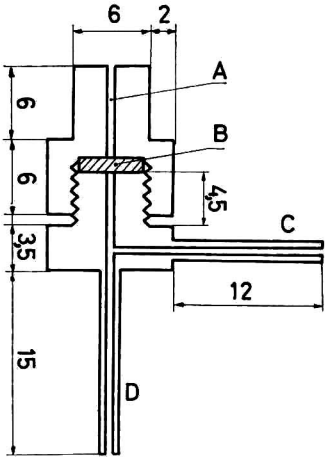
The sample was dissolved in a suitable solvent and about 5–50 μg in about 5 μl was injected into the column (usually about 10 mm below the gel surface at the top of the column). The flow rate was kept at 0.5–0.6 ml/h.

Vitamins K_2 appearing in the effluent were determined by measurement of the absorption at 270 nm. With the capillary columns, U.V. measurements did not permit the collection of fractions small enough to make use of the full separating efficiency of the columns. At a later stage of the work Dr. E. HAAHTI kindly lent us one of his platinum chain–flame ionization detectors[2]. This permitted a continuous monitoring of the effluent from the capillary columns.

Results

Several different solvent mixtures were tried. Satisfactory results were obtained with chloroform–methanol–heptane, 1:1:2. The separation of vitamins $K_{2(40)}$, $K_{2(20)}$ and $K_{2(10)}$ on a 25 g column is shown in Fig. 2. Since only small amounts of vitamins K_2 were available to us the capacity of the columns could not be tested.

The separation of vitamins $K_{2(40)}$–$K_{2(10)}$ on a capillary column is shown in Fig. 3. It is possible that a higher column efficiency would be obtained with methyl-



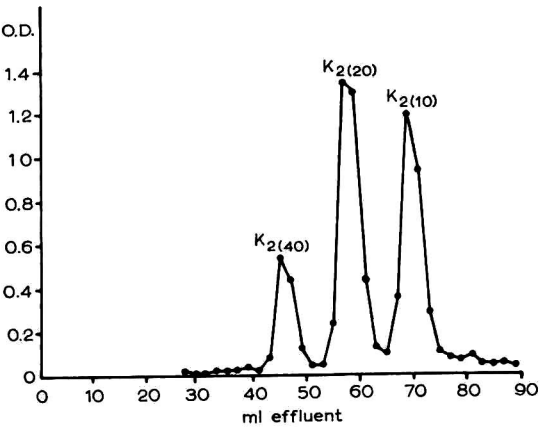


Fig. 1. Schematic drawing of stainless steel injection port used for capillary columns. All dimensions are in mm. (A) 0.6 mm hole guiding the 50-mm needle of a Hamilton syringe during sample injection; (B) silicone rubber membrane; (C and D) capillary tubing, $^1/_{16}$ in. O.D. 0.6 mm I.D., connected to the teflon column (D) and to the solvent reservoir (C).

Fig. 2. Separation of 0.2–0.3 mg each of vitamins $K_{2(40)}$, $K_{2(20)}$ and $K_{2(10)}$ on a 30 × 2 cm column of methylated Sephadex G-25 in chloroform–methanol–heptane (1:1:2). Flow rate: 0.7 ml/min.

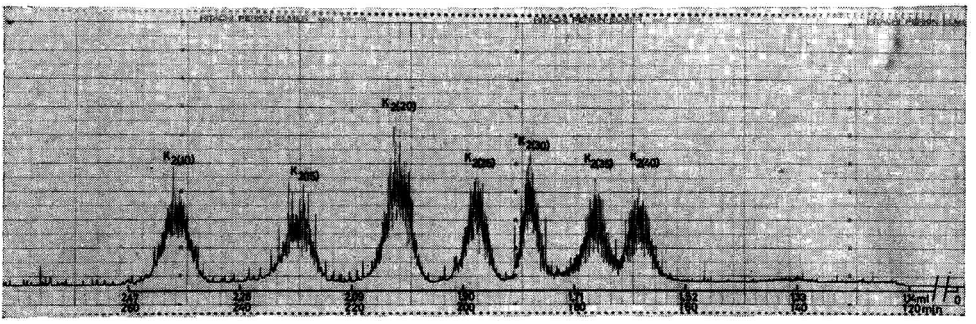


Fig. 3. Separation of about 20 μg each of vitamins $K_{2(40)}$–$K_{2(10)}$ on a 1750 × 1.5 mm column of methylated Sephadex G-25, superfine, in chloroform–methanol–heptane (1:1:2). The effluent was monitored with a flame ionization detector[2].

ated Sephadex having a more uniform particle size than the superfine grade used in this column.

The mechanisms responsible for the separations have not been elucidated. The compounds are eluted in an order of decreasing molecular weight and in an order of increasing polarity. From this and previous studies[1] it appears reasonable to assume that the separations are effected through a combination of gel filtration and partition chromatography between a stationary gel–solvent phase and a less polar mobile phase.

Several methods for the separation of vitamins K_2 have been published previously (for a review see ref. 3). The present technique using methylated Sephadex could be used as a complement to these methods. It is non-destructive, and the separations can be carried out rapidly on a micro- or macroscale. The columns can be used repeatedly over long periods of time and in this respect chromatography on methylated Sephadex can be compared with gas–liquid chromatography which is less suitable for the separation of vitamins K_2.

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Column chromatography of some methionine peptides

Column chromatography is useful for the separation of peptides, but little systematic information is available to help predict elution time. The present note is a step in this direction; it describes the elution behavior and color yield of 22 methionine peptides.

Experimental

Compounds. Samples of chromatographically homogeneous L-peptides, analyzed for C, H, N and S and with specific rotation data, were obtained from Mann Research Laboratories, Inc., New York City.

Chromatography. Chromatography was done with a commercial semiautomatic analyzer system (Technicon Corp., Ardsley, N.Y.) by the regular procedure recommended by the manufacturer, salient details of which are given elsewhere[1]. Compounds were run repeatedly, in amounts varying between 0.01 and 0.80 μmole and the peaks independently measured on two instruments, both by dot-count and automatically with a CRS-10A digital integrator (Infotronics Corp., Houston, Texas). Cysteic acid was used as the standard[2]. It emerges with the sample front, at one void volume, and elution times were measured from this peak. Molar color yields are in terms of the ratio of peak area for peptide to peak area for cysteic acid.

TABLE I

ELUTION TIME AND COLOR YIELD OF 22 METHIONINE PEPTIDES

Peptide	*Elution time (min after cysteic acid)*	*Molar color yield peptide/ cysteic acid*	*Emerges near or between*
MetAsp	494	1.22	Met-Ileu
MetSer	540	1.12	Ileu-Leu
MetGlu	581	1.11	Leu-Tyr
MetGlyGly	614	1.08	
MetGly	614	0.91	Tyr-Phe
GlyMet	619	0.61	
AlaMet	623	1.34	
MetAla	636	1.08	Tyr-βAIB
MetMetAla	664	0.91	βAIB-Eth-NH_2
MetGlyMetMet	683	0.64	
ValMet	684	0.24	
MetGlyMet	690	0.82	Eth-NH_2
MetLeuGly	706	1.0*	
MetHis	715	0.8*	NH_3
MetVal	718	1.2*	
MetMet	723	1.08	OH–Lys
MetMetMet	751	0.93	
MetLeu	771	0.97	
MetTyr	832	0.95	Orn
MetPhe	870	0.90	
MetPheGly	870	0.88	Lys
PheMet	887	0.92	Try

* Approximate; ammonia interference.

Results and discussion

Table I shows the results obtained. The nearest amino acids are indicated, for comparison (methionine emerges at 470 min). A number of elution data for other peptides are available in the literature[3,4], the methods used being sufficiently similar to, but not exactly the same, as that used here.

Inspection of the assembled information suggests some tentative empirical generalizations concerning peptide chromatography on cation exchange resins. (*a*) Neutral dipeptides having the same N-terminal substituent appear in the same sequence as the C-terminal substituent would if it were free, but later (MetHis is an apparent exception). With HAMILTON's[3] system the time difference is regularly about 210 min; with the Technicon system the difference decreases with increasing elution time and ranges from 350 to 220 min. If the N-terminal substituent is glutamyl, the order is generally the same, but the dipeptide may appear before either residue would if it were free or, with increasing elution time for the carboxyl substituent, between the positions the two would occupy as free amino acids. Glu-β-amino acid peptides emerge earlier than their sequence as free amino acids would suggest. (*b*) Neutral dipeptides with the same C-terminal residue appear in the sequence the N-terminal residue would if it were free, but 120–300 min later. (*c*) Reversal of substituents of neutral dipeptides (for example, AlaMet and MetAla) does not greatly affect elution time. (*d*) Neutral di- and tripeptides of similar structure (for example, MetAla and MetMetAla) emerge closely. (*e*) Molar color yield decreases with increasing chain length. (*f*) Effect of increasing chain length on elution time is not empirically predictable.

It will be useful to see how well future results with other peptides bear out these suggestions.

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Separation of aliphatic dicarboxylic acids by thin-layer chromatography (TLC)

For the determination of the position of the double bond in monoethenoid fatty acids, the most commonly employed method is oxidation with permanganate–periodate reagent and subsequent identification of the dicarboxylic acid fragment. Melting point determinations are not often sharp even after several recrystallizations. PHATAK *et al.*[1] and SUBBARAM[2] among others[3–5] used paper chromatographic methods for this purpose, but these are rather slow and tedious, and recently quicker TLC procedures have been used. Thus BRAUN AND GEENEN[6] separated the dicarboxylic acids from oxalic to sebacic on silica gel plates using an alcohol–ammonia–water mixture for development. PETROWITZ AND PASTUSKA[7] used two different solvent systems, consisting of benzene–dioxane–acetic acid and benzene–methanol–acetic acid. Separations obtained by these two methods are satisfactory for C_2–C_5 acids but only marginal for C_6–C_{10} acids. The use of polyethylene glycol (molecular weight 1000) as stationary phase, sodium diethyldithiocarbaminate as antioxidant and a mixture of di-isopropyl ether–formic acid–water as developing solvent gave good separations[8]. Both the stationary phase and antioxidant are not readily available. In this note we describe a TLC method on a silica gel G plate for the separation of aliphatic dicarboxylic acids. The developing solvent used here is a composite of various solvent mixtures earlier employed by KALBE[4] in paper chromatographic separations. The present procedure has been in routine use in this laboratory for two years for structure determinations of unsaturated fatty acids, and for location of hydroxyl group position in a carbon chain following catalytic hydrogenation of epoxy fatty acids.

Experimental

All compounds were of high purity. The acids in either methanol or methanol–water (1:1) solution were spotted on a 20 × 20 cm glass plate coated with silica gel G (250 μ) and developed (1 h) with a solvent system consisting of *n*-butanol, xylene, phenol, formic acid and water (10:70:30:8:2, v/v). The plates were then removed, dried at 150° for 30 min, cooled and sprayed with an alcoholic solution of bromocresol

TABLE I

TLC OF DICARBOXYLIC ACIDS OF C_2–C_{10} CHAIN LENGTH

Solvent system: Xylene–phenol–butanol–formic acid–water (70:30:10:8:2, v/v).

Acid	*Carbon chain length*	$R_F \times 100$ *observed*
Oxalic	2	0
Succinic	4	15
Glutaric	5	20
Adipic	6	25
Pimelic	7	31
Suberic	8	38
Azelaic	9	44
Sebacic	10	49

purple containing a little alkali, when the acids appeared immediately as yellow spots on a purple background. The exact position of the spots was outlined quickly before the colour contrast lessened by fading.

Results

Table I shows the results. The dicarboxylic acids separate well with the solvent system mentioned (Table I), the $R_F \times 100$ values increasing from the lower to the higher homologues in a regular manner with a difference of about 6 units between adjacent members. For specific separation of dicarboxylic acids of C_8–C_{13} chainlength, which are the most commonly encountered, a better spread of R_F values was sought by alterations in the proportions of the solvent components. The proportions of formic acid and water in the combined solvent system were kept constant, and the effect of

TABLE II

TLC OF HIGHER DICARBOXYLIC ACIDS WITH VARYING SOLVENT PROPORTIONS

	Solvent system, v/v					
	1	*2*	*3*	*4*	*5*	*6*
Xylene	70	70	70	90	65	65
Phenol	30	30	70	20	50	50
Butanol	10	20	50	10	15	15
Formic acid	8	8	8	8	8	8
Water	2	2	2	2	2	2
Ether	—	—	—	—	—	7

Acid		$R_F \times 100$ *values*					
		1	*2*	*3*	*4*	*5*	*6*
Suberic	C_8	38	56	57	42	48	48
Azelaic	C_9	44	62	64	45	56	56
Sebacic	C_{10}	49	67	70	50	62	63
Brassylic	C_{13}	—	—	—	—	75	76

alterations in the proportions of the remaining solvents examined (Table II). Increase in the proportion of either butanol or phenol, especially the former, caused a strong forward movement. Increase of phenol and butanol together rather surprisingly had only a small effect. Increase of xylene and addition of ether as an extra component had little effect on movement. Solvent systems 5 or 6 yielded the best spread in the spots.

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Quantitative Bestimmung von Harnstoff in Serum

Spektralphotometrische Bestimmung nach dünnschichtchromatographischer Abtrennung auf Celluloseschichten

In einer früheren Arbeit[1] beschrieben wir eine quantitative Bestimmung des Harnstoffgehaltes im Harn. Die Methode kann auch auf Serum angewandt werden.

Hierzu wird das Serum zunächst enteiweisst. In einem Zentrifugenglas werden 2.00 ml Serum mit 4 ml 96%igem Äthanol gemischt und zentrifugiert. Die überstehende Flüssigkeit wird in ein Glasschälchen abgegossen und das Zentrifugenglas mit etwas Äthanol nachgespült. Die vereinigten Flüssigkeiten werden vorsichtig abgedampft. Der Rückstand wird in 0.2 ml Wasser (genau abgemessen mit der Agla-Pipette) aufgenommen. 0.03 ml dieser Lösung und ebenso 0.03 ml Standardlösung werden in der gleichen Weise behandelt wie bei der Bestimmung des Harnstoffgehaltes im Harn beschrieben.

Der Gehalt wird entweder mit Hilfe der Standardlösung bestimmt oder der Eichkurve entnommen, die man erhält, wenn man jeweils 0.03 ml verschiedener Verdünnungen einer wässrigen Lösung von 1.8000 g Harnstoff/100.00 ml aufträgt. Die weitere Bestimmung erfolgt gemäss den in der oben zitierten Arbeit gemachten Angaben.

Die wiedergefundene Menge betrug 90.2 % (± 1.39 %).

Harnstoff-Standardlösung: 0.1000 g Harnstoff werden in Wasser zu 100.00 ml gelöst.

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Quantitative Bestimmung von Harnsäure in Harn und Serum

Spektralphotometrische Bestimmung nach dünnschichtchromatographischer Abtrennung

Die in der Literatur zur Bestimmung des Harnsäuregehalts von Harn und Serum angegebenen Methoden beruhen z.T. auf der reduzierenden Wirkung der Substanz. Da aber auch andere Stoffe die verwendeten Reagenzien reduzieren können, sind die Bestimmungen mit Fehlermöglichkeiten behaftet.

Um sie auszuschalten, versuchten wir, die Harnsäure zunächst aus Harn und Serum dünnschichtchromatographisch abzutrennen. Das gelang uns durch Verwendung von Cellulose-Schichten (mit Fluoreszenzzusatz) und Isopropanol–10 %ige wässrige Piperazinlösung (1:2, V/V) als Fliessmittel.

Nach Extraktion und Umsetzung mit Arsenphosphorwolframsäurereagenz wurde der Gehalt spektralphotometrisch bestimmt.

Material

Sorptionsschicht. Zur Herstellung der Sorptionsschicht wurden 15 g Cellulosepulver F_{254} (Macherey, Nagel & Co.) mit 90 ml Wasser 2 Min. lang in einem elektrischen Mixgerät homogenisiert und mit Hilfe eines Streichgeräts auf die Platten gebracht (ausreichend für 5 Platten 200 × 200 mm, Schichtdicke 0.25 mm). Die beschichteten Platten wurden an der Luft getrocknet.

Fliessmittel. Als Fliessmittel diente Isopropanol–10%ige wässrige Piperazinlösung (1:2, V/V).

Es wurde bei Raumtemperatur und Kammersättigung chromatographiert. Die Laufzeit betrug 4 Stunden bei einer Trennstrecke von 150 mm. Nach dem Entwickeln wurden die Platten an der Luft getrocknet. Der Nachweis der Harnsäure erfolgte im kurzwelligen U.V.-Licht. Sie war als dunkler Fleck (Fluoreszenzlöschung) auf fluoreszierendem Untergrund zu erkennen.

Die Reagenzien zur quantitativen Bestimmung wurden folgendermassen hergestellt:

Glyzerin–Silikat-Reagenz. 6 Vol. Teile 10 %ige wässrige Natriumsilikatlösung wurden mit 1 Vol. Teil Glyzerin gemischt.

Arsenphosphorwolframsäurereagenz. 100 g Natriumwolframat wurden in 600 ml Wasser gelöst und mit 50 g Arsenpentoxid, 25 ml 85 %iger Phosphorsäure und 20 ml konzentrierter Salzsäure versetzt. Die Mischung wurde 20 Min. lang unter Rückfluss gekocht. Nach dem Erkalten wurde mit Wasser auf 1 l aufgefüllt.

Harnsäure-Standardlösungen. Für die Bestimmung des Harnsäuregehalts im Harn wurde eine Lösung von 0.0200 g Harnsäure/100.00 ml in 10 %iger wässriger Piperazinlösung als Standardlösung verwendet.

Als Standardlösung für die Bestimmung des Gehalts im Serum diente eine Lösung von 0.0150 g Harnsäure/100.00 ml in 10 %iger wässriger Piperazinlösung.

Bestimmung des Harnsäuregehalts im Harn

0.05 ml Harn wurden mit einer Agla-Mikrometerspritze bandförmig auf die Platten aufgetragen, ebenso 0.05 ml Standardlösung. Nach dem Entwickeln wurden die Platten an der Luft getrocknet. Die Flecke wurden im kurzwelligen U.V.-Licht

markiert und ausgeschabt. Als Blindwert diente ein gleich grosser Fleck Cellulose. Nun wurde jeweils mit 4 ml Wasser extrahiert und unter Druck durch eine Mikroglasfilternutsche G1 in ein 5 ml-Messkölbchen filtriert. Nach Zugabe von 5 Tropfen einer kalt gesättigten wässrigen Piperazinlösung wurde mit Wasser genau bis zur Marke aufgefüllt.

2.00 ml dieser Lösungen wurden mit 2.60 ml Glyzerin–Silikat-Reagenz und 0.40 ml Arsenphosphorwolframsäure-Reagenz versetzt. Nach 15 Min. war die maximale Farbintensität der Lösungen erreicht, die 10 Min lang bestehen blieb. Die Messung erfolgte bei 700 mμ (Zeiss Spektralphotometer PMQ II; 1 cm-Küvetten).

Der Gehalt wurde an Hand der in gleicher Weise behandelten Standardlösung bestimmt. Er konnte aber auch einer Eichkurve entnommen werden, zu deren Aufstellung jeweils 0.05 ml verschiedener Verdünnungen einer Lösung von 0.2400 g Harnsäure/100.00 ml in 10 %iger wässriger Piperazinlösung aufgetragen und weiterbehandelt wurden wie oben beschrieben.

Der mittlere Fehler der Bestimmungen betrug ± 0.90 % (mittlerer Fehler des Mittelwerts aus 10 Bestimmungen).

Bestimmung des Harnsäuregehalts im Serum

In einem Zentrifugenglas wurden 3.00 ml Serum mit 6 ml 96 %igem Äthanol gemischt und zentrifugiert. Die überstehende Flüssigkeit wurde in ein Glasschälchen abgegossen und das Zentrifugenglas mit etwas Äthanol nachgespült. Die vereinigten Flüssigkeiten wurden vorsichtig abgedampft. Der Rückstand wurde in 0.2 ml 10 %iger wässriger Piperazinlösung (genau abgemessen mit einer Agla-Mikrometerspritze) aufgenommen.

0.05 ml dieser Lösung wurden mit einer Agla-Mikrometerspritze bandförmig auf die Platten aufgetragen, ebenso 0.05 ml Standardlösung. Die weitere Bestimmung erfolgte in der gleichen Weise, wie sie oben für Harn beschrieben wurde.

Den Gehalt berechneten wir mit Hilfe der Standardlösung oder entnahmen ihn einer Eichkurve. Zu ihrer Aufstellung trugen wir jeweils 0.05 ml verschiedener Verdünnungen einer Lösung von 0.1800 g Harnsäure/100.00 ml in 10 %iger wässriger Piperazinlösung auf und arbeiteten nach den bei der Bestimmung im Harn gemachten Angaben weiter.

Die wiedergefundene Menge betrug 80.3 % (± 1.8 %).

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Separation of the methyl esters of benzenecarboxylic acids by thin-layer chromatography

Benzenecarboxylic acids, resulting from vigorous oxidation of alkylated benzenes and polynuclear compounds have frequently been utilized to provide structural evidence regarding the parent substance.

These acids were formerly isolated by conversion to methyl esters, followed by separation *via* fractional crystallization or distillation. This method is not well suited to a qualitative and quantitative analysis of mixtures of ^{14}C-labelled benzenecarboxylic acids.

The methyl esters of benzene mono-, di-, tri-, tetra-, penta- and hexacarboxylic acids have been well resolved by gas chromatography, but the separation of the methyl esters of isomeric di-, tri- and tetracarboxylic acids has been incomplete[1].

This communication describes the application of thin-layer techniques to the separation of the methyl esters of benzenecarboxylic acids.

Experimental

The acids were obtained commercially with the exception of the 1,2,3,4-benzenetetracarboxylic acid, which was prepared as described by READ AND PURVES[2]. The acids were converted to the corresponding methyl esters by treatment with diazomethane generated from "Diazald". The resulting liquid esters were purified by distillation while the solid esters were recrystallized to constant melting point from methanol.

Preparation of plates. A slurry of "Camag" (30 g) in water (65 ml) was applied to 5 × 20 and 20 × 20 cm glass plates using a Brinkmann spreader. The plates were dried at 110° for 45 min.

Chromatography. Several systems were examined and the following proved to be most satisfactory for the separation of the mixture.

TABLE I

R_T VALUES OF METHYL ESTERS OF BENZENECARBOXYLIC ACIDS

Solvent: 100% chloroform.

$$R_T = \frac{\text{migration distance of methyl ester}}{\text{migration distance of the trimethyl ester of 1,3,5-benzenetricarboxylic acid}}$$

No.	*Methyl ester of*	*R_T value*
1	Benzoic acid	1.78
2	Phthalic acid	1.13
3	Isophthalic acid	1.41
4	Terephthalic acid	1.39
5	1,2,3-Benzenetricarboxylic acid	0.59
6	1,2,4-Benzenetricarboxylic acid	0.87
7	1,3,5-Benzenetricarboxylic acid	1.00
8	1,2,3,4-Benzenetetracarboxylic acid	0.47
9	1,2,3,5-Benzenetetracarboxylic acid	0.63
10	1,2,4,5-Benzenetetracarboxylic acid	0.69
11	Benzenepentacarboxylic acid	0.53
12	Benzenehexacarboxylic acid	0.50

A solution (1 %) of each isomer in methanol–chloroform (20:80) was prepared. The resulting solutions were spotted (10 μl; 100 μg) on a thin layer of silica gel containing a fluorescent indicator as prepared by STAHL. The chromatoplate was developed with chloroform (100 %) and observed under a 254 mμ U.V. light in the dark. Blue spots of varying intensity appeared against a greenish background.

Discussion

Chromatographic data for various methyl esters of benzenecarboxylic acids are summarized in Table I. Migration rates are given as R_T values (where R_T is the migration distance of the ester divided by the migration distance of the trimethyl ester of 1,3,5-benzenetricarboxylic acid) rather than as R_F values.

While gas chromatography has provided an effective method for the qualitative analysis of these esters, it has been of limited value in effecting a quantitative separation. The TLC technique described has provided a means of overcoming this limitation.

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Thin-layer chromatography of imino-acids

Imino-acids in plants are of three main types, based on the 4-, 5-, and 6-membered ring systems of azetidine, pyrrolidine and piperidine, respectively. Azetidine-2-carboxylic acid is widely distributed in the *Liliaceae* and occurs in a few species of *Agavaceae*, but is otherwise a rare plant component[1]. Of the pyrrolidine compounds, 4-hydroxyproline is a constituent of a few plant proteins, and proline is of widespread occurrence. Several piperidine compounds have been isolated from plants, including piperidine-2-carboxylic acid[2,3], 5-hydroxypiperidine-2-carboxylic acid[4], 4-hydroxypiperidine-2-carboxylic acid[5] and 1,2,3,6-tetrahydropyridine[3]. In several of the papers describing the characterisation of these piperidine compounds, evidence has been obtained by comparing the chromatographic characteristics of the unknown compound with synthesised reference compounds[2–6]. The chromatographic methods described have been with paper, but the solvent systems used have given only small differences in R_F values between the compounds, and hence the time of development required for effective separation of the compounds was lengthy. In recent work on *Salix fragilis* L. leaf galls, a new imino-acid was encountered, probably based on piperidine. For the study of this compound, thin-layer chromatographic methods were developed which gave discrete and rapid separations of the different imino-acids.

Experimental and results

Air dried silica gel G (Merck) layers, 250 μ thick, on glass plates 20 × 20 cm were used. The test compounds, in aqueous solution, were applied near one corner of the plate, the spot was dried with a hot air blower and developed using the supersaturated method of STAHL[7]. Two-way chromatograms were prepared using solvent systems that have been used successfully for the separation of amino-acids[8]. For the first direction, development was with chloroform–methanol–17 % ammonia (2:2:1, v/v), and for the second direction the solvent used was either (a) propanol–water (64:36, w/w), (b) *n*-butanol–acetic acid–water (60:20:20, w/w), or (c) phenol–water (4:1, w/w). Development in each direction was for 15 cm from the point of application of the spot. The spots were detected with an 0.1 % w/v solution of ninhydrin in acetone and with an 0.2 % w/v solution of isatin in *n*-butanol containing 4 % acetic acid. The colours were developed by heating the plates at 100° for 10 min (Table I). The compounds examined were piperidine-2-, piperidine-3-, and piperidine-4-carboxylic acids;

TABLE I

IMINO-ACID COLOURS PRODUCED WITH ISATIN AND NINHYDRIN

Compound	*Isatin*	*Ninhydrin*
Piperidine-2-carboxylic acid	blue-green	mauve to blue
Piperidine-3-carboxylic acid	blue-green	mauve to blue
Piperidine-4-carboxylic acid	blue-green	mauve to blue
4-Hydroxypiperidine-2-carboxylic acid	weak green	yellow changing to blue
5-Hydroxypiperidine-2-carboxylic acid	blue-green	mauve to blue
Compound from *Salix fragilis* galls	blue-green	mauve to blue
4-Hydroxyproline	blue-green	yellow changing to pink
Proline	green-blue	yellow

4-hydroxy- and 5-hydroxypiperidine-2-carboxylic acids; 4-hydroxyproline, proline and an imino-acid from *Salix fragilis* leaf galls, probably based on piperidine. Using the two-way chromatographic systems described, all the imino-acids tested were easily separated and clearly identified (Fig. 1).

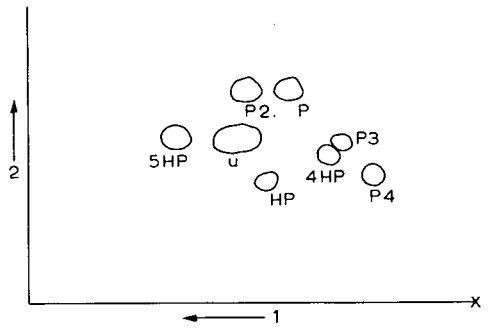

Fig. 1. Thin-layer chromatogram of imino-acids. 5HP = 5-hydroxypiperidine-2-carboxylic acid; 4HP = 4-hydroxypiperidine-2-carboxylic acid; P2 = piperidine-2-carboxylic acid; P3 = piperidine-3-carboxylic acid; P4 = piperidine-4-carboxylic acid; P = proline; HP = 4-hydroxyproline; u = compound from *S. fragilis* galls. Solvent systems; (A) chloroform–methanol–17% ammonia (2:2:1, v/v) and (B) phenol–water (4:1, w/w).

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We wish to thank Prof. A. VIRTANEN for the gift of samples of 4-hydroxy- and 5-hydroxypiperidine-2-carboxylic acid and Prof. CLARK-LEWIS for a sample of 4-hydroxypiperidine-2-carboxylic acid.

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An improved method for charring lipids on thin-layer chromatograms

Lipids separated on thin-layer plates may be visualised by destructive "charring" with one of the dehydrating acids (sulphuric, phosphoric or chromic). This is normally sprayed on to the thin-layer plate; the plate is then heated to 120–200° to char lipids present.

The major advantage of this method is that the position of the lipid is determined unambiguously; disadvantages are that further analysis is impossible; the surface of the thin layer is pitted by the small but heavy droplets of acid and spraying may be uneven. The latter two facts caused some concern during our attempts to quantitate lipids by a method[1] involving thin-layer separation on microscope slides, charring, and determination of resultant optical density.

We therefore attempted to generate the acid on the thin layer by interaction of a volatile halide or oxide with water vapour. Any of the following compounds can be hydrolysed to the acid named:

(1) $PCl_5 + H_2O \longrightarrow POCl_3 + 2\ HCl$
Phosphorus pentachloride (solid, sublimes 162°) → Phosphorus oxychloride

$POCl_3 + 3\ H_2O \longrightarrow H_3PO_4 + 3\ HCl$
Phosphorus oxychloride (b.p. 105°C) → Phosphoric acid

(2) $CrO_2Cl_2 + 2\ H_2O \longrightarrow H_2CrO_4 + HCl$
Chromyl chloride (liquid, b.p. 117°) → Chromic acid

(3) $SO_2Cl_2 + 2\ H_2O \longrightarrow H_2SO_4 + 2\ HCl$
Sulphuryl chloride (liquid, b.p. 69°) → Sulphuric acid

(4) $SO_3 + H_2O \longrightarrow H_2SO_4$
Sulphur trioxide (α-form liquid, b.p. 44.5°; β-form solid, sublimes 50°; α-form is slowly converted to β-form on standing) → Sulphuric acid

As we perform sensitive phosphorus analyses in our laboratory, the use of volatile phosphorus compounds was contra-indicated. Chromyl chloride was satisfactory from the aspect of application but on hydrolysing and charring the whole thin layer was blackened.

Sulphuryl chloride and sulphur trioxide worked equally well, and in view of the difficulty in both obtaining and handling sulphur trioxide or fuming sulphuric acid, we decided on the former compound for simplicity.

Thin-layer chromatograms on microscope slides (3 in. × 1 in.) were placed in a Coplin jar, in the bottom of which 1 ml of sulphuryl chloride was placed. Slides did not touch the liquid. After 2-min exposure to the vapour, slides were rapidly removed and held over a steaming water bath for 30 sec. They were then placed on a hot plate,

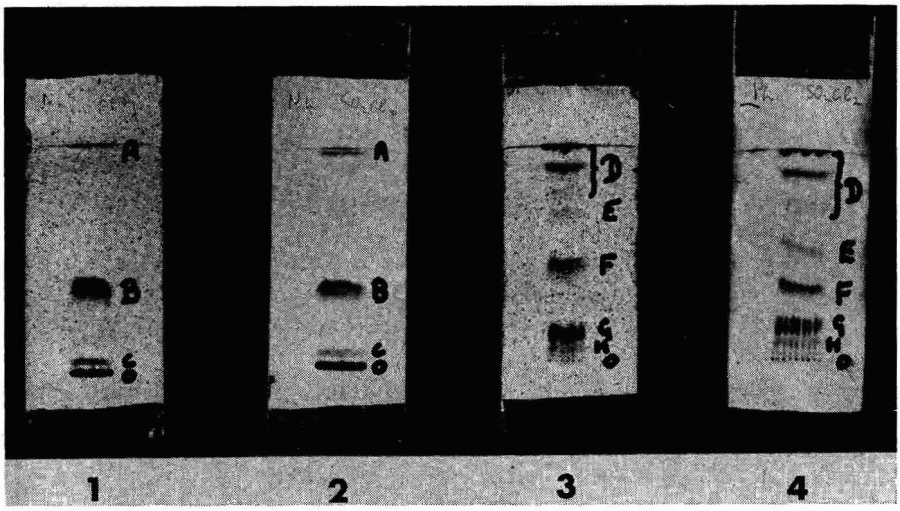

Fig. 1. Neutral lipids (1 and 2) and phospholipids (3 and 4) from chicken liver. Slides 1 and 3 were sprayed with concentrated sulphuric acid. Slides 2 and 4 were exposed to sulphuryl chloride vapour. Identity of lipids — Slides 1 and 2: A = cholesterol esters; B = triglycerides; C = cholesterol; O = origin (containing phospholipids). Slides 3 and 4: D = neutral lipids; E = phosphatidic acid (?); F = phosphatidyl ethanolamine; G = phosphatidyl choline; H = sphingomyelin; O = origin.

thermostatically controlled at 200°. Lipids were charred in a few seconds (Fig. 1).

This method therefore overcomes the disadvantage of uneven spraying and poor surface.

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Biochemistry of sphingolipids

VII. Separation of isomeric sphingosine bases as their dinitrophenyl derivatives

At present a great variety of conditions has been used for the cleavage of sphingolipids. The most commonly employed reagent, acidic methanol, generally gives good yields of total long-chain base but also leads to the formation of undesirable by-products of sphingosine. During our comparative study on the formation of some degradation products in various hydrolytic procedures of sphingolipids special interest was taken in the erythro- and threo-sphingosines[1].

It seems likely that some threo-isomer is formed by inversion during acid hydrolysis. However, this does not rule out the possibility that some of this isomer occurs naturally.

Several authors [2–4] have reported the separation of the free bases of erythro- and threo-sphingosine. In earlier papers[5–7] we have described the use of thin-layer chromatography for the characterization of DNP (dinitrophenyl)-derivatives of sphingosines and phytosphingosines on aluminium oxide. Although this technique is

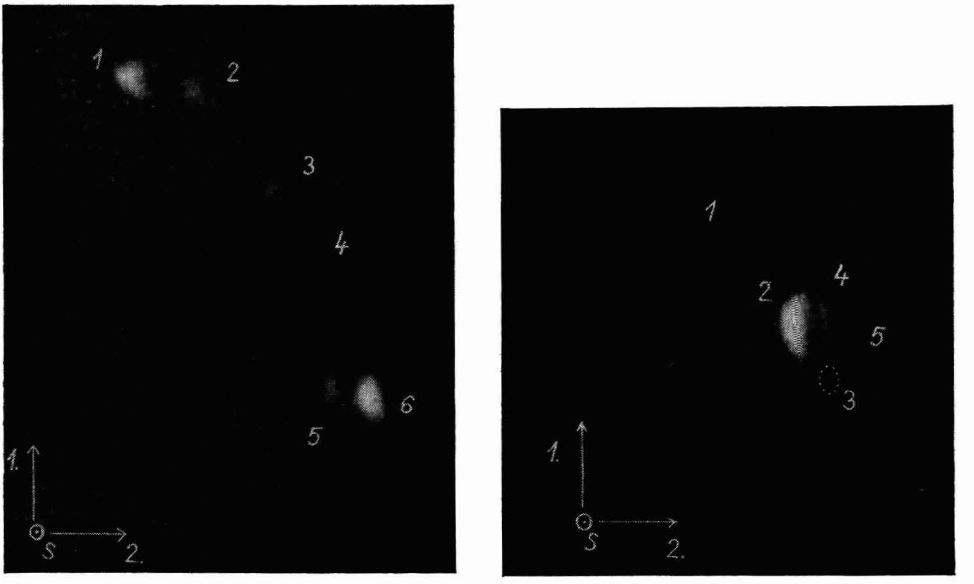

Fig. 1. Two-dimensional thin-layer chromatography of DNP-derivatives of erythro- and threo-sphingosines. Adsorbent: silica gel G impregnated with sodium tetraborate; 1st dimension: chloroform–methanol (90:10); 2nd dimension: methanol–tetralin–water (90:10:10) (upper phase) after impregnation of the layer with 5% tetralin. Detection: U.V. light (254 nm). DNP-derivatives: 1 = C_{20}-dihydrosphingosine; 2 = C_{18}-dihydrosphingosine; 3 = erythro-C_{18}-sphingosine; 4 = threo-C_{18}-sphingosine; 5 = C_{20}-phytosphingosine; 6 = C_{18}-phytosphingosine; S = start.

Fig. 2. Sphingosine bases of human blood serum sphingomyelins after aqueous methanolic HCl hydrolysis according to GAVER AND SWEELEY[8]. Experimental conditions are the same as in Fig. 1. 1 = C_{18}-dihydrosphingosine; 2 = erythro-C_{18}-sphingosine; 3 = threo-C_{18}-sphingosine; 4 = C_{17}-sphingosine; 5 = C_{16}-sphingosine; S = start.

very useful for the identification of these substances no resolution of isomeric sphingosines was obtained.

A two-dimensional thin-layer chromatographic method is reported here for the qualitative separation of isomers on silica gel impregnated with sodium tetraborate.

The borate-impregnated plates (18 × 18 cm) were prepared from a slurry of silica gel G (E. Merck) and a half-saturated solution of sodium tetraborate in water. The plates were dried at room temperature overnight. The solvent systems used for developing the chromatograms were chloroform–methanol (90:10) in the first dimension and methanol–tetralin–water (90:10:10) after impregnation with tetralin in the second dimension as described earlier[5–7]. The spots were visualized under ultra-violet light (Fig. 1).

The two-dimensional system here described was superior to the system on Al_2O_3 layers in which no separation of the isomers was obtained. Separations on non-borate silica gel were also unsuccessful. The excellent resolving power, rapidity and simplicity makes this modification very useful for the analysis of mixtures of isomeric sphingosines with other long-chain bases.

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Thin layer chromatography of polynitrophenols, nitrosophenols, nitrohydroquinones and related compounds

Polynitrophenols, nitrosophenols, nitrohydroquinones and their esters are useful in the synthesis of high-energy fuels for rockets and also for explosives. Their quick separation and microestimation will be of advantage in the study of the course of chemical reactions during the synthesis of new explosives, for quality control in their manufacture and storage. TLC of phenols, mononitrophenols and hydroquinones has been carried out by different workers[1–17]. The colorimetric estimation of some phenols and hydroquinone on TLC using *p*-nitrobenzenediazonium fluoroborate has been reported by SEEBOTH AND GORSCH[18]. LIPINA[19] employed diazotized *p*-nitroaniline for the quantitative estimation of some phenols.

In the present work a few polynitrophenols and nitrosophenols, nitrohydroquinones and some of their esters were studied employing thin-layer chromatography. The suitability of various adsorbents for the separation of these compounds is compared. 2,4,6-Trinitroresorcinol, α-nitroso-β-naphthol, β-nitroso-α-naphthol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, nitrosothymol and trinitrothymol were estimated colorimetrically by employing diazotised sulphanilic acid as a reagent[20]. 2,6-Dinitrohydroquinone was estimated by the method of BELYAKAV AND KOMIS[21] for hydroquinone, employing phloroglucinol and sodium carbonate. The individual compounds after separation and elution were estimated colorimetrically.

Methods and materials

All solvents used were dry and freshly distilled. The temperature of irrigation was 25 ± 2°; unless otherwise specified. Kodak photographic glass plates (22 × 22 cm) were used. The distance travelled by the solvent front in each case was 18 cm. A Hilger Spekkar Absorptiometer was employed for the colorimetric measurements.

Adsorbents

1. Silica gel G (200 mesh, B.S.S.).
2. Cellulose powder, ashless (Whatman, 100 mesh, B.S.S.), containing 5% calcium sulphate (200 mesh).
3. Cellulose acetate, containing 5% calcium sulphate (200 mesh, B.S.S.). Cellulose acetate was prepared by treating ashless cellulose powder (Whatman) with acetic anhydride for 3 h at room temperature, washing with distilled water, drying at 100° (30 h) and passing through a 200 mesh sieve.
4. Neutral alumina, containing 20% calcium sulphate (200 mesh, B.S.S.). Neutral alumina was prepared from Brockman alumina by REICHSTEIN AND SHOPPEE'S[22] method.
5. Neutral alumina (M. Woelm, 200 mesh, B.S.S.), containing 20% calcium sulphate (200 mesh).
6. Acidic alumina (M. Woelm, 200 mesh, B.S.S.), containing 20% calcium sulphate (200 mesh).
7. Amberlite ARI/400/Cl/AR (200 mesh, B.S.S.).
8. Amberlite IR/45/OH/AR (200 mesh, B.S.S.).

Compounds

2-Nitrohydroquinone-4-benzoate (m.p. 96°) was prepared from hydroquinone monobenzoate according to the method of KEHRMANN, SANDOZ AND MONNIER[23]. 2,6-Dinitrohydroquinone-4-acetate (m.p. 98°) was prepared from hydroquinone diacetate by following the procedure of NIETZKI[24]. 2,6-Dinitrohydroquinone (m.p. 136°) was prepared by the hydrolysis of 2,6-dinitrohydroquinone-4-acetate by the procedure of PRIDEAUX AND NUNN[25]. Nitranilic acid (86–87°, decomposition) was prepared by TOWN's method[26]. 2,3,6-Trinitrothymol (m.p. 110°) was prepared by nitration of thymol[27], and nitrosothymol (m.p. 163–164°) by following the method of KREMERS, WAKEMAN AND HIXON[28]. Other phenols were crystallised twice from ethanol. *p*-Toluenesulphonates of 2,6-dinitrohydroquinone, nitrosothymol, and *ortho*-, *meta*-, and *para*-nitrophenols were prepared by heating together nitrohydroquinone or the respective phenol (1 mole), *p*-toluenesulphonyl chloride (1.25 mole) and pyridine (3 mole) in a boiling tube on a water bath for 1.5 h. The mixture was poured into iced water and the resultant crystalline mass was filtered, washed with 5% sodium hydroxide followed by water and repeatedly crystallised from ethyl alcohol.

In this way the following compounds were obtained: 2,6-dinitrohydroquinone-4-*p*-toluenesulphonate (m.p. 194–195°; $C_{13}H_{10}O_7N_2S$; S = 9.32 % found, 9.46 % calculated), nitrosothymol-*p*-toluenesulphonate (m.p. 87–88°; $C_{17}H_{19}O_4NS$; S = 9.53 % found, 9.60 % calculated), *o*-nitrophenol-*p*-toluenesulphonate (m.p. 83°; $C_{13}H_{11}O_5NS$; S = 10.81 % found, 10.92 % calculated), *m*-nitrophenol-*p*-toluenesulphonate (m.p. 113°; $C_{13}H_{11}O_5NS$; S = 10.75 % found, 10.92 % calculated), and *p*-nitrophenol-*p*-toluenesulphonate (m.p. 97°; $C_{13}H_{11}O_5NS$; S = 10.88 % found, 10.92 % calculated).

Preparation of thin layer plates, application of the compounds and irrigation of the plates

A slurry of the adsorbent in a suitable solvent was prepared and poured on to the glass plates. By tilting the plates from side to side a uniformly thin coating was obtained. The plates were left at room temperature (25 ± 2°) overnight and activated or dried at the appropriate temperature in an oven. The plates were weighed before

TABLE I

DETAILS OF TLC COATINGS AND THEIR ACTIVATION

Coating	*Wt. of adsorbent (g)*	*Solvent*	*Activation temperature (°C) and time (h)*	*Average coating of adsorbent (mg/cm²)*
Neutral silica gel G	30	Chloroform–methanol (2:1), 100 c.c.	110, 0.5	7.7
Buffered silica gel G	30	0.5 *N* Oxalic acid, 20 c.c., and methanol, 70 c.c.	110, 2	7.0
Cellulose–$CaSO_4$	26.25	Water, 100 c.c.	110, 1	7.6
Cellulose acetate–$CaSO_4$	26.25	Water, 100 c.c.	105, 1	7.4
Neutral/acid alumina–$CaSO_4$	30	Water, 60 c.c.	180, 0.5	10.7
Amberlite ARI/400/Cl/AR	25	Ethyl acetate, 100 c.c.	60, 1	4.8
Amberlite IR/45/OH/AR	25	Ethyl acetate, 100 c.c.	60, 1	4.8

spotting and the average coating of the adsorbent noted. Table I gives the quantities of adsorbent for five plates (22 × 22 cm) and other details.

An ethanolic solution of each sample (5 μl containing 5 μg) was applied on the chromatoplates with a standard capillary. Except when ion exchange resins were used, chromatograms were irrigated by ascending technique.

In the case of Amberlite resins, the plates were kept slanting at 30° angle, solvent was fed through cotton and the descending technique employed. The chromatoplates after irrigation were either viewed under U.V. light or sprayed with a chromogenic reagent (see below under Detection). Table II gives the R_F values of the various compounds.

Solvent systems

A. Chloroform
B. Benzene
C. Chloroform–xylene (4:1)

TABLE II

R_F VALUES OF THE VARIOUS COMPOUNDS IN VARIOUS SOLVENT SYSTEMS AND ON VARIOUS ADSORBENTS

Adsorbents		*Silica gel G*				*Buffered silica gel G*				*Cellulose–$CaSO_4$*			
Sample No.	*Compound*	*A**	*B*	*C*	*D*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	2-Nitrohydroquinone-4-benzoate	0.85	0.91	0.74	0.68	0.91	0.92	0.64	0.64	0.37	0.53	0.81	0.49
2	2,6-Dinitrohydroquinone-4-acetate	0.68	0.37	0.51	0.16	0.35	0.43	0.32	0.27	0.31	0.40	0.72	0.94
3	2,6-Dinitrohydroquinone	0.48	0.00	0.13	0.06	0.33	0.27	0.07	0.05	0.23	0.36	0.64	0.75
4	2,4,6-Trinitroresorcinol	0.10	0.34	0.20	0.05	0.69	0.55	0.28	0.28	0.36	0.18	0.33	0.81
5	α-Nitroso-β-naphthol	0.85	0.52	0.53	0.27	0.92	0.41	0.32	0.29	0.95	0.89	0.77	0.48
6	β-Nitroso-α-naphthol	—	—	0.20	0.04	0.48	0.15	0.13	0.61	0.81	0.95	0.84	0.51
7	4-(p-Nitrosophenylazo)-resorcinol	0.00	0.04	0.14	0.08	0.33	0.06	0.31	0.04	0.92	0.95	0.78	0.04
8	2,4-Dinitrophenol	0.85	0.86	0.62	0.48	0.94	0.98	0.56	0.53	0.79	0.74	0.77	0.76
9	Nitranilic acid	—	—	—	—	—	—	—	—	—	—	—	—
10	Nitrosothymol	0.56	0.08	0.22	0.16	0.38	0.15	0.13	0.10	0.91	0.91	0.92	0.53
11	Trinitrothymol	0.28	0.15	0.16	0.06	0.93	0.58	0.49	0.54	0.87	0.91	0.89	0.71
12	Picramic acid	0.49	0.33	0.37	0.18	0.65	0.24	0.18	0.16	0.67	0.64	0.75	0.61
13	2,4,6-Trinitrophenol	0.00	0.19	0.03	0.11	0.97	0.09	0.41	0.10	0.96	0.80	0.86	0.82
14	2,6-Dinitrohydroquinone-4-p-toluenesulphonate	0.98	0.80	0.00	0.03	1.00	0.71	—	0.40	0.98	0.94	0.89	0.84
15	Nitrosothymol-p-toluenesulphonate	0.99	0.56	0.59	0.30	1.00	0.40	0.41	0.61	0.98	0.95	0.92	0.00
16	o-Nitrophenol-p-toluenesulphonate	0.97	0.77	0.56	0.36	1.00	0.67	0.45	0.41	0.98	0.94	0.92	0.00
17	m-Nitrophenol-p-toluenesulphonate	0.99	0.84	0.63	0.44	1.00	0.75	0.52	0.52	0.97	0.93	0.91	0.00
18	p-Nitrophenol-p-toluenesulphonate	0.98	0.89	0.67	0.50	1.00	0.83	0.57	0.52	0.87	0.53	0.91	0.00

* For key to solvents, see text.

D. Chloroform–xylene (1:1)
E. *n*-Butanol saturated with 5 *N* ammonium hydroxide
F. *n*-Butanol–3 *N* ammonium carbonate–3 *N* ammonium hydroxide (4:3:3)
G. *n*-Butanol–ethanol–3 *N* ammonium carbonate (40:11:19)
H. 3 % sodium chloride solution
I. *n*-Butanol–water (86:14)
J. *n*-Butanol saturated with water
K. Ethanol–water (2:3)
L. Acetone–water (2:3)
M. Petroleum ether (b.p. 40–60°)–ether (1:1) (irrigation was done at 0°).
N. *n*-Butanol–water–acetic acid (6:2:1).
O. Cyclohexane–ethyl acetate–acetic acid (5:1:1).

Detection

The spots could be detected by: (1) use of an ultraviolet lamp "Chromatolite"

Cellulose acetate–$CaSO_4$				*Alumina (neutral)–$CaSO_4$*			*Alumina (neutral)–$CaSO_4$*			*Alumina (acidic)–$CaSO_4$*			*Amberlite ARI/400/-Cl/AR*	*Amberlite 45/OH/AR*
I	*J*	*K*	*L*	*M*	*B*	*C*	*M*	*B*	*C*	*M*	*B*	*C*	*N*	*O*
0.74	0.91	0.79	0.91	0.00	0.11	0.00	0.32	0.03	0.16	0.42	0.13	0.46	0.02	0.08
0.86	0.63	0.88	0.93	0.07	0.00	0.05	0.02	0.00	0.00	0.15	0.00	0.00	0.04	0.00
0.83	0.61	0.86	0.91	0.06	0.00	0.05	0.02	0.00	0.00	0.20	0.00	0.00	0.23	0.00
0.64	0.48	0.91	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.83	0.87	0.79	0.73	0.06	0.00	0.06	0.13	0.00	0.06	0.11	0.06	0.05	0.37	0.00
0.87	0.96	0.71	0.85	0.05	0.00	0.00	0.02	0.00	0.00	0.10	0.00	0.05	0.42	0.00
0.86	0.89	0.56	0.46	0.04	0.00	0.29	0.00	0.00	0.08	0.09	0.00	0.00	0.42	0.00
0.82	0.74	0.91	0.91	0.02	0.06	0.06	0.03	0.00	0.00	0.04	0.04	0.06	0.16	0.00
—	—	0.97	0.55	0.00	0.00	0.00	0.03	0.00	0.00	—	0.06	0.00	—	—
0.92	0.91	0.89	0.74	0.75	0.16	0.22	0.77	0.04	0.18	0.86	0.09	0.30	0.59	0.00
0.66	0.70	0.92	0.91	0.00	0.00	0.93	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.12
0.84	0.72	0.83	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00
0.71	0.74	0.86	0.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
0.79	0.90	0.84	0.88	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.62	0.13
0.87	0.96	0.60	0.60	0.68	0.70	0.86	0.92	0.72	0.72	0.53	0.57	0.86	1.00	0.00
0.87	0.95	0.78	0.68	0.69	0.69	0.87	0.82	0.76	0.68	0.48	0.58	0.78	0.71	0.00
0.87	0.91	0.78	0.65	0.81	0.79	0.93	0.88	0.84	0.72	0.63	0.67	0.80	0.85	0.00
0.87	0.86	0.78	0.56	0.88	0.79	0.93	0.96	0.85	0.78	0.86	0.68	0.83	—	0.37

for short-wave (2537 Å) radiation, which revealed all the compounds; (2) spraying the plates with 2 % phosphomolybdic acid solution and then treating with ammonia vapour; and (3) spraying with sulphanilic acid reagent (solution A, followed by solution B). Solution A is an 0.5 % solution of sulphanilic acid in *N* HCl and solution B a 5 % solution of sodium nitrite.

Estimation of phenols

Cellulose–$CaSO_4$ plates were used in all cases. The spotting as well as the irrigation in the case of nitrosothymol and trinitrothymol were carried out at 0 ± 2°. A number of spots of each compound (5 to 20 μl) having different amounts were spotted and irrigated with *n*-butanol–3 *N* ammonium hydroxide–3 *N* ammonium carbonate (4:3:3). After observing the spots under U.V. light they were scraped off, extracted with 50 % ethanol (5 c.c., 1 h) and filtered. At 0° 2 c.c. of 0.2 % sulphanilic acid containing 0.7 c.c. concentrated hydrochloric acid, 1 c.c. of 0.2 % sodium nitrite solution, and 2.5 c.c. of 0.25 % sodium hydroxide solution were added to the filtrate. Before addition all solutions were kept at 0°, and the mixture also maintained at the same temperature. After 3 min the colour intensities were read in the colorimeter using a suitable filter (see Table III) and compared with the standard curves previously plotted for the individual compounds.

Estimation of 2,6-dinitrohydroquinone

2,6-Dinitrohydroquinone, after spotting, irrigation and detection under U.V., as in the case of phenols, was extracted from the adsorbent with 50 % ethanol (5 c.c., 1 h). The extract was filtered, and the filtrate added to a mixture of 0.05 % phloroglucinol (2 c.c.) and 1.5 % sodium carbonate (4 c.c.). After leaving the solution for 1 h the colour intensities (percentage transmission) were noted using the appropriate filter (see Table III).

TABLE III

LIMITS OF ESTIMATIONS OF THE COMPOUNDS AND THE FILTERS EMPLOYED

Sample No.	*Compound*	*Filter (mμ)*	*Lower limit of estimation (μg)*
1	2,6-Dinitrohydroquinone	540	5
2	2,4,6-Trinitroresorcinol	425	3.3
3	α-Nitroso-β-naphthol	425	10
4	β-Nitroso-α-naphthol	425	3.3
5	2,4-Dinitrophenol	425	5
6	2,4,6-Trinitrophenol	425	5.5
7	Nitrosothymol	425	6
8	Trinitrothymol	425	16

Discussion

The technique of coating the glass plates with various adsorbents by pouring a known volume of the slurry on to the plates and then tilting them from side to side produced thinner coatings compared to other conventional methods. It was observed that the thinner the coating, the quicker and better is the resolution of the com-

pounds. The coatings of Amberlite resins were found to crack if dried for periods longer than 1 h at 60°.

Buffering of silica gel G with 0.5 *N* oxalic acid had a marked effect on the R_F values of some of the polynitrophenols, *viz.* 2,4,6-trinitroresorcinol, 2,4-dinitrophenol, trinitrothymol, and 2,4,6-trinitrophenol.

On cellulose and cellulose acetate plates the use of 2 % starch instead of calcium sulphate as binder resulted in major tailing of the compounds.

A reversed phase system using cellulose acetate–calcium sulphate produced better resolutions compared to cellulose–calcium sulphate in the case of nitrohydroquinone and its esters, 2,4,6-trinitroresorcinol, 2,4-dinitrophenol, picramic acid and *p*-toluenesulphonates.

Neutral alumina–calcium sulphate coatings in general gave low R_F values compared to silica gel G. However, alumina was found to be the best adsorbent for the resolution of *p*-toluene sulphonates. In general, the R_F values of phenols on acid alumina (Woelm) were higher than on neutral alumina (Woelm). On the other hand, acid alumina (Woelm) lowered the R_F values of *p*-toluene sulphonates compared to neutral alumina (Woelm).

Basic ion exchange resins had a powerful binding effect on polynitrophenols.

In view of their slow vaporisation from chromatoplates the spotting, irrigation and elution of nitrosothymol and trinitrothymol had to be carried out at low temperature (0 $\pm$ 2°).

In the estimation of phenols with sulphanilic acid reagent, a time of 3 min gave maximum colour development and was rigidly adhered to; longer periods produced erroneous results. All the compounds were found to observe linearity in their colours developed with the chromogenic reagent with respect to their weights. The lower limits of detection are given in Table III.

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A method for quantitative thin-layer chromatography

In this communication, a convenient method is described for the determination of substances on thin-layer chromatograms by means of a gas-chromatographic technique of elementary analysis.

Fig. 1 shows a sketch of an apparatus consisting of a combustion furnace and gas chromatograph. After separation of a compound containing nitrogen from a sample of known volume by thin-layer chromatography, the spot is scraped off with the adsorbent (aluminum oxide or silica gel) into a platinum boat. As soon as the

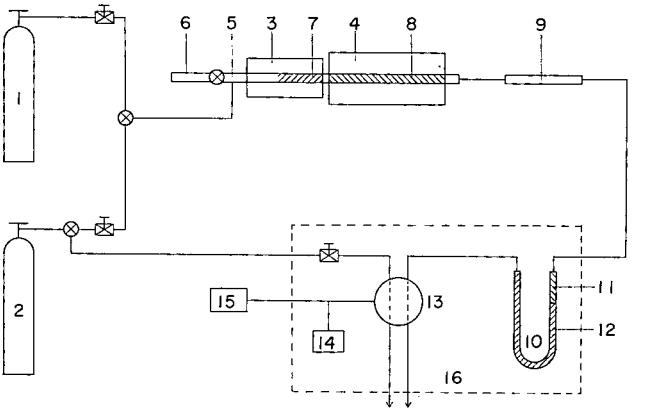

Fig. 1. Outline of apparatus. 1 = He containing 2 % O_2; 2 = He; 3 = electric furnace I (900°); 4 = electric furnace II (500°); 5 = combustion tube; 6 = preparation chamber; 7 = cupric oxide; 8 = reduced copper; 9 = anhydrone; 10 = U-type column (ø 4 mm × 75 cm); 11 = MnO_2; 12 = activated carbon; 13 = T.C. detector; 14 = recorder; 15 = electronic integrator; 16 = gas chromatograph. Gas-chromatographic conditions: He flow rate, 30 ml/min; column temperature, 100°; detector temperature, 100°.

platinum boat is inserted into the preparation chamber of the combustion tube, the atmosphere is replaced with helium gas containing 2 % oxygen by turning the cock. The atmosphere is purged completely, followed by insertion of the platinum boat into the furnace, then the sample is burned instantly. The resulting combustion products are swept through cupric oxide, reduced copper, anhydrone and manganese dioxide, and led into an activated carbon column at 100°. Gas-chromatographic separation of nitrogen and carbon dioxide is recorded and simultaneously the peak area of the nitrogen is determined by an electronic integrator.

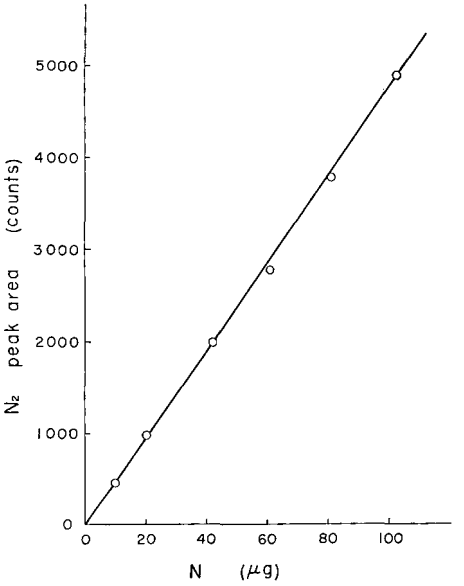

Fig. 2. Relationship between peak area and quantity of nitrogen.

Based on a calibration curve of the amount of nitrogen *vs.* its peak area, the amount of nitrogen due to the sample substance is found, and the content of the substance in an original sample may be determined.

Fig. 2 shows the relationship between the amount of nitrogen and its peak area.

TABLE I

DETERMINATION OF *p*-NITROANILINE*

Sample (μl)	$N_{calc.}$ (μg)	$N_{obs.}$ (μg)**		*Error* (%)
			Average	
2.5	12.5	12.7, 13.1, 12.3	12.7	+1.6
5.0	25.0	25.3, 25.1, 24.8	25.1	+0.4
10.0	50.0	49.8, 50.6, 50.6	50.3	+0.6
20.0	100.0	99.0, 101.0, 99.9	100.0	0.0

* Methanol solution of *p*-nitroaniline (5.00 μg N/μl solution) was spotted on thin layers (300 mμ aluminum oxide on 5 × 20 cm glass plate), and developed with benzene–ethanol (9:1). R_F 0.74.

** Blank value of 0.5–2.0 μg depending on each spot area was used as correction.

TABLE II

DETERMINATION OF *p*-NITROANILINE AND *p*-BROMOACETANILIDE*

Sample (*μl*)	*p-Nitroaniline*				*p-Bromoacetanilide*			
	$N_{calc.}$ (*μg*)	$N_{obs.}$ (*μg*)	*Average*	*Error* (%)	$N_{calc.}$ (*μg*)	$N_{obs.}$ (*μg*)	*Average*	*Error* (%)
5.0	12.5	12.1 11.9 11.9	12.0	−4.0	3.125	3.1 3.2 3.2	3.2	+3.2
10.0	25.0	25.7 25.2 24.5	25.1	+0.4	6.25	6.4 6.4 6.6	6.5	+4.0
15.0	37.5	37.1 36.7 36.2	36.7	−2.1	9.375	9.4 9.7 9.2	9.4	0.0

* Mixed methanol solution of *p*-nitroaniline and *p*-bromoacetanilide (2.50 *μg* N as *p*-nitroaniline/*μl* solution, 0.625 *μg* N as *p*-bromoacetanilide/*μl* solution) was spotted and developed with benzene–ether (19:1). R_F values: *p*-nitroaniline 0.34, *p*-bromoacetanilide 0.18.

For example, Table I shows data on *p*-nitroaniline alone developed on an aluminum oxide layer, and Table II shows data on a mixture of *p*-nitroaniline and *p*-bromoacetanilide.

The convenient technique described above is useful for the determination of a compound containing nitrogen which has a low vapor pressure and/or is thermally unstable, consequently cannot be isolated by gas chromatography. Work concerning compounds not containing nitrogen is in progress.

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Reagents for the detection of antioxidants on thin layers of silica

Antioxidants are used in foodstuffs and plastic materials to prevent autoxidation. Because only a few antioxidants are permitted in foodstuffs and food packaging materials, methods for their detection became necessary. Usually the antioxidants are extracted from the food or plastic with suitable solvents, followed by separation on thin layers of silica gel[1–5]. The detection and identification may be hindered by the fact that the reagents used are not specific for antioxidants: they may also produce coloured spots with other extracted substances or they may not react with all antioxidants. Most investigators therefore use a range of reagents.

In this paper some reagents are suggested and in Table I the colours obtained with reagents that produce different colours with different antioxidants are listed for a number of antioxidants. In these cases 20 μg of antioxidant were spotted and the silica-covered chromatoplate was sprayed after elution. (For solvents see refs. 3 and 5.) The colours may be different when other quantities of antioxidant are used.

Detection reagents

Reagents which can be used for the detection of antioxidants can be divided into groups as follows:

(A) Chemicals which produce colours when reduced; since antioxidants are used to prevent oxidation they are reducing substances.

(B) Substances which can be coupled to phenols (many antioxidants are substituted phenols): (1) diazo-compounds; (2) aromatic aldehydes; (3) Gibbs reagent, which forms indophenols that form coloured salts with alkali.

(C) Stable free radicals, which accept a hydrogen radical from the antioxidant.

(D) Substances that form coloured addition compounds.

Group A

(1) *Potassium permanganate.* Potassium permanganate produces yellow spots on a pink background with all oxidisable substances. It is therefore not specific for antioxidants.

It is prepared by dissolving a few grains of potassium permanganate in 10 ml acetone.

(2) *Ferric–ferricyanide.* A mixture of ferric sulphate and ferricyanide is turned into Prussian blue by reducing agents. It is specific for antioxidants but gives the same blue colour with nearly all of them. A disadvantage is that some time after spraying the whole chromatoplate turns blue.

It is prepared by mixing one volume of an 0.5 % ferric sulphate (anhydrous) solution in 1 N H_2SO_4 with one volume of an 0.2 % aqueous solution of potassium ferricyanide.

(3) *Dipyridyl.* BURTON[6] suggested a modification of type of reaction with the ferric–ferricyanide reagent by using dipyridyl, which gives a permanent white background. The spots with all antioxidants are red-brown.

One volume of an 0.5 % ferric sulphate (anhydrous) solution is mixed with one volume of an 0.5 % solution of α,α-dipyridyl in methanol.

(4) *Phosphomolybdic acid.* This reagent develops blue spots with reducing agents;

TABLE I

COLOURS OBTAINED WITH DIFFERENT ANTIOXIDANTS ON SILICA COVERED CHROMATOPLATES AFTER SPRAYING WITH DIFFERENT REAGENTS

Antioxidant	*Colour with reagent number*								
	2	*4*	*5*	*6*	*7*	*8*	*9*	*11*	*12*
Propyl gallate	blue	blue	yellow	red	pink	pink	purple	grey	brown
Octyl gallate	blue	blue	yellow	chocolate	pink	pink	brown	grey	brown
Dodecyl gallate	blue	blue	yellow	orange	pink	pink	grey	grey	brown
Nordihydroguaiaretic acid	blue	blue	rustbrown	brick red	purple	pink	brown	grey	chocolate
Gum guaiac	blue	blue	orange	redbrown	light purple	purple	brown	brown	chocolate
2,4,5-Trihydroxybutyrophenone	blue	blue	ochre	brown	grey	ochre	brown	brown	chocolate
Stearoyl-*p*-aminophenol	blue	blue	grey	brown	grey	—	purple	—	ochre
4,4′-Butylene-bis-(6-*tert.*-butyl-3-methylphenol)	blue	blue	brown	rustbrown	violet	purple	brown	pink	violet
4,4′-Thio-bis-(16-*tert.*-butyl-3-methylphenol)	blue	blue	brown	rustbrown	claret	red	brown	rustbrown	brown
2,2′-Dihydroxy-3,3′-dicyclohexyl-5′5-dimethyldiphenylmethane	blue	purple	light purple	pink	brown	grey	yellow	yellow	black
Dilauryl thiodipropionate	blue*	—	—	yellow	—	bluish	grey	yellow	—
Butylidene-2,2-bis-(octylthioglycolate)	blue*	—	—	yellow	—	bluish	—	yellow	—
Diphenyl-*p*-phenylenediamine	blue	blue	violet	ochre	yellow	green	grey	ochre	grey
2-Naphthyl-*p*-phenylenediamine	blue	blue	rustbrown	redbrown	green	green	pink	brownish	blue
2-*tert.*-Butyl-4-hydroxyanisole	blue	blue	brick red	violet	brown	brown	violet	—	pink
4,4′-Cyclohexylidene-bis-(2-cyclohexylphenol)	blue*	blue	orange	brown	buff	rustbrown	violet	grey	chocolate

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4,4′, 4″-Tris-(6-*tert.*-butyl-*m*-cresol)-1,3,3-crotonaldehyde	blue*	blue	orange	brown	violet	violet	brown	pink	brown
2-Hydroxy-4-alkylbenzophenones	blue	—	yellow	orange	yellow	brown		yellow	yellow
2-(2′-Hydroxy-5′-alkylphenyl)-benzotriazoles	blue	—	ochre	violet	yellow	—	grey	yellow	light orange
Phenyl-α-naphthylamine	blue	blue	rustbrown	brown	yellow	greenish yellow	rustbrown	ochre with blue rim	blue
Phenyl-β-naphthylamine	blue	blue	violet	purple	yellow	yellow	blue	yellow	green
2,2′-Methylene-bis-(4-methyl-6-*tert.*-butylphenol)	blue	violet	yellow	ochre	green	red	brown	violet greenish	brown
2,2′-Thio-bis-(4-methyl-6-*tert.*-butylphenol)	blue	blue	rustbrown	brick red	brown	brown	bluish	grey	brown
2,6-Di-*tert.*-butylphenol	green	blue	yellow	yellow	purple	violet	grey	grey	brown
2,6-Di-*tert.*-butyl-4-methylphenol	blue	purple	—	yellow	purple	violet	grey**	grey	brown
2,2′-Propylene-bis-(4,4′,6,6′-*tert.*-butylphenol)	blue	blue	yellow	yellow	buff	pink	bluish grey	—	purple
1,3,5-Trimethyl-2,4,6-tris-(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-benzene	bluish purple	purple	yellow	yellow	violet	blue	buff	—	red
n-Octadecyl β-(4′-hydroxy-3,5-di-*tert.*-(butylbenzene)-propionate	blue	blue	yellow	pink	violet	blue	brownish violet	—	brown
Tris-(nonyl-phenyl)-phosphite	blue	blue	buff	claret	pink	pink	purple*	—	brown
Di-octylphenylphosphite	blue	blue	buff	orange	pink	pink	blue	—	brown
2,2′-Methylene-bis-[6-(2-methylcyclohexyl)-4-methylphenol]	blue	violet	ochre	pink	purple	buff	brown	grey	brown

* Colour develops only after heating the chromatoplate.
** Mixed colour of green, brown and pink.

it is not specific for antioxidants and though the colour may differ from purple to green-blue for various substances it generally gives dark blue spots.

10 g of phosphomolybdic acid are dissolved in 90 % ethanol. The plate is heated for 10 min at 105°.

Group B(1)

(5) *Diazo-reagent.* Diazotized *p*-nitroaniline has been used for a long time for the detection of aromatic compounds; with antioxidants it forms yellow to brown spots.

800 mg *p*-nitroaniline are dissolved in a mixture of 250 ml water and 20 ml hydrochloric acid (25 %). A 5 % sodium nitrite solution is added dropwise until the solution is colourless.

(6) *Red salt.* Some typical colours are obtained with some types of antioxidant when red salt is used, *e.g.* orange spots are given by benzophenone derivatives that are used as U.V. absorbers[7] in plastics and claret spots by phosphites.

It is prepared by dissolving successively in 10 ml water 100 mg sodium acetate and 200 mg red salt A.L. (C.I. 37275).

Group B(2)

Coupling of antioxidants with aromatic aldehydes has the disadvantage that the colour is not very reproducible, but it may be useful because different bright colours can be obtained.

(7) *Anisaldehyde.* This is prepared by dissolving 500 mg *p*-methoxybenzaldehyde (anisaldehyde) in a mixture of 10 ml glacial acetic acid and 85 ml methanol and adding 5 ml concentrated sulphuric acid.

The plate is heated for 10 min at 105°.

(8) *Vanillin.* 400 mg vanillin are dissolved in a mixture of 95 ml methanol and 5 ml concentrated sulphuric acid. The plate is heated for 10 min at 105°.

Group B(3)

(9) *Gibbs reagent.* Though it is not specific for antioxidants it develops very typical colours, particularly with some antioxidants which do not give specific colours with other reagents (*e.g.* butylhydroxytoluene).

It consists of 100 mg 2,6-dichloro-*p*-benzoquinone-4-chlorimine in 100 ml ethanol. Spraying of the chromatoplate with this solution is followed by a spray of a 2 % borax solution in 50 % ethanol.

Group C

(10) *α,α-Diphenyl-β-picrylhydrazyl.* WOGGON *et al.*[4] described this reagent for the quantitative determination of antioxidants on chromatoplates: the violet-coloured stable free radical α,α-diphenyl-β-picrylhydrazyl is decolorised stoichiometrically by antioxidants. All the spots are yellow against a deep violet background.

It is prepared by dissolving 100 mg α,α-diphenyl-β-picrylhydrazyl in 100 ml 96 % ethanol.

Group D

(11) *Palladium chloride.* Palladium chloride forms addition compounds with many aromatic compounds, the colours of which may differ.

150 mg palladium chloride are dissolved in 100 ml 0.2 *N* hydrochloric acid.

(*12*) *Antimony pentachloride.* 20 ml antimony pentachloride are mixed with 80 ml carbon tetrachloride. The plate is heated for 10 min at 105°.

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The cytogenetics of *Lotus*

XII. Thin-layer chromatography in the separation of secondary phenolic compounds in *Lotus* (Leguminosae)

The successful separation of secondary phenolic compounds in *Lotus* through the use of the Shandon thin-layer chromatographic equipment and silica gel G as the coating material has recently been reported by GRANT AND WHETTER[1]. The availability of commercially prepared coated plates would eliminate the initial time needed to learn the technique required in order to obtain a satisfactory coating on the plates as well as the time required for the messy preparation of the silica gel coating on the plates. This note reports the results obtained in the separation of secondary phenolic compounds in *Lotus* using the techniques reported in the earlier paper by GRANT AND WHETTER[1] but using prepared Eastman Chromagram sheets, Type K301R with a fluorescent indicator, and the Eastman Chromagram Developing Apparatus (Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York).

Preliminary tests

Samples of fresh leaves of *Lotus* were prepared by weighing out 0.08 g and leaving them in 0.5 ml of 1 % hydrochloric acid in methanol at room temperature, in the dark, overnight. The plates were prepared for development by applying an approximately 7 μl spot of sample solution with a micropipette at a distance of 2.0 cm from the base. Two spots were run for each sample. Ascending development was carried

out at room temperature in the Eastman Chromagram developing apparatus. Cyclohexane–ethyl acetate (1:1, v/v) was used as the first solvent and allowed to pass twice up the layer to a height of 15 cm, the plate being dried between runs. A second solvent, methanol–chloroform (30:70) was then allowed to run up only to R_F 0.5. The plates were examined between runs for spots, but the results were very unsatisfactory as the color was extremely faint and very poor resolution was obtained.

Final tests

From the preliminary results it was considered that the leaf extract used was too weak. Plates were then prepared by using extracts from both 0.08 g and 0.16 g of fresh leaves (in 0.5 ml of 1 % hydrochloric acid in methanol) and spotting the plates with 7, 10 and 15 μl from each extract. When the plates with the highest concentration of extract (0.16 g and 15 μl) were examined the fluorescence was still only approximately half as bright as that from the glass plates prepared with silica gel G. Consequently, the chromagrams were considered unsatisfactory for proper diagnosis. Depending on the species of *Lotus*, three to nine leaves (including leaflets) are required for the preparation of 0.08 g of extract. If the quantity is exceeded beyond the doubled amount, that is 0.16 g, then this would require most of the leaves on a young plant, and certainly would not be satisfactory for the analyses of herbarium material[1]. Therefore, we do not consider the Eastman Chromagram sheets suitable for our particular requirements, as to use increased amounts of leaf material would with certain species, such as *Lotus micranthus*, use up most of the leaves on a single plant. In addition, we found that it required 1.5 h for each single development with the Eastman Chromagram sheets using the Eastman Chromagram developing apparatus, while a single development with the glass plates and silica gel G required only 30 to 40 min. Therefore, for our analyses of the phenolic compounds of *Lotus* we have abandoned the commercially prepared sheets in favor of the standard glass plates and the silica gel G coating method which was reported previously[1].

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Detection and thin-layer chromatography of isomeric chlorophenols and their derivatives

I. N-Trichloroacetyl carbamates

Isomeric chlorophenols and their esters have exhibited a broad range of utility as nematicides[1–3], germicides[4], herbicides[5–6], growth regulators[7–8], and insecticides[9].

The analysis of chlorophenols has been accomplished by procedures such as infrared[10–11] and ultraviolet[12] spectrophotometry, non-aqueous titrations[13], potentiometric titrations[14] as well as a number of chromatographic techniques that include ion-exchange[15], gas–liquid[16–19], paper[20–23], pH-paper[24], and silica-gel column chromatography[25].

The purpose of this investigation was to determine the utility of pi-electron acceptors for the detection of isomeric chlorophenols and their derivatives, *e.g.* N-trichloroacetyl carbamates on thin-layer chromatograms and concomitantly elaborate the effect of structure on their chromatographic behavior in several solvent systems.

Experimental

Thin-layer chromatography. The chromatoplates, silicic acid layers, were prepared according to the method of MORLEY AND CHIBA[26]. Silica-gel DF-5* was applied on 8 × 8 in. plates to a thickness of 280 μ. After air-drying, the plates were activated in an oven for 30 min. Acetone solutions (1–2 μl containing 1–10 μg/μl) of test substance were applied along a line 2.5 cm from the lower end of the plate and developed by the ascending method. After evaporation of the solvent, the spots were located on the plate by U.V. detection, then sprayed with one of the chromogenic reagents and the initial color development as well as subsequent color changes noted.

The sprayed plates were then exposed briefly to ammonia vapors with the results described in Table I.

The developing solvent systems utilized in this work were:

A Chloroform–acetic acid (5:1)

B Benzene–acetic acid (5:1)

C 2.5 % acetone in benzene

D Iso-amyl alcohol–ammonia–water (30:15:5)[21].

Detecting reagents. (1) DDQ reagent[27], 2 % 2,3-dichloro-5,6-dicyano-1,4-benzoquinoneimine in benzene. (2) TCNE reagent[27], 2 % tetracyanoethylene in benzene. (3) Chloranil, 1 % tetrachloro-*p*-benzoquinone in benzene. (4) Gibbs reagent, 2 % 2,6-dibromo-N-chloro-*p*-benzoquinoneimine in benzene.

Materials. The isomeric chlorophenols (compounds 1–13) were obtained from Aldrich Chemical Co., Milwaukee, Wisc., U.S.A. The N-(trichloroacetyl) carbamates (compounds 14–49) were prepared via the reaction of trichloroacetyl isocyanate with the appropriate alcohol or chlorophenol and recrystallized from petroleum ether (30–60°). Trichloroacetyl isocyanate was obtained from Distillation Industries, Rochester, N.Y., U.S.A.

* Obtained from Camag, Muttenz, Switzerland.

TABLE I

R_F VALUES × 100 AND SPOT COLORS OF ISOMERIC CHLOROPHENOLS ON SILICA-GEL DF-5

(A) After detector application; (B) after detector application and exposure to ammonia vapors.
Designation of colors developed at room temperature: B = blue; Bg = beige; Bn = brown; C = crimson; Cr = cream; G = green; L = lilac; O = orange; P = purple; Pk = pink; R = rose; T = tan; V = violet; Y = yellow.

No.	*Compound*	*(A) Before NH_3 exposure*				*(B) After NH_3 exposure*				*Solvent systems*			
		1	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*A*	*B*	*C*	*D*
1	*o*-Chlorophenol	L	O–C	Y–O	O	Pk/B*	R	B–V	O	72	63	58	69
2	*m*-Chlorophenol	Cr	O	Y	O	B–L/B*	Pk → L	V	Y–O	65	59	53	65
3	*p*-Chlorophenol	B	O–C → Bn	O	O–C	Y–G/Y–G*	O → YG	B–G	O	62	56	48	63
4	2,3-Dichlorophenol	O	O	Y	Y	Y–G/B*	Y–G	B–V	Y–G → O	74	62	52	66
5	2,4-Dichlorophenol	B	Pk	Y	Pk–O	Y/B–G*	Bg	B	Y–G → O	74	60	50	64
6	2,5-Dichlorophenol	O–C	Y–O	Y–T	Y–O	B/B*	B	B–V	G–B → O	71	56	46	61
7	2,6-Dichlorophenol	T	O	T	Y	B–G/B*	R–O	B	G–B → O	68	51	42	57
8	3,4-Dichlorophenol	B–Bk	O–C	O–Y	O–C	T/B*	Y	V	O	62	49	43	54
9	3,5-Dichlorophenol	O–P	O	Y	Y–O	Bg/B*	O	V	O	65	50	44	59
10	2,4,5-Trichlorophenol	B–Gr	O–Bn	T	O	Gr–G	B–G	B	V–G → O	72	57	48	60
11	2,4,6-Trichlorophenol	T–P	G	Y	Cr	B–Gr	Bn–G → Bg	B	B → T	76	60	52	64
12	2,3,4,6-Tetrachlorophenol	T–P	G	Y	Cr	B–Gr	Bn–G → Bg	B	B → Bg	67	53	47	58
13	Pentachlorophenol	O	G	Y	Y	G–T	Bn–G → Bg	Bg	B → Bg	64	50	45	55

* Fluorescence after spraying.

Results and discussion

Table I depicts the spot colors (as well as the R_F values) of the isomeric chlorophenols on silica-gel DF-5 plates obtained with four detecting reagents before and after exposure to ammonia vapors. Tables II and III depict the R_F values of the N-(trichloracetyl) carbamates of isomeric chlorophenols and miscellaneous alkyl and aryl derivatives, respectively.

A number of salient observations can be made in regard to the chromogenic behavior of the isomeric chlorophenols as well as the overall utility of pi-electron detectors employed:

1. The monochlorophenols can be distinguished chromogenically from one another utilizing either DDQ or TCNE reagents with the former being the detector of choice. Differentiation of the above isomers can also be effected utilizing detectors 1, 2 or 3 followed by brief exposure of the plate to ammonia vapors as illustrated in Table I.

2. The isomeric dichlorophenols, *viz.*, 2,3, 2,4, 2,5, 2,6, 3,4 and 3,5 can be distinguished from one another utilizing preferentially the DDQ reagent or secondarily the TCNE reagent followed by subsequent exposure of the sprayed plate to ammonia vapors.

3. The isomeric trichlorophenols, *viz.*, 2,4,5 and 2,4,6 can be differentiated utilizing any of the detecting reagents 1–4 with reagents 1 or 2 preferred.

4. The mono- and dichlorophenols can further be distinguished from the tri-, tetra- and penta-chlorophenols tested utilizing the DDQ reagent followed by exposure of the chromatogram to ammonia vapors. Only the mono- and di-derivatives yield fluorescent spots (see Table I).

5. Overall, in order of decreasing utility for the detection and differentiation of the isomeric chlorophenols (compounds 1–13): DDQ, TCNE > Chloranil > Gibbs.

The utility of the strongest pi-electron acceptors screened in this study, *viz.* DDQ and TCNE (oxidative potential *ca.* 1.0 V) has been previously demonstrated for the detection of 3,4-methylenedioxyphenyl derivatives[27], sulfoxides, sulfones and sulfides[28] and aromatic hydrocarbons[29].

Correlation of R_F values with structure. In addition to the solvents enumerated in Table I (solvent systems A–D), the following solvent systems were screened and rejected because of their less overall utility in effecting separations of at least 0.03 with the majority of the chlorophenols studied:

chloroform–acetic acid–water (4:1:1)

chloroform–acetone–acetic acid (48:2:1)

benzene–acetic acid–water (125:72:3).

1. The isomeric monochlorophenols have been best separated in this study on silica-gel DF-5 plates utilizing solvent C (2.5 % acetone–benzene). The order of R_F values for the monochlorophenols in all the solvent systems tested was $o > m > p$. This same order of separation for the isomeric monochlorophenols has been observed on paper[30] and absorption silica-gel chromatography[31].

2. Separation of isomeric dichlorophenols has best been accomplished utilizing solvent A (chloroform–acetic acid, 5:1). For both solvent systems A and D the following R_F relationship of the above isomers prevails: 2,4 > 2,5 > 3,5 > 3,4. It has not been found possible in this study to effect the separation of 2,3- from 2,4-dichlorophenol with any of the solvent systems employed.

TABLE II

R_F VALUES × 100 OF N-(TRICHLOROACETYL) CARBAMATES OF ISOMERIC CHLOROPHENOLS

No.	*R*	*m.p.* (°C)	*Solvents*			
			A	*B*	*C*	*D*
14	*o*-Chlorophenyl	94– 95	73	58	54	64
15	*m*-Chlorophenyl	92– 93	68	52	50	58
16	*p*-Chlorophenyl	105–106	65	49	46	53
17	2,3-Dichlorophenyl	84– 85	71	54	52	60
18	2,4-Dichlorophenyl	49– 50	69	53	52	58
19	2,5-Dichlorophenyl	95– 97	65	50	49	53
20	2,6-Dichlorophenyl	81– 82	61	46	44	50
21	3,4-Dichlorophenyl	55– 56	54	42	40	46
22	3,5-Dichlorophenyl	123–124	57	44	39	48
23	2,4,5-Trichlorophenyl	55– 56	70	55	56	62
24	2,4,6-Trichlorophenyl	98– 99	75	60	62	66
25	2,3,4,6-Tetrachlorophenyl	58– 59	78	64	67	71
26	Pentachlorophenyl	122–123	74.	60	65	68

TABLE III

R_F VALUES × 100 OF N-(TRICHLOROACETYL) CARBAMATES

$$Cl_3—C(=O)—C(=O)—N(H)—C(=O)—O—R$$

No.	*R*	*m.p.* (°C)	*Solvents*			
			A	*B*	*C*	*D*
27	Methyl	100–101	57	41	37	48
28	Ethyl	57– 58	60	46	42	52
29	*n*-Propyl	44– 45	64	49	47	55
30	Isopropyl	73– 74	60	45	43	52
31	Allyl	32– 33	68	54	51	60
32	Propynyl	54– 55	67	53	52	62
33	*n*-Butyl	38– 39	67	53	52	59
34	*sec.*-Butyl	61– 63	63	50	47	57
35	Isobutyl	80– 81	63	51	46	58
36	*tert.*-Butyl	99–100	60	47	43	54
37	*n*-Amyl	34– 35	73	57	56	60
38	Heptyl	28– 29	80	60	59	67
39	2-Chloroethyl	62– 63	67	49	45	55
40	2-Bromoethyl	54– 55	65	46	49	52
41	2,2,2-Trifluoroethyl	95– 97	62	45	41	50
42	2,2,2-Trichloroethyl	101–102	69	48	44	53
43	2,2,2-Tribromoethyl	124–125	66	46	46	51
44	Cyclohexyl	89– 90	64	50	48	58
45	Cyclopentyl	65– 66	61	46	44	54
46	Piperonyl	88– 89	80	64	59	70
47	Phenyl	108–109	68	53	47	59
48	Benzyl	83– 85	73	56	50	63
49	Phenethyl	43– 44	77	60	54	66

3. The isomeric trichlorophenols, *viz.* 2,4,5 and 2,4,6 have been separated utilizing solvents A–D with the order of R_F values being 2,4,6 > 2,4,5. The observed enhancement of the R_F values due to the *ortho* effect shown in this study for mono- and trichlorophenols has been previously reported for halogenated phenols in paper[30], absorption silica-gel[31] and pH chromatography[24].

4. In regard to the N-(trichloroacetyl) carbamates of the isomeric chlorophenols, analogous chromatographic behavior of these derivatives to their parent precursors was observed, *e.g.* the R_F values were as follows: $o > m > p$; 2,4 > 2,5 > 2,6 > 3,5 > 3,4; 2,4,6 > 2,4,5.

5. Utilizing solvent systems A or D, there is essentially a linear relationship when the R_F values are plotted against the number of substituted carbon atoms for the N-trichloroacetyl alkyl carbamates with the R_F values increasing with increasing chain length.

Although the separation of *n*-, *iso*-, and *tert.*-butyl derivatives could be best affected by solvent systems A and C neither system could resolve the respective N-trichloroacetyl *iso*- and *sec.*-butyl carbamates.

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Concentrating compounds by continuous horizontal thin-layer chromatography

In preparative TLC, the common technique is to place the sample in a small band on the starting line of the plate, and separate the components by single or multiple development, followed by removal of the strip of adsorbent containing one of the compounds, and extraction with a suitable solvent[1–8]. The thus isolated compound can then be identified or determined. Because of band widening during the elution process a relatively large amount of adsorbent must be removed, so that the solution obtained after extraction always contains finely divided adsorbent particles. In quantitative work this is a serious disadvantage, only partly overcome by high-speed centrifuging (15.000 r.p.m.). It is practically impossible to prepare a clear solution which is sufficiently free of background adsorbance for measurement in the U.V. region.

A second disadvantage in quantitative work is the presence of organic compounds in nearly all commercial adsorbents[9]. Dependent upon the elution system, these are more or less eluted, so that on extraction of the separated compounds the solution will be contaminated. It is therefore necessary to pretreat the plates by several elutions with chlorohydrocarbons (*e.g.* trichloroethylene). As the organic compounds are thus concentrated at one end of the plate, their interference in the final solution can largely be obviated in this manner.

The effect of finely divided particles of adsorbent can also be reduced substantially by drastic reduction of the amount of adsorbent required. This, at the same time, would lead to more rapid and more effective extraction.

In the following, a method is described which concentrates separated compounds on a very small surface area by two-dimensional chromatography. Thus only a very small amount of adsorbent need be extracted. Use, in this connexion, is made of the continuous horizontal TLC method according to BRENNER AND NIEDERWIESER[10,11]. Though this method has mainly been applied to the separation of compounds differing very little in R_F value, it appeared to be excellently suited to our purpose: the amount of adsorbent to be extracted could be reduced to 1/50–1/60 that of the amount in normal procedures on 20 × 20 cm plates.

Experimental

As shown schematically in Fig. 1, the set-up for continuous horizontal TLC consists of a chromatoplate covered partly by a second glass plate, kept 2 mm apart

by means of strips along the edge, the space between the plates forming the development chamber. A strip of chromatography paper serves as a wick to transfer the eluant from its reservoir to the lower plate over its full width. After the elution has proceeded to the end of the plate, which is exposed to the air, the solvent evaporates, so that the eluant can be fed continuously.

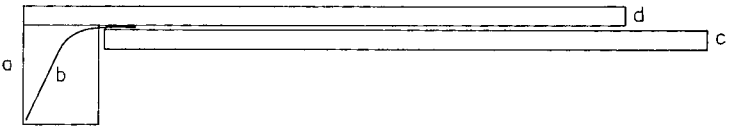

Fig. 1. Continuous horizontal layer chromatography. a = Solvent reservoir; b = paper wick; c = lower plate carrying the adsorbent layer; d = upper plate with edge strips (not drawn).

In our tests we used glass plates of 20 × 20 cm, the lower one covered with a 0.2 mm layer of Kieselgel G-HR (Macherey & Nagel). By way of illustration a mixture of 250 μg of Fettrot 7 B, 250 μg of dimethyl yellow, and 250 μg of indophenol was applied on a line 4 cm from the left side of the lower plate; the latter was covered with the upper plate to form an exposed strip of 2 cm. The paper wick was then positioned to reach to 1 cm from the starting line. The reservoir was filled with chloroform. Elution was started, and continued even after the front of the first band, which was about 12 mm wide, was outside the chamber. Because of evaporation of the chloroform, which can be speeded up by means of a hot air blower, the front of the band did not proceed any further, whereas the rear of the band was still moving. Finally, all the material was concentrated in a straight, very narrow band less than 1 mm wide (see Fig. 2).

Even with irregularities in the shape of the bands during the elution process, the compounds could be concentrated in a straight narrow band. After thus concentrating the first compound, the upper plate was shifted 2 cm backwards, so that a 4 cm wide strip of adsorbent was now exposed. Elution was then continued until the second compound was concentrated. Shifting of the upper plate must be done very carefully and without interruption of the elution process, which is unnecessary, however, if the upper plate is partly replaced by a number of small glass strips laid close together and of widths corresponding with the distances between the separated bands. In this case one strip is removed after concentration of the first compound, and so on.

After concentration of the two dyes elution was stopped, and the upper plate removed. The eluant in the layer was evaporated, and the adsorbent removed to within a distance of 0.5 cm on both sides of the narrow bands containing the concentrated compounds. The plate was then chromatographed at right angles in the same manner, now using a paper wick 1 cm wide. The result was that the compounds were concentrated on a very small area at the side of the plate, as shown in Fig. 3.

In this example the coloured regions are clearly visible. In the case where colourless compounds are to be concentrated, two possibilities arise, *viz.*:

(a) When the compounds show U.V. absorbance, a fluorescent adsorbent may be used and the free end of the lower plate exposed to U.V. radiation. As soon as a compound reaches the exposed area, fluorescence is extinguished. This procedure was followed in the separation and concentration of isomeric aromatic diamines.

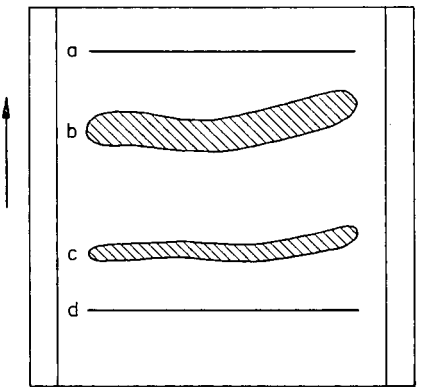

Fig. 2. Concentration in first direction. a = Concentrated in narrow band; b and c = not concentrated; d = start.

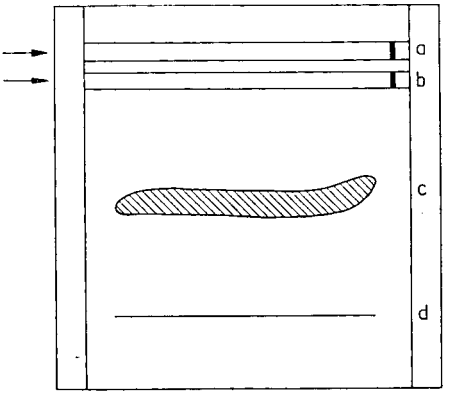

Fig. 3. Concentration in second direction. a and b = Concentrated on small surface areas; c = not concentrated; d = start.

(b) When the compounds to be separated are non-absorbing, it must previously be determined when they will leave the elution chamber and enter the free area. This can be done by running the compound together with a dyestuff under normal TLC conditions and, after development and coloration of the compound, measuring the distance between the spots. With the same dye as a reference in continuous TLC, the upper plate is shifted the moment the dye spot reaches a distance from the end corresponding with that determined in the preliminary test[12].

After scraping off the adsorbent the concentrated compounds can be separated by micro-extraction for identification or further analysis. By this technique solutions with very low background absorbance are obtained without centrifuging, while for the very small amounts of material to be extracted the desorption process is more quantitative.

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Thin layer chromatography of aliphatic nitramines

Availability of numerous cyclic and linear nitramines resulting from recent acetolysis and nitrolysis studies[1] in these laboratories encouraged an investigation of their thin layer chromatographic behaviour. The results, which extend studies of HARTHON[2], HANSSON[3], and FAUTH AND ROECKER[4], have been applied to the separation of many complex reaction mixtures.

Experimental

Materials. Most of the nitramines examined were prepared by published methods (references are given in Table I) although some compounds were obtained from our departmental collection. Purity of samples was established by measuring melting points and infrared spectra.

Analar-grade solvents were used; solvent mixtures containing nitromethane (redistilled) were kept overnight over molecular sieve 4A before use.

Method. Thin layers (0.3 mm) of Silica gel G on glass plates (10 × 20 cm) were dried in air overnight, then activated at 110° for 30 min and stored in Camag drying racks over calcium chloride. Nitramines were applied in acetone (5 μl of 1 % solution), then the thin layer chromatograms were developed with one of the following solvent systems:

A Benzene–nitromethane (2:1)
B Chloroform–nitromethane (10:1)
C Light petroleum (b.p. 40–60°)–acetone (2:1.2)
D Ether–acetone (10:1).

One or more spots of 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) were placed on each plate to check operating conditions. Development took place in filter paper-lined chambers previously saturated with solvent vapour for 1 h. After solvents had travelled 10 cm up the plates, the chromatograms were dried in a current of warm air. Chromatograms developed with solvent mixtures A, B, or C were replaced in the appropriate chamber and subjected to a second development.

The dried plates were sprayed with diphenylamine in ethanol (1 % solution) and irradiated with U.V. light[5].

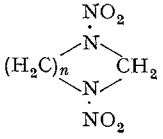
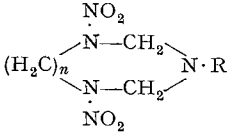
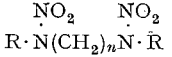
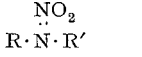
Results and discussion

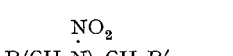
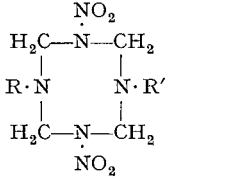
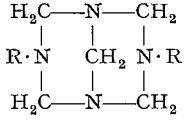
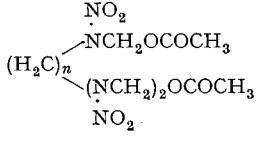
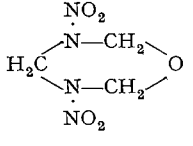
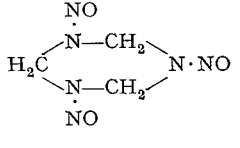
The spray reagent varied in sensitivity towards the compounds listed in Table I. After being irradiated for 30 min most of the nitramines gave purple-grey spots on a tan-coloured background. Compounds containing the nitroxy group (*e.g.* compounds nos. 14, 24 and 26) gave distinctive blue colours which developed rapidly under U.V. light. Only poorly-defined spots accompanied by considerable streaking were obtained with 1,9-dinitroxy-2,4,6,8-tetranitro-2,4,6,8-tetrazanonane (compound no. 36) and it was difficult to identify this compound in mixtures. Difficulty was also experienced in detecting the presence of dinitropentamethylenetetramine (compound no. 47); the compound appeared as a colourless spot on a brown background after prolonged irradiation. Nitrosamines gave a characteristic yellow-brown colour which developed rapidly.

Use of light petroleum–acetone (solvent C) was previously recommended by HARTHON[2] for separating aliphatic nitramines on Silica gel G plates. The present

TABLE I

THIN LAYER CHROMATOGRAPHY OF ALIPHATIC NITRAMINES

Nitramine							*Distance of travel (mm)*					
Formula	*No.*	*n*	*R*	*R′*	*Trivial name*	*Ref. to preparation*	*A* *	*B*	*C*	*D*	*Alumina G* *A*	*Alumina G* *B*
	1	2				6	70	69				
	2	3				1	71	73				
	3	4				1	69	71				
	4	1	NO_2		RDX	7	67	48	44	41	67	49
	5	1	NO			1, 8	67	48	41	39		
	6	1	$COCH_3$		TAX	9, 10	32	15	31			
	7	2	NO_2		Homo-RDX	11	63	43				
	8	2	NO			1	65	54	49	65		
	9	2	$COCH_3$			1, 11	25	12				
	10	3	NO_2			1, 12	57	27				
	11	3	NO			1	49	25	20	14		
	12	3	$COCH_3$			1	17	5				
	13	1	H		MEDNA	13	37 (c)**	16 (c)				
	14	1	CH_2ONO_2		COX	14	79	78				
	15	1	CH_2OCOCH_3		TEX	15, 16	59	65				
	16	1	CH_2OCH_3			17	62	69				
	17	2	H		EDNA	18	24 (c)	6 (c)				
	18	2	CH_2OCOCH_3			11	49	47				
	19	2	CH_2OCH_3			15	56	58				
	20	3	H			15	32 (c)	8 (c)				
	21	4	H			15	25 (c)	6 (c)				
	22		CH_3	H		19	54	40				
	23		n-C_4H_9	H		20	69	65				
	24		CH_2ONO_2	CH_2ONO_2		21	81	82				
	25		CH_2OCOCH_3	CH_2OCOCH_3		9	62	72				
	26	3	ONO_2	ONO_2	ATX	9	77	70				
	27	3	$OCOCH_3$	$OCOCH_3$	BSX	22, 23	56	58	55	82	71	78
	28	3	OCH_3	OCH_3	"104"	9	60	66				
	29	3	OC_2H_5	OC_2H_5		9	73	77				
	30	3	$OCOCH_3$	OCH_3		1	57	60				
	31	3	$OCOCH_3$	OC_2H_5	"107"	9	64	70				

	32	3	H	ONO_2	NSX	24, 25	71	68				
	33	3	H	$OCOCH_3$	MSX	24, 25	61	63				
	34	3	H	OCH_3		24, 25	61	65				
	35	3	H	OC_2H_5		26	69	72				
	36	4	ONO_2	ONO_2	"106"	27	75 (c)	0				
	37	4	$OCOCH_3$	$OCOCH_3$	AcAn	27	46	42	50 (c)	(c)	64	73
	38	4	OCH_3	OCH_3		27	57	56				
	39	4	OC_2H_5	OC_2H_5		27	68	70				
	40	4	$OCOCH_3$	OCH_3		1	50	46				
	41	4	$OCOCH_3$	OC_2H_5		1	59	55				
	42		NO_2	NO_2	HMX	23	48	9	30	13	22	5
	43		NO_2	NO	MNX	28	46	10	24	11		
	44		NO_2	$COCH_3$	SEX	28, 29	19	3	13	6		
	45		NO	NO		1	40	7	18	7		
	46		NO	$COCH_3$	QNX	28	14	2	9	4		
	47		NO_2		DPT	21, 23	43		37			
	48		NO		Dinitroso-PT	28	17	13	24	28		
	49				Homo-BSX	11	45	39				
	50				Cyclonite oxide	17, 30	71	72				
	51***					28	64	54	46	50		

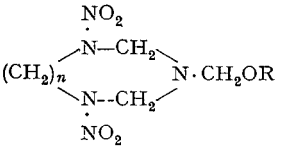
* The letters refer to the solvent systems and development methods used (p. 253).
** Spots accompanied by comet formation are indicated by (c).
*** This compound is included for comparison purposes.

work, whilst confirming HARTHON's results, shows that the solvent mixture is not satisfactory for separating mixtures containing 1,9-diacetoxy-2,4,6,8-tetranitro-2,4,6,8-tetrazanonane (compound no. 37); even when applied to the plate as a single compound this diacetate gave a poor spot with extensive comet formation. The compound showed better chromatographic behaviour on plates developed with benzene–nitromethane (solvent A) and this solvent mixture was suitable for most of the compounds in Table I.

Chloroform–nitromethane (solvent B) and ether–acetone (solvent D) were examined as alternative solvent systems for some separations. The former was particularly useful for separating mixtures of 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclo-octane (compound no. 42) and 1,9-diacetoxy-2,4,6,8-tetranitro-2,4,6,8-tetrazanonane (compound no. 37), compounds not separated by benzene–nitromethane. Cyclic nitramines containing nitrosamine groups were best separated by ether–acetone (solvent D); well-defined spots were obtained and only one development was necessary.

Examination of the results shows that rates of movement of linear nitramines on chromatograms developed with solvents A and B are related to chain length and the nature of the end groups. For a given chain length, distances of travel, when solvent A is used, follow the order: nitroxy- > ethoxy- > methoxy- > acetoxy-terminated chains; with solvent B, the order is: ethoxy- > nitroxy- > methoxy- > acetoxy-terminated chains. Shorter chain compounds travel more rapidly in both solvents, but the effect of chain-length is more marked with solvent B. The exceptional behaviour of 1,9-dinitroxy-2,4,6,8-tetranitro-2,4,6,8-tetrazanonane (compound no. 36) with solvent B may be due to a solubility effect.

Unsuccessful attempts were made to determine distances of travel for compounds of the general formula:



n	1	1	1	1	2	2	2	3	3
R	CH_3CO	CH_3	C_2H_5	t-C_4H_9	CH_3CO	CH_3	C_2H_5	CH_3	C_2H_5
Ref. to preparation	9	1,10	1,10	1	1,11	1	11	1	1

These nitramines and the related eight-membered ring compound, 1-acetoxy-methyl-3,5,7-trinitro-1,3,5,7-tetrazacyclo-octane (PHX)[31], failed to give detectable spots on plates developed with solvents A or B. The method was also unsuitable for detecting 3,5-dinitro-1,3,5-triazacyclohexane nitrate (PCX)[32]; the compound, or its decomposition products, gave a coloured spot at the origin of the developed chromatogram.

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Preparation of a firm polyamide adsorbent for thin-layer chromatography

A thin-layer chromatographic procedure which accomplishes the separation of isomeric flavanones has been reported[1]. The adsorbent employed is Polyamide Woelm* applied to glass plates as a slurry of 1 part polyamide in 7.5 parts benzene–methanol (2:3, v/v). However, due to the fragility of this adsorbent layer, the handling of the plate, removal of areas by scraping, marking and application of sample must be performed with extreme care. Furthermore, the flakiness of this adsorbent layer seriously reduces its use in autoradiography. This flakiness also increases the probability of instrument contamination when using a radiochromatogram scanner for locating radioactive areas.

The purpose of this investigation was to find a method which would give a firm polyamide layer and retain the polyamide's resolving power. Such a procedure has been found and studied with various flavonoids and under conditions necessary for the detection of radioactive areas.

* Use of a company and/or product name by the department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Experimental

Preparation of plates. A mixture of 0.8 g of rice starch[2–4], 0.4 g of silica gel (Fisher No. 1 impalpable powder*) and 9 ml of water in a covered 20 ml beaker was heated for 40 min on a steam bath with occasional stirring. Water (1–2 ml) was added as needed during the heating to prevent caking on the sides of the beaker. This mixture was rinsed with 3 ml of water into a 100 ml beaker containing 5.5 g of Woelm polyamide powder and 35–40 ml of methanol. This mixture was stirred and then blended in a Waring blendor* microcup for 3 min. The resulting mixture was spread as a 250 μ thick layer on 20 × 20 cm glass plates and allowed to dry 2 h at room temperature before use.

Results and discussion

The procedure described above resulted in an adsorbent layer which was as firm as starch-bound silica gel[2]. While the starch is sufficient to produce a firm polyamide adsorbent layer, the addition of silica gel gives a surface which can be easily written upon with a dull pencil. The use of a Waring blendor gave a slurry which formed a smoother surface than could be obtained by simply stirring the ingredients. After completion of the chromatogram, areas of interest may be readily scraped from the glass plate without loss of material. The firmness of this adsorbent layer allows the plates to be used with a radiochromatogram scanner without instrument contamination.

The resolution of different flavonoids including isomeric flavanones such as naringin, naringenin rutinoside, hesperidin and neohesperidin on polyamide–rice starch–silica gel was comparable to that obtained on polyamide alone[1].

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* Use of a company and/or product name by the department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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A colour reaction for psychotropic drugs on thin layer chromatograms

Evolving from a selective test for organosulphur compounds[1] a procedure has been described for the colorimetric estimation of the anthelmintic phenothiazine which depends on treatment with bromine in the presence of aniline[2]. It was shown that the obviously mixed reddish colour resulting from treatment with bromine alone[3] was due to a series of coloured products whose formation was controlled primarily by the amount of phenothiazine present. The function of the aniline was to direct the course of reaction to the appearance of one main product.

The present note describes a logical extension of the test to the detection on chromatograms of psychotropic drugs whose structure is based on the phenothiazine nucleus. In accordance with the results previously described use is made of differences in colour depending on whether the material is treated with bromine directly or in the presence of aniline.

The opportunity has been taken to modify the method of aniline treatment. Previously incorporation of this on to chromatograms was achieved by dipping in solutions in light petroleum. In the present work this has been done conveniently by exposing to aniline vapour.

Experimental

Materials. Commercial samples of pharmaceutical quality were used without further treatment.

Procedure. Thin layer chromatography was carried out with plates of silica gel G (0.25 mm thickness) using a mixture of ethanol–water–acetic acid (20:20:1) as developing solvent. Materials (50 μg) were loaded on to the chromatogram either from a chloroform or an alcohol solution (in the case of prochlorperazine and thioproperazine).

Spots were visualized by exposure to bromine vapour directly or after previous exposure to aniline vapour for periods up to 30 min.

Results

Results are presented in Table I.

Discussion

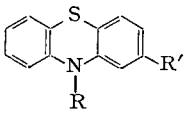
Results show that the materials may be divided into two groups—those which in the direct test give a predominantly brown colour changing to green (promethazine and ethopropazine) compared to the red colours given by the second group. In the latter a repeatable variety of shades is given and the descriptions crimson, rose pink, and orange pink are very meaningful.

This division into two groups is sustained in the aniline test. Here the predominant colour given by the first group is green, particularly after standing, while the remaining materials give a mauve colour.

Structural influence on the sensitivity of the test appears with the perazines, characterized by the N-methylpiperazine substitution in the main side chain. The colours in both the direct and aniline tests, while easily recognizable, are much weaker. With uniform loading of the chromatograms this difference in sensitivity itself may

J. Chromatog., 24 (1966) 259–261

TABLE I

CHROMATOGRAPHY OF PSYCHOTROPIC DRUGS OF THE TYPE: 

Spot No.	Material*	Structure		Colour		R_F**
		R	R′	With bromine	With bromine after aniline	
1	Promethazine	—CH_2—CH(Me)—$N(Me)_2$	H	Green-brown changing to green	Green	0.41
2	Ethopropazine	—CH_2·CH(Me)·$N(Et)_2$	H	Brown changing to green	Green, intensifying with time	0.36
3	Chlorpromazine sample 1	—CH_2·CH_2·CH_2·$N(Me)_2$	Cl	Crimson	Mauve-purple	0.39
4	Chlorpromazine sample 2	—CH_2·CH_2·CH_2·$N(Me)_2$	Cl	Crimson	Mauve-purple	0.39
5	Trimeprazine (tartrate)	—CH_2·CH(Me)·CH_2·$N(Me)_2$	H	Rose-pink	Mauve-purple	0.41
6	Prochlorperazine (maleate)	—CH_2·CH_2·CH_2·N(piperazine)N·Me	Cl	Crimson	Mauve-purple	0.25
7	Trifluoperazine	—CH_2·CH_2·CH_2·N(piperazine)N·Me	—CF_3	Orange-pink	Mauve-purple	0.28
8	Thioperazine (methanesulphonate)	—CH_2·CH_2·CH_2·N(piperazine)N·Me	—$SO_2N(Me)_2$	Pink	Pale mauve	0.25
9	Methotrimepazine	—CH_2·CH(Me)·CH_2·$N(Me)_2$	—OMe	Blue	Deep mauve changing to blue	0.42

* As hydrochloride unless otherwise stated.
** The R_F value is subject to change with temperature.

be used to assist identification being so markedly associated with the structure of the drug.

It should be noted that the strong test given by other members of the series permits reduction (to 20 μg) in the amount used.

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Thin-layer chromatography of some phenothiazine derivatives

Thin-layer chromatography (TLC) is increasingly being used to identify pharmaceutical mixtures[1] and also to determine quantitatively their composition. This technique has been used successfully in the separation of many phenothiazine derivatives. The separation of hydroxy derivatives, chlorophenothiazine and bromopromazine has, nevertheless, remained a problem. Their separation with the aid of TLC is reported below.

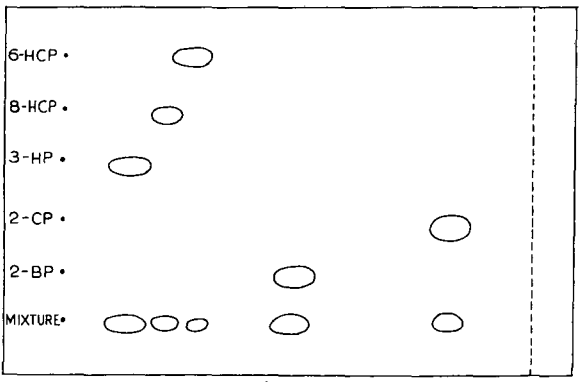

Fig. 1. Separation of 6-hydroxychloropromazine (6-HCP), 8-hydroxychloropromazine (8-HCP), 3-hydroxypromazine (3-HP), 2-chlorophenothiazine (2-CP) and 2-bromopromazine (2-BP).

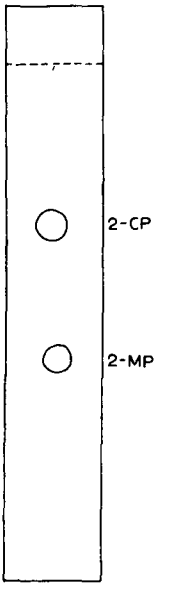

Fig. 2. Separation of 2-chlorophenothiazine (2-CP) and 2-methoxyphenothiazine (2-MP).

TABLE I

R_F AND COLOUR OF THE SPOTS WITH DRAGENDORFF'S REAGENT OF SOME PHENOTHIAZINE DERIVATIVES

Solvent systems: $CHCl_3$–C_2H_5OH (70:30) for compounds 1–5, benzene for compounds 6 and 7.

No.	*Name of chemical*	$R_F \times 100$	*Colour of spot*
1	6-Hydroxychloropromazine	27	Orange
2	8-Hydroxychloropromazine	23	Orange
3	3-Hydroxypromazine	17	Orange change to brick red
4	2-Chlorophenothiazine	73	Green
5	2-Bromopromazine	46	Orange
6	2-Methoxyphenothiazine	38	Violet
7	2-Chlorophenothiazine	66	Green

A mixture in $CHCl_3$ of 6-hydroxychloropromazine, 8-hydroxychloropromazine, 3-hydroxypromazine, 2-chlorophenothiazine and 2-bromopromazine was separated on a glass plate (15 cm × 10 cm) coated with silica gel G, thickness 250 μ (activated at 110° for half an hour). The resolution of the mixture was best afforded by a solvent system chloroform–ethanol (70:30) (Fig. 1). 2-Methoxyphenothiazine could not be separated from the mixture of the above-mentioned compounds with chloroform–ethanol (70:30) because of its very close proximity with the R_F value of 2-chlorophenothiazine. However, 2-methoxyphenothiazine and 2-chlorophenothiazine can be separated on silica gel G with benzene (Fig. 2). The R_F values and the colour of the spots with Dragendorff's reagent are given in Table I.

During the TLC study of these phenothiazine derivatives it was observed that hydroxy derivatives of phenothiazine are highly absorbed on silica gel, their migration being very small with nonpolar solvents as compared with the halo derivatives and 2-methoxyphenothiazine. R_F values in C_6H_6 and $CHCl_3$, respectively, are: 2-methoxyphenothiazine, 0.43, 0.59; 2-chlorophenothiazine, 0.63, 0.68; 2-bromopromazine 0, 0.023. Hydroxy derivatives did not move from the starting line.

The limit of detection of the Dragendorff reagent on a TLC plate is 0.2 μg for chloropromazine.

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Separation of hydroxocobalamin and cyanocobalamin by thin-layer chromatography

Recently hydroxocobalamin has been used extensively for therapeutic purposes. It has haematopoietic activity approximately equivalent to that of cyanocobalamin, but is a more desirable form of vitamin B_{12} for human use than cyanocobalamin because of its more effective retention by the body, its greater reactivity and high ability to form stronger bonds with proteins[1,2]. Hydroxocobalamin, isolated from microbial fermentations[3,4], or obtained from cyanocobalamin (by irradiation[5] or by catalytic hydrogenation[6,7]) is accompanied by other cobalamins, very often by cyanocobalamin.

There have been reports[8,9] on the separation of hydroxocobalamin from cyanocobalamin on silica thin layers, but the suggested methods are complicated and time consuming.

A new, very rapid and simple method for the separation of hydroxocobalamin and cyanocobalamin on thin layers of dry alumina is reported here. Advantage was taken of the property of hydroxocobalamin to form a compound of the cobalichrome group with NH_4OH[10]. Under these conditions cyanocobalamin does not change, so that both compounds can be easily separated, and the individual cobalamins can then be determined quantitatively by known techniques[11].

Experimental and results

The experiments were performed with aqueous solutions of cyanocobalamin, hydroxocobalamin and mixtures thereof whose pH had been adjusted to 8.5 with dilute NH_4OH.

Different mixtures of isobutanol, *n*-butanol, isopropanol and water with the addition of NaOH, NH_4OH and CH_3COOH were examined as solvent systems. The best separation, with good compact spots, was obtained with the mixture isobutanol–isopropanol–water, 1.5:1:1.25 (with addition of NH_4OH until the pH was 8.5). This is due probably to the fact that only under these conditions will hydroxocobalamin form a cobalichrome with NH_4OH, since with the same solvent system, but with the pH adjusted to 8.5 with dilute NaOH, the spots were elongated and not clearly separated.

Neutral alumina (second degree of acitivity according to Brockmann) was the most suitable as the adsorption medium. The R_F values in this case are: hydroxocobalamin, 0.30 and cyanocobalamin, 0.46.

When basic alumina was used, the spots were not well defined, and acid alumina did not give any separation.

Thin layers of dry alumina (about 1 mm thick) were prepared according to the usual method[12] on frosted glass plates, 30 × 8 cm.

The solutions of the cobalamins were applied 2–3 cm from the edge of the plate. After the spots had been dried, the plate was placed in an inclined position (20°) in an airtight chromatographic chamber, saturated previously with solvent system, 1–2 cm of the lower edge of the plate dipping into the solvent. The development of the chromatogram was effected for 4 h (temperature, 20 ± 0.5°) until the solvent had run about 24–26 cm.

The minimum amount of each compound detectable was 0.5 μg.

The method described for the separation of hydroxocobalamin and cyanocobalamin is quick and simple, can be easily carried out, and can be used for quantitative determination.

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Ion exchange chromatography of some acidic and aromatic amino acids*

Available methods for separating phosphorylated and other acidic amino acids by ion-exchange column chromatography are time-consuming and do not provide good resolution. In view of the significance of glutamic acid and its metabolites in neural function and metabolism, and because the phosphoamino acids serve as the active sites of enzymes involved in phosphorus metabolism, it was desirable to develop an effective method for their separation.

Experimental

About 500 g of Dowex 1-X8 (AG 200–400 mesh BIO-RAD) was suspended in 2 l of water in a 3 l beaker and allowed to settle for five minutes. Approximately three-fourths of liquid containing the finer particles was decanted for subsequent use. After washing in the usual manner, the resin was converted to the acetate from the chloride form using 2 *M* sodium acetate.

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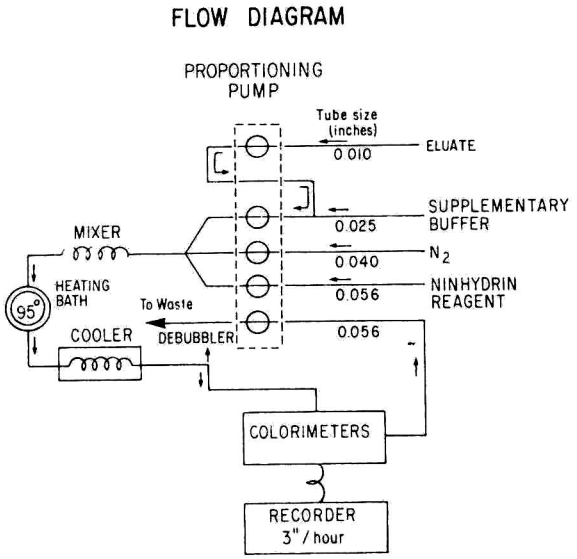

Fig. 1. Flow diagram.

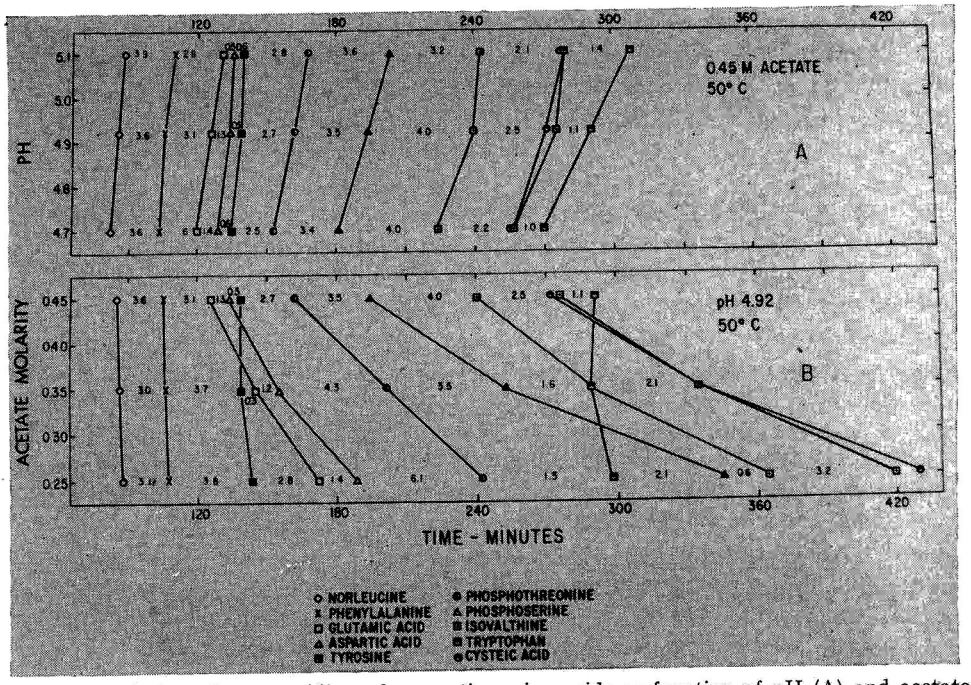

Fig. 2. Resolution of some acidic and aromatic amino acids as function of pH (A) and acetate concentration (B).

A 0.9 × 60 cm column was prepared with the resin and equilibrated at 50° for 2 h with the same acetate buffer used as an eluent in each run. The column was attached to the Technicon Auto Analyzer, and the amino acid analysis was conducted according to the standard micro-method recommended by the manufacturer with the following modifications: A Milton Roy mini pump was adjusted to supply 20 ml of buffer per hour, which was the maximal rate permitted to obtain a clear separation with the column used. The eluate from the column was channeled through an orange-green Tygon manifold tube where it was supplemented with acetate buffer. (Supplementation was necessary because of the inadequate flow rate.) The combined mixture was passed through an orange-white Tygon tube, mixed with the ninhydrin reagent, segmented with nitrogen gas, and incubated in the heating bath for 15 min at 95°. The speed of the chart recorder was 3 in./h. Technicon's certified 4 *N* sodium acetate buffer pH 5.51 was used to prepare the eluent, after appropriate dilution and adjustment of pH with glacial acetic acid. Standard amino acids were obtained from Sigma Chemical Company. Isovalthine (L-isovalthine + L-alloisovalthine) was a gift from Dr. S. OHMORI.

Results

The chromatographic separation of acidic and aromatic amino acids was studied as a function of pH and concentration of acetate buffer (Figs. 2A and B). With an increase in pH in the range 4.7–5.1 at an acetate concentration of 0.45 *M*, there was a slight retardation in the elution but no significant improvement in the resolution of the amino acids examined. Its hould be noted that isovalthine possesses two peaks, the second of which is overlapped completely with that of cysteic acid (see "Discussion"). The numerical value between the plots of the amino acids in the figure refers to the resolution ratio[1]. Although the distances between adjacent peaks were sufficiently great, the later peaks (Figs. 2A and B) were too broad to permit a high resolution ratio. Fig. 2B refers to results obtained when the pH was held constant at 4.92 while the acetate concentration was varied. With the exception of the aromatic amino acids, the position of each amino acid was markedly dependent upon the acetate concentration. The inability of varying acetate concentration to affect the elution of aromatic amino acids may be due to their specific adsorption to the resin. Again, the second peak of isovalthine completely overlapped with that of cysteic acid. A clear resolution of glutamic acid from aspartic acid could be obtained with the 0.20 *M* acetate buffer pH 5.0; however, elutions of amino acids appearing after tryptophan, which was eluted separately immediately after aspartic acid, were greatly retarded. A temperature of 50° was necessary to obtain a good separation, while phosphothreonine and phosphoserine, which are more labile, were eluted separately with 0.25 *M* acetate at 38°. It has not yet been possible to clearly separate the amino acids by a single chromatographic run; however, certain amino acids could be resolved within individual groups. For example, norleucine, phenylalanine, tyrosine, glutamic acid and aspartic acid can be separated by using 0.25 *M* acetate buffer pH 4.6. The resolution ratios of adjacent peaks were 3.3, 4.0, 3.8 and 2.2, respectively. Phosphothreonine and phosphoserine are best separated by 0.40–0.45 *M* acetate buffer pH 4.7. Although cysteic acid was poorly separated from tryptophan and the second peak of isovalthine, its analysis is made possible by virtue of the fact that the tryptophan can be destroyed by acid hydrolysis, while isovalthine

is only present in trace amounts, if at all. Amino acids such as phenylalanine, tyrosine, glutamic acid, aspartic acid and cysteic acid were quantitatively recovered, while the recovery of phosphothreonine, phosphoserine, tryptophan and isovalthine was 75.5, 86.0, 78.5 and 57.0 per cent respectively. The reproducibility of the position of each peak was within ± 1.0 per cent and the reproducibility of the areas of the peaks ± 4.0 per cent. No decrease in the exchange capacity of the resin was noted after use for two months.

Discussion

Although KENNEDY *et al.*[2] used a Dowex 1 acetate column to isolate phosphoserine from a partial protein hydrolysate by employing a gradient of acetate buffer, the time required for the elution of phosphoserine was 26 h, as compared to 4 h in the method described. If a finer spherical resin were to become available, the flow rate could then be increased to permit a still faster and better separation.

The decomposition of dicarboxylic amino acids on the Dowex 1 acetate column which was described by HIRS *et al.*[3] was not observed. The loss of significant quantities of phosphoserine, phosphothreonine, tryptophan, and, especially, isovalthine would seem to be related to the high temperature used as well as to some characteristic of the resin. Isovalthine was completely resolved into its components, L-isovalthine and L-alloisovalthine[4] by the present method. The first peak was presumed to be L-isovalthine and the second L-isoallovalthine from chromatography on a strongly acidic cation exchanger[4].

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The detection of amino compounds on paper chromatograms with diazotized *p*-nitroaniline

Diazotized aromatic amines have long been used to detect phenols on paper chromatograms[1]. However, they are not specific for phenols since sulphanilic acid and *p*-nitroaniline give colours with indoles[1], and *p*-nitroaniline with the ammonium ion[2]. These observations together with others arising in the course of studies on cocoa bean phenols prompted this investigation into the specificity of diazotized *p*-nitroaniline, sulphanilic acid and *o*-dianisidine, all of which are used for the detection of phenols[3]. The first was prepared by WHITEFIELD's method[2] and the remaining two by LINDSTEDT's method[4].

The compounds to be tested were made up in aqueous or aqueous alcoholic solution, spotted on filter paper and silica gel thin layers, and sprayed first with the diazotised amine and then with 20 % aqueous sodium carbonate solution. Sulphanilic acid and *o*-dianisidine gave brown colours only with aromatic amines and histidine. *p*-Nitroaniline, on the other hand, gave colours with a wide range of amino compounds. On paper, thirteen common amino acids, putrescine and methylammonium chloride gave mauve or purple spots, histidine a purple-brown spot, tyrosine a grey spot, hydroxyproline and pyrrolidine yellow spots, creatinine and *o*-phenylenediamine brown spots, and *p*-aminobenzoic acid a deep red spot. There was no colour with urea, hydrazine hydrochloride, betaine hydrochloride, hydroxylamine hydrochloride and nicotinamide, and only a faint yellow colour with creatine. All the compounds giving mauve or purple colours gave yellow spots on silica gel and yellow spots on paper (sometimes very faint) when the carbonate spray was omitted. The purple colours with amino acids are more stable than those of ninhydrin but fade on prolonged exposure to light. The sensitivity of the reagent for amino acids is similar to that of ninhydrin.

It is clear that diazotised *p*-nitroaniline reacts readily with many amino compounds, both aliphatic and aromatic, and it has now been shown by the usual chromatographic techniques that a number of spots appearing on chromatograms of cacao extracts (spots 17–26, Fig. 4, ref. 5) are not due to phenols but to amino acids.

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Contamination of paper chromatograms by scissors

In recent paper chromatography work dealing with the analysis of aqueous solutions of trace amounts of Fe(II) and Fe(III) it was observed that the act of cutting the wet chromatograms with clean ferrous-alloy scissors released minute amounts of iron to the paper. It seems that the mechanical shearing force exerted at the point of contact between the paper and the scissor blades combined with the oxidizing and/or solvent action of the solution in the wet paper creates an environment favorable to the release of iron.

Experiments were conducted using paper which had been previously washed free of iron. The paper was then saturated with either distilled water or dilute sulfuric acid. Cuts of varying length were made using clean scissors. The cut paper was then eluted with dilute sulfuric acid, the solution concentrated, and the total iron determined colorimetrically as the ferrous-1,10-phenanthroline complex. The amounts found were of the order of 10^{-9}–10^{-8} mole/cm. The contamination was not detected when cuts were made in dry paper.

Stainless steel, nickel-plated and chrome-plated ferrous-alloy scissors were all found to participate in this phenomenon. Close examination of the "plated" scissors indicated that the nickel or chromium film had been either ground off during the sharpening process or destroyed because of frequent use, thus exposing a surface containing a high concentration of iron atoms. The contamination problem was effectively eliminated by replating the sharpened, commercially available scissors with nickel.

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Note by the editor

This contamination was also observed in work with carrier-free radioisotopes, and therefore scissors made of Perspex were used, which, however, did not work well with wet paper. We never did determine the quantity left on the paper.

^{14}C-Labeled pyrrolidonecarboxylic acid as a contaminant in chromatography*

Chromatographic techniques are employed frequently to separate amino acids, keto acids and related compounds.

In recent separations of ^{14}C-labeled amino acids from brain tissue extracts, we used both chromatographic and ionophoretic techniques. After two-dimensional paper chromatography with water-saturated phenol and water-saturated lutidine[1] as the first and second solvent systems, respectively, we found that the "alanine spot" on the chromatogram was quite highly labeled. Ionophoretic separation[2], on the other hand, yielded an "alanine spot" with no appreciable radioactivity.

When the spot of "radioactive alanine" on the paper chromatogram was cut out, eluted with water, concentrated and rerun ionophoretically (pH 1.9), a radioactive, ninhydrin negative spot remained close to the origin whereas the ninhydrin positive unlabeled alanine migrated a long distance.

The radioactive spot was again eluted, hydrolyzed[3] and rechromatographed. It yielded a ninhydrin-positive radioactive compound which migrated like glutamic acid. The ninhydrin-negative radioactive component had the same R_F as authentic pyrrolidonecarboxylic acid in four different chromatographic solvent systems[4] and in electrophoretic separations under two different pH conditions. It appears probable, therefore, that the contaminant is pyrrolidonecarboxylic acid.

We have detected this cyclized ninhydrin-negative derivative of glutamic acid and glutamine in many brain tissue extracts, particularly when ethyl alcohol was used as a deproteinizing agent and extracts were concentrated by evaporation. Pyrrolidonecarboxylic acid may have been formed as an artifact during sample preparation or represents a normal constituent of the tissues[5].

Investigators working with tissue extracts containing radioactive glutamate-^{14}C, glutamine-^{14}C, glutathione-^{14}C or other γ-glutamyl amino acids would be well advised to check separately the migration of pyrrolidonecarboxylic acid-^{14}C in their chromatographic, electrophoretic or column systems so as to avoid erroneous results due to the unknown coincidence of pyrrolidonecarboxylic acid-^{14}C with other compounds.

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Two-dimensional paper chromatography interspersed with reaction on the paper*

Many situations arise in which it is desired to examine the effect of a reagent or enzyme on the individual constituents of a chromatographically separable mixture. The use of more or less specific spray or dip reagents is commonplace for both paper and thin-layer chromatography. Endless variations are possible, as in the use by FRENCH AND WILD of a phosphorylase–glucose-1-phosphate spray to reveal specific primers for phosphorylase[1]. The present communication deals with a modification of the FRENCH AND WILD technique. In this modification, the mixture is applied near one corner of a large sheet of paper, as for two-dimensional chromatography. Following irrigation in one dimension, the area containing the separated or partially separated constituents is treated with a specific reagent or enzyme, and reaction is allowed to occur on the paper. Finally the paper is dried and subjected to chromatographic irrigation in a direction at right angles to the original direction. The rationale of the method, as applied to structure analysis of ε-dextrin, is illustrated in Fig. 1a.

We here report application of this technique to enzyme action on starch oligosaccharides. The method is particularly useful to rapidly survey enzyme action on individual members of homologous series. In some of the following examples, we have also used it as a convenient method for identifying and characterizing constituents of mixtures.

Experimental

Chromatography has been carried out on Whatman No. 1 paper at room temperature[2,3] or Whatman 3 MM paper at about 80°[4]. The latter was washed with water and ethanol to remove interfering impurities. Irrigating solvents employed for separation of starch oligosaccharides have been mainly 80 % *n*-PrOH, and *n*-BuOH–pyridine–water (6:4:3 or 6:4:4, v/v)[3,4]. Multiple ascent (2–6 times) is particularly effective in resolving higher starch oligosaccharides in the range up to 15 D-glucose units. The mixture of oligosaccharides is applied in a single spot near the corner of a square or rectangular sheet of paper, dried, and subjected to irrigation in the first direction. After the desired amount of irrigation, the paper is dried and the area containing the resolved components is sprayed with an appropriate reagent or enzyme solution. With reactions that require more than a few minutes, the damp paper may be suspended in a damp chamber to prevent it from drying out. No serious difficulties have been encountered from diffusion during the damp reaction, although overspraying leads to "bleeding" and distortion of the finished chromatogram. After the reaction has gone for the desired length of time, the paper is dried and again subjected to irrigation in a direction at right angles to the original direction.

Revelation of spots may be made by any suitable method, for example, the silver nitrate–alkali method[2] which is very effective for reducing sugars. Results may be improved by substituting sodium carbonate for sodium hydroxide, and dipping the

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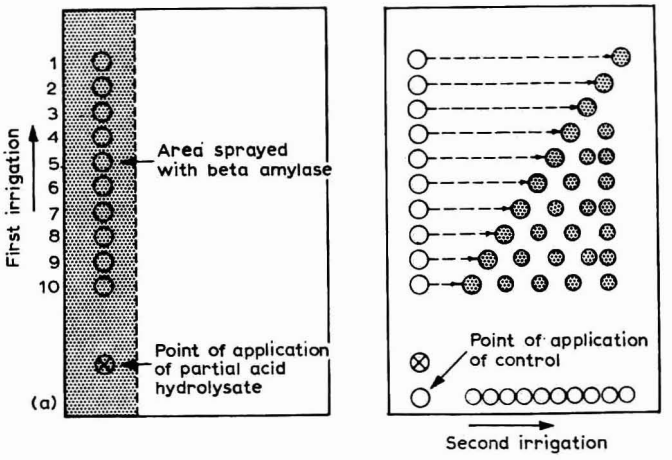
papers into the reagents rather than spraying. Following adequate revelation of the carbohydrate zone, further darkening is prevented by briefly treating with photographic fixer (Kodak F-24) and water washing.

Example 1. Action of beta amylase on partial acid hydrolysis products of epsilon dextrin. Crystalline ε-dextrin[5] (1.35 mg) was partly hydrolyzed by heating in a sealed tube at 100° with 0.15 ml of 0.1 *N* HCl for 60 min. It was neutralized with pyridine and applied near one corner of a paper. After four ascents with *n*-BuOH–



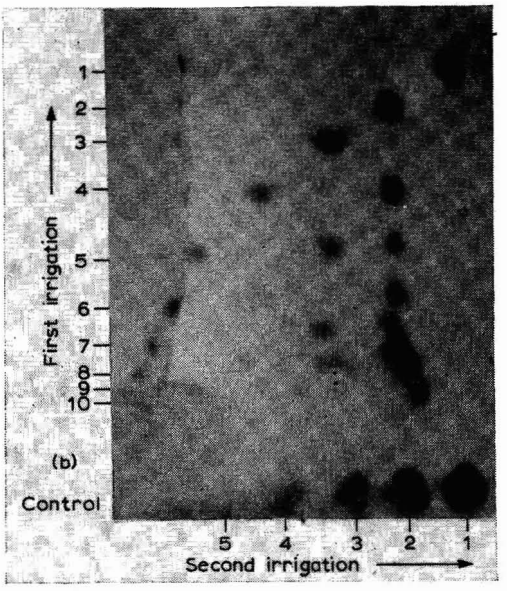


Fig. 1. Action of β-amylase on products of partial acid hydrolysis of ε-dextrin. Fig. 1 a is the schematic representation, and Fig. 1 b is the actual experimental result. The original sample was applied at ⊗. Overspraying with β-amylase solution in the lower left-hand part of Fig. 1 b has resulted in some distortion of the chromatogram in the G_8–G_{10} region. A control was applied at ○ just prior to rirrigation in the second dimension. The numbers 1–10 indicate G_1–G_{10}.

pyridine–water (6:4:4) the paper was dried and sprayed with a solution of β-amylase (Worthington Biochemical Corp., Freehold, N.J.; 0.01 ml of suspended crystals per ml of water). After standing 60 min in a damp atmosphere, the paper was dried and again subjected to chromatography (2 ascents) in the perpendicular direction. After drying, the reducing sugars were revealed using the silver dip method. Fig. 1a represents the rationale in schematic form and Fig. 1b the actual experimental result.

The enzyme β-amylase is very specific for linear starch chains[6]. Therefore, susceptibility to β-amylase is convincing evidence that the oligosaccharides (and hence the parent cyclic molecule) must contain only α-1 → 4-D-glucosidic links. Moreover, because β-amylase is very specific in removing maltose from the non-reducing terminus of starch chains, from the intermediate and final products one can easily tell how many D-glucose units are present in each individual oligosaccharide even though these may not be clearly resolved on the one-dimensional chromatogram. For example, from maltopentaose (G_5) one can clearly see unchanged G_5, as well as G_3 and G_2 in at least approximately equal amounts. In the range G_8–G_{12} where the one-dimensional resolution is impaired after enzyme reaction and perpendicular chromatography there is an obvious pattern of "even" and "odd" oligosaccharides produced by partial action of beta amylase.

The finished chromatogram clearly shows that the original series of starch oligosaccharides terminated very sharply with G_{10}, and hence the ε-dextrin must have contained 10 D-glucose units. This result could not have been obtained by conventional one- or two-dimensional chromatography inasmuch as the unchanged ε-dextrin interfered seriously with the resolution of oligosaccharides in the critical range of 8–12 glucose units.

Example 2. Radioactive oligosaccharides formed during photosynthesis in the presence of $^{14}CO_2$. The formation of radioactive oligosaccharides during photosynthesis in $^{14}CO_2$ was brought to our attention by BASSHAM AND CALVIN[7], who kindly furnished radioactive algal oligosaccharides. Action of β-amylase on the isolated individuals indicated that they were starch oligosaccharides[8]. Similar compounds were produced by photosynthesis in soybean leaves with $^{14}CO_2$ as described by FISHER[9]. The deionized, neutral leaf extracts containing radioactive oligosaccharides were subjected to chromatography in one dimension, sprayed as in Example 1 with β-amylase and again subjected to chromatography in the perpendicular direction. The resulting chromatogram was subjected to radioautography (Fig. 2). As in Example 1, susceptibility to β-amylase confirmed that the original radioactive compounds are linear starch oligosaccharides. Moreover, by counting the radioactivity in the individual maltose and glucose spots, it was possible to infer the distribution of radioactivity in the oligosaccharides. In this experiment, quantities of material too small for ordinary chemical work were identified by their behavior with a specific enzyme, and some indication was obtained regarding the distribution of radioactivity in the individual compounds.

Example 3. Starch oligosaccharides produced by action of malt α-amylase on waxy maize starch. This mixture was thought to consist of linear as well as branched components. The mixture was subjected to chromatography as in Example 1, treated with pullulanase[10,11] (an enzyme specific for α-1 → 6-D-glucosidic links), and again subjected to chromatography in the perpendicular direction (Fig. 3). Linear oligo-

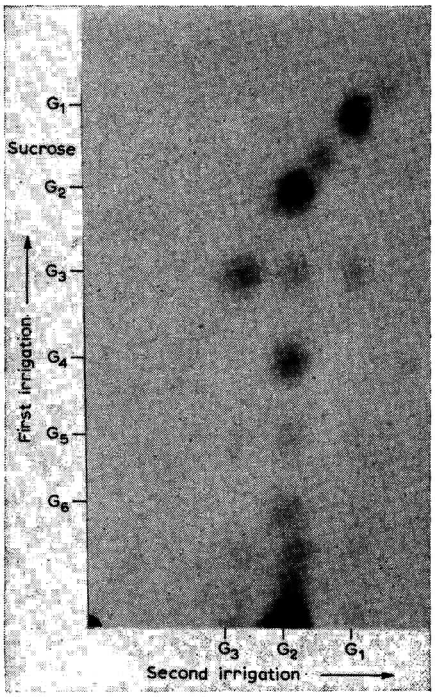
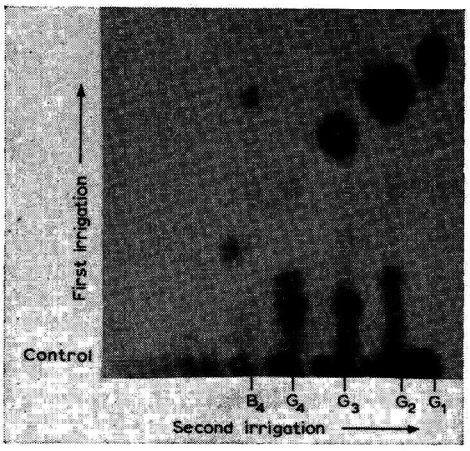


Fig. 2. Identification of radioactive starch oligosaccharides by action of β-amylase. Solvents: first irrigation, 2 ascents in BuOH–pyridine–H_2O (6:4:4); second irrigation, one ascent in the same solvent.



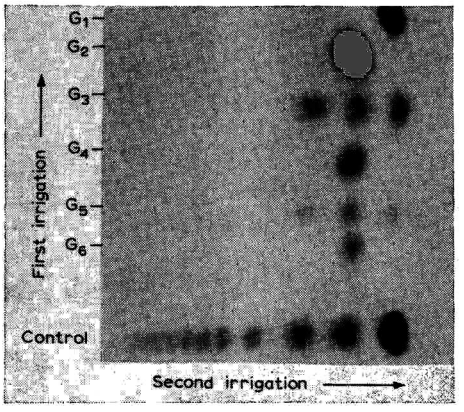


Fig. 3. Action of pullulanase on malt enzyme dextrins.

Fig. 4. Action of β-amylase on products of malt enzyme acting upon maltohexaose.

saccharides were not cleaved by pullulanase; oligosaccharides containing 1 → 6 links could be identified by their cleavage products. Unexpectedly, maltose gave a tetrasaccharide indicating that a condensation reaction had occurred[12].

Example 4. Starch oligosaccharides produced by action of a malt enzyme on maltohexaose. A malt α-amylase sample was prepared from barley malt by heat treatment and glycogen precipitation[13]. The preparation was suspected to contain a transferase in addition to an α-amylase. Hence, products of its action on maltohexaose might contain α-1 → 6-linked oligosaccharides, which would be at least partly resistant to β-amylase. The oligosaccharide mixture was treated as in Example 1, and the results are presented in Fig. 4. All oligosaccharides in the sample were degraded to give maltose (even-membered oligosaccharides) or maltose plus glucose and maltotriose (odd-membered oligosaccharides). Thus the original sample contained only glucose and linear α-1 → 4-linked oligosaccharides, and there cannot have been any appreciable 1 → 4; 1 → 6 transferase activity in the malt enzyme preparation.

Example 5. Branched oligosaccharides produced by action of salivary amylase on glycogen. Shellfish glycogen (purchased from Sigma Chemical Co., St. Louis, Mo.) was extensively treated with crystalline salivary amylase[14] to give products as shown in the one-dimensional chromatogram (Fig. 5a). Chromatographically visible products include glucose, maltose, singly branched oligosaccharides (B_4–B_7) and putative doubly-branched oligosaccharides (BB)[8,15]. The mixture of products was applied to the corner of a paper as in Example 1, separated in one dimension (3 ascents), dried,

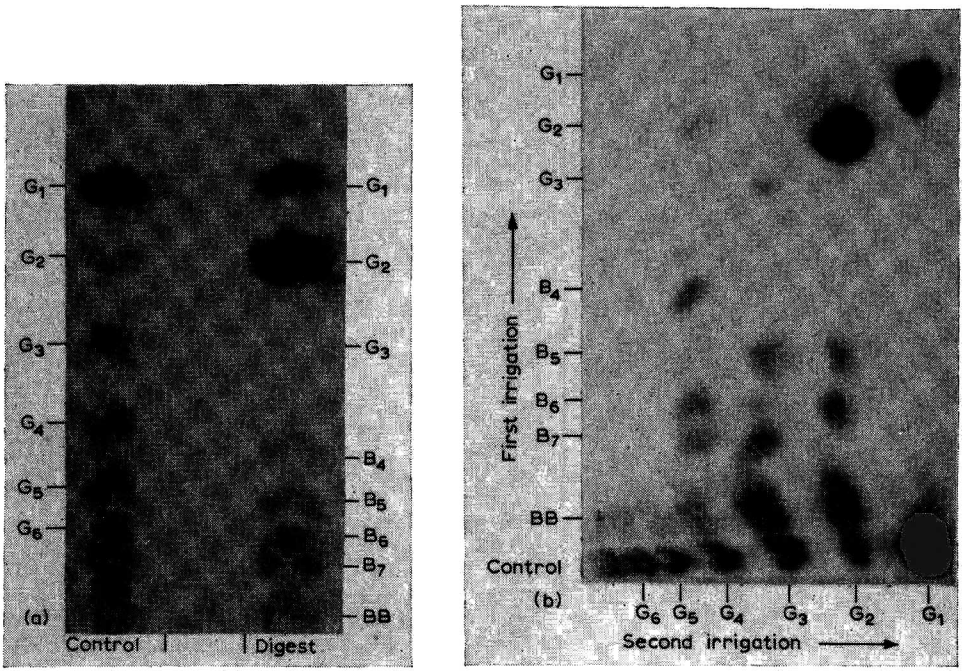


Fig. 5. (a) One-dimensional chromatogram of products of salivary amylase action on shellfish glycogen. B_4–B_7: singly-branched oligosaccharides; BB: doubly-branched oligosaccharides. Control contains only linear oligosaccharides. (b) Two-dimensional chromatogram showing products of pullulanase action on the sample of Fig. 5a.

and sprayed with a solution of pullulanase. After standing overnight in a damp atmosphere, the paper was dried and submitted to chromatography in the perpendicular direction (2 ascents). The results (Fig. 5b) indicating the susceptibility of the salivary amylase branched limit dextrins to pullulanase action as follows. All the singly and doubly branched oligosaccharides, except B_4 and a trace component of B_5, are degraded to form linear oligosaccharides, mainly maltose, maltotriose and maltotetraose. The doubly-branched oligosaccharides produce in addition small amounts of higher oligosaccharides in the range G_5–G_8 and possibly higher (unresolved in this chromatogram).

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Detection of *tert.*-butyloxycarbonyl derivatives on paper and thin-layer chromatograms

Since the *tert.*-butyloxycarbonyl (*t*-Boc) group was first introduced as an amino nitrogen protecting group in peptide synthesis it has gained very wide usage and has become second only to the benzyloxycarbonyl group[1]. Recently it has been shown that *tert.*-alkyloxycarbonyl derivatives of amino acids and peptides, besides being very sensitive towards acid, are also heat sensitive. The *tert.*-alkyloxycarbonyl group can be removed thermally either in the fused state or in aqueous solution to give the corresponding free amino acid or peptide in over 90 % yield[2].

Under the usual conditions, both paper and thin-layer chromatograms of *tert.*-butyloxycarbonyl derivatives of amino acid and peptides give a negative ninhydrin test[3]. We have found that a positive ninhydrin test is obtained if the paper or the thin-layer plate was heated at 125–130° for some time, before or after spraying with the ninhydrin reagent.

TABLE I

R_F VALUES OF *tert.*-BUTYLOXYCARBONYL DERIVATIVES ON PAPER CHROMATOGRAMS

Derivative	*Solvent A (butanol–acetic acid–water)*[4]	*Solvent B (sec.-butanol–ammonia*[5]*)*
t-Boc·Gly·OH	0.90	0.53
t-Boc·Leu·OH	0.95	0.74
t-Boc·Try·OH	0.93	0.66
t-Boc·Tyr(OBz)·OH	0.85	0.95
t-Boc·Lys(*t*-Boc)·Ala	0.82	0.78
t-Boc·Lys(Z)·Lys(Z)·Ala·OMe	0.89	0.91
t-Boc·Leu·Lys(Z)·Lys(Z)·Ala·OMe	0.92	0.92

In the case of paper chromatograms the best results were obtained by first spraying the dry paper with the ninhydrin solution and then heating it in an oven at 125–130° for 25 min. R_F's of several *tert.*-butyloxycarbonyl derivatives, which have been obtained using Whatman No. 1 filter paper, are given in Table I.

After heating thin-layer chromatograms of *tert.*-butyloxycarbonyl derivatives at 125–130° for 25 min, positive ninhydrin spots are obtained upon spraying the hot plates with an 0.25 % solution of ninhydrin in butanol. Some of the R_F's which have been obtained using 20 × 20 cm glass plates coated with an 0.25 mm layer of silica gel G (E. Merck and Co., Darmstadt, Germany) are given in Table II.

TABLE II

R_F VALUES OF *tert.*-BUTYLOXYCARBONYL DERIVATIVES ON THIN-LAYER CHROMATOGRAMS

Derivative	*Solvent A (methanol–water (9:1))*	*Solvent B (dioxan–water (6:4))*	*Solvent C (sec.-butanol–ammonia)*[5]
t-Boc·Gly·OH	0.62	0.83	0.37
t-Boc·Leu·OH	0.61	0.74	0.49
t-Boc·iLeu·OH Z	0.62	0.83	0.49
t-Boc·Lys·OH *t*-Boc	0.57	0.81	0.52
Z·Lys·OH	0.62	0.87	0.77
t-Boc·Thr·O⁻DCHAH⁺	0.65	0.84	0.40
t-Boc·Try·OH	0.70	0.84	0.45
t-Boc Lys·Ala *t*-Boc	0.63	0.81	0.61
Z·Lys·His·OMe Z Z	0.75	0.84	0.73
t-Boc·Lys·Lys·Ala·OMe Z Z	0.82	0.92	0.77
t-Boc·Leu·Lys·Lys·Ala·OMe	0.83	0.89	0.85

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De l'adénosine tétraphosphate

IV. Chromatographie bidimensionelle des nucléotides sur diéthylaminoéthyl-cellulose*

L'adénosine tétraphosphate (AtétraP) est un nucléotide de l'adénine contaminant certaines préparations commerciales d'adénosine triphosphate[1–3] (ATP), dont il ne diffère que par la présence d'un phosphate supplémentaire en boût de chaîne[4–7].

Ce nucléotide peut être aisément séparé des autres nucléotides de l'adénine par chromatographie sur papier[8]. Cette méthode ne permet pas de le séparer de certains autres nucléotides, en particulier de l'inosine monophosphate (IMP), du guanosine diphosphate (GDP) et du cytidine triphosphate (CTP).

Les mono-, di- et triphosphates des ribonucléosides se laissent aisément séparer sur couche mince de diéthylaminoéthyl-cellulose (DEAE-cellulose) à l'aide d'un développement à l'acide chlorhydrique dilué (RANDERATH[9]). Ce procédé permet également de séparer l'adénosine tétraphosphate de l'ATP mais non de l'uridine triphosphate (UTP) et de l'inosine triphosphate (ITP). Cette séparation peut être obtenue à l'aide d'un autre solvant utilisé par RANDERATH[10] qui, par contre, ne sépare pas correctement l'adénosine tétraphosphate de l'ATP et du guanosine triphosphate (GTP).

Ce travail décrit brièvement comment utiliser ces deux développements pour réaliser une séparation satisfaisante de l'adénosine tétraphosphate des autres ribonucléoside triphosphates, au cours d'une chromatographie bidimensionnelle sur DEAE-cellulose. Le procédé permet l'analyse quantitative.

Ce travail a fait l'objet d'une communication préliminaire[11].

Méthodes

Produits utilisés. Tous les nucléotides proviennent de Sigma Chemical Company (St. Louis, Mo., Etats-Unis d'Amérique). La cellulose pure, sans gypse (MN 300) et la DEAE-cellulose (MN 300) proviennent de Macherey, Nagel & Co. (Düren, Allemagne). Tous les autres réactifs sont des produits pro analysi à l'exception de l'acétone toute pure et de la triéthylamine B.D.H. (Poole, Angleterre).

Préparation des plaques. 8 g de DEAE-cellulose sont mis en suspension dans 250 ml HCl *N*, lavés plusieurs fois à l'aide de HCl et d'eau puis additionnés de 2 g de cellulose et amenés à 100 ml (voir STAHL[12] et BÖRNIG ET REINICKE[13]). La pâte est appliquée sous une épaisseur de 0.3 mm. Les plaques, de 20 cm de côté, sont séchées à température ordinaire, activées 40 min à 50° et conservées en présence de silicagel.

Pose des échantillons. La quantité de nucléotide appliquée est de 10 à 15 n-moles; il importe peu que la solution soit diluée (environ 1 m*M*) ou plus concentrée. La zone dans laquelle se répand l'échantillon peut atteindre 5 mm de diamètre sans inconvénient.

Développements. Le premier solvant, HCl 0.08 *N*, a été utilisé à la chambre froide (4°) pour éviter toute hydrolyse, pendant près de 2 h durant lesquelles le front se déplace de 12 à 13 cm. Le second solvant, conçu par MARKHAM ET SMITH[14] et modi-

* Ce travail a été réalisé grâce à l'aide financière du Fonds national de la Recherche scientifique et du Fonds de la Recherche scientifique médicale de Belgique.

fié par RANDERATH[10], est fait de $(NH_4)_2SO_4$ sat.–NaAc *M*–isopropanol (80:18:2) et a été appliqué dans une direction perpendiculaire à celle du premier, à la température du laboratoire, pendant 10 à 12 min durant lesquelles le front se déplace de 8 à 9 cm. De meilleures séparations sont obtenues lorsque ce solvant a été préparé au moins un jour à l'avance.

Les plaques acidifiées par le premier solvant sont neutralisées et lavées avant application du second solvant: par la triéthylamine à 8 vol. % dans l'acétone (1 fois) puis par l'acétone (3 fois). Il est indispensable d'effectuer cette neutralisation en laissant la solution acétonique de triéthylamine parcourir la plaque dans le sens qui sera suivi par le second solvant.

Extraction. Les tâches révélées par leur fluorescence en lumière ultraviolette (lampe "Mineralight" de Ultra-Violet Products, Inc., San Gabriel, Calif., Etats-Unis d'Amérique), sont prélevées à l'aide d'une lame de rasoir et transférées dans un petit tube de centrifugeuse auquel on ajoute 2 ml HCl 0.2 *N*. On extrait par inversions brusques et répétées sur le doigt recouvert de parafilm, centrifuge et filtre la solution surnageante sur verre poreux (Jena G4). L'absorbance du filtrat est mesurée à 257 et 290 nm (voir DEVIGNE *et al.*[8]), un échantillon témoin sans nucléotide étant traité de la même manière.

Résultats et discussion

Nous avons confirmé que l'acide chlorhydrique très dilué (0.02 *N*) déplace essentiellement les monophosphates des ribonucléosides, tandis que l'acide chlorhydrique plus concentré (0.04 *N*) déplace les mono- et les diphosphates; la concentration de HCl doit atteindre 0.08 *N* pour qu'on observe un déplacement appréciable des triphosphates, à froid tout au moins.

Les valeurs de R_F que nous observons sont généralement inférieures, pour ce solvant, à celles décrites par RANDERATH[9]. Nous avons constaté en particulier que le lavage répété de la DEAE-cellulose s'accompagne d'une augmentation des valeurs de R_F.

Comme on le voit au Tableau I, l'adénosine tétraphosphate se sépare correctement de l'ATP, mais migre à la vitesse de l'UTP lorsque HCl 0.08 *N* est utilisé comme seul agent de développement. Le second solvant distingue par contre l'adénosine tétraphosphate des autres nucléosides triphosphates à l'exception de l'ATP.

TABLEAU I

R_F DES NUCLÉOSIDE TRIPHOSPHATES ET DE L'ADÉNOSINE TÉTRAPHOSPHATE

Le premier solvant est HCl 0.08 *N*. Le second solvant est fait d'un mélange de sulfate d'ammonium saturé, d'acétate de sodium *M* et d'isopropanol (dans les proportions 80:18:2).

Nucléotide	*Premier solvant*	*Second solvant*
Adénosine triphosphate	0.28	0.53
Cytidine triphosphate	0.36	0.91
Guanosine triphosphate	0.25	0.73
Inosine triphosphate	0.20	0.83
Uridine triphosphate	0.10	0.90
Adénosine tétraphosphate	0.09	0.59

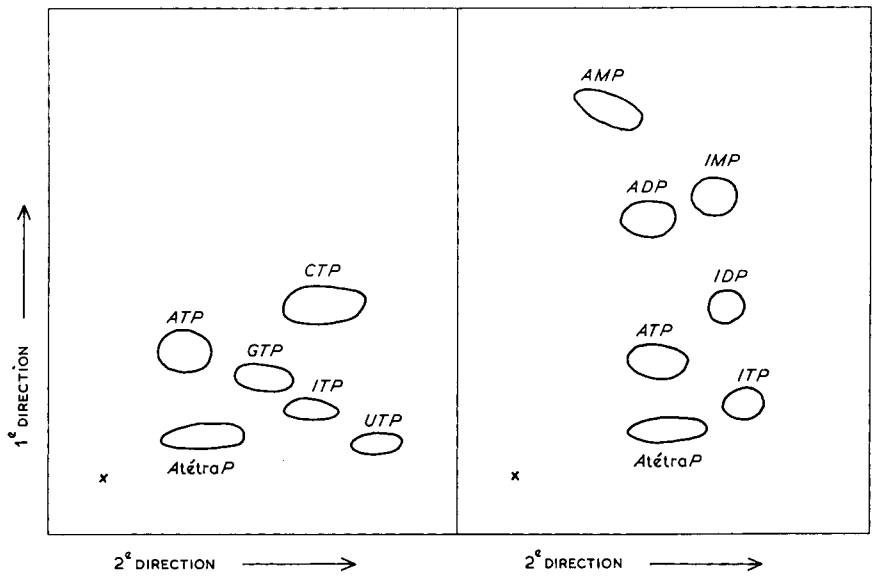

Fig. 1. Séparation des ribonucléotides sur DEAE-cellulose. Solvants utilisés: 1e direction = 0.08 *N*; 2e direction = $(NH_4)_2SO_4$ sat.–NaAc *M*–isopropanol (80:18:2). Abréviations utilisées: AMP, ADP, ATP et AtétraP pour les mono-, di-, tri- et tétraphosphates d'adénosine; IMP, IDP et ITP pour les mono-, di- et triphosphates d'inosine; GTP, CTP et UTP pour les triphosphates de guanosine, de cytidine et d'uridine.

Il en résulte que l'application successive et perpendiculaire des deux développements (avec neutralisation et lavage intermédiaire) doit permettre de séparer tous les constituants envisagés. C'est ce qu'illustre la Fig. 1 dans sa partie gauche; celle-ci montre aussi, dans sa partie droite, la répartition de l'adénosine tétraphosphate et des mono-, di- et triphosphates d'inosine et d'adénosine après chromatographie bidimensionnelle.

TABLEAU II

ANALYSE D'UN MÉLANGE DE NUCLÉOTIDES DE L'ADÉNINE

Nucléotide	*Chromatographie sur*		
	*Papier** (%)	*DEAE-cellulose***	
		A (%)	*B* (%)
Adénosine monophosphate	20.0	21.0	20.8
Adénosine diphosphate	21.4	19.5	21.2
Adénosine triphosphate	50.0	51.0	50.1
Adénosine tétraphosphate	7.0 } 8.6	8.5	7.9
Adénosine pentaphosphate	1.6 }		

* Solvant[20] fait d'acide isobutyrique, d'ammoniaque *N* et d'acide éthylènediamine tétraacétique 0.1 *M* (dans les proportions 100:60:1.6): récupération de l'ordre de 100%.

** Developpement (A) dans une seule direction à l'aide de HCl 0.08 *N*: récupération de 98–99%; (B) comme en (A) suivi, après lavage, par un développement dans la seconde direction à l'aide d'un mélange de sulfate d'ammonium saturé, d'acétate de sodium *M* et d'isopropanol (dans les proportions 80:18:2): récupération de 90–98%.

Le premier solvant sépare en fonction de la charge électrique (monophosphates en tête); le second solvant sépare en fonction de la nature de la base azotée du nucléotide.

La méthode proposée sépare sur couche mince, comme les méthodes de chromatographie bidimensionnelle sur papier[15,16] ou les méthodes combinant l'électrophorèse sur papier et la chromatographie sur papier[17,18].

Une autre méthode de chromatographie bidimensionnelle sur couche mince de poly(éthylèneimine)-cellulose, plus récemment proposée par RANDERATH ET RANDERATH[19], n'aurait pas permis l'isolement de l'adénosine tétraphosphate.

Le Tableau II montre la comparaison des analyses d'un mélange de nucléotides par chromatographie sur papier et par chromatographie sur DEAE-cellulose. Les résultats sont tout à fait comparables. La chromatographie sur papier permet toutefois la séparation des tétra- et pentaphosphates d'adénosine.

En résumé, l'adénosine tétraphosphate et les nucléotides en général peuvent être aisément séparés et dosés par chromatographie bidimensionnelle sur couche mince de DEAE-cellulose.

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A simple technique for detecting homogentisic acid on paper chromatograms

Paper chromatography has proved useful in the identification of homogentisic acid in the urine of alcaptonuric subjects[1]. Such chromatograms are usually stained with Pauly's reagent or ammoniacal $AgNO_3$. The use of the latter reagent has its own limitations. It gives rise to extra spots apart from homogentisic acid and its products, and the chromatogram turns brown after prolonged exposure. These defects have been largely eliminated by the use of a simple and specific colour reaction for homogentisic acid developed in this laboratory[2] and the results of this investigation are presented in this communication.

Collection and subsequent processing of the urine samples of alcaptonuric subjects are carried out as outlined earlier[2].

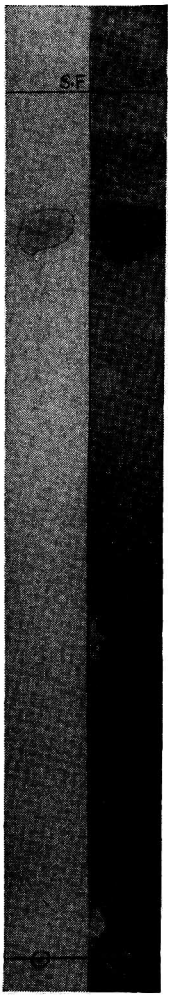

Fig. 1. Paper chromatogram of the urinary extract of an alcaptonuric subject. Left: developed with the new reagent; right: developed with ammoniacal $AgNO_3$.

Paper chromatography of the urinary extract is carried out on Whatman No. 1 paper in butanol–acetic acid–water (4:1:5).

Chromatograms were stained with ammoniacal $AgNO_3$ and with the reagent developed by us. This reagent is prepared by mixing 5 ml of 0.01 % ethanolic $CuSO_4$ with 20 ml of ethanol and 10 ml of 0.01 *N* ethanolic NaOH.

The dried chromatograms were dipped into this reagent and allowed to remain there for 10 min for the development of a pinkish brown colour. The chromatograms were then air-dried.

It is clear from Fig. 1 that the new reagent developed by us for homogentisic acid has a distinct advantage over ammoniacal $AgNO_3$. The chief objections to the use of ammoniacal $AgNO_3$ for developing the chromatograms, *viz.* the appearance of extra spots unrelated to homogentisic acid and its products and the turning of the chromatogram to brown colour after prolonged exposure, have been virtually eliminated by the use of this new developer. The chromatogram developed with the new reagent showed only one pinkish brown spot corresponding to homogentisic acid and which was clearly seen on a perfectly white background. However, the R_F value of homogentisic acid in our experiment was 0.85, which was slightly higher than reported earlier[1].

In earlier experiments, we tried the use of this reagent in aqueous medium. This resulted in the complete washing out of the spot corresponding to homogentisic acid. This has necessitated the use of the ethanolic solution of the reagent. The use of ethanol in this reagent is helpful in some manner in fixing the homogentisic acid in the paper during colour development.

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Double spot formation in chromatography of imidazolepropionic acid

Recently more attention has been given in biology and medicine to imidazolepropionic acid. BALDRIDGE AND TOURTELLOTTE[1] found the substance in rat urine after a histidine load was given. AUERBACH *et al.*[2] identified imidazolepropionic acid in the urine of an infant with histidinemia. SEN *et al.*[3] showed that imidazolepropionic acid is a normal constituent of human urine, and in the rat is derived from urocanic acid. Its role in bacterial metabolism has been investigated[4,5].

In our laboratory one of the solvents previously used for this compound[1,3] has consistently produced double zones on paper chromatograms. Since it is usually assumed that pure organic compounds give single spots on paper chromatograms, we first questioned the purity of the compound and then explored the possibility of of multiple spot formation. This report will describe evidence that imidazole propionate forms a double spot in the solvent *tert.*-butanol–acetone–formic acid–water (160:160:1:39), using Whatman No. 1 filter paper. The double spot is avoided by the use of acid-washed paper. Investigators may waste time and effort if they are not aware of the distinctive double spot formed by this imidazole. The phenomenon of multiple zone formation has been reviewed and a theoretical treatment has been presented by KELLER AND GIDDINGS[6].

Methods

Ascending chromatography was carried out on Whatman No. 1 filter paper. Solvents were commercial analytical reagent grade. Chromatograms were formed into cylinders and developed 23–26 cm from origin at 25–30°. Imidazole propionate was obtained from Calbiochem (m.p. 207–208°), Koch-Light Laboratories (m.p. 208–209°) and by hydrogenation of urocanic acid with palladium catalyst (m.p. 210–211°) as described by KRAML AND BOUTHILLIER[7]. It was recrystallized from water–ethanol–acetone (1:1:1). The compound was dissolved in deionized water for chromatography unless additions are stated. After drying, the paper was sprayed with diazotized sulfanilic acid. Imidazolepropionic acid was determined as described by TABOR[8].

Results and discussion

The double spot produced by imidazolepropionic acid is shown in Fig. 1. The two spots are identical in color. The double spot occurred when either of the two commercial products were used. When the compound was synthesized in our laboratory, both spots appeared and increased together as the synthesis proceeded. The spots are clearly and significantly separated, and are joined by a diffuse area of color as described by KELLER AND GIDDINGS[6]. The faster spot contains 10 % of the total as determined after elution. The R_F values for the two spots are 0.35 and 0.23. The faster and smaller spot corresponds to the value of 0.36 given in the literature[1,3]. The double spot was also found when imidazole propionate was added to urine and the mixture chromatographed and when descending chromatography was employed.

The R_F values of several imidazoles were found to be histidine, 0.00; urocanic acid, 0.58; imidazolelactic acid, 0.05; imidazoleacetic acid, 0.05; and histamine, 0.03. Imidazolepropionic acid is the only imidazole tested which gave a double spot in this solvent. Other imidazoles which might be contaminants do not correspond to either of the R_F values for the double spot.

The pH of the imidazolepropionic acid placed at the origin has a marked effect on the occurrence of multiple spots. The pH of the solution was altered by addition of NaOH and HCl. At pH 12 a bottom spot (R_F 0.04) predominates with a middle spot leading and touching the intense bottom spot. As pH is gradually lowered the middle spot predominates (R_F 0.16 to 0.24) and becomes compact and the bottom spot fades out. At the same time a trace of the upper spot begins to appear (R_F 0.3 to 0.35), until at pH 1 a single spot is observed (R_F 0.30 to 0.35).

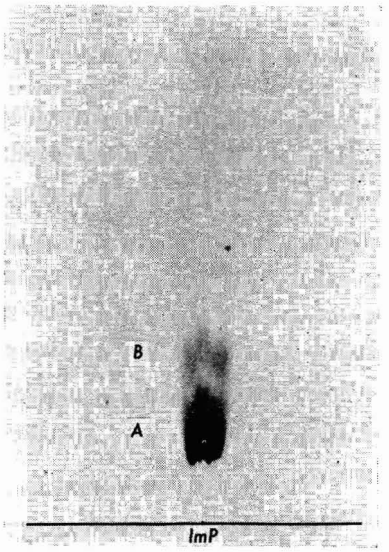

Fig. 1. Double spot formation by imidazolepropionic acid; 0.4 μmole applied to Whatman No. 1 paper; solvent: *tert.*-butanol–acetone–formic acid–water (160:160:1:39); spray: diazotized sulfanilic acid.

Evidence that the formic acid content of the solvent system affected the multiple spot formation was obtained. As the formic acid is increased from 0 to 20 ml (water content adjusted accordingly) the apparent quantity of imidazolepropionic acid in the slow spot changes from 100 % to 0 % as more and more of the compound appears in the fast spot.

In a bidimensional chromatogram using the same solvent in both dimensions one expects four spots if there are two forms of the compound in equilibrium, and two spots if two separate compounds are present. However, three spots were found. On the first pass the imidazole propionate produces two spots. On the second pass, the slower spot (A) splits again into two spots (A and B), but the faster spot (B) remained as one discrete spot, and none of the slower spot could be detected. The slower substance (A) is converted by a slow reaction to the faster substance (B), but after the conversion B did not revert to A on the second pass. To examine this further, two bands (A and B) from a chromatogram were eluted by descending chromatography with water, concentrated under reduced pressure and each rechromatographed. The lower band (A) broke up into two spots when rerun. However, the upper band gave only one discrete fast spot. It appears that imidazolepropionic acid (A) is converted to a new compound (B) by development of the chromatgram. This type of result is discussed by KELLER

AND GIDDINGS[6]. This new compound (B) was stable and was not reconverted to the original (A) during rechromatography or by altering pH. It is not likely, therefore, that we were dealing with two ionic species of the same compound.

It was possible to prevent double-spot formation by washing the paper in 2 *N* acetic acid followed by a rinse in deionized water. Commercially acid-washed papers also were effective, including Whatman No. 40, Schleicher & Schüll (S and S) 589 Black and 589 White. In order to determine if inhibition of double-spot formation was due to acidification of the paper, Whatman No. 1 was washed in deionized water. A single wash prevented double-spot formation, although considerable "heading" of the spot was observed. Whatman No. 1 paper from another package obtained from another laboratory also gave distinctive double spots, as did an unwashed S and S paper, 2041. HANES AND ISHERWOOD[9] working with inorganic phosphate attributed ghost spots to calcium and magnesium ions in the paper, and found that acid washing of the paper eliminated multiple spots. In order to see if the addition of ions at the origin of washed-paper chromatograms would restore double-spot formation, we placed the following inorganic compounds at separate origins at two concentrations (0.02 μmole and 0.05 μmole): $MgCl_2$, $CaCl_2$, KCl, NaCl, $CuCl_2$, $ZnSO_4$. Imidazole-propionic acid (0.1 μmole) was also placed at the origins. No double spots occurred. In a similar experiment with unwashed paper, inorganic ions did not change the double-spot formation.

The conversion of A to B is finite. The reverse reaction is slow or does not occur. A and B have different mobilities which are affected by the pH of the applied solution and by the formic acid concentration in the chromatographic solvent. Chromatography of A produces two spots and chromatography of B produces one spot whether done by elution or by two dimensional chromatography. An unknown component of the paper is responsible for the reaction since washing the paper prevents the double spot. Other imidazoles either do not undergo the reaction or the two forms have the same mobility.

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Variation of R_F of vitamin B_6 group with pH*

The R_F values of some members of the vitamin B_6 complex have been reported to vary from run to run[1]. A reinvestigation of these variable results in our laboratory revealed that the R_F values of this group are a function of pH of the developing solvent at constant temperature. All six members were tested: pyridoxol, pyridoxamine, pyridoxal, and their corresponding 5-phosphates. All data were subjected to statistical analysis.

Methods and materials

The solvent used was pyridine–butanol–water (1:2:saturated). The pH of the solvent was adjusted with either HCl or NH_4OH and verified with the aid of pHydrion paper (low range buffer). Twenty-five double strips of Whatman No. 1 chromatographic paper were used for each member of the group; this paper had the most uniform structure of several, thus giving the least variability in the background absorption[2]. Single compounds, dissolved in water, were applied three times, and the successive spots were dried completely; each composite spot contained 10 μg of B_6. Origins were visualized by fluorescence under U.V. light at 3660Å and outlined.

The pH was checked and adjusted when necessary before each run, and the solvent inside each hydrometer jar was changed to avoid contamination with previous compounds. In a darkened laboratory, the strips were saturated for fifteen minutes, then dipped and kept in contact with the solvent for one hour, removed, and dried with a heat gun. The pH range tested was from 5.0 to 9.0.

The spot was located with the aid of the U.V. light. For pyridoxal, fluorescence was greatly enhanced by exposure of the strip to NH_4OH (conc.) vapor, not so for pyridoxol and pyridoxamine; fluorescence in these two compounds was, in fact, slightly inhibited. This method was followed with both individual components and mixtures of components of B_6.

Results and discussion

R_F values were computed for each pH. Table I gives the mean $R_F \pm$ one standard deviation. Analysis of the data was carried out with the aid of the Y-test[3]. These results are charted on Figs. 1, 2, 3 and 4.

Four of the six components of the B_6 group can be separated by paper chromatography at any one pH. The separation of pyridoxol from its 5-phosphate can be accomplished only at pH 9; this may be due to hydrolysis of the phosphate at other acidities.

It is difficult to distinguish the phosphate of pyridoxal from that of pyridoxamine. When a mixture of the B_6 group was chromatographed at any pH, the minimum R_F found was 0.12, which is a value in between the R_F values of the 5-phosphates of pyridoxamine and pyridoxal. In spite of this difficulty, it is always possible to separate pyridoxol, pyridoxal hydrochloride, pyridoxamine hydrochloride, and either the phosphate of pyridoxal or pyridoxamine.

Our experimental observations confirm the report that the area of the chromatographed spot decreases with increase of pH of the solvent system[4]. Thus, starting

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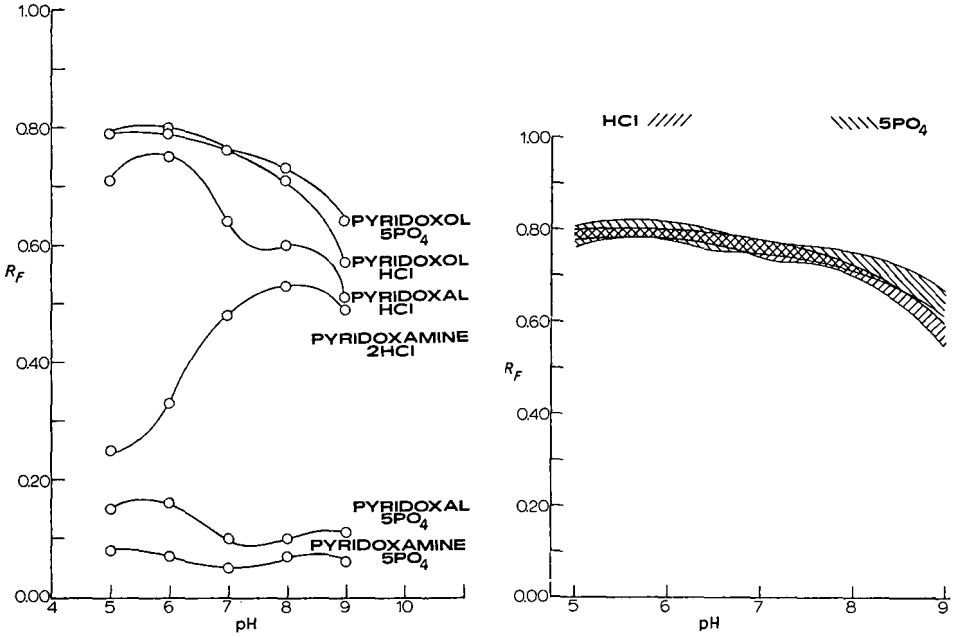

Fig. 1. R_F values of vitamin B_6 cogeners at various pH.

Fig. 2. Pyridoxol confidence bands.

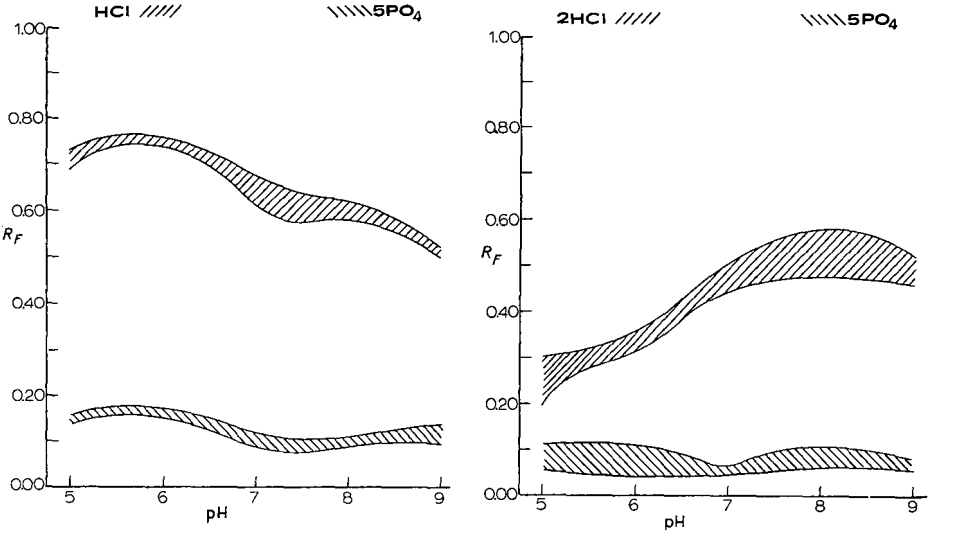

Fig. 3. Pyridoxal confidence bands.

Fig. 4. Pyridoxamine confidence bands.

TABLE I

pH DEPENDENCE OF R_F VALUES OF B_6 COMPOUNDS

pH	*Pyridoxol*	*Pyridoxal*	*Pyridoxamine*	
5	0.79 ± 0.01	0.71 ± 0.02	0.25 ± 0.05	
	0.79 ± 0.02	0.15 ± 0.01	0.08 ± 0.03	($5PO_4$)
6	0.79 ± 0.01	0.75 ± 0.01	0.33 ± 0.02	
	0.80 ± 0.02	0.16 ± 0.01	0.07 ± 0.03	($5PO_4$)
7	0.76 ± 0.02	0.64 ± 0.03	0.48 ± 0.03	
	0.76 ± 0.02	0.10 ± 0.02	0.05 ± 0.01	($5PO_4$)
8	0.71 ± 0.01	0.60 ± 0.02	0.53 ± 0.05	
	0.73 ± 0.02	0.10 ± 0.01	0.07 ± 0.01	($5PO_4$)
9	0.57 ± 0.03	0.51 ± 0.01	0.49 ± 0.03	
	0.64 ± 0.03	0.11 ± 0.02	0.06 ± 0.01	($5PO_4$)

with an average area of the original spot of 2.4 cm^2, the chromatographed spot at pH 5 acquired an average area of 9.3 cm^2, while at pH 9 the average area of the chromatographed spot was 6.0 cm^2. A relationship can be established between the area of the chromatographed spot and the pH: area $= C/pH$ (where C is a constant inherent to the pH).

The problem of separation was investigated with a probabilistic conjecture[5], and the estimated confidences were from 95 % to 99 % for pH 5, 6 and 7; the confidence limits dropped considerably at pH 9.

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Über organische Rhodanverbindungen*

16. Mitteilung. Papierchromatographische Verteilung und Nachweis von Rhodanphenolen, Rhodanphenoläthern und Rhodanphenolketonen

In einer früheren Mitteilung berichteten wir über die Möglichkeiten der Identifizierung von organischen Rhodanverbindungen nach papierchromatographischer Verteilung mit Hilfe von Quecksilber–Fluorescein-Reagens oder von Natriumsulfid- und Eisen(III)-chlorid-Lösung[1].

Ausgehend von diesen Erfahrungen, versuchten wir in Zusammenhang mit präparativen Arbeiten das papierchromatographische Verteilungsverhalten einer Reihe von Rhodanphenolen, Rhodanphenoläthern und Rhodanphenolketonen und ihren entsprechenden rhodanfreien Ausgangsstoffen näher zu untersuchen.

Als stationäre Phase bewährte sich auch in diesen Fällen ein mit Dimethylformamid imprägniertes Papier (25 %ige Lösung in Methanol). Auf Grund der sehr unterschiedlichen Löslichkeit der untersuchten Verbindungen war es allerdings unvermeidbar, als mobile Phase 9 verschiedene Lösungsmittelsysteme (System *a* bis *i*) heranzuziehen. Die Laufzeiten liegen bei 2–5 Std.; sie betragen nur bei System *f* 9 Std. Die ermittelten R_F-Werte (Tabellen I, II und III) stellen Richtwerte für die einzelnen Substanzen dar und sind als Mittel aus je 10 Chromatogrammen berechnet worden. Es ist daraus deutlich zu erkennen, dass sich die synthetisierten organischen Rhodanverbindungen unter Einsatz eines bestimmten Lösungsmittelsystems von ihren Ausgangsstoffen trennen lassen. Um reproduzierbare R_F-Werte zu erhalten, ist es vor allem wichtig, als polares Imprägnierungsmittel ein Dimethylformamid gleicher Dichte zu verwenden.

Eine Ausnahme machen allerdings die Salicylsäuren, die auf dimethylformamidimprägniertem Papier bei Verwendung der Lösungsmittelsysteme *a* bis *i* als mobile Phasen hinsichtlich des chromatographischen Verhaltens keine befriedigenden Ergebnisse zeigen. Teils waren kaum Unterschiede in den R_F-Werten von rhodanierter und rhodanfreier Substanz festzustellen und zum anderen neigen die Salicylsäuren zur Streifenbildung, wahrscheinlich bedingt durch unterschiedliches Dissoziationsvermögen in den einzelnen Zonen des dimethylformamidimprägnierten Papiers.

Für die Trennung von Salicylsäurederivaten eignen sich dafür sehr gut phosphatgepufferte Papiere und das Gemisch von *n*-Butanol–Wasser (1:1, v/v) als mobile Phase (System k). Die Laufzeit beträgt 7 Std. (Tabelle I).

Interessanterweise konnten wir auch einige Flüssigkeiten wie Phenetol (nicht Anisol!), *n*-Propoxybenzol, Aminophenoläther, Rhodanphenoläther, 3-Rhodanacetophenon u.a. (nicht Acetophenon und Propiophenon!) chromatographieren, ohne sie vorher in feste Derivate zu überführen; es müssen lediglich etwas grössere Mengen an Substanz aufgetragen werden.

Der Nachweis der Verbindungen auf dem Papier richtet sich nach ihrem chemischen Reaktionsvermögen. Die rhodanhaltigen Verbindungen sowie die Benzthiazole, ferner Phenol, 2-Aminophenol, 2-Kresol, PAS, *o*-Vanillin und alle Isothiocyanate lassen sich durch Besprühen mit Fluorescein–Quecksilber-Reagens[1] sichtbar machen. Man erhält rote Flecke auf gelblichrotem Untergrund, unter U.V.-Licht schwarze Flecke auf gelbfluoreszierendem Grund.

* 15. Mitt., siehe Zit. 1.

TABELLE I

R_F-WERTE VON PHENOLEN

Substanz	*R_F-Wert*
System f (Laufzeit 9 Std.)	
Hydroxy-benzol (Phenol)*	0.29
1-Hydroxy-4-rhodan-benzol (4-Rhodan-phenol)	0.19
1-Hydroxy-2-methyl-benzol (2-Kresol)*	0.49
1-Hydroxy-4-rhodan-2-methyl-benzol	0.32
System c (Laufzeit 4 Std.)	
1-Hydroxy-2-amino-benzol (2-Amino-phenol)*	0.18
1-Hydroxy-5-rhodan-2-amino-benzol	0.33
System e (Laufzeit 2.5 Std.)	
1-Hydroxy-2-methoxy-benzol (Guajakol)*	0.32
1-Hydroxy-2-methoxy-4-rhodan-benzol	0.18
System a (Laufzeit 3 Std.)	
1-Hydroxy-2-methoxy-6-formyl-benzol (*o*-Vanillin)*	0.30
1-Hydroxy-2-methoxy-4-rhodan-6-formyl-benzol	0.06
System k (Laufzeit 7 Std.)	
1-Hydroxy-2-carboxy-benzol (Salicylsäure)*	0.52
1-Hydroxy-4-rhodan-2-carboxy-benzol (5-Rhodan-salicylsäure)	0.76
1-Hydroxy-5-amino-2-carboxy-benzol (PAS)*	0.16
1-Hydroxy-5-rhodan-2-carboxy-benzol (4-Rhodan-salicylsäure)	0.73
System d (Laufzeit 2 Std.)	
1,3-Dihydroxy-benzol (Resorcin)*	0.04
2,6-Dioxo-benzo [1,2-*d*:5,4-*d'*]bis[1,3]oxathiol	0.96

* Ausgangssubstanz.

TABELLE II

R_F-WERTE VON PHENOLÄTHERN

Substanz	*R_F-Wert*
System a (Laufzeit 3 Std.)	
1-Methoxy-2-amino-benzol (2-Anisidin)*	0.41
1-Methoxy-2-rhodan-benzol (2-Rhodan-anisol)	0.85
1-Methoxy-5-rhodan-2-amino-benzol	0.12
1-Methoxy-3,5-dirhodan-2-amino-benzol	0.06
4-Methoxy-6-rhodan-2-amino-benzthiazol	0
1-Methoxy-2,5-dirhodan-benzol	0.60
1-Methoxy-2,3,5-trirhodan-benzol	0.15
4-Methoxy-2,6-dirhodan-benzthiazol	0.26
1-Methoxy-3-amino-benzol (3-Anisidin)*	0.11
1-Methoxy-3-rhodan-benzol (3-Rhodan-anisol)	0.85
1-Methoxy-4-amino-benzol (4-Anisidin)*	0.11
1-Methoxy-4-rhodan-benzol (4-Rhodan-anisol)	0.79

(*Fortsetzung S. 293*)

TABELLE II (*Fortsetzung*)

Substanz	R_F-*Wert*
1-Äthoxy-2-amino-benzol (2-Phenetidin)*	0.44
1-Äthoxy-2-rhodan-benzol (2-Rhodan-phenetol)	0.83
1-Äthoxy-5-rhodan-2-amino-benzol	0.27
1-Äthoxy-2,5-dirhodan-benzol	0.84
1-Äthoxy-2-isothiocyanato-benzol	0.97
1-Äthoxy-3-amino-benzol (3-Phenetidin)*	0.20
1-Äthoxy-3-rhodan-benzol (3-Rhodan-phenetol)	0.84
1-Äthoxy-4,6-dirhodan-3-amino-benzol	0.04
1-Äthoxy-3,4,6-trirhodan-benzol	0.53
1-Äthoxy-3-isothiocyanato-benzol	0.97
Äthoxy-benzol (Phenetol)*	0.14
1-Äthoxy-4-amino-benzol (4-Phenetidin)*	0.23
1-Äthoxy-4-rhodan-benzol (4-Rhodan-phenetol)	0.92
n-Propoxy-benzol*	0.14
1-*n*-Propoxy-4-amino-benzol*	0.22
1-*n*-Propoxy-4-rhodan-benzol	0.96
x-Brom-*n*-propoxy-benzol	0.99 (Front)
System e (*Laufzeit 2.5 Std.*)	
1-Methoxy-2-amino-benzol (2-Anisidin)*	0.55
1-Methoxy-5-rhodan-2-amino-benzol	0.32
1-Methoxy-3,5-dirhodan-2-amino-benzol	0.21
4-Methoxy-6-rhodan-2-amino-benzthiazol	0.03
1-Methoxy-2,3,5-trirhodan-benzol	0.49
4-Methoxy-2,6-dirhodan-benzthiazol	0.55
1-Methoxy-4,6-dirhodan-3-amino-benzol*	0.10
1-Methoxy-3,4,6-trirhodan-benzol	0.57
1-Äthoxy-2-amino-benzol (2-Phenetidin)*	0.75
1-Äthoxy-5-rhodan-2-amino-benzol	0.48
1-Äthoxy-3,5-dirhodan-2-amino-benzol	0.41
4-Äthoxy-6-rhodan-2-amino-benzthiazol	0.04
1-Äthoxy-2,5-dirhodan-benzol	0.95
1-Äthoxy-3-amino-benzol (3-Phenetidin)*	0.45
1-Äthoxy-4,6-dirhodan-3-amino-benzol	0.24
1-Äthoxy-3,4,6-trirhodan-benzol	0.88
1-*n*-Propoxy-4-amino-benzol*	0.54
6-*n*-Propoxy-2-amino-benzthiazol	0.09
1-*n*-Butoxy-4-amino-benzol*	0.72
6-*n*-Butoxy-2-amino-benzthiazol	0.17
System h (*Laufzeit 5 Std.*)	
1-Methoxy-3-amino-benzol (3-Anisidin)*	0.43
1-Methoxy-6-rhodan-3-amino-benzol	0.33
1-Methoxy-4,6-dirhodan-3-amino-benzol	0.47
5-Methoxy-6-rhodan-2-amino-benzthiazol	0.20
System g (*Laufzeit 2 Std.*)	
1-Methoxy-4-amino-benzol (4-Anisidin)*	0.70
6-Methoxy-2-amino-benzthiazol	0.45
6-Methoxy-4-rhodan-2-amino-benzthiazol	0.82
6-Methoxy-2-rhodan-benzthiazol	0.92
1-Äthoxy-4-amino-benzol (4-Phenetidin)*	0.80
6-Äthoxy-2-amino-benzthiazol	0.64
6-Äthoxy-4-rhodan-2-amino-benzthiazol	0.86
6-Äthoxy-2-rhodan-benzthiazol	0.95

* Ausgangssubstanz.

TABELLE III

R_F-WERTE VON PHENOLKETONEN

Substanz	R_F-*Wert*
System e (*Laufzeit 2.5 Std.*)	
2-Amino-1-acetyl-benzol (2-Amino-acetophenon)*	0.06
5-Rhodan-2-amino-1-acetyl-benzol (5-Rhodan-2-amino-acetophenon)	0.25
2-Rhodan-1-acetyl-benzol (2-Rhodan-acetophenon)	0.65
2-Isothiocyanato-1-acetyl-benzol (2-Isothiocyanato-acetophenon)	0.99
3-Amino-1-acetyl-benzol (3-Amino-acetophenon)*	0.06
6-Rhodan-3-amino-1-acetyl-benzol (6-Rhodan-3-amino-acetophenon)	0.02
3-Rhodan-1-acetyl-benzol (3-Rhodan-acetophenon)	0.56
3-Isothiocyanato-1-acetyl-benzol (3-Isothiocyanato-acetophenon)	0.96
4-Amino-1-acetyl-benzol (4-Amino-acetophenon)*	0.02
4-Rhodan-1-acetyl-benzol (4-Rhodan-acetophenon)	0.67
ω-Rhodan-acetyl-benzol (ω-Rhodan-acetophenon)	0.43
3-Rhodan-4-amino-1-acetyl-benzol (3-Rhodan-4-amino-acetophenon)	0.04
3,4-Dirhodan-1-acetyl-benzol (3,4-Dirhodan-acetophenon)	0.47
2-Rhodan-6-acetyl-benzthiazol	0.62
2-Amino-6-propionyl-benzthiazol*	0.02
2-Rhodan-6-propionyl-benzthiazol	0.86
3-Amino-1-propionyl-benzol (3-Amino-propiophenon)*	0.14
6-Rhodan-3-amino-1-propionyl-benzol (6-Rhodan-3-amino-propiophenon)	0.04
3-Rhodan-1-propionyl-benzol (3-Rhodan-propiophenon)	0.78
3-Isothiocyanato-1-propionyl-benzol (3-Isothiocyanato-propiophenon)	0.97
4-Amino-1-propionyl-benzol (4-Amino-propiophenon)*	0.06
4-Rhodan-1-propionyl-benzol (4-Rhodan-propiophenon)	0.89
3-Rhodan-4-amino-1-propionyl-benzol (3-Rhodan-4-amino-propiophenon)	0.10
3,4-Dirhodan-1-propionyl-benzol (3,4-Dirhodan-propiophenon)	0.75
System b (*Laufzeit 2 Std.*)	
4-Amino-1-acetyl-benzol (4-Amino-acetophenon)*	0.66
3-Rhodan-4-amino-1-acetyl-benzol (3-Rhodan-4-amino-acetophenon)	0.91
2-Amino-6-acetyl-benzthiazol	0.55
System i (*Laufzeit 3.5 Std.*)	
4-Amino-1-propionyl-benzol (4-Amino-propiophenon)*	0.62
3-Rhodan-4-amino-1-propionyl-benzol (3-Rhodan-4-amino-propiophenon)	0.85
2-Amino-6-propionyl-benzthiazol	0.38
System d (*Laufzeit 2 Std.*)	
3-Rhodan-4-amino-1-acetyl-benzol (3-Rhodan-4-amino-acetophenon)*	0.42
3,4-Dirhodan-1-acetyl-benzol (3,4-Dirhodan-acetophenon)	0.95
3-Rhodan-4-amino-1-propionyl-benzol (3-Rhodan-4-amino-propiophenon)*	0.72
3,4-Dirhodan-1-propionyl-benzol (3,4-Dirhodan-propiophenon)	0.98
System a (*Laufzeit 3 Std.*)	
3-Rhodan-4-amino-1-propionyl-benzol (3-Rhodan-4-amino-propiophenon)*	0.02
3,4-Dirhodan-1-propionyl-benzol (3,4-Dirhodan-propiophenon)	0.39
2-Rhodan-6-propionyl-benzthiazol	0.64
ω-Brom-acetyl-benzol (ω-Brom-acetophenon)*	0.73
ω-Rhodan-acetyl-benzol (ω-Rhodan-acetophenon)	0.19

* Ausgangssubstanz.

Die Aminophenylalkyläther, Bromphenylalkyläther, Phenylalkyläther und Guajakol können mit Millons Reagens[2] als braune (Phenoläther), rote (Halogenphenoläther) bis violette (Aminophenoläther) Flecke bzw. die Aminophenylalkylketone und das ω-Bromacetophenon mit 2,4-Dinitrophenylhydrazin-Reagens[2] als gelbe Flecke nachgewiesen werden. Zur Unterscheidung auf Isothiocyanate wird mit Jodazid-Reagens[2] besprüht. Isothiocyanate geben innerhalb kurzer Zeit weisse Flecke. Salicylsäure ist besonders gut unter U.V.-Licht durch die blaue Fluoreszenz zu erkennen. Diese papierchromatographischen Arbeitsweisen waren uns übrigens bei der Synthese der in den Tabellen I–III wiedergegebenen organischen Rhodanverbindungen in verschiedener Richtung von nutzen. Durch die Verteilung fast aller Roh- und mehrmals umkristallisierten Reinprodukte war es auf diese bequeme Weise möglich, praktisch jede Substanz zusätzlich zur Elementaranalyse auf Identität und Reinheit zu prüfen.

Bei der direkten Rhodanierung von einfachen Phenoläthern (Anisol, Phenetol usw.) entstehen neben den Rhodanderivaten (4-Rhodan-anisol, 4-Rhodan-phenetol, usw.) als Nebenprodukte immer x-Bromalkoxybenzole*. Diese lassen sich mit dem Lösungsmittelsystem *e* verteilen und mit Millons Reagens identifizieren. Am besten gelingt der Nachweis der x-Bromalkoxybenzole bei einer Laufstrecke von nur 15 cm (Laufzeit nicht über 1 Std.!) in kleinen Glaszylindern, da diese Verbindungen bei längerer Laufstrecke und -zeit sehr stark ausgewaschen werden. Bei der Einführung der Rhodangruppe über die Diazoniumsalze gelang es, in allen Rohprodukten ebenfalls auf dem Wege der Papierchromatographie neben den entsprechenden Rhodanverbindungen die Bildung von Isothiocyanaten nachzuweisen.

Versuchsteil

Papierchromatographische Verteilung. Die Imprägnierung der Papierbogen 29 × 30 cm (Schleicher & Schüll 2043 bMgl) erfolgt, wie bereits an anderer Stelle beschrieben[1]. Danach werden Lösungen entsprechend 20 μg Substanz (Ausnahmen: 80 μg bei 2-Anisidin, 2-, 3- und 4-Phenetidin, 4-Methoxy-6-rhodan-2-amino-benzthiazol, 4-Rhodan-phenol, 1-Hydroxy-4-rhodan-2-methyl-benzol und ω-Rhodan-acetophenon sowie 200 μg bei Phenol, 2-Kresol, Guajakol und n-Propoxy-benzol. Phenetol und die x-Bromalkoxybenzole wurden zu je 0.002 ml rein aufgetragen) in Methanol (Ausnahmen: 4-Methoxy-6-rhodan-2-amino-benzthiazol und 2-Amino-6-acetyl-benzthiazol werden in Eisessig gelöst) punktförmig aufgetragen. Insgesamt 10 Min. nach der Imprägnierung wird ohne vorherige Sättigung in den üblichen zylinderförmigen Glasgefässen (Höhe 34 cm, Durchmesser 19 cm), auf deren Boden sich eine Petrischale (Durchmesser 14 cm) zur Aufnahme von 50 ml mobiler Phase befindet, aufsteigend bei 20° (± 2°) und bei einer Laufstrecke von 20 cm chromatographiert.

Als mobile Phase werden wahlweise folgende Systeme angewandt:

System: *a* Cyclohexan
b Benzol
c Tetrachlorkohlenstoff
d Cyclohexan–Benzol (1:1)
e Cyclohexan–Benzol (5:1)
f Cyclohexan–Pyridin (10:1)

* Die Ergebnisse dieser und auch der übrigen synthetischen Arbeiten werden an anderer Stelle veröffentlicht.

g Benzol–Paraffin (10:1)
h Tetrachlorkohlenstoff–Paraffin (10:1)
i Tetrachlorkohlenstoff–Benzol (5:1).

Trennung der Salicylsäuren. Papierbogen 29 × 30 cm werden mit 0.1 *M* wässriger Dinatriumhydrogenphosphat- und Kaliumdihydrogenphosphatlösung imprägniert, und nach völligem Trocknen werden die zu chromatographierenden Substanzen aufgetragen. Eine Nacht wird das Chromatographiergefäss (mit Chromatogramm) mit der Unterphase der Mischung *n*-Butanol–Wasser (1:1, v/v) gesättigt und danach mit der Oberphase als Laufmittel (System k) aufsteigend bei 20° (±2°) und bei einer Laufstrecke von 20 cm chromatographiert.

Sichtbarmachen auf dem Papier. Es werden die getrockneten Chromatogramme wahlweise mit Quecksilber–Fluorescein-Reagens[1], Millons Reagens (Quecksilber und rauchende Salpetersäure, 1:2)[2], mit 2,4-Dinitrophenylhydrazin-Reagens[2] oder mit Jodazid-Reagens[2] besprüht. Salicylsäure kann besonders gut unter U.V.-Licht als blaufluoreszierender Fleck erkannt werden.

Bei Millons Reagens wird das Chromatogramm leicht besprüht, anschliessend mit dem Föhn kurze Zeit heiss getrocknet und erneut mit dem Reagens behandelt. Dieser Prozess wird 2–3 mal wiederholt, bis die Flecke sichtbar sind. Es färben sich Aminophenoläther (violett) sofort, dagegen Phenoläther (braun) und Halogenphenoläther (rot) erst nach einiger Zeit. Die Flecke werden im übrigen etwa 2 Min. nach dem Besprühen nachgezeichnet; beim Quecksilber–Fluorescein-Reagens unter U.V.-Licht.

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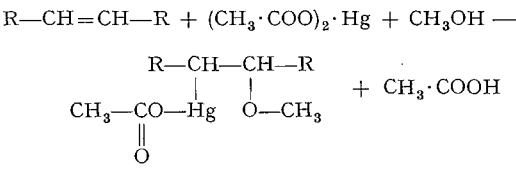
Über die Papierchromatographie einiger Styrolderivate

Im Rahmen unserer Untersuchungen zur Analytik des dehydrierten Diäthylbenzols[1,2] testeten wir auch die von HUBER[3] 1960 veröffentlichte papierchromatographische Methode zur Trennung, Identifizierung und quantitativen Bestimmung von Olefinen. Dieser Autor überführte dabei verschiedene Vinylverbindungen durch Umsetzen mit Quecksilberacetat in Methanol in Quecksilberadditionsverbindungen, die sich wegen ihrer spezifischen Eigenschaften für derartige Untersuchungen vorzüglich eignen.



HUBER bestimmte auf diese Art eine Reihe von R_F-Werten von Olefinen und Diolefinen, wobei auch der Wert für Styrol in einer der Tabellen angegeben[3] wird.

Dies gab Veranlassung zu untersuchen, wie sich nach dieser Methode die Alkylderivative des Styrols, *p*-Methylstyrols und *p*-Äthylstyrols sowie des *p*-Divinylbenzols verhalten. Diese Verbindungen sind u. a. Inhaltsstoffe des dehydrierten Diäthylbenzols.

Die Vinylverbindungen wurden nach der von MARTIN[4] angegebenen Weise in ihre Quecksilberaddukte überführt. Die Entwicklung* erfolgte absteigend und aufsteigend zweiphasig mit einer Kombination von *n*-Butanol einerseits und 1.5 *N* NH_3- und 1.5 *N* $(NH_4)_2CO_3$-Lösung andererseits. Zum Kennzeichnen der Flecken wurden die Papierstreifen zuerst an der Luft getrocknet; anschliessend folgte die Zersetzung der Quecksilberaddukte durch HCl-Dämpfen. Das freigesetzte Quecksilber lässt sich durch Besprühen mit Dithizon nachweisen.

TABELLE I

R_F-WERTE FÜR STYROLDERIVATE

	Absteigende Methode	*Aufsteigende Methode*
Styrol	0.56 ± 0.01	0.56 ± 0.01
p-Methylstyrol	0.62 ± 0.02	0.63 ± 0.01
p-Äthylstyrol	0.65 ± 0.02	0.66 ± 0.02
p-Divinylbenzol	0.07 ± 0.01	0.06 ± 0.01
Styrol-Wert nach HUBER[3]	0.56 ± 0.03	— —

In der Tabelle sind die für Styrol, *p*-Methylstyrol, *p*-Äthylstyrol und *p*-Divinylbenzol ermittelten R_F-Werte nach der absteigenden und aufsteigenden Methode angeführt. Die Werte stellen den Durchschnitt von 9 Messungen dar.

* Papier WF 1, VEB Feinpapierwerk Niederschlag.

Es zeigte sich, dass mittels der aufsteigenden papierchromatographischen Trennung besser reproduzierbare Ergebnisse zu erzielen sind.

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J. Chromatog., 24 (1966) 297–298

A new method of detection of inorganic phosphate on paper

BELEN'SKII AND RISKIN[1] described the formation of a stable alkaline violet lake resulting from mixing of methyl violet and phosphotungstomolybdic acid. AKAMATSU AND IMAI[2] have described a method for the colorimetric determination of inorganic phosphate based on the reaction of phosphate with methylene violet and ammonium molybdate in diluted hydrochloric acid. A simple and sensitive method of detection of inorganic phosphate on paper based on similar principles and suitable for chromatography is now described.

Reagents

1. Methyl violet, Geigy, 1.0 g, is dissolved in methanol and made up to 100 ml; before use this solution is diluted with diethyl ether 1:3.

2. Ammonium molybdate, 12 g, is dissolved in 150 ml of water by heating, and 35 ml 10 *N* HCl is added to this solution.

Procedure

The chromatogram is rinsed in solution 1 and after about 30 sec sprayed with solution 2 until the background becomes yellow-green. (The former step can be substituted by intensive spraying.) Inorganic phosphate gives blue spots.

When detecting phosphate esters or polyphosphates (which do not give the reaction mentioned) the dry chromatogram must be previously subjected to acid hydrolysis by commonly known methods. This process can also be realised by spraying with solution 2 followed by 30 min heating at 60–70°. Those conditions were sufficient for ATP, ADP, AMP and pyrophosphate, but the hydrolysis of α- and β-naphthyl phosphate was incomplete.

Fig. 1. Detection of inorganic phosphate (from left to right: 0.5, 0.15 and 0.05 μg of P) on chromatogram. System: formic acid 85%–water–dimethyl ketone (15:25:60), ascending development 45 min, paper Ederol 208.

About 0.1 μg of PO_4^{3-} ions can be detected on the chromatograms (Fig. 1). The method is suitable for testing the purity of enzymatic substrates containing phosphate as well as in qualitative analysis, where low quantities of phosphate must be detected.

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Separation of inorganic phosphates by molecular-sieve chromatography

Chromatographic separation of macromolecules with Sephadex, which is a cross-linked polysaccharide, has recently been developed in the field of biochemistry. The basic principle of this method has been explained in terms of the molecular sieving effect in the gel phase[1–4]. As shown in eqn. (1) the effluent peak volume V_e of a given solute in the column operation is related to the void volume V_o of a solvent in the gel bed and the internal volume V_i of the gel phase available for the solvent. K_d is defined as a fraction of the internal volume available for the solute.

$$V_e = V_o + K_d V_i \tag{1}$$

The present work describes the application of this method to the separation of inorganic phosphates. An elution curve for a series of polyphosphates using a column of Sephadex G-25 (fine) and 0.1 M potassium chloride solution as eluent is shown in Fig. 1. The amount of phosphate and tritiated water (THO) in each 1 ml fraction was determined by colorimetry and with a liquid scintillation counter, respectively.

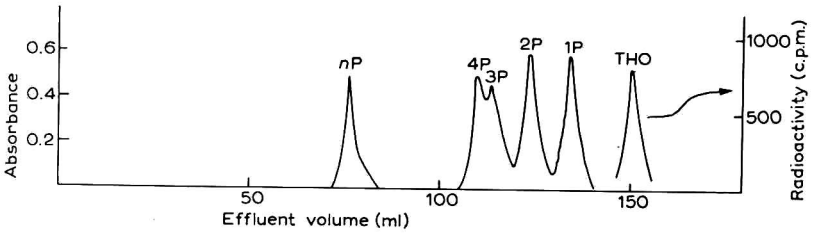

Fig. 1. Elution curve of inorganic phosphates and tritiated water. Column bed: 1.5 × 98 cm; flow rate: 18 ml/h. THO = tritiated water; 1P = orthophosphate; 2P = diphosphate; 3P = triphosphate; nP = Kurrol's salt.

The effluent peak volume of a Kurrol's salt[5] $(KPO_3)_n$ was the same as that of Blue Dextran (mol. wt. = 2,000,000) which is considered to be completely excluded from the gel phase. Peak positions of the other phosphates were in the order of decreasing molecular weights. K_d values of phosphates were calculated according to eqn. (1) in which V_o and V_i were determined by the use of Blue Dextran and tritiated water as standard materials of $K_d = 0$ and 1.

K_d values were also determined by a batch method, eqn. (2), which is based on the assumption that the solute concentration within the gel pore is essentially the same as that in the external liquid phase. Then,

$$K_d = 1 - \frac{V_o' + V_i'}{V_i'} \frac{C_2 - C_1}{C_2} \tag{2}$$

where V_o' is the void volume of the solvent and V_i' is the internal volume. C_2 is the concentration of a solute in equilibrium with a certain amount of Sephadex and C_1 is the concentration of it when no Sephadex is added. In the present work, 0.1 M potassium chloride solution was used as solvent and 3 g of dry Sephadex was equilibrated with 30 ml $= V_o' + V_i'$) of the solvent containing a known concentration

(= C_1) of each phosphate. To determine V_i' in eqn. (2) Kurrol's salt was used as a standard material of $K_d = 0$.

As shown in Fig. 2 there is no marked difference between K_d values of each phosphate obtained by the column and batch methods. This fact supports that the separation mechanism of phosphates in the column of Sephadex may be ascribed to the molecular sieving effect.

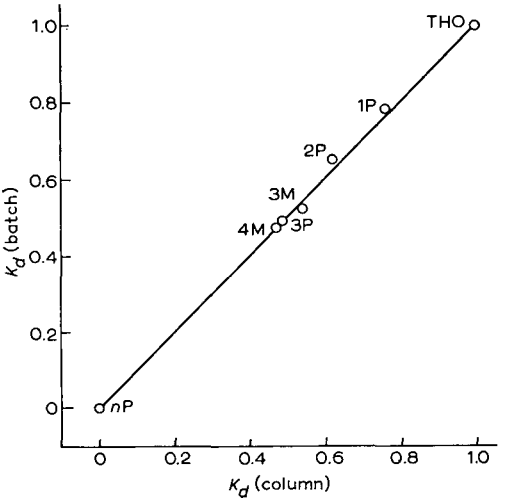

Fig. 2. Comparison between K_d values of phosphates obtained by the column and batch methods. 3M = trimetaphosphate; 4M = tetrametaphosphate. For other abbreviations, see legend Fig. 1.

Details concerning the behavior of various polyphosphates and the correlation between K_d values and molecular weights of phosphates will be discussed later.

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Detection of metals on paper chromatograms with Rhodamine B

It is well known that some metals react with Rhodamine B (R) in hydrochloric acid solution to give red or violet compounds of the type $(RH)Me^{III}X_4$ and $(RH)Me^{V}X_6$, which can be extracted by suitable solvents and the metals can then be determined qualitatively or quantitatively[1–4]. An aqueous solution of 0.01 % R has been used for the detection of Sb(V) on paper and in visible light[5].

The possible use of this reaction for detecting small quantities of metals on strips of paper was studied. Under U.V. light, R (in hydrochloric acid solution) was found to give intensive blue or violet spots with Au(III), Bi(III), Cd(II), Fe(III), Hg(II), Mo(VI), Sb(V), Tl(I, III), V (V) and W(VI) in the presence of Br^- or I^- ions. The background becomes rose, pink or orange fluorescent according to the hydrochloric acid concentration.

Experimental

20 μg amounts of each metal were applied on Whatman No. 1 paper strips in the usual way. The spots were sprayed with solution of varying concentrations of hydrochloric or sulphuric acid, oversprayed with either 10 % KBr or 10 % KI solution, and then observed in visible and U.V. light (Analytic Quartzlamp, Hanau, λ =

TABLE I

SPOT TESTS OF METALS UNDER U.V. LIGHT WITH HCl, KBr AND KI

Cd, Hg and Tl give no coloured spots under U.V. light.
Colours of spots: b = blue, br = brown, c = carmine, g = gray, r = red, v = violet, y = yellow, d = deep, l = light. Parentheses indicate that the colours may be neglected.

Metal (20 µg)	*Acid*	*Acid KBr*	*Acid KI*		*Kind and concentration of acid*
	U.V.	*U.V.*	*V*	*U.V.*	
Au(III)	gb	—	br	v ⟶ dc	1 *N* HCl
	(gb)	v	br	v ⟶ dc	3 *N* HCl
	gb	br	br	rv ⟶ dc	2 *N* H_2SO_4
Bi(III)	—	lv	y	v ⟶ dc	1 *N* HCl
	—	lv	y	v ⟶ dc	3 *N* HCl
	—	v	y	rv ⟶ dc	2 *N* H_2SO_4
Fe(III)	gb	gb	br	v ⟶ dc	1 *N* HCl
	v	v	br	v ⟶ dc	3 *N* HCl
	gb	(lg)	br	rv ⟶ dc	2 *N* H_2SO_4
Mo(VI)	(gb)	—	—	—	1 *N* HCl
	(gb)	—	—	—	3 *N* HCl
	g	—	—	—	2 *N* H_2SO_4
Sb(V)	—	—	br	rv ⟶ dc	1 *N* HCl
	(lg)	—	br	v ⟶ dc	3 *N* HCl
	(lg)	—	br	br.r	2 *N* H_2SO_4
V(V)	(gb)	gb	br	rv ⟶ dc	1 *N* HCl
	(gb)	(lv)	br	v ⟶ dc	3 *N* HCl
	(lg)	—	br	rv ⟶ dc	2 *N* H_2SO_4
W(VI)	gb	g	—	(g)	1 *N* HCl
	(lv)	v	—	(lv)	3 *N* HCl
	g	gb	—	(g)	2 *N* H_2SO_4

360 nm). Except for Bi, Fe, Sb, V and Au, after spraying with 10 % KI (Table I) the other metals gave no coloured spots or were only very slightly coloured. The sensitivity of this procedure is very low with the exceptions mentioned above.

Another set of metal spots were sprayed consecutively with an 0.025 % R solution in 1 *N* HCl and 10 % KBr, or 10 % KI. After each operation the wet spots were observed in visible and U.V. light. The same experiment was then performed with 3 *N* HCl instead of 1 *N* HCl. The results are given in Table II. The intensity of all spots was compared visually and expressed as values ranging from + to 5 +.

The following practical procedure is recommended: spray the metal spots with a 1:1 mixture of 0.05 % R, in 2 *N* or 6 *N* HCl, and 20 % KBr (the mixture is stable for a week). Examine the spot in visible and U.V. light and then overspray it with 10 % KI and repeat the observation. The last step is necessary if very small amounts of metal are present. The ions which oxidized KI are relatively more sensitive. The results are presented in Table III.

The spots retain their detectability for a longer period (except Bi, Cd, Hg and Mo) if the paper is dried, but they are more distinct when it is wet. After spraying with KI solution the paper becomes brown in time due to iodine formation. The most

TABLE II

SPOT TESTS WITH RHODAMINE B UNDER DIFFERENT CONDITIONS OF SPRAYING

Colours as in Table I. — = negative reaction; ± = slightly positive reaction; + = positive reaction; the intensity of the colour was compared visually from + to 5 +.

Metal 20 µg	*Colour of spot with 0.025 % Rhodamine B in 1 N HCl**						*Colour of spot with 0.025 % Rhodamine B in 3 N HCl***					
	–		+ *KBr*		+ *KI*				+ *KBr*		+ *KI*	
	V	*U.V*	*V*	*U.V.*	*V*	*U.V.*	*V*	*U.V.*	*V*	*U.V.*	*V*	*U.V.*
Au(III)	rv	db 4 +	rv →y	db 4 +	br	db 5 +	rv	b 4 +	rv	db 4 +	br	db 5 +
Bi(III)	—	—	rv	db 3 +	y	db 5 +	—	—	—	g +	y	db 5 +
Cd(II)	—	—	rv	b 2 +	—	b 3 +	—	—	—	—	—	—
Fe(III)	—	dg 3 +	—	—	ybr	db 5 +	—	dg 3 +	—	g +	br	db 5 +
Hg(II)	rv	—	rv	b 2 +	rv	db 5 +	—	—	rv	—	rv	b 4 +
Mo(VI)	rv	db 4 +	rv	b 3 +	rv	rv 3 +	r	b 2 +	rv	rv +	rv	rv 3 +
Sb(V)	rv	db 4 +	rv	db 4 +	br	db 5 +	rv	db 4 +	rv	b 4 +	br	db 5 +
Tl(I)	—	b 2 +	rv	db 4 +	rv	db 4 +	—	—	rv	b +	rv	b 3 +
Tl(III)	—	b 2 +	rv	db 4 +	rv	db 4 +	—	—	rv	b 2 +	rv	b 3 +
V(V)	r	—	—	g ±	br	db 5 +	—	g ±	—	g ±	br	db 5 +
W(VI)	rv	db 4 +	rv	db 4 +	rv	db 3 +	r	b 2 +	rv	rv +	rv	rv 2 +

* The background is pink coloured and gives rose fluorescence under U.V. light.

** The background is light orange coloured and gives yellow-orange fluorescence under U.V. light.

TABLE III

SPOT TESTS WITH THE RECOMMENDED REAGENT RHODAMINE B

— = negative reaction; ± = slightly positive reaction; + = positive reaction; the intensity of the colour was compared visually from + to 5 +.

Metal 20 µg	*Colour of spot with 0.025% Rhodamine B 10% KBr in 1 N HCl**				*Colour of spot with 0.025% Rhodamine B 10% KBr in 3 N HCl***				*Suitable concentration of acid (N)*
			+ *KI*				+ *KI*		
	V	*U.V.*	*V*	*U.V.*	*V*	*U.V.*	*V*	*U.V.*	
Au(III)	drv	db	br	db	drv	b	br	db	
		5 +		5 +		5 +		5 +	1
Bi(III)	rv	dv	y	db	—	gb	y	db	
		3 +		5 +		+		5 +	1
Cd(II)	rv	b	rv	b	—	—	rv	b	
		±		4 +				3 +	1
Fe(III)	—	g	dbr	db	—	g	dbr	db	
		±		5 +		2 +		5 +	3
Hg(II)	rv	v	rv	b	—	—	rv	b	
		2 +		4 +				2 +	1
Mo(VI)	rv	v	—	b	r	b	r	b	
		3 +		4 +		±		±	1
Sb(V)	rv	v	br	db	drv	b	dbr	db	
		3 +		5 +		5 +		5 +	3
Tl(I)	rv	dv	rv	db	rv	b	rv	b	
		5 +		5 +		3 +		3 +	1
Tl(III)	rv	dv	rv	db	rv	b	rv	db	
		5 +		5 +		3 +		4 +	1
V(V)	—	g	ybr	db	—	g	br	db	
				5 +				5 +	3
W(VI)	rv	v	rv	bv	—	g	—	gb	
		3 +		2 +		2 +		2 +	1

suitable concentration of HCl for detecting Fe, Sb and V is 3 *N*, while for Au, Bi, Cd, Hg, Mo and Tl it is 1 *N*. However, the most sensitive procedure for Au, Bi, Cd and Hg on paper is spraying with 0.025 R–10 % KBr in water and in the case of very small amounts of these metals is overspraying with 10 % KI. We observed red-violet coloured spots in visible light, deep violet ones under U.V. light or deep blue when KI was applied. Tungsten is best detected when the reagent is used in 2 *N* H_2SO_4. The colour developed by thallium lasts about 1–3 min. The method is suitable for chromatography of metals on paper.

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News

Forthcoming Event

THE PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

EXPOSITION OF MODERN LABORATORY EQUIPMENT

Preliminary announcement of 1967 Pittsburgh Conference

The Eighteenth PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY, INC. will be held at the Penn-Sheraton Hotel in Pittsburgh, Pennsylvania, U.S.A., March 6–10, 1967. Approximately 250 papers on all phases of analytical chemistry and spectroscopy will be presented. Symposia on the following subjects are now being arranged.

1. Laser Excitation Raman Spectroscopy
2. Information Retrieval and Data Handling
3. Biomedical Applications of Gas Chromatography
4. Coblentz Symposium on Infrared Spectroscopy
5. Analytical Chemistry in Air and Water Pollution
6. Computer Applications in Analytical Chemistry
7. X-Ray Analysis of Light Elements
8. Analytical Techniques in Nonaqueous Systems
9. Carbon-13 NMR Spectroscopy
10. Emission Spectroscopy in the Vacuum Ultraviolet.

Original papers on all phases of analytical chemistry and spectroscopy are invited. A brief abstract (150 words) of each paper will be printed in the program. Three copies of this abstract, with a letter listing the names of the authors, the laboratory in which the work was done, and the current addresses of the authors, should be addressed to: Dr. GERALD L. CARLSON, Program Chairman, The Eighteenth Pittsburgh Conference, Inc., Mellon Institute, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, U.S.A.

The final date for receipt of abstracts is October 15, 1966.

In addition to the program of technical papers, there will be an exhibition of the newest analytical instrumentation. More than 200 companies will display instruments, chemicals, and equipment. A complete program of activities for wives and lady attendees at the Conference is also being planned for the 1967 Pittsburgh Conference.

VARIAN AEROGRAPH BASIC GAS CHROMATOGRAPHY COURSES

Three day training courses in gas chromatography are being held every month at the European headquarters in Basel, Switzerland. They offer basic theory and practical laboratory sessions.

Please write for your registration to the course secretary, Varian Aerograph AG., Pelikanweg 2, Basel, Switzerland.

Apparatus

A *laboratory analogue computer* has been designed to resolve overlapping peaks in spectral and other experimental curves, by E. I. du Pont de Nemours and Co., Wilmington, Del. 19898, U.S.A.

The 310 curve resolver duplicates peaks corresponding to Gaussian, Lorentzian, and other distributions. Individual peak parameters of height, width, and ordinate position are varied independently by controls of the instrument to produce a summation curve that is equivalent to the original curve. This duplicated curve is then reduced electronically to its component parts. The 310 consists of an oscilloscope, controls and up to 10 function generator channels. An integrator, or an X-Y recorder, are available as accessories.

An *electronic paper tape data logger*, which enables automatic interpretation of the most complex gas chromatographic analyses using the power of a computer has recently been announced by Perkin-Elmer Corporation, Norwalk, Conn., U.S.A. An important feature of this equipment is its ability to carry out all the calculations and corrections needed in the reduction of gas chromatographic data. The system will observe and make allowances for drifting baselines, but eliminate the consideration of transient baseline movements and shifts due to attenuation. The system simultaneously handles the outputs of any number of gas chromatographs, and prints out the data in a form suitable for readout in a computer. By conversion of the data to paper tape format it is possible to use computers normally used in business or commercial operations. The system comprises one or more Data Acquisition Units, each of which accepts analogue voltage signals from a gas chromatograph and records them in a modified form on magnetic tape. In turn, a Data Translation Unit, which is able to handle a number of Data Acquisition Units, accepts the magnetic tape and converts it into a suitable paper tape form for a computer. When read into the computer the data are processed and printed out as a chromatographic analysis. The intermediate calculations can be simple or complex, obviously depending on the requirements of the application. Over-all accuracy is determined by the operation of a very stable voltage-to-frequency converter. After the signal has been changed to a frequency, all data handling is by the very accurate method of counting pulses. The fundamental accuracy of the converter is enhanced by use of an automatic scale change, which increases the dynamic range of the over-all system, and improves the accuracy for small signals. The conventional potentiometric recorder is not needed with this system, but one may be used to monitor the results if required.

An *integrator* which automatically computes and presents on printed paper tape, in digital form, peak areas which are a function of mass, concentration or structure has recently been developed by the Nester/Faust Manufacturing Corporation. The Summatic integrator is capable of reliably transforming input signals from 0–1 V to a high speed mechanical counter-printer which operates at 4800 counts per minute at 1 mV. Analyses of gas, liquid and paper chromatography may be accomplished in a fraction of the time previously required. This instrument uses the well-known voltage-to-frequency principle to convert DC input for summation. The integration process is triggered by the leading edge of the peak and finishes when the slope goes from negative to zero. Thus unresolved peaks and shoulders are therefore separately integrated. A ranging device attenuates the input signal by a factor of 10 at three decades (1, 10, 100 mV) for wider dynamic range. An accuracy of 0.7 % is claimed for 20 mV peaks of 10 sec duration. A recorder signal with attenuator is used so that when a 1 mV recorder is employed the pen remains on scale with input signals up to 1 V DC. For further details contact Nester/Faust Manufacturing Corporation, 2401 Ogletown Road, Newark, Del., U.S.A.

Recently developed is a *syringe with a threaded plunger* so that the user can deliver a constant volume by advancing the plunger with a screw-like motion. When used with a drive motor this device will deliver a given volume over a given period of time. It offers a positive means for delivering viscous and similar heavy samples. The plunger is twist-locked onto the syringe, providing a quick method of disconnection, and easy filling by normal pumping action. Hamilton Company, P.O. Box 307B, Whittier, Calif., U.S.A.

New Chemicals

The New Whatman Plant and Products

For a number of years, ion-exchange cellulose have found an increasingly important application in the field of polyelectrolyte separation. Studies carried out by the Whatman marketing and research groups, over a number of years, have indicated the basic requirement and have formulated completely new and improved forms of cellulose ion-exchangers.

Applications are found at all levels of requirements from the research laboratory to the manufacture of pharmaceutical materials, and the new plant has been designed to cater for the anticipated overall requirement for many years ahead. Whilst only two chemical substitutions, diethylaminoethyl cellulose and carboxymethyl cellulose, are at present being manufactured in the new advanced form, the manufacturing facility is sufficiently flexible to accommodate other chemical forms and other variants which are still in the research and development stage.

A new plant has been designed which makes possible the controlled substitution of ion-exchange groups into natural cellulose on a large scale, and to produce modified celluloses in the new and improved forms which have been developed by the Research

and Development departments. A considerable amount of "shopping around" was necessary, in order to acquire suitable equipment for the scale-up from pilot plant level, and the present set-up is the result of months of careful trials, both at equipment manufacturers' premises and at Maidstone. The final assembly is a unique manufacturing facility for ion-exchangers.

One of the main considerations, when the new facility was conceived, was to control chemical substitution in such a way that functional group distribution on the cellulose chains could be made in a more consistent and predictable manner than has been previously possible at the degree of substitution suitable for an ion-exchange cellulose.

A combination of careful process planning and selection of equipment has enabled a much higher degree of control to be achieved. Consequently, it is now possible to avoid the gross variations in specificity, resolution, and required elution conditions which have on occasion been associated with materials of this type, not only from one source to another but from one batch to another.

The plant is capable of producing ion-exchange celluloses in a number of physical forms. The range at present consists of:

Special derivatives for papermaking
Fibrous powder for column chromatography
Very fine fibrous powders for thin layer chromatography
A microgranular form
A swollen microgranular form.

The major improvements introduced with these new products may be summarized as follows:

(1) Improved fibrous forms which give a higher effective capacity, faster kinetics and superior flow characteristics than previous products.

(2) An entirely new microgranular form produced by molecular rearrangement and cross-linking of the cellulose. This gives rise to an even higher effective capacity, faster kinetics, and improved resolution.

(3) An entirely new feature is the availability of a pre-swollen form of the microgranular derivatives, which does not require precycling.

(4) A high standard of reproducibility achieved by careful design and construction of an entirely new plant for cellulose modification.

H. Reeve Angel & Co. Ltd.
14 New Bridge Street, London, E.C.4

Reagent for dimethylsilyl ethers

The reagent, Di-Sil-Prop, consisting of a mixture of tetramethyldisilazane, dimethylchlorosilane, and pyridine, is useful for the preparation of dimethylsilyl ethers. Availability should permit gas–liquid chromatography of high boiling point materials.

Applied Science Laboratories, Inc., P.O. Box 440, State College, Pa., U.S.A.

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Paper Chromatography

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Gas Chromatography

1. REVIEWS AND BOOKS

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2. FUNDAMENTALS, THEORY AND GENERAL

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HU, LI-ZHI AND PENG, SHAO-YI.: (Thinly coated gas–solid chromatography for the separation of hydrocarbon gases). *Acta Focalia Sinica*, 6 (1965) 113–121 — alumina was wetted with aluminium isopropoxide in benzene solution, and alcoholate was then heated to 600° for activation; analysis up to butadiene in one min.

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3c. Apparatus, accessories and materials for GC

JOHNSON, E. G.: Production of pure helium carrier gas by diffusion. *J. Chromatog.*, 22 (1966) 175–176 — He with an impurity below 0.5 p.p.m. is obtainable by diffusion through quartz; at 1500 p.s.i. inlet pressure and 600° maximally 450 ml/min of pure He could be obtained at atmospheric pressure; normal operation at 500 p.s.i. and 375° provided 70 ml He/min.

SHARMA, R. K., MCLEAN, D. R. AND BARDWELL, J.: Apparatus for analysis of combustion products from oxidation of hydrocarbons. *Indian J. Technol.*, 3 (1965) 206–208.

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GUILLEMIN, C. L.: Large diameter columns in preparative chromatography. *J. Gas Chromatog.*, 4 (1966) 104–109 — fluidised columns were used; they gave 500 TP/m at 6 cm in. diameter for injection of 0.25 cm^3.

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CABICAR, J. AND POSPÍŠIL, M.: (A rapid method for the investigation of gas–solid reactions). *Chem. Listy*, 60 (1966) 528–532 — a type of reaction gas chromatography where the carrier gas undergoes the reaction.

COTGREAVA, T.: A detector for liquid chromatography. *Chem. Ind. (London)*, (1965) 689 — GC detector coupled with column chromatography; the transfer mechanism is described.

KIM, YOU SUN, SON, YOUN SOO AND CHOI, Q. WON: (New gas-chromatographic method of organic elementary analysis). *J. Korean Chem. Soc.*, 8 (1964) 188–191 — combustion of organic substance to CO_2 and H_2O; water is converted to C_2H_2; errors for C ± 0.5 % and for H ± 0.33 %.

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STOUFFER, J. E., OAKES, P. L. AND SCHLATTER, J. E.: A liquid chromatographic system for the analysis of macromolecules. *J. Gas Chromatog.*, 4 (1966) 88–92.

4e. Automation

PUZNIAK, T. J. AND LICHTENFELS, D. H.: Gas chromatography is moving more and more from lab to plant. *Oil Gas J.*, 63, No. 32 (1965) 88–90.

4f. Measurement of physico-chemical and related values

DUTY, R. C. AND MAYBERRY, W. R.: A study of vapor pressures and boiling points of hydrocarbon solutions by gas–liquid chromatography. *J. Gas Chromatog.*, 4 (1966) 115–120.

WAKSMUNDZKI, A., RUDZINSKI, W. AND SUPRYNOWICZ, Z.: On the possibility of determination of adsorption potential. Distribution on adsorbent surface by gas chromatography. *J. Gas Chromatog.*, 4 (1966) 93–95 — surface interval in % was calculated for given adsorption potentials in cal/mol. from retention data.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

ORTMAN, G. C.: Monitoring methane in atmosphere with a flame ionization detector. *Anal. Chem.*, 38 (1966) 644–646 — frontal GC on charcoal.

TAGO, S. AND KONDO, O.: (Gas chromatographic determination of small amounts of acetylene hydrocarbons in the C_4 fraction). *Bull. Japan Petrol. Inst.*, 7 (1965) 1–6 — 2–3 p.p.m. on formamide and β,β′-oxydipropionitrile.

5b. Other hydrocarbons

BOGDANOVA, T. A., LIPOVICH, T. V. AND KALECHITS, I. V.: (Conversion of aromatic bicyclic hydrocarbons under the condition of destructive hydrogenation). *Neftekhimiya*, 6 (1966) 27–34.

EVDOKIMOVA, S. P., NEMTSOV, M. S. AND ISAKOVA, N. A.: (Identification of some by-products in the industrial synthesis of isoprene). *Neftekhimiya*, 6 (1966) 75–79 — preparative and analytical GC is used.

HÁLA, S., KURASH, M. AND LANDA, S.: (Isolation of adamantane from a paraffinic crude). *Neftekhimiya*, 6 (1966) 3–8 — on Apiezon L at 220°.

POP, A., BARBUL, M. AND BESCHEA, C.: (The determination by gas–liquid distribution chromatography of naphthalene and some methylnaphthalenes in oils and dealkylated products). *Rev. Chim. (Bucharest)*, 15 (1964) 686–689.

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SHUĬKIN, N. I., NAYRYSHKINA, T. I. AND RASHCHUPKINA, Z. A.: (Dehydrogenation of decalin and tetralin in the presence of activated charcoal). *Neftekhimiya*, 6 (1966) 22–26 — on PEGA at 190°.

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5c. Halogen derivatives of hydrocarbons

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8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

GRLIČ, L.: (Recent advances in the chemical research of cannabis). *Bull. Narcotics, U.N. Dept. Social Affairs*, 16, No. 4 (1964) 29–38 — a review with 56 references, some of which are GC.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

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DI STEFANO, F., VERCILLO, A. AND BONIFORTI, L.: (Gas-chromatographic determination of sorbic acid in wine). *Boll. Lab. Chim. Provinciali (Bologna)*, 15 (1964) 523–531 — on DEGS at 110° with ethyl nonanoate as internal standard.

MAERKER, G. AND HAEBERER, E. T.: Direct cleavage of internal epoxides with periodic acid. *J. Am. Oil Chemists' Soc.*, 43 (1966) 97–100 — on XE-60 and SE-30 at 200° or by PTGC 80–270°, 5°/min.

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21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

MACGEE, J.: Determination of nucleic acid base ratios by gas–liquid chromatography. *Anal. Biochem.*, 14 (1966) 305–314 — cytosine, uracil, adenine and guanine were separated after methylation with tetramethyl ammonium salt on Carbowax 20M by PTGC 143–221°.

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26. METALLO-ORGANIC COMPOUNDS

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29. INSECTICIDES AND OTHER PESTICIDES

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31. PLASTICS AND THEIR INTERMEDIATES

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GUDZINOWICZ, B. J.: Analysis of psychoactive phenothiazine drugs by gas chromatography. *J. Gas Chromatog.*, 4 (1966) 110–114 — on SE-30 at 237°.

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33. INORGANIC SUBSTANCES

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KARLSSON, B. M.: Heat treatment of molecular sieves for direct separation of argon and oxygen at room temperature by gas chromatography. *Anal. Chem.*, 38 (1966) 668–669 — mol. sieve 5A and 13X is heated to 450–500° for 5–24 h in an argon stream purge with a humidity of 100 p.p.m.; if a helium stream is applied, only 10 p.p.m. of water vapour can be used.

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33b. Volatile inorganic compounds

KUZ'MINA, A. V., KUDRYAVTSEVA, N. A., LULOVA, N. I. AND TARASOV, A. I.: (Rapid chromatographic method for the determination of hydrogen sulfide in gases and crude petroleum). *Khim. Tekhnol. Topliv i Masel*, No. 4 (1966) 57–60.

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Errata

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Page 529. Ninth line: For "diacetoxydihydroxyacetone" read "diacetoxyacetone".

Page 530. Table I, footnote: For "M = mauve" read "M = mauve-red".

Page 533. Fourth line: For "α-ketoacid" read "ketoacid".

Page 534. Legend to Fig. 6: For "α-ketoacids" read "ketoacids".

Page 535. Third and seventh lines from bottom: For "acetoxyglycolaldehyde DNPH" read "glycolaldehyde acetate DNPH".

Page 539. Twelfth line: For "α-ketoacids" read "ketoacids".

A STUDY OF THE QUANTITATIVE MEASUREMENT OF CERTAIN METABOLIC ACIDS BY GAS–LIQUID CHROMATOGRAPHY*,**

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INTRODUCTION

In recent years, gas–liquid chromatography (GLC) has been utilized for the separation and identification of organic acids involved in the tricarboxylic acid (TCA) cycle and certain other organic acids which perform vital functions in carbohydrate, fat, and protein metabolism[1,6,12,14]. The acids have been readily chromatographed as their methyl or ethyl ester derivatives. The GLC technique appears to be more applicable than previous methods which are time consuming, require meticulous attention to details, and are limited by compounds which interfere with the analytical measurement of these acids in biological material. Unfortunately, there is a paucity of reports[4,5,8,13] in the literature pertaining to the measurement of these metabolic intermediates by GLC, possibly because of the considerable amount of time required to study the quantitative aspects of the technique. If a useful and efficient GLC method was developed for quantitatively measuring these acids in biological material, it would be a useful tool with which to ascertain the complexities of intermediary metabolism in both plants and animals.

The objective of this research was to develop a quantitative GLC method for measuring certain metabolic acids, which could be readily applied to biological samples on a routine basis. Because the authors have previously reported on the separation and identification of the acid esters involved in this study[12], the qualitative aspects of the GLC technique will not be discussed in this paper except to outline the GLC instrumentation and columns used. None of the operating conditions for chromatographing the acid esters were changed from those specified in the previous report; however, it was necessary to change the esterification procedure to increase analytical precision.

EXPERIMENTAL

Chromatography

The instrumentation for chromatographing the organic acids consisted of a model 600 Aerograph Hy-Fi§ gas chromatograph equipped with a gold-plated

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§ Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, Calif.

hydrogen flame ionization detector and a Speedomax H* Leeds and Northrup recorder. Hydrogen gas for the detector was supplied by a model A-650 hydrogen generator** and a commercial source of high purity nitrogen was used as the carrier gas. The hydrogen gas line was kept dry by periodically regenerating the molecular sieve filter at 100° and an ultrasonic water bath was used to clean the flame detector. A loosely packed 1.5-in. long glass wool plug was placed inside the glass liner of the injection block next to the chromatographic column. A clean liner and plug were inserted periodically to minimize contamination of the system.

Methyl lactate, methyl oxalate, dimethyl malonate, dimethyl fumarate, and dimethyl succinate were separated with a 9-ft., 1/8-in. O.D. stainless steel column containing 15 % diethylene glycol succinate (DEGS) on 60–80 mesh, acid-washed Chromosorb W. These esters were separated isothermally at 140°. The same column at 200° resolved dimethyl malate and dimethyl α-ketoglutarate. Trimethyl citrate was most conveniently separated with a 5-ft. column containing the same packing material operated isothermally at 200°. The detector response for each ester appeared on the recorder chart as an individual, symmetrical peak.

Complete details of the GLC resolution and identification of these organic acid esters have been reported[12], including retention times and examples of representative chromatograms.

Methylation

The organic acids were converted to the corresponding methyl esters using concentrated hydrochloric acid (HCl) as the esterifying catalyst. Solutions of individual organic acids and of organic acid mixtures were prepared by dissolving the undissociated acids in absolute methanol (Fisher Scientific, 99.9 %, ACS). Ten-milliliter aliquots of the acid solutions were placed in dry reaction tubes (20 × 125 mm culture tubes with screw caps), 0.5 ml concentrated HCl was added to each tube, and the tubes were sealed and shaken in a shaker water bath at 55° for 4 h. At the end of the reaction period, the samples were stored in a refrigerator in the tightly capped reaction tubes until chromatographed.

A 3-μl aliquot of a sample was injected at each isothermal setting using a 10-μl Hamilton No. 701 N syringe. Prior to each injection the attenuator of the electrometer was set at 250 and was kept at this position until approximately 0.75 min after the solvent (methanol) peak. The attenuator was then adjusted to settings of 2, 4, 8, or 16, depending on the amount of acid esters. Attenuation changes were made only between peaks and the recorder pen was adjusted to the baseline before the next peak appeared, thus minimizing the error in peak area as determined with a Disc integrator***.

Titration

The undissociated acids used as standards were of the highest purity available commercially, but absolute purity values did not accompany the acids. Rather than assuming the acids to be 100 % pure, their purities were estimated by titrating them with a standardized sodium hydroxide (NaOH) solution. Titration was also used to determine the per cent methylation of each organic acid by comparing the

* Leeds and Northrup, Inc., 4901 Stenton Ave., Philadelphia, Pa.
** Wilkens Instr. and Research, Inc., P.O. Box 313,. Walnut Creek, Calif.
*** Disc Instruments, Inc., 3014-B South Halladay St., Santa Ana, Calif.

amount of organic acid left after esterification to the original amount. This technique for determining per cent methylation was reported to compare favorably with a direct determination which involved weighing the esterified acids[11]. The estimates of purity and per cent methylation were used to determine the m*M*/l of acid ester measured by the gas chromatograph.

Quantification

A standard solution of a known concentration for each organic acid and a solution containing a mixture of the acids were prepared by dissolving the acids in absolute methanol. A series of dilutions was then prepared from each solution. Triplicate 10-ml samples were taken at each concentration from the solutions containing single acids and duplicate samples from solutions containing a mixture of acids. All samples in a given series were chromatographed once, then rechromatographed on the following day. The data from the first day were compared with those of the second day to detect any effects or changes in the operating conditions of the chromatograph and column with time. These data were also used to study the relationship between peak area and methyl ester concentration and to study the effect of methylating the organic acids individually and in a mixture.

Commercial esters were obtained from Eastman Kodak for all acids except lactic. A standard solution of each ester and a solution containing an ester mixture were prepared by dissolving the esters in absolute methanol. A series of dilutions for each solution was prepared, chromatographed, and standard curves constructed. These data were compared to the previous data, obtained from standard acids methylated in the laboratory, to study the effect of methylation on the quantification of these acids.

Lyophilization

The direct esterification of organic acids is a reversible reaction, with water as one of the end-products. Thus, the presence of excess water during the methylation reaction will prevent maximum production of the acid esters. This problem must be considered when adapting this method to biological samples and aqueous extracts. In the present study, lyophilization was investigated as a means of dehydrating and concentrating samples prior to methylation. It was necessary to determine if any organic acids were lost during lyophilization and the effect of pH on these losses.

A water solution and a methanol solution containing 10 m*M*/l of each acid were prepared. The water solution was adjusted to pH 7.0 with NaOH to dissolve the acids. Ten 20-ml samples of the aqueous solution were placed in 8-oz. narrow-mouth polyethylene bottles. Five were adjusted to pH 10.0 with NaOH and 5 to pH 4.5 with HCl. Four layers of cheesecloth were placed over the opening of each bottle. The bottles were closed with screw caps and frozen in liquid nitrogen. While frozen, the screw caps were removed and the bottles were placed in the vacuum chamber of a Stokes model 21 lyophilizer.* The cheesecloth acted to trap any flakes of material that might float to the mouth of the bottle while in the lyophilizing chamber.

Following lyophilization, 20 ml of methanol and 0.5 ml of concentrated HCl were added to the dried residue of each sample. The bottles were tightly capped and shaken in a shaker water bath at 55° for 4 h. After methylation, each sample was

* F. J. Stokes Corp., Philadelphia 20, Pa.

chromatographed in duplicate. The results were compared to similar samples from the alcohol solution which had not been lyophilized.

RESULTS AND DISCUSSION

Methylation

The first requirement for quantitatively measuring these organic acids was to establish a reliable method for converting the acids to their esters. Methanol and HCl were tried as a possible methylating agent because it has received wide application for methylating low molecular weight carboxylic acids with no apparent difficulties.[3] This method was used by earlier workers[9] for separating acids involved in the TCA cycle by fractional distillation and it was found equally applicable in the present study. Although the qualitative phase of this procedure used concentrated sulfuric acid as the methylating catalyst, changing to concentrated HCl did not pose any chromatographic problems. On the basis of comparisons with standard acids, the chromatographic results of samples methylated with these two catalysts were identical except that analytical variation was less when HCl was used.

The conditions for methylation as outlined in the experimental section were optimal for the organic acids studied. They were determined by varying the amount of concentrated HCl added and the length of time in the water bath at 55°. Maximal methylation of the organic acids occurred when sufficient HCl was added to lower sample pH below the pK's of each acid. Also, maximal methylation was not affected by adding HCl in an amount which lowered the pH to 1.0. The lowest pK was for oxalic acid (pK = 1.19). Thus, methylation of all acids at pH 1.0 permitted carrying out the procedure under the same conditions. At pH 1.0, maximal methylation of the organic acids occurred within 4 h at 55° in a shaker water bath.

Under the conditions in our laboratory, this method was superior to the sodium-methylate method[2] and the boron-trifluoride method.[7] Also, when 2,2-dimethoxypropane was used as a water scavenger, as reported by RADIN *et al.*[11] and PRICE[10], no measurable benefit was obtained. This suggested that under the conditions of the present study, the water endogenous to the reagents, and that produced during methylation, had an insignificant effect on the efficiency of esterification.

Titration

Once the optimal conditions for methylation were established, acid titration was used to quantitate the amount of each acid which was converted to its ester. The percentage of esterification of each acid is presented in Table I. The range values represent the lowest and highest of three determinations. Table I also contains estimates of acid purity as determined by titration. The standard errors of titration were 0.36 and 0.90 for per cent methylation and per cent purity, respectively. Although these percentage estimates may be subject to error, most of them were within a realistic range and, because of their constancy, they were used to develop the quantitative aspects of measuring these organic acids by GLC. Different lots of acids were not used in the study.

Quantification

Investigations of the quantitative aspects of measuring the organic acids consisted of studying each acid individually and as part of a mixture of acids. Thus,

TABLE I

PER CENT METHYLATION AND PURITY OF ORGANIC ACIDS AS DETERMINED BY TITRATION

Acid	*Methylation*		*Purity*	
	Average	*Range*	*Average*	*Range*
Lactic	98.7	98.5– 98.9	75.1	74.1– 75.5
Oxalic	42.6	42.6– 45.5	84.4	82.9– 85.5
Malonic	97.6	97.5– 98.2	77.4	76.6– 78.3
Fumaric	93.3	93.2– 93.4	104.0	103.1–104.8
Succinic	99.4	99.0– 99.8	97.6	96.8– 98.5
Malic	100.5	100.2–100.8	93.8	92.8– 94.1
α-Ketoglutaric	83.6	82.8– 84.1	94.8	94.0– 95.7
Citric	91.6	91.4– 91.8	103.1	101.5–103.4

any differences between methylating and chromatographing the organic acids separately and in a mixture could be defined. The per cent methylation and per cent purity values obtained by titration were used to determine the methyl ester concentration of each acid so that the data could be subjected to statistical analyses and standard curves could be established. Table II summarizes the statistical significance of the effects of ester concentration and time interval on the chromatographic response measured as peak area by a Disc integrator. This information represents the type of concentration *versus* response curve obtained for the methyl ester of each acid, the curves for the esters in a mixture being more important because biological samples contain organic acid mixtures. The fact that very similar curves were obtained for the esters measured individually and in a mixture indicated that the organic acids were chemically independent of each other when carried through the procedure as a mixture.

Linear curves fit by the least squares procedure were obtained for lactic, malonic, fumaric, succinic, and citric acids. Although the actual data tended to be non-linear at the lower concentrations, this deviation from linearity was not significant at the $P = 0.05$ level. Plotting the curve for α-ketoglutarate revealed a linear relationship between 6 and 24 m*M*/l. A cubic relationship was obtained when the entire concentration range was considered because of the significant curvilinear response below 6 m*M*/l which was the lower quarter of the concentration range. A similar non-linear response in the lower portion of the curve for individual methyl citrate explains its cubic relationship between concentration and response.

The curves for methyl malate and methyl oxalate obtained from the acid mixture were quadratic. This agrees with data on oxalic acid reported by SHARPLESS[13], who also reported non-linear curves for malonate and succinate but did not report any statistical treatment of the data. In the present study the type of curvilinear response for oxalic acid measured individually and in a mixture did not agree; however, when samples of commercially prepared methyl oxalate were analyzed, both individually and as part of a mixture, they followed a quadratic trend. Although the curves for malic acid were non-linear, there was considerable variation not accounted for by regression and the curves may have been linear.

It is difficult to make direct comparisons of this study with other studies found in the literature because of the differences in instrumentation and column materia

TABLE II

STATISTICAL SUMMARY OF THE EFFECTS OF CONCENTRATION AND DAYS ON THE CHROMATOGRAPHIC RESPONSE TO ORGANIC ACID ESTERS

Source	*Lactic*		*Oxalic*		*Malonic*		*Fumaric*		*Succinic*		*Malic*		*α-Ketoglutaric*		*Citric*	
	I[a]	*M*[b]	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*
Concentration	**[c]	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Linear	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Quadratic	o[d]	o	**	**	o	o	o	o	o	o	**	**	o	**	**	o
Cubic	o	o	**	o	o	o	o	o	o	o	o	o	o	*	**	o
Quartic	o	o	o	o	—	o	—	o	o	o	o	o	o	o	o	o
Quintic	o	—[f]	—	—	—	—	—	—	o	—	o	—	o	—	o	—
Days	*[e]	o	o	o	o	o	o	o	o	o	o	o	o	o	o	**
Days × concentration	*	*	o	o	o	o	o	o	o	o	o	**	o	*	o	**
Linear	**	**	o	o	o	o	o	o	o	o	*	**	o	**	o	**
Quadratic	o	*	*	o	o	*	*	o	o	o	o	**	o	**	o	o
Cubic	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Quartic	o	o	o	o	—	o	—	o	o	o	o	o	o	o	o	o
Quintic	o	—	—	—	—	—	—	—	o	—	o	—	o	—	o	—

[a] Organic acids methylated and chromatographed individually.
[b] Organic acids methylated and chromatographed as part of a mixture.
[c] $P < 0.01$
[d] Statistically non-significant at $P = 0.05$.
[e] $P < 0.05$.
[f] Sources of variation which could not be tested with the data.

which play an important role in the analysis of compounds by GLC. SHARPLESS[13] obtained alterations in peak area response as increasing amounts of the methyl esters of oxalate, malonate, and succinate were chromatographed. The esters of these acids were separated using DEGS as the liquid phase and measured with an argon B-ray detector. MIROCHA AND DEVAY[8] found this same non-linear response between peak area and concentration with fumaric acid using DEGS as the liquid phase and a hydrogen flame ionization detector; however, fumarate was separated as its diethyl ester. A linear response for the methyl esters of succinate, oxalacetate, malate, α-ketoglutarate, *cis*-aconitate, citrate, and fumarate was obtained by KUKSIS AND VISHWAKARMA[4] using a flame-ionization detector and Carbowax as the liquid phase. It appears that because of the differences in chromatographic systems, one should be concerned with the type and reproducibility of the standard curves in establishing a quantitative procedure and should not expect the ideal linear relationship between concentration and chromatographic response in all cases. However, it may be possible to choose concentration ranges within which the response is linear.

TABLE III

REGRESSION EQUATIONS REPRESENTING THE STANDARD CURVES FOR ORGANIC ACID ESTERS OBTAINED FROM AN ACID MIXTURE

Ester	*Concentration range (mM/l)*	*Day*	*Equation*
Lactic	1.19–19.06	1	$Y^a = -258.47 + 212.95(X)^b$
		2	$Y = -443.64 + 253.61(X)$
Oxalic	0.45– 1.81	1	$Y = -151.30 + 592.55(X)$
		2	$Y = -148.76 + 588.74(X)$
	1.81– 7.25	1	$Y = -461.25 + 752.47(X)$
		2	$Y = -552.44 + 798.52(X)$
Malonic	1.14–18.29	1	$Y = -153.17 + 394.89(X)$
		2	$Y = -192.56 + 400.15(X)$
Fumaric	1.45–23.20	1	$Y = -527.44 + 605.01(X)$
		2	$Y = -391.77 + 580.92(X)$
Succinic	1.38–22.20	1	$Y = -231.48 + 599.94(X)$
		2	$Y = -172.85 + 588.39(X)$
Malic	1.43– 5.73	1	$Y = -184.78 + 141.91(X)$
		2	$Y = -134.32 + 123.36(X)$
	5.73–22.92	1	$Y = 35.43 + 114.62(X)$
		2	$Y = -200.00 + 133.00(X)$
α-Ketoglutaric	1.50– 6.00	1	$Y = -116.77 + 114.02(X)$
		2	$Y = -52.05 + 90.67(X)$
	6.00–24.00	1	$Y = -276.24 + 147.71(X)$
		2	$Y = -546.76 + 168.34(X)$
Citric	1.37–21.98	1	$Y = -234.89 + 243.32(X)$
		2	$Y = -581.55 + 327.40(X)$

[a] Y = peak area.
[b] X = ester concentration.

Linear equations which represent the standard curves for the acid esters are shown in Table III. For the esters which gave a linear response with concentration by showing no significant (at $P = 0.05$ level) departure from linearity, an equation is presented which covers the entire range. For the esters giving a non-linear response the concentrations were divided into linear segments and linear equations are presented for each segment. In all cases, an equation representing each concentration range is presented for each of two days. Thus, these equations can be used to plot the standard curves obtained for the mixture of esters in this study to give an idea of the variation obtained due to analyzing the same set of standard solutions on different days. There were some differences due to days which is shown in Table II by the significant concentration–day interactions. This day effect was due primarily to changes in the operating conditions of the chromatograph or changes within the chromatographic columns.

Commercially prepared esters for all the acids except lactic were obtained and standard curves prepared with these esters compared favorably with the esters prepared in the laboratory. This indicated that any curvilinear response was due to the chromatographic instrumentation and not the esterification of the acids.

The coefficients of variation for lactic, oxalic, malonic, fumaric, succinic, malic, α-ketoglutaric, and citric acids when analyzed in a mixture were 8.1, 2.7, 3.1, 2.8, 1.7, 1.8, 2.0, and 3.6, respectively. These coefficients were determined using the average peak area of the concentration range of each acid studied. Above the mid-point of each concentration range, the coefficient of variation was constant; but as the concentrations were decreased below the mid-points, the proportional amount of random variation increased. The coefficients of variation range from 5 to 10 % at the lower contration levels.

Lyophilization

Before applying this method to biological tissues and fluids which contain large amounts of water, a method had to be developed for removing the water prior to the esterification step. The use of lyophilization was suggested by THIMANN AND BONNER[15]

TABLE IV

EFFECT OF pH ON LOSSES DURING LYOPHILIZATION

	Loss in mM/l					*Recovery* (%)
	Non-lyophilized		*Lyophilized*		$\bar{S}x$	
	pH 10.0	*pH 4.5*	*pH 10.0*	*pH 4.5*		
Lactic	10.0	10.5	10.2	9.9	0.71	98.3
Oxalic	10.0	9.7	11.0	9.7	0.32	105.2[b]
Malonic	10.0	9.6	6.7	6.3	0.29	66.3[c]
Fumaric	10.0	9.4	10.1	9.9	0.28	103.0
Succinic	10.0	9.3	9.7	9.5	0.23	99.4
Malic[a]	—	10.0	—	10.2	0.49	101.5
α-Ketoglutaric	—	10.0	—	9.6	0.64	95.7
Citric	10.0	9.8	10.1	9.5	0.38	98.4

[a] The effect of pH on malic acid was studied later with α-ketoglutaric acid.
[b] $P < 0.05$.
[c] $P < 0.01$.

for drying biological materials with a minimum of organic acid loss. Table IV contains a summary of the data from the present investigation comparing lyophilized to non-lyophilized samples at a high and low pH. All recoveries during lyophilization were near 100 % except for malonic acid, which was consistently lost in a significant amount. One sample at a pH of 10.0, which gave an unusually high detector response, caused an apparent increase in oxalic acid during lyophilization; but an error in its preparation could not be found. Although there was a tendency for the recoveries of the other acids to be lower at pH 4.5, the differences attributable to pH were insignificant.

TABLE V

EFFECT OF pH ON THE LOSS OF MALIC AND α-KETOGLUTARIC ACIDS DURING LYOPHILIZATION

Acid	*Loss in mM/l*					
	pH 4.5	*pH 6.0*	*pH 7.0*	*pH 8.5*	*pH 10.0*	$S\bar{x}$
Malic	10.0	9.6	10.4	12.8	11.8	0.68
α-Ketoglutaric	10.0	9.3	10.7	—	—	1.14

α-Ketoglutaric acid was completely lost at a pH of 10.0 during lyophilization. Therefore, this acid was studied at pH's of 4.5, 6.0, 7.0, 8.5, and 10.0 and found to be completely lost above pH 7.0 (Table V). From pH 4.5 to 7.0, α-ketoglutarate was not significantly lost. Also, under the conditions of this study, malic acid was not lost by changing the pH. Malic acid was studied with α-ketoglutaric acid instead of the other organic acids because it was chromatographed with α-ketoglutarate.

Thus, the data indicated that freeze-drying was applicable for removing water from biological samples and extracts. Although α-ketoglutarate was completely lost at pH's above 7.0, there was no loss between 4.5–7.0, which represents a wide working range.

This technique also allowed standard acid solutions to be made with water instead of methanol. Therefore, pure organic acid salts, which were easily obtained for some of the organic acids, could be incorporated into standard solutions and used for subsequent quantitative measurements. It was also noted that purities of the free organic acids as determined by titration were in close agreement with their purities determined chromatographically by comparing the free acids to the salts. The purities of lactic, oxalic, fumaric, succinic, and citric acids were 75.1, 84.4, 104.0, 97.6, and 103.1 % respectively when determined by titration and 81.9, 86.8, 97.6, 95.6 and 101.1 % when determined chromatographically.

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SUMMARY

Various factors in the quantitative measurement of metabolic organic acids by GLC were studied. Conditions were established for quantitatively converting lactic,

oxalic, malonic, fumaric, succinic, malic, α-ketoglutaric, and citric acids to their methyl esters using methanol and concentrated HCl, and the esters were measured by GLC using a DEGS column. Not all of the esters gave a linear response with increasing concentration levels, and the non-linearity appeared to be an effect of the GLC system. Lyophilization was suitable for dehydrating aqueous samples. This method of drying represents a convenient means of adapting the GLC method to the analysis of biological samples or aqueous extracts.

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SUPPORT-COATED OPEN TUBULAR COLUMNS

IV. SELECTED APPLICATIONS*

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In our previous publications on support-coated open tubular columns[1–3], the advantages of these columns as compared with classical, wall-coated open tubular columns were discussed in detail. As outlined there, the β-value (the ratio of the volumes of the gas and liquid phase in the column) of the new column type is decreased; thus, the number of theoretical plates required for the separation of a given peak-pair—particularly for early peaks—is also decreased. At the same time, due to the increased liquid phase volume, the sample capacity of support-coated open tubular columns is significantly increased as compared with the classical, wall-coated open tubular columns; in fact, the sample capacity of the new columns is of the same order of magnitude as that of the standard 1/8 in. O.D. (0.085 in. I.D.) packed columns.

In the present paper, the influence of sample size on peak resolution is explored in more detail. In addition to this, the minimum detectability of systems incorporating wall-coated and support-coated open tubular columns is compared and the relative performance of support-coated and conventional packed columns is investigated. Finally, a few practical applications are shown.

INSTRUMENTATION

All analyses reported here were carried out with the Model 880 gas chromatograph of the Perkin-Elmer Corporation. The instrument was equipped with a flame ionization detector and connected to a Leeds & Northrup Speedomax "G" 1 or 5 mV recorder. With a 1 mV recorder, this particular system corresponded to a full scale pen deflection of $2.1 \cdot 10^{-12}$ A at attenuation $\times$ 1.

The columns used were prepared from 0.020 in I.D. copper tubes in the usual way, as discussed in our earlier publications[1–3]. Helium was used as the carrier gas. The flow rates reported were measured at column outlet and ambient temperature. The average linear gas velocities ($\bar{u}$) were calculated with help of the following equation:

$$\bar{u} = \frac{L}{t_M} \tag{1}$$

where L is the column length (cm) and t_M the retention time of methane (sec).

* Presented at the 17th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Pittsburgh, Pa., February 23, 1966.

Samples were injected with help of Hamilton 1 μl syringes. As discussed in our previous paper[2], support-coated columns could permit direct sample injection into the column. However, for convenience, we generally split the injected sample with a low split ratio (1/10–1/20). The sample volumes listed in the figures and in the text always refer to the actual sample entering the column.

SAMPLE SIZE *vs.* PEAK RESOLUTION

The best way to illustrate the influence of increased sample amounts on peak resolution is to select certain peak pairs representing a difficult separation and to investigate how the actual separation will change with increased sample size. For this study, we have selected two test mixtures.

Our first test mixture consisted of hexene-1 and *n*-hexane, in about 1:1 ratio and it also contained an impurity which emerged—under the conditions used—between the two major peaks. The calculated composition of the sample was:

Hexene-1: 50.2 vol. %
Impurity: 0.8 vol. %
n-Hexane: 49.0 vol. %.

This sample was analyzed on a 150 ft long support-coated column prepared with squalane liquid phase, at 100°C, with a carrier gas flow of 2.76 ml/min ($\bar{u}$ = 20 cm/sec). The respective partition ratio values were measured as 1.52, 1.67 and 1.73. The relative retention (α) of the two major peaks is 1.138 while the relative retention calculated for the second major peak and the impurity peak is 1.036.

Fig. 1 shows the chromatograms obtained with various sample sizes. The volume of the total sample entering the column and the recorder attenuation are

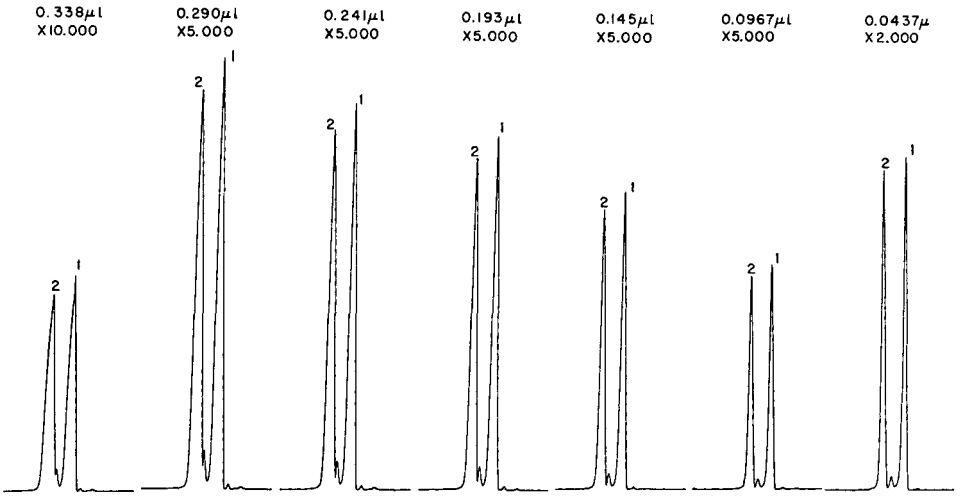

Fig. 1. Chromatograms of a sample containing (1) hexene-1 and (2) *n*-hexane and an impurity, obtained with various sample sizes. Column: 150 ft. × 0.020 in. I.D. support-coated open, tubular, prepared with squalane liquid phase. Column temperature: 100°. Carrier gas (He) flow rate at column outlet: 2.76 ml/min. The numbers above the peaks refer to the total sample volume entering the column and the recorder attenuation used.

listed above each chromatogram. As seen, the separation of the small peak from the second major peak is quite good up to about 0.15 μl total sample volume but the small peak can still be seen even at 0.388 μl total sample.

Fig. 2 plots the values of peak heights, peak area, and resolution, against the volume of a single component entering the column, for the two major peaks (α = 1.138). The resolution (R_h) was calculated from the peak width at half height (w_h):

$$R_h = \frac{2\,\Delta t}{w_{h1} + w_{h2}} \tag{2}$$

where Δt is the distance of the two peak maxima. Values calculated in this way are by a factor of 1.7 larger than the resolution values (R) calculated from the peak width at base, in the usual way[4]:

$$R_h = 1.7\,R \tag{3}$$

In Fig. 2, dotted lines indicate the values corresponding to base line separation (R = 1.5) and about 85 % separation (R = 1.0).

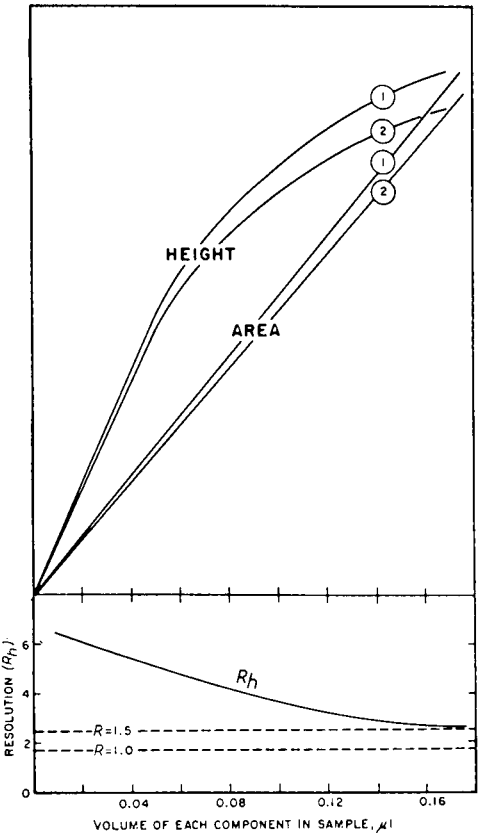

Fig. 2. Plot of the values of peak heights, peak area and resolution against the volume of each component, for the chromatograms shown in Fig. 1.

As seen in Fig. 2, the peak area values are linear over the whole range investigated while the peak heights start to be non-linear at about 0.06 μl (single component). This simply means that for quantitative purposes, one should use peak area rather than peak height above this level. Such an observation can be found for any column above a certain point. Base line separation or better could be obtained up to about 0.16 μl of a single component.

The second test mixture consisted of 3-methylhexane and cyclohexane with a respective concentration of 51.8 and 48.1 vol. %. This sample was analyzed on a 150 ft. long support-coated column prepared with DC-550 phenylsilicone oil liquid phase, at 75°, with a carrier gas flow of 2.7 ml/min ($\bar{u}$ = 17.7 cm/sec). At the given temperature, the two peaks represent a relative retention (α) of 1.079. The values of the partition ratio are 3.70 and 3.99, respectively.

Fig. 3 shows the chromatograms obtained with various sample sizes. The volume of the total sample entering the column and the recorder attenuation are listed above each chromatogram. As seen, the first peak starts to become skewed at about 0.3 μl total sample volume but even there, the separation is still quite good.

Fig. 4 plots the values of peak heights, peak area (for the second peak) and resolution against the volume of a single component entering the column, for the chromatograms shown in Fig. 3. Again, the peak heights start to become non-linear at about 0.06 μl of a single component. The area of the second peak was linear in the whole range investigated; since the first peak skewed significantly with higher sample load, we did not calculate its area. Base line separation or better could be obtained up to about 0.12 μl of a single component.

MINIMUM DETECTABILITY

For the investigation of the minimum detectability, we used ASTM-grade (99.5 % + vol. %) *n*-heptane as a typical sample because this analysis is the best illustration for a case where high column performance and the determination of trace impurities has to be combined. The problems involved in this analysis were discussed in detail in our previous paper[2].

Fig. 5 shows the chromatogram of an ASTM-grade *n*-heptane sample, obtained on a 300 ft. × 0.010 in. I.D. classical wall-coated open tubular column prepared with squalane liquid phase, at 37° using a 1 mV recorder with attenuation × 1. We have added 600 p.p.m. cyclohexane and 100 p.p.m. each of cyclopentane and 2-methylpentane to the sample in order to have an indication of the concentration levels of the individual peaks and to be able to calculate minimum detectability. For this analysis, we have introduced $13.2 \cdot 10^{-3}$ μl sample into the column which is about a factor of 5 larger than specified in the original ASTM-method[5]. As a comparison, Fig. 6 shows the analysis of the same sample, on a 150 ft. × 0.020 in. I.D. support-coated open tubular column prepared with squalane liquid phase, at 85°. The volume of sample introduced into the column was now 0.21 μl.

Table I lists the comparative data for these two chromatograms. A peak with 5 mm height obtained on a 1 mV recorder at attenuation × 1 was taken as the minimum detectable limit. As shown, this value was as low as 0.4 p.p.m. with the support-coated column, under the conditions listed.

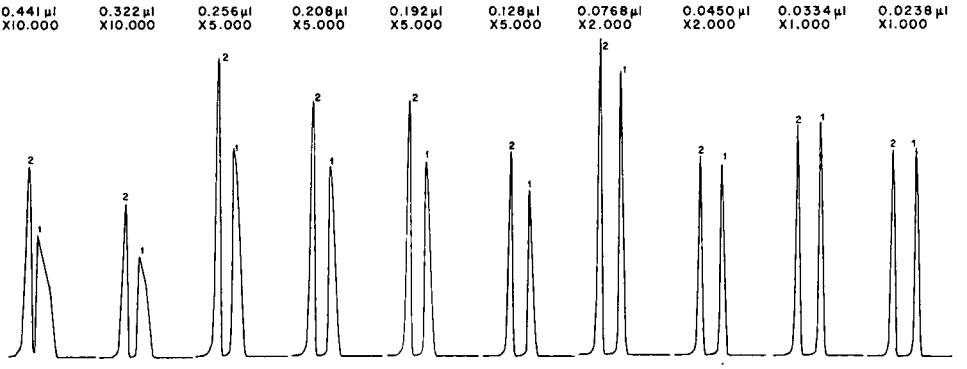

Fig. 3. Chromatograms of a sample containing (1) 3-methylhexane and (2) cyclohexane, obtained with various sample sizes. Column: 150 ft. × 0.020 in. I.D. support-coated, tubular, prepared with DC-550 phenylsilicone oil liquid phase. Column temperature: 75°. Carrier gas (He) flow rate at column outlet: 2.7 ml/min. The numbers above the peaks refer to the total sample volume entering the column and the recorder attenuation used.

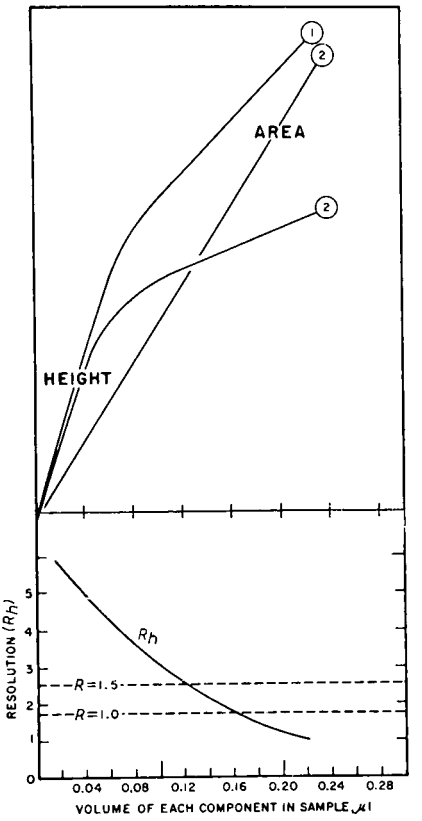

Fig. 4. Plot of the values of peak heights, peak area and resolution against the volume of each component, for the chromatograms shown in Fig. 3.

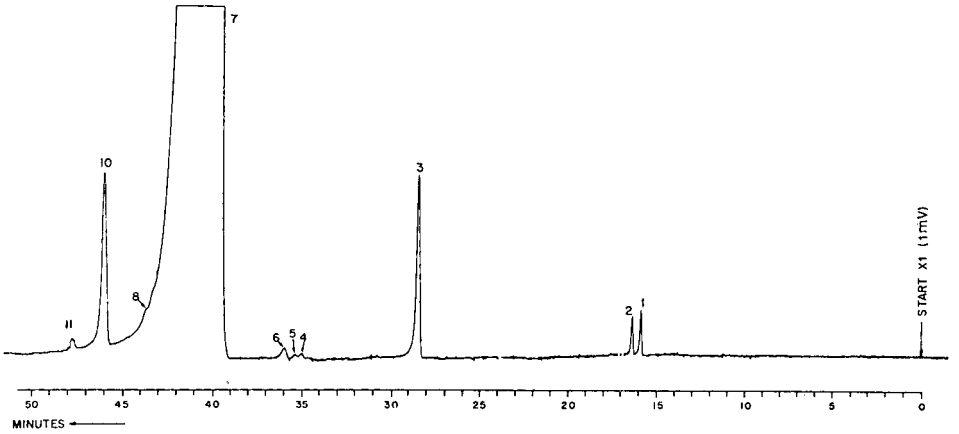

Fig. 5. Analysis of an ASTM-grade *n*-heptane, I. Column: 300 ft. × 0.010 in. I.D. standard wall-coated open tubular. Liquid phase: squalane. Column temperature: 37°. Carrier gas (He) flow rate at column outlet: 1 ml/min. Sample volume: $13.2 \cdot 10^{-3}$ μl. 1 mV recorder. Peaks: 1 = cyclopentane (100 p.p.m.; added), 2 = 2-methylpentane (100 p.p.m.; added), 3 = cyclohexane (600 p.p.m.; added), 7 = *n*-heptane.

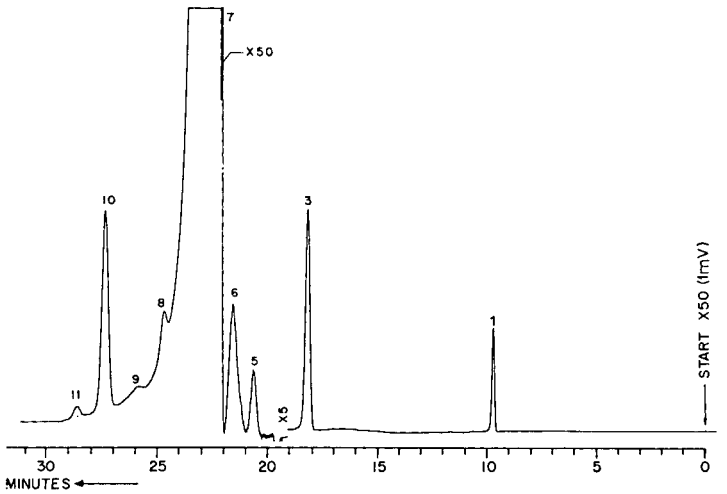

Fig. 6. Analysis of an ASTM-grade *n*-heptane, II. Column: 150 ft. × 0.020 in. I.D. support-coated open tubular. Liquid phase: squalane. Column temperature: 85°. Carrier gas (He) flow rate at column outlet: 3 ml/min. Sample volume: 0.21 μl. 1 mV recorder. Peaks: 1 = cyclopentane + 2-methylpentane (100 p.p.m. each; added), 3 = cyclohexane (600 p.p.m.; added), 7 = *n*-heptane.

TABLE I

COMPARATIVE DATA FOR THE CHROMATOGRAMS SHOWN IN FIGS. 5 AND 6

		Open tubular column	
		Wall-coated	*Support-coated*
Column length	(ft.)	300	150
Column internal diameter	(in.)	0.010	0.020
Sample volume	(10^{-3} μl)	13.2	210
Column temperature	(°C)	37	85
Carrier gas (He) flow rate	(ml/min)	1.0	3.0
Total number of theoretical plates for cyclohexane		116,160	43,540
HETP for cyclohexane	(mm)	0.79	1.05
Minimum detectable limit (cyclohexane) in the sample	(p.p.m.)	18.5	0.4
Retention time of the last peak (No. 11)	(min)	47.7	28.7
Concentration of cyclohexane in the carrier gas at column outlet	(p.p.m.)	4	40

Table I also gives the concentration of cyclohexane in the carrier gas at column outlet. The relatively larger concentration with the support-coated column is advantageous if concentration-sensitive detection devices are used.

COMPARISON WITH PACKED COLUMNS

Until now, support-coated columns were only compared with the standard wall-coated open tubular columns. Below, a comparison with packed columns follows. Since the sample capacity of the usual 1/8 in. O.D. (I.D. 2.2 mm) packed columns and the support-coated open tubular columns is about the same order of magnitude, we will primarily investigate comparative data concerning analysis time, the necessary carrier gas inlet pressure, and column performance.

For this comparison, we took the support-coated column used for the analysis shown in Fig. 6, the conditions of which are listed in Table I. This column is characterized by a β-value of 21.9 and by a specific permeability (B_0) of $583 \cdot 10^{-7}$ cm^2. The partition ratio for cyclohexane was 4.66 and 43,540 theoretical plates were obtained for this peak; this value corresponds to an effective plate number of 29,490. As discussed by DESTY *et al.*[6], the correlation between the number of theoretical plates (n) and effective plates (N) is expressed by the following equation:

$$N = n \left(\frac{k}{k + 1}\right)^2 \tag{4}$$

The number of effective plates is a value which permits a direct comparison of column performance. If one obtains a certain effective plate value on a column, then for a similar separation, the same effective plates are necessary on any column, regardless of its type, prepared with the same liquid phase.

According to DAL NOGARE AND CHIU[7], the optimum β-value for a packed column with 80–100 mesh diatomaceous-earth support is 19. If this column is used at 85°, the value of the partition ratio for cyclohexane is 5.37, on squalane liquid phase.

Thus, the number of theoretical plates on this column corresponding to 29,490 effective plates will be 41,490. Assuming an HETP of 0.6 mm for this column, its length can be calculated as 24.89 m.

Let us assume an outlet flow rate of 40 ml/min for the packed column with 1/8 in. O.D. and 2.2 mm I.D. Using a value of 0.40 for the interparticle porosity (ε) this corresponds to an outlet velocity (u_0) of 43.85 cm/sec. According to DAL NOGARE AND JUVET[8], the specific permeability of a packed column with 80–100 mesh Chromosorb support is $1.96 \cdot 10^{-7}$ cm^2. If the column is operated at 85° with helium carrier gas (viscosity at 85°: $2.2 \cdot 10^{-4}$ poise), the carrier gas inlet pressure (p_i) can be calculated from the KOZENY–CARMAN[9] equation:

$$B_o = 2\,\eta\varepsilon L \frac{p_o}{p_i^2 - p_o^2} u_o \tag{5}$$

where η is the carrier gas viscosity, L the column length and p_0 is the column outlet pressure (in this calculation, it is assumed as atmospheric pressure). Knowing the inlet pressure and the compressibility correction factor, the average linear gas velocity can be calculated. On the other hand, from the column length (L), the average gas velocity ($\bar{u}$), and the partition ratio (k), the retention time of a certain peak (t_R) can be obtained:

$$t_R = \frac{L}{\bar{u}}(1 + k) \tag{6}$$

In a similar way, we also calculated the retention time of peak No. 11 which is the last peak on the chromatogram.

A packed column with an HETP of 0.6 mm represents very high efficiency, and columns used in practice usually have a higher HETP value. Therefore, we also carried out the same calculation but now for a column with an HETP value of 1 mm. Table II summarizes the values obtained for the support-coated open tubular column and the two packed columns.

As listed in Table II, the retention time of the last peak was only 28.7 min on the support-coated open tubular column while it would be 64.5 and 137.9 min on the packed columns, with respective HETP values of 0.6 and 1.0 mm. The (absolute) carrier gas inlet pressure with the support-coated column was only 1.41 atm; its respective values would be 9.95 and 12.82 atm on the two packed columns.

Finally, we would also like to compare true column performance. As discussed in detail by DESTY *et al.*[6], the true column performance should be expressed as *the number of effective plates produced in unit time*:

$$\frac{N}{t_R} = \frac{\bar{u}}{h} \frac{k^2}{(1 + k)^3} \tag{7}$$

where h is the HETP of the respective peak.

As listed in Table II, for the cyclohexane peak, this value was found as 1630 with the support-coated open tubular column while only 730 and 340 respectively with the two packed columns (expressed as effective plates/min).

TABLE II

COMPARATIVE DATA ON SUPPORT-COATED OPEN TUBULAR AND PACKED COLUMNS PREPARED WITH SQUALANE LIQUID PHASE

		Support-coated open tubular column	*Packed column*	
			No. 1	*No. 2*
Column length	(m)	45.720	24.894	41.490
Column internal diameter	(mm)	0.50	2.2	2.2
Column temperature	(°C)	85	85	85
Partition ratio of cyclohexane		4.66	5.37	5.37
Partition ratio of peak No. 11		7.94	9.15	9.15
HETP of the cyclohexane peak	(mm)	1.05	0.60	1.00
Number of theoretical plates for the cyclohexane peak		43,540	41,490	41,490
Number of effective plates for the cyclohexane peak		29,490	29,490	29,490
Carrier gas flow rate at column outlet	(ml/min)	3.0	40.0	40.0
Carrier gas outlet velocity	(cm/sec)	28.93	43.85	43.85
Carrier gas average velocity	(cm/sec)	23.78	6.53	5.09
Carrier gas inlet pressure (abs.)	(atm)	1.41	9.95	12.82
Carrier gas outlet pressure (abs.)	(atm)	1.00	1.00	1.00
Pressure drop along the column	(atm)	0.41	8.95	11.82
Compressibility correction factor		0.822	0.149	0.116
Specific permeability	(10^{-7} cm^2)	586	1.96	1.96
Retention time of cyclohexane	(min)	18.14	40.47	86.54
Retention time of peak No. 11	(min)	28.7	64.5	137.9
Effective plate number/retention time	(min^{-1})	1,630	730	340

EXAMPLES FOR APPLICATIONS

We would like to show four chromatograms on the practical application of support-coated open tubular columns to the analysis of complex samples.

Fig. 7 demonstrates the analysis of a gasoline fraction. A 50 ft. long support-coated column with DC-550 phenylsilicone oil liquid phase was used for this analysis and the carrier gas flow rate was as high as 20 ml/min. The volume of the sample entering the column was 0.2 μl.

Fig. 8 illustrates the analysis of a peppermint oil sample on a 50 ft. long support-coated column prepared with Carbowax 1540 poly(ethylene glycol) liquid phase. The individual peaks in this chromatogram were identified in an integrated GC-MS system[10].

Finally, Figs. 9 and 10 show the separation of C_6–C_9 saturated isomeric hydrocarbons on a 150 ft. long support-coated open tubular column with squalane liquid phase. Two samples were analyzed, the first containing mainly the C_7 while the second the C_8 isomers; the column temperatures were 90 and 110° respectively. These higher temperatures were again made possible by the low β-value; on classical wall-coated open tubular columns prepared with squalane liquid phase, similar samples have to be analyzed at 20–50°, resulting in a much longer analysis time.

We calculated the retention index value[11,12] of each peak and compared these values with the data published recently by the Data Subcommittee of the English Gas Chromatography Discussion Group[13]. Based on this comparison, most peaks

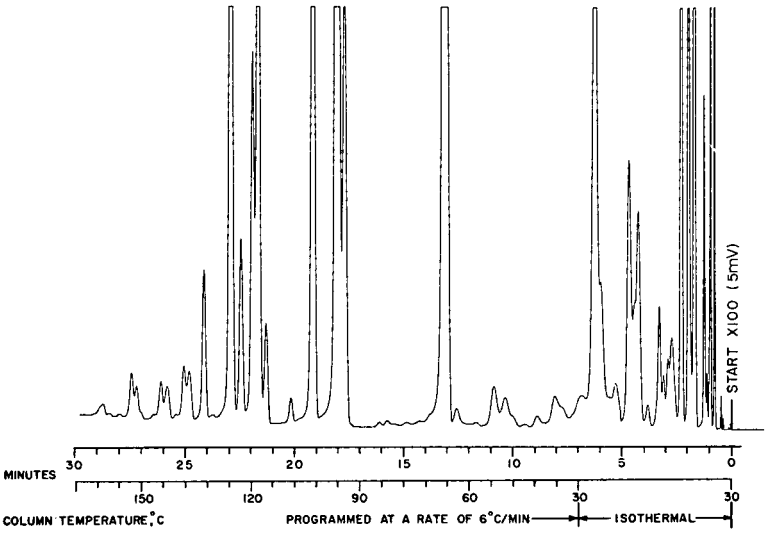

Fig. 7. Analysis of a gasoline fraction. Column: 50 ft. × 0.020 in. I.D. support-coated open tubular. Liquid phase: DC-550 phenylsilicone oil. Carrier gas/flow (He) rate at column outlet: 20 ml/min. Sample volume: 0.2 μl; 5 mV recorder.

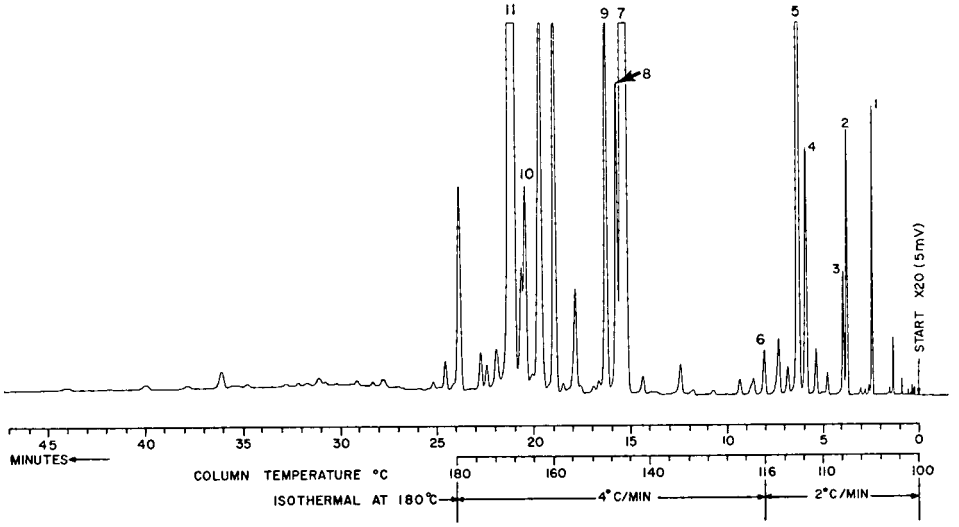

Fig. 8. Analysis of a peppermint oil sample. Column: 50 ft. × 0.020 in. I.D. support-coated open tubular. Liquid phase: Carbowax 1540 poly(ethylene glycol). Carrier gas (He) flow rate at column outlet: 3 ml/min. Sample volume: 0.02 μl. 5 mV recorder. Peaks: 1 = α-pinene; 2 = β-pinene; 3 = myrcene (?); 4 = limonene; 5 = eucalyptol; 6 = *p*-cymene; 7 = menthone; 8 = menthofurane; 9 = isomenthone; 10 = caryophyllene; 11 = menthol.

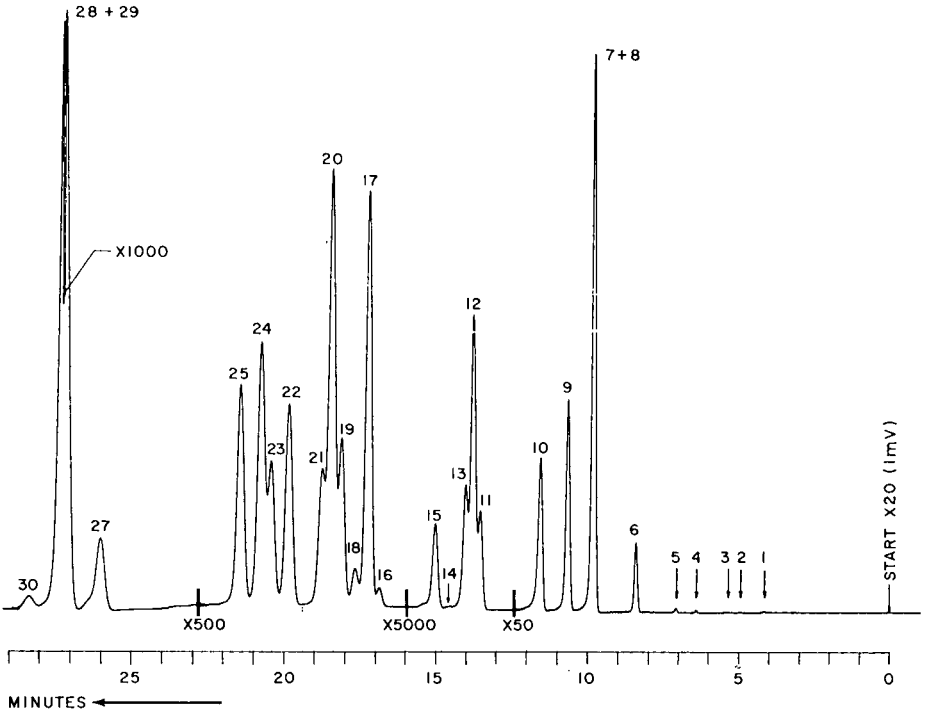

Fig. 9. Analysis of saturated isomeric hydrocarbons, I. Column: 150 ft. × 0.020 in. I.D. support-coated open tubular. Liquid phase: squalane. Column temperature: 90°. Carrier gas (He) flow rate at column outlet: 2.64 ml/min. Sample volume: 0.1 μl. 1 mV recorder. For peak identification, see Table III.

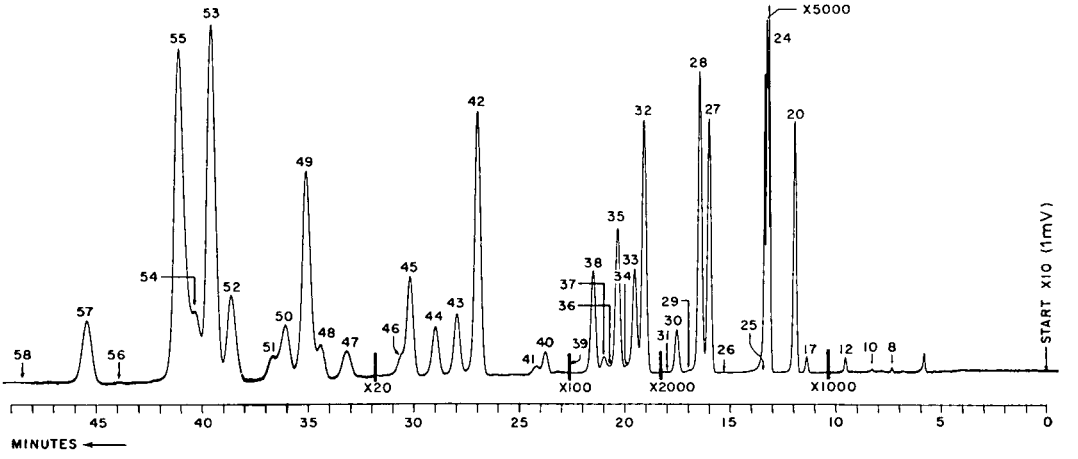

Fig. 10. Analysis of saturated isomeric hydrocarbons, II. Column as in Fig. 9. Column temperature: 110°. Carrier gas (He) flow rate at column outlet: 2.7 ml/min. Sample volume: 0.05 μl. 1 mV recorder. For peak identification, see Table III.

could be identified tentatively. Table III lists the retention index values of the individual peaks and the corresponding substances, based on this tentative identification.

TABLE III

RETENTION INDEX VALUES OF THE PEAKS IN FIGS. 9 AND 10 AND THEIR TENTATIVE IDENTIFICATION

Peak No.	*Substance*	*Retention index at*	
		90°C	*110°C*
5	*n*-Pentane	500	500
6	2,2-Dimethylbutane	536	—
7	2-Methylpentane + 2,3-Dimethylbutane	569	572
8	Cyclopentane		—
9	3-Methylpentane	585	—
10	*n*-Hexane	600	600
11	2,2-Dimethylpentane	628	—
12	2,4-Dimethylpentane	631	633
13	Methylcyclopentane	633	—
14	?	640	—
15	2,2,3-Trimethylbutane	645	—
16	3,3-Dimethylpentane	664	—
17	2-Methylhexane	667	669
18	Cyclohexane	671	—
19	2,3-Dimethylpentane	675	—
20	3-Methylhexane	678	678
21	Dimethylcyclopentane isomer (?)	680	—
22	3-Ethylpentane	688	—
23	Dimethylcyclopentane isomer (?)	693	—
24	2,2,4-Trimethylpentane	695	696
25	*n*-Heptane	700	700
26	2,2-Dimethylhexane	—	723
27	2,5-Dimethylhexane	728	731
28	2,4-Dimethylhexane		736
29	Methylcyclohexane + 2,2,3,3-Tetramethylbutane	735	741
30	2,2,3-Trimethylpentane	741	767
31	3,3-Dimethylhexane	—	751
32	2,3,4-Trimethylpentane	—	761
33	2,3-Dimethylhexane	—	765
34	2-Methylheptane	—	
35	2-Methyl-3-ethylpentane + 4-Methylheptane	—	772
36	2,3,3-Trimethylpentane	—	
37	3-Methylheptane + 3,4-Dimethylhexane + 3-Ethylhexane	—	777
38	2,2,5-Trimethylhexane	—	781
39	3-Methyl-3-ethylpentane	—	789
40	2,2,4-Trimethylhexane (?)	—	797
41	*n*-Octane	800	800
42	(?)	—	817
43	(?)	—	823
44	(?)	—	828
45	(?)	—	835
46	(?)	837	837
47	(?)	—	849

(*continued on p. 347*)

TABLE III (*continued*)

Peak No.	*Substance*	*Retention index at*	
		90°C	*110°C*
48	(?)	—	855
49	(?)	—	858
50	(?)	—	862
51	(?)	—	864
52	(?)	—	872
53	(?)	—	876
54	(?)	—	879
55	(?)	—	881
56	(?)	—	891
57	(?)	—	896
58	*n*-Nonane	900	900

SUMMARY

It was demonstrated that with support-coated open tubular columns, critical separations (base line resolution) can be achieved even with sample volumes as high as 0.15 μl of a single component. The minimum detectable limit with such a column was as low as 0.4 p.p.m., about a factor of 50 lower than with a standard wall-coated capillary column. Comparison with long packed columns of comparable sample capacity showed that their utilization makes high inlet pressures necessary while the time of analysis is increased many times. The relative performance of support-coated open tubular columns—expressed as effective plates per time—is much higher than even the best packed column.

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EVALUATION OF AN ASSEMBLY FOR AUTOMATED COLUMN CHROMATOGRAPHY*

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With the introduction of substituted celluloses, cross-linked dextran gels, and a variety of adsorbents for the exchange or exclusion chromatography of macromolecules, techniques for the separation of proteins, nucleic acids, polysaccharides and peptides have been phenomenally advanced. Concomitant with this progress have been technological advances in the formulation and design of equipment capable of detecting the material as it is released from the column as well as separating the eluate into discrete fractions. When ion-exchange chromatography is used for the partition of complex mixtures, selective separation is achieved by appropriate choice of buffers, added either singly in a discontinuous fashion, or, *via* programmed gradients as obtained by a device similar to the "varigrad" of PETERSON AND SOBER[1]. In either case, the pH and conductivity of each fraction should be recorded so that appropriate changes in gradient on rechromatography may be designed to afford the best resolution of the desired fractions. In general, proteins are thermolabile and provision should be made to refrigerate both columns and column eluates.

Specific instrumentation which can perform the aforementioned processes has been available for some time in the form of modular components which are modified by the individual laboratories to suit their specific needs. Recently, the Spinco Division of the Beckman Instrument Company made available a unitized apparatus (Spectrochrom, Model 130) capable of performing all the above-cited functions in a completely automated and integrated fashion. As is often the case in newly-developed apparatus, promise and fruition may be at variance. The purpose of this report is to evaluate the Spectrochrom as a useful and reliable device for the separation of blood and urinary proteins using a variety of chromatographic adsorbents.

METHODS

In general, the chromatographic procedures cited fall into three categories: (a) gradient-programmed separations on DEAE-cellulose**, (b) gel filtration with several types of Sephadex***, and (c) ion-retardation studies with AG-11A8§. This latter material is composed of spherical polymer beads containing paired anionic and

* The statements contained herein are the opinions of the authors and do not reflect any endorsement or recommendation by the Office of the Surgeon General, Department of the Army.

** Obtained from Sigma Chemical Company, St. Louis, Mo.

*** Obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

§ Obtained from Bio-Rad Laboratories, Richmond, Calif.

TABLE I

ADSORBENTS, COLUMN DIMENSIONS, AND BUFFERS USED FOR ELUTION

Column packing	*Number of separations* with the column dimensions (cm):*			*Eluting fluids*
	1.5 × 15	*1.5 × 50*	*1.5 × 150*	
DEAE-cellulose	68	41		TRIS–PO_4 buffers
AG-11A8	2	5		Distilled water
Sephadex G-25			107	PO_4–NaCl buffers, water
Sephadex G-100		22		PO_4–NaCl buffers
Sephadex G-200		49		PO_4–NaCl buffers

* Total number of separations: 294.

cationic exchange sites. The mixtures separated on various columns include whole human serum and fractions thereof, urinary protein extracts, and "protein-free" filtrates of human plasma. Table I indicates the packing material, the average size of the columns employed, the solvents used for elution as well as the number of separations. In all cases, temperature of the column jackets was maintained at 4° and the fraction collector compartment was held at 6–8°. Fractions were collected by drop-count where conducting buffers were used or by timed collection where distilled water was the eluting medium. The absorption of the eluates was monitored at wave lengths varying from 220 through 405 mμ.

Overall views of the apparatus as well as location of some specialized components are shown in Figs. 1 and 2. A schematic representation of the Spectrochrom is shown in Fig. 3. Buffers are pumped from the reservoir bottles, singly or admixed, through the left arm of the dual light path cuvette (2.5 mm and 10 mm) contained

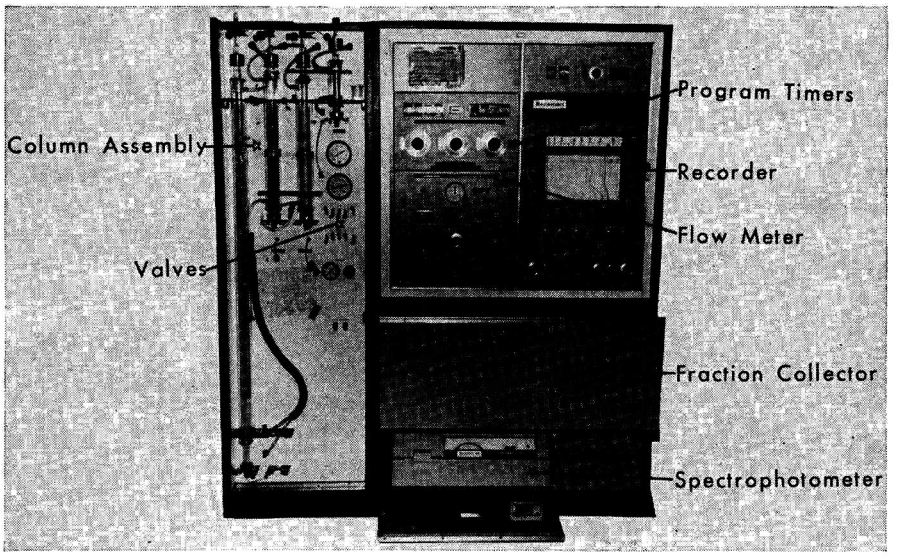


Fig. 1. Spectrochrom, Model 130, front view. Chromatography columns are shown to the left, recording and collecting systems are contained within the right side of the apparatus.

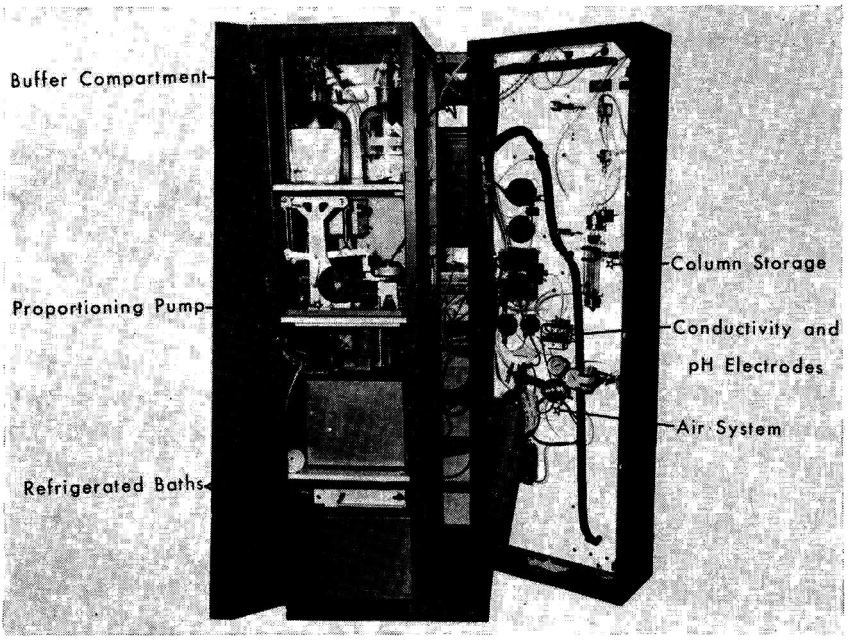
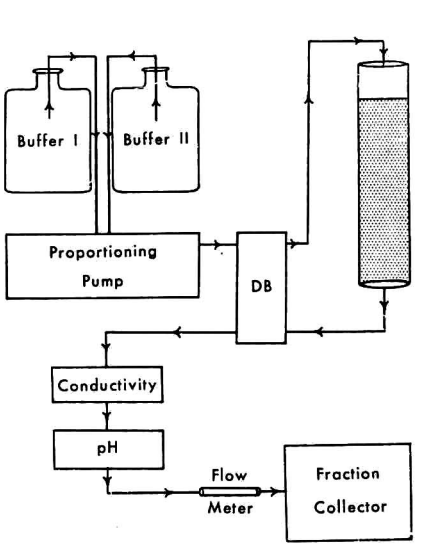


Fig. 2. Spectrochrom, Model 130, left side exposed.



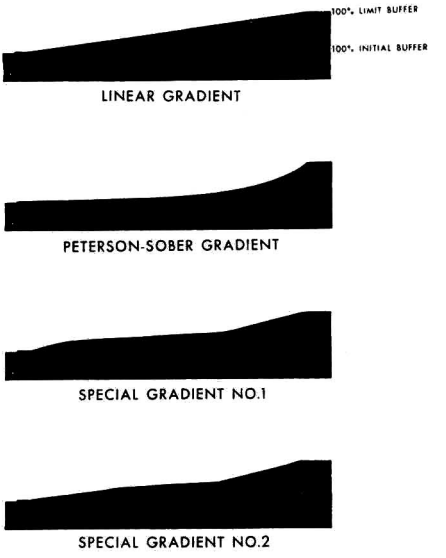


Fig. 3. Schematic view of the flow path within the Spectrochrom.

Fig. 4. Program cams for the proportioning pump.

within the DB Spectrophotometer, on to the top of the chromatographic column. The column outflow is returned to the right side of the cuvette, through the conductivity, pH, and flow meters, and then finally to the fraction collector. Three- and four-way valves permit switching any of the systems in or out of the flow circuit. The monitored variables (pH, conductivity, multiple absorbance) appear as discreet colored dots on a Bristol Recorder. The absorbance is indicated on the Bristol chart paper in a logarithmic rather than a linear fashion. Changes in fraction are indicated by slash marks in the lower portion of the chart. Programming of the buffer flow and shutdown of the apparatus can be accomplished by time-set mechanisms. If desired, programmed changes in the jacket temperature of the columns may be instituted.

When subtle continuous changes of buffer are required, program cams cut to the requisite changes are inserted in the proportioning pump. Control in the amount of each buffer added is by means of a rocker arm whose excursions are limited by the shape of the cam. The linear program cam, another that mimics the PETERSON–SOBER gradient system, and two of our own design are shown in Fig. 4.

RESULTS AND DISCUSSION

Representative chromatographic separations (taken from our routine fractionation programs) have been examined in some detail with view toward evaluation of component function. Included are unsatisfactory records representative of specific malfunctions.

Sephadex G-200 (Fig. 5a)

In this separation, approximately 180 mg of a crude kaolin extract of proteins from post-menopausal urine were dissolved in 10 ml of phosphate–saline, pH 7.4, and applied to a 1.5 × 50 cm column; a flow rate of 18 ml/h was established and 5 ml aliquots were collected[2]. Chart speed was set at 1 in./h and the absorbance at 260, 280 and 405 mμ was recorded at two light paths, along with pH and conductivity of the eluate. The initial buffer was used throughout the elution. This pattern and all subsequent figures were directly photographed from the charts; the blue grid background was faded out to increase the resolution of the spectral absorbance lines. The absorption is indicated on the abscissa, the fractions on the ordinate. As the pattern indicates, the separation and its record are satisfactory.

Sephadex G-100 (Fig. 5b)

One ml of human serum was applied to a 1.5 × 50 cm column with phosphate -saline, pH 7.4, used as the eluting fluid. The absorbance was monitored at 220, 260 and 280 mμ at a flow rate of 27 ml/h. In this study, the fraction collector was bypassed and no aliquots were collected. The column and monitoring apparatus worked quite well as evidenced by the detailed resolution of the components.

Sephadex G-25 (Fig. 5c)

The protein-free filtrate from 5 ml of plasma (prepared by addition of perchloric acid, then neutralization with potassium hydroxide) was applied to a 1.5 × 150 cm column. The absorbance of the distilled water eluate (38 ml/h, 5 ml aliquots) was followed at 225, 260 and 280 mμ. The use of multiple absorbance settings permits the

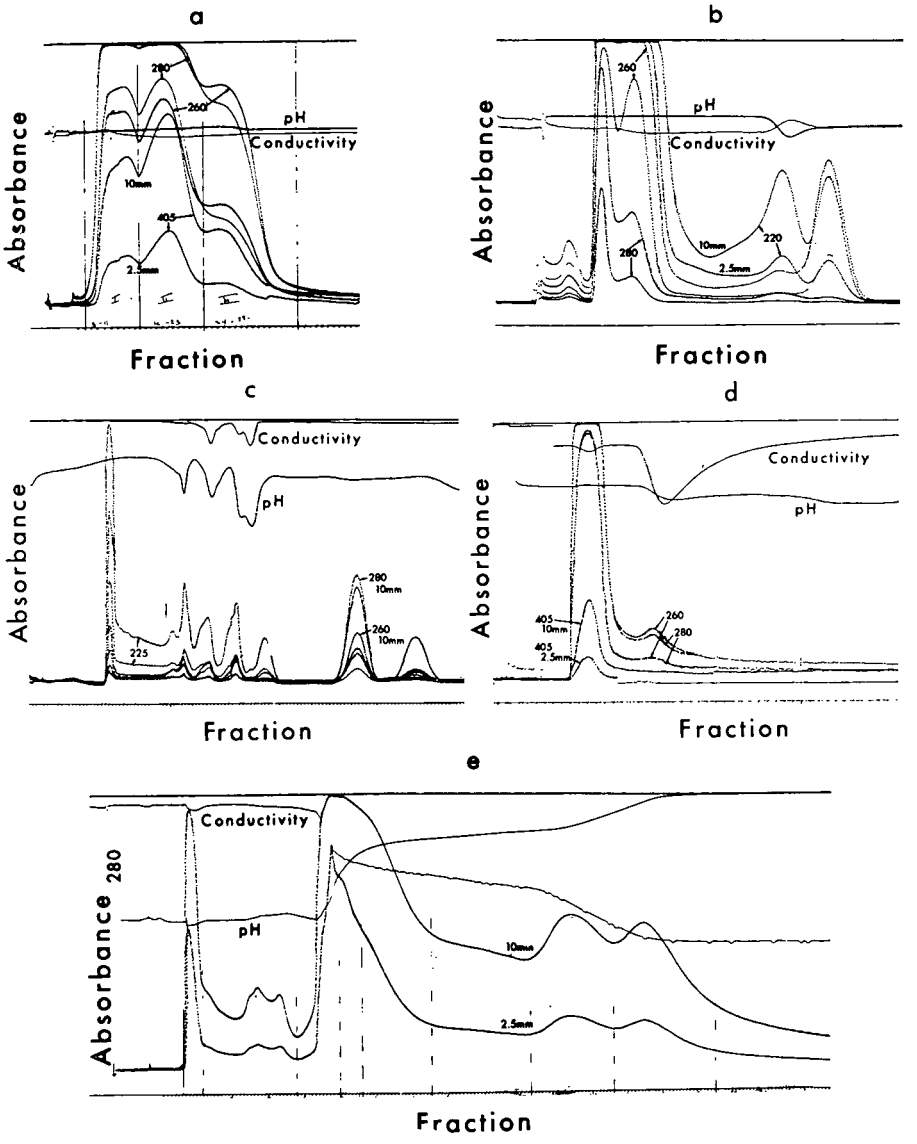


Fig. 5. Representative separations on varying chromatographic adsorbents: (a) Sephadex G-200; (b) Sephadex G-100; (c) Sephadex G-25; (d) AG-11A8; and (e) DEAE-cellulose.

detection of small amounts of material and additionally aids in the identification of general classes of materials separated by virtue of their relative absorption at selected wave lengths. As the salts originally contained in the sample are released from the column, associated changes in the pH and conductivity of the eluate are shown on the chart.

AG-11A8 (Fig. 5d)

The material is a polymer with weak exchange properties and is commonly used for desalting protein solutions (ion retardation). In this separation, 2 ml of human plasma, diluted to 10 ml with saline, were applied to a 1.5 × 50 cm column and elution performed with distilled water (flow rate, 32 ml/h, 10 ml aliquots collected). The column eluate was monitored at 260, 280 and 405 mμ. As noted from the pattern, an increase in conductivity appears after the major protein peak has passed through the column and would correspond to the release of retarded salts from the column.

DEAE-cellulose (Fig. 5e)

All the separations previously discussed represented single buffer elution and did not fully utilize the proportioning pump. In this study, 480 mg of urinary protein (identical to that applied to the G-200 column of Fig. 5a) were dissolved in 30 ml of a 0.04 *M* TRIS–0.005 *M* PO_4, pH 8.6 buffer, and then applied to a 1.5 × 50 cm DEAE-cellulose column[3]. The initial buffer was maintained until fraction 21 (210 ml), then the program device was set in operation and a limit buffer consisting of 0.5 *M* TRIS–PO_4, pH 4.0, was added in a gradual and predetermined fashion. The gradient (Special No. 1) used is shown in Fig. 4. To reinforce the absorbance recording all wave length calibrations were set at 280 mμ. Since cellulose columns permit high flow rates, elution was done at 120 ml/h. The change in pH as well as variation in conductivity are clearly shown on the chart. Although the pH changes, as shown, are accurate and in accord with the programmed gradient, the conductivity determinations are at variance from true values. This departure will be discussed in a later section.

Figs. 6a and 6b are illustrative of non-satisfactory records which result from some malfunction in the system.

Chromatographic "failures"

Fig. 6. illustrates faulty records obtained with protein mixtures separated on columns of Sephadex G-200 and DEAE-cellulose, respectively. The upper recording is a separation identical to that shown in Fig. 5a. In this case, the completely erratic pattern resulted from air bubbles trapped within the flow-through cuvette. This has occurred more often with Sephadex columns and may, in part, be related to slow flow rates and non-refrigeration of the lines leading from the chilled column to the fraction collector. Bubbles formed in the eluate upon rewarming can exceed the bubble trap capacity, lodge in the cuvette, and produce an illegible record. Fig. 6b shows a urinary protein mixture separated on DEAE-cellulose in a manner identical to that previously described (Fig. 5e). In this case, the change in absorbance was not due to eluted protein, but faulty operation of the recording spectrophotometer occasioned by misalignment of the optical system as well as changes in the automatic slit control. In many of our early separations, a number of poor records were obtained

due to faulty spectrophotometer operation. Change of the spectrophotometer with incorporation of suggested changes in the replacement considerably reduced the failure rate. Despite the detailed instructions given in the manual, optical alignment is best left to the professional.

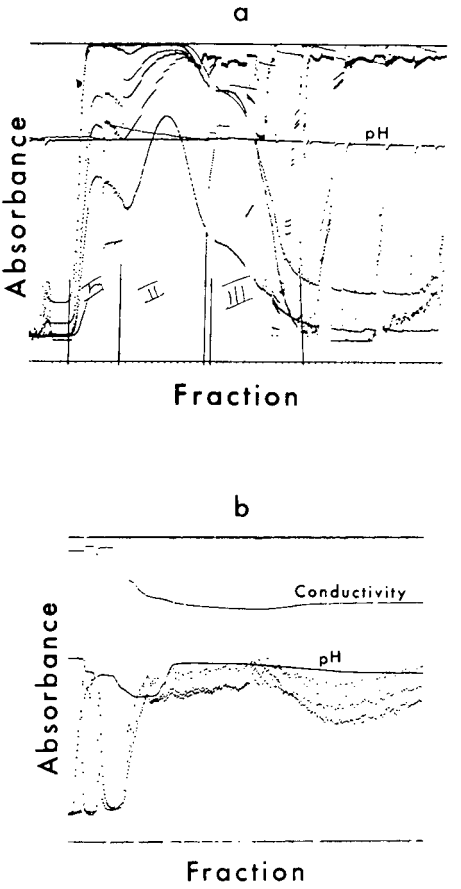


Fig. 6. Representative recordings indicating malfunction of the apparatus: (a) Sephadex G-200 and (b) DEAE-cellulose.

Summary of the major chromatographic separations obtained to date

The figures shown in Table I represent the number of separations performed under the conditions stated. Apart from the 294 listed, this laboratory has done an additional fifty runs of varying type using other adsorbents and column sizes. Initially (the first fifty separations) the failure rate was in excess of 25%, but added familiarity with the apparatus as well as revisions in various components have reduced this rate to less than 10%. In the larger number of these "failures", malfunction was evidenced in the recording, but the separations were obtained as programmed and the fractions could be read manually. The series reviewed were done over a period of 20 months.

Evaluation of the components

Some of the strengths and weaknesses of this automated system are apparent from the preceding discussion. In some cases, entirely new components were specifically designed for this apparatus; in other cases, they are standard commercial items minimally altered to suit the new equipment. The chromatography columns are well made, easy to pack, clean, and use. However, the metal screw at the top of the column can become frozen when salt-encrusted and accordingly difficult to remove. This problem might be avoided if holes were drilled to accommodate a spanner-type wrench or if nylon or teflon were substituted for the metal parts. Vacuum jackets are a necessity since the initial jackets supplied with the unit developed excessive condensation. Column cooling, as well as refrigeration of the fraction collector, worked well, although connection of the cold-water circulating lines to the column jackets is such that change of columns cannot be easily accomplished without fluid loss. In addition, provision should be made for refrigerating the buffer compartment as well as insulating the lines leading throughout the system.

For single buffer operation, the proportioning pump has been evaluated at delivery volumes ranging from 12 to 120 ml/h and found to be extremely reproducible. When gradients elutions were employed, the pump functioned well, and apparently accomplished the work of the "Varigrad" in a far simpler fashion. Care should be taken to bleed the delivery pistons free of any air to prevent admixture of buffer.

Although the response of the electrode to change in pH is somewhat slow, the values obtained on the chart were comparable to the measurements obtained with a laboratory meter. The manipulations required to change the electrodes are somewhat difficult, but changes are not often necessary. The conductivity meter, purported to record in a linear fashion, does not perform as described. Although changes in conductivity are clearly indicated, they do not represent actual values. However, the curve shape is useful in following gradient changes. To our knowledge, this component is undergoing complete re-design.

As indicated earlier, the largest number of our initial problems were encountered with the spectrophotometer. A complete change of unit in addition to the substitution and relocation of a heat-resistant capacitor did away with much of the early instability. The dual-path flow-through cell is quite ingenious and immeasurably adds to versatility of the apparatus. If both visible and U.V. ranges are scanned in a single run, adjustment of condensing mirror and alignment of the optical system are critical. Moreover, these adjustments should be periodically checked.

The fraction collector is a standard unit which has been directly incorporated into the present apparatus. Although some initial difficulty was encountered as the result of faulty soldering, the collector has worked well on both drop and timed collections. The volumetric collecting head has been used with another fraction collector, and performed satisfactorily. Since drop and volume systems depend on electrically conducting fluids, it is necessary to use timed collections for all distilled water operations.

Systems which permit automatic, unattended operation are those which lend greatest appeal to laboratories doing a large number of chromatographic separations. These have functioned reliably and well, permitting buffer changes, specified shutdowns, etc.

The Spectrochrom uses a multi-point Bristol recorder, which is quite easy to

ink, possesses good stability and marks in a clear manner. Due to the placement of the door, gear changing for paper speed control is very awkward. It is our understanding that a recorder attachment is available from the Bristol Company which reduces chart-speed changes to control of a single knob. We would recommend that it be incorporated into the present apparatus.

The air supply system is extremely useful for rapid application of samples on to cellulose columns as well as providing pressure for the regeneration buffers. This permits preparation of unused columns during standard operations. The distilled water flush system is also air-controlled. The bubble flowmeter functions well and adds to the general utility of the apparatus.

On the basis of what has been described, we feel that a completely automated device such as the Spectrochrom is an asset to laboratories doing a large number of chromatographic separations. The limiting features of this particular apparatus should be amenable to repair or re-design. At the present time, the machine is kept operative for 60–70 hours a week. Some of the initial difficulty encountered would have been obviated had the apparatus been factory tested for a longer period prior to shipment. Additionally, the instrument should be placed under regular service so that appropriate preventive maintenance can be instituted. The cleaning of pump pistons, adjustments of the spectrophotometer and general overhaul are better done by service engineers. However, the techniques associated with actual use of the apparatus are not difficult and training of personnel is easily accomplished.

ACKNOWLEDGEMENT

The authors would like to acknowledge the help of an unsung hero, the Beckman Field Service Engineer, Mr. EDWIN OGIBA, who, for the sake of the Spectrochrom, has oft long overstayed his appointed rounds.

SUMMARY

An automated apparatus for column chromatography (Spectrochrom, Model 130) has been evaluated for performance and reliability. Although certain deficiencies exist, these would appear to be capable of correction. This apparatus has permitted us to greatly increase our scope in preparative and analytical column chromatography, and the feasibility of completely automating the techniques would appear to be justified.

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THIN-LAYER CHROMATOGRAPHY OF HOMOLOGUES AND VINYLOGUES OF HIGHER FATTY ALDEHYDES, DIMETHYL ACETALS, AND 2,4-DINITROPHENYLHYDRAZONES

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INTRODUCTION

Plasmalogens are present in a variety of animal tissues. The aldehydes bound in them have been analyzed by gas–liquid chromatography (GLC) as their dimethyl acetals by a number of investigators[1–5]. Methanolysis of mixtures of acyl esters and plasmalogens with methanolic HCl or BF_3–methanol yields a mixture of methyl esters of fatty acids and aldehyde dimethyl acetals (DMAs). Some investigators have found it necessary to effect a separation of the DMAs from the methyl esters before analyzing them by GLC. FARQUHAR[3] made this separation by chemical means, and ENG *et al.*[4] by thin-layer chromatography.

Caution should be exercised in the interpretation of results obtained by GLC of DMAs from methanolyzed lipids. The presence of free fatty aldehydes existing in such mixtures either due to incomplete conversion of fatty aldehydes to the DMAs[2] or by reversion of DMAs to the aldehydes[5] should be verified by independent methods. Further, the behavior of DMAs on stationary liquid phases used in GLC needs further investigation in the light of observations by several investigators[2,5,6], who have reported that DMAs are not found or partially decomposed in columns commonly used in the GLC analysis of methyl esters of fatty acids. TLC should prove to be a valuable adjunct to GLC in these cases.

Separations by TLC of homologues and vinylogues of free aldehydes and DMAs have not been reported in the literature. Usually aldehyde separations have been made in the form of their 2,4-dinitrophenylhydrazones (DNPHs). Even with this approach, separations of DNPHs of longer chain saturated and unsaturated aldehydes have not been described. In 1952, ONOE[7] separated the DNPHs of *n*-aliphatic aldehydes up to C_{10} on silica gel plates, using benzene saturated with water as developing solvent. KAUFMANN AND KIRSCHNEK[8] reported the separation of DNPHs of saturated even numbered (C_8–C_{18}) and unsaturated C_{18} aldehydes (oleyl, linoleyl and linolenyl) by reversed-phase paper chromatography. Use was made of mercuric acetate adducts in separating unsaturated aldehydes and critical pairs. URBACH[9] separated the DNPHs of *n*-aliphatic aldehydes (C_1–C_{14}) by multiple development on Kieselguhr G plates impregnated with 2-phenoxyethanol. LIBBEY AND DAY[10] separated the DNPHs of the same aldehydes on Silica Gel G plates impregnated with mineral oil.

This papers describes a simple procedure for separation of mixtures of aldehydes, DMAs and methyl esters into classes by TLC. The separation of individual fatty alde-

hydes, their DMAs and DNPHs according to chain length and degree of unsaturation is described also.

EXPERIMENTAL

Materials

The various aldehydes, DMAs and DNPHs were prepared as described previously[11,12]. Solvents were reagent grade and redistilled.

Preparation of plates

Using a thin-layer applicator (Desaga, Heidelberg), glass plates (20 × 20 cm) were coated as usual with a well stirred suspension of silica Gel G or Kieselguhr G (E. Merck, Darmstadt; 30 g in 60 ml water) to give a layer approximately 250 μ in thickness. The plates were air-dried at room temperature for 15–30 min and activated at 110° for 1 h.

Siliconized plates for reversed-phase chromatography were made by dipping the activated Kieselguhr G plates in a 10 % (v/v) solution of silicone (Dow Corning 200 fluid) in petroleum ether (30–60°). The solvent was removed by exposing the plates to the atmosphere for about 5 min.

Silver nitrate impregnated plates were made by dipping activated Silica Gel G plates in a saturated solution of silver nitrate in 95 % aqueous methanol. After removal of the solvent by evaporation at room temperature, the plates were activated at 110° for 30 min and allowed to cool (10 min) before applying the solutions.

Development of chromatograms

The compounds were dissolved in petroleum ether, except the DNPHs, which were dissolved in ethyl ether. One μl of 0.1 % solution of each of the several compounds were spotted on the plates 2 cm from the base. Each spot, therefore, contained 1 μg of the individual compound. The spotted plates were dried in a stream of nitrogen and developed by the ascending technique in a tank lined with filter paper soaked with the solvent. Spots of aldehydes and their DMAs were made visible on siliconized plates by spraying the plates with a saturated solution of $K_2Cr_2O_7$ in 70 % (by volume) sulfuric acid[13] and charring at 160°. The spots on $AgNO_3$ impregnated plates were made visible by spraying with 50 % aqueous H_2SO_4 and charring. The DNPH spots were clearly visible as yellow spots against the white background.

RESULTS

Separation into classes

Fig. 1 shows a representative two-dimensional chromatogram demonstrating the separation of a mixture containing methyl esters of fatty acids, DMAs and aldehydes into classes using Silica Gel G plates. A solution of the mixture was spotted on the lower left hand corner of a plate 2 cm away from each of the nearest edges. The plate was first developed in toluene until the solvent was 12 cm above the spot (20 min). This resulted in the separation of the DMAs from the methyl esters and the aldehydes. In order to separate the methyl esters and the aldehydes, the plate was dried in a stream of nitrogen for 5 min and developed in the second dimension (90

degree rotation counterclockwise) with petroleum ether–ethyl ether–acetic acid, (90:10:1, v/v); the new solvent front was allowed to move for 18 cm (45 min). Mixtures containing 1 μg of each class could be detected.

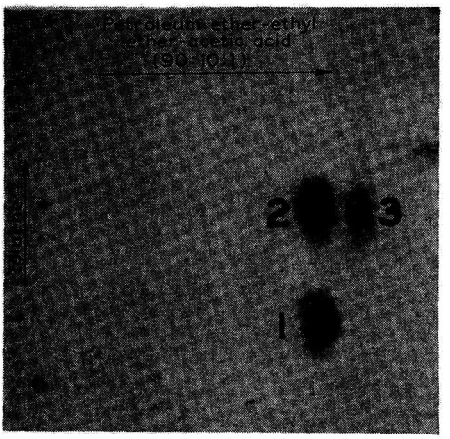

Fig. 1. Photograph of a two-dimensional adsorption thin-layer chromatogram of methyl esters, aldehydes and dimethyl acetals on Silica Gel G. The first dimension was developed for 20 min with toluene (12 cm) and the second dimension, with petroleum ether (b.p. 30–60°)–ethyl ether–acetic acid (90:10:1) for 45 min (18 cm). 1 = Dimethyl acetals; 2 = aldehydes; 3 = methyl esters.

Separation of aldehydes

Fig. 2A shows the separation of the saturated and unsaturated aldehydes achieved by reversed-phase partition chromatography. The plate was developed with 85 % aqueous acetone saturated to 90 % with silicone. It was then removed from the tank, dried in a stream of nitrogen for 5 min, and redeveloped once more. The saturated aldehydes were separated from one another, as were also the unsaturated aldehydes. The critical pairs, palmitaldehyde and oleylaldehyde, and myristaldehyde and linoleylaldehyde, had identical R_F values and could not be separated by this technique. However, they were separated by adsorption chromatography using $AgNO_3$–impregnated Silica Gel G plates.

Fig. 2B shows a separation of the aldehydes according to unsaturation achieved by $AgNO_3$–Silica Gel G plates. The plates were developed with petroleum ether–ethyl ether (76:24, v/v). The saturated aldehydes as a class moved to the solvent front. The R_F values of the unsaturated aldehydes varied with the degree of unsaturation; the lower the degree of unsaturation, the higher the R_F value. The saturated aldehydes were recovered from the plate by extracting the relevant portion with ether, and separated from one another by reversed-phase partition chromatography as shown in Fig. 2A. Thus, by a combination of $AgNO_3$-impregnated adsorption chromatography and reversed-phase partition chromatography, a complete separation of all the aldehydes was made.

Separation of dimethyl acetals

The separation of the DMAs of the saturated and unsaturated aldehydes made by reversed-phase partition chromatography is shown in Fig. 3A. The developing solvent was 85 % aqueous acetone saturated to 90 % with silicone. The plate was

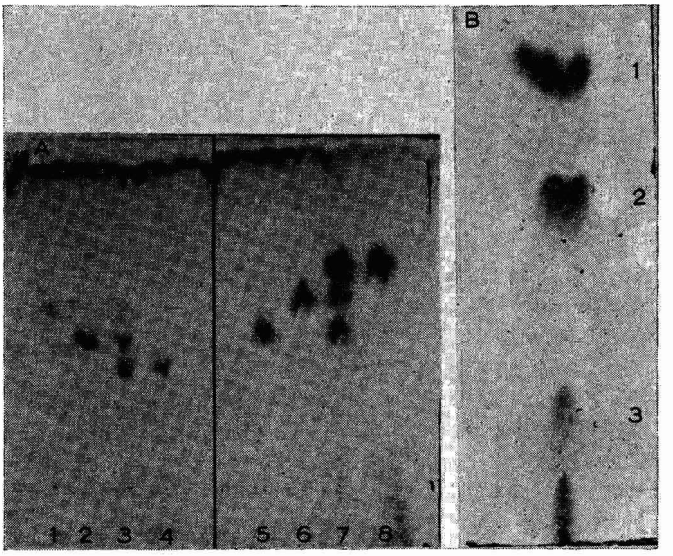

Fig. 2. (A) Photograph of a reversed-phase thin-layer chromatogram of fatty aldehydes. Support: Kieselguhr G. Impregnation: 10% silicone in petroleum ether. Solvent: 85% aqueous acetone saturated to 90% with silicone. Conditions: the mobile solvent was allowed to ascend 16 cm from the starting line two times (2 h). 1 = Myristaldehyde; 2 = palmitaldehyde; 3 = mixture of 1, 2, and 4; 4 = stearaldehyde; 5 = oleyl aldehyde; 6 = linoleyl aldehyde; 7 = mixture of 5, 6 and 8; 8 = linolenyl aldehyde. (B) Separation of fatty aldehydes according to degree of unsaturation by $AgNO_3$–Silica Gel G TLC. Solvent: petroleum ether–ethyl ether (76:24, v/v). Conditions: The solvent was allowed to ascend once for 16 cm from the starting line (30 min). 1 = Mixture of saturated aldehydes; 2 = oleyl aldehyde; 3 = linoleyl aldehyde; 4 = linolenyl aldehyde.

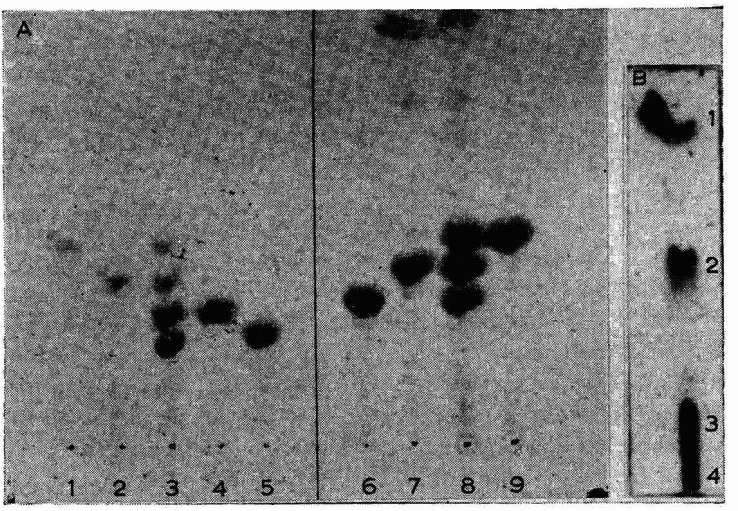

Fig. 3. (A) Photograph of a reversed-phase thin-layer chromatogram of dimethyl acetals. Support and solvent used were the same as in Fig. 2 A. Conditions: The solvent was allowed to ascend 16 cm from the starting line three times (3 h). 1 = Lauraldehyde dimethyl acetal; 2 = myristaldehyde dimethyl acetal; 3 = mixture of 1, 2, 4 and 5; 4 = palmitaldehyde dimethyl acetal; 5 = stearaldehyde dimethyl acetal; 6 = oleyl aldehyde dimethyl acetal; 7 = linoleyl aldehyde dimethyl acetal; 8 = mixture of 6, 7 and 9; 9 = linolenyl aldehyde dimethyl acetal. (B) Separation of the dimethyl acetals according to degree of unsaturation by $AgNO_3$–Silica Gel G TLC. Solvent: petroleum ether–ethyl ether (88:12, v/v). Conditions were the same as those in Fig. 2 B. 1 = Saturated fatty aldehyde dimethyl acetals; 2 = oleyl aldehyde dimethyl acetal; 3 = linoleyl aldehyde dimethyl acetal; 4 = linolenyl aldehyde dimethyl acetal.

developed thrice in the same direction with the same solvent, after drying each time for 5 min in a stream of nitrogen. As in the case of the free aldehydes, the separation of critical pairs of DMAs by reversed-phase alone is difficult.

Fig. 3B shows the separation of the DMAs on $AgNO_3$–Silica Gel G plates on the basis of unsaturation. Like the aldehydes, the saturated DMAs moved to the solvent front and the unsaturated DMAs separated in the same order as the corresponding aldehydes. The saturated DMAs were recovered from the plate and rechromatographed by the reversed-phase method to give separations as shown in Fig. 3A.

Separation of DNPHs

Fig. 4A shows the separation of the DNPHs of the saturated fatty aldehydes (C_{10}–C_{18}) and the unsaturated aldehydes, oleyl, elaidyl, linoleyl and linolenyl. Critical pairs also occur among such derivatives. In addition, only a slight difference in R_F values between the *cis* and *trans* pairs, oleyl and elaidyl-DNPHs, was observed in this system.

Fig. 4B shows the separation of the DNPHs on the basis of unsaturation on $AgNO_3$–Silica Gel G layers. The solvent was petroleum ether–ethyl ether (85:15, v/v). A well-defined separation of the geometrical isomers, oleyl and elaidyl-DNPHs, was obtained.

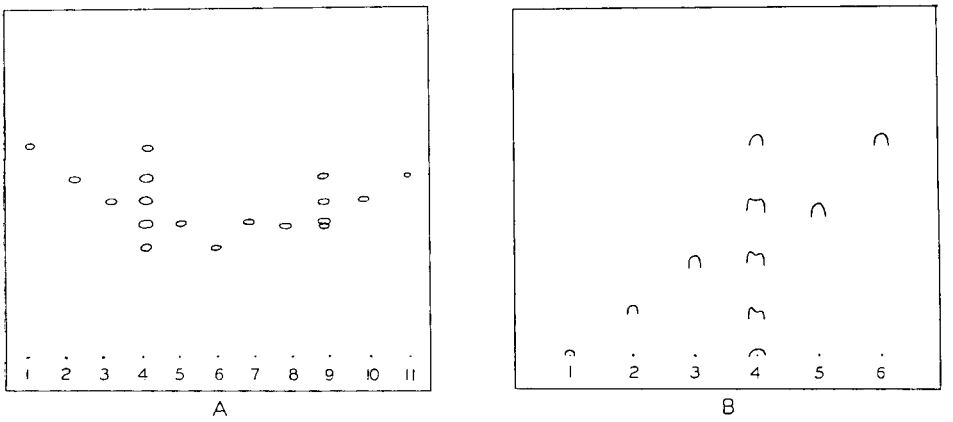

Fig. 4. (A) Tracing of a thin-layer chromatogram of 2,4-DNPHs of saturated and unsaturated fatty aldehydes. Support and solvent were the same as those in Fig. 2A. The solvent was allowed to ascend once 16 cm from the starting line (40 min). DNPHs of 1 = capryl aldehyde; 2 = lauryl aldehyde; 3 = myristyl aldehyde; 4 = mixture of 1, 2, 3, 5 and 6; 5 = palmityl aldehyde; 6 = stearyl aldehyde; 7 = oleyl aldehyde; 8 = elaidyl aldehyde; 9 = mixture of 7, 8, 10 and 11; 10 = linoleyl aldehyde; 11 = linolenyl aldehyde. (B) Tracing of a thin-layer chromatogram of 2,4-DNPHs of fatty aldehydes according to degree of unsaturation of $AgNO_3$–Silica Gel G plates. Solvent: petroleum ether–ethyl ether (85:15, v/v). Conditions: The solvent was allowed to ascend 16 cm (40 min). DNPHs of 1 = linolenyl aldehyde; 2 = linoleyl aldehyde; 3 = oleyl aldehyde; 4 = mixtures of 1, 2, 3, 5 and 6; 5 = elaidyl aldehyde; 6 = mixture of saturated aldehydes.

DISCUSSION

Separations of free fatty aldehydes and their dimethyl acetals by TLC have not been previously reported in the literature. By a combination of reversed-phase partition chromatography and $AgNO_3$-impregnated Silica Gel G adsorption chromato-

graphy, complete separations of the saturated and unsaturated aldehydes and of geometrical isomers of DNPHs of monounsaturated aldehydes were accomplished. The dimethyl acetals were also separated by a combination of these techniques.

The free aldehydes apparently were not oxidized to the corresponding carboxylic acids during chromatography, since spraying them with the 2,4-dinitrophenylhydrazone reagent[14] gave yellow spots characteristic of the aldehydes. Further, spraying with the sulfuric acid reagents and charring yielded only single spots. Spots corresponding to acids were not obtained. Aldehydes lower than C_{14} chain length could not be detected in less than 1 μg amounts, possibly due either to failure to char or to high volatility. DMAs could be detected down to C_{12} chain length in μg amounts. However, the DNPHs of even lower chain aldehydes could be detected, even in smaller amounts.

In contrast to the DNPH derivatives which were separated from one another by reversed-phase technique with only one development, the free aldehydes required two developments and the dimethyl acetals required three developments. The number of developments increased with the reduction in polarity of these classes.

In the reversed-phase system used in this study the higher the number of carbon atoms in the free aldehyde, the DMA and the DNPH, the slower was the migration. URBACH[9] separated the DNPHs of C_1–C_{14} *n*-alkanals on Kieselguhr G plates impregnated with 2-phenoxyethanol. The separation was achieved by developing the plates four times with light petroleum. In that system, the higher members of the series moved the fastest. LIBBEY and DAY[10] separated the DNPHs of the same aldehydes on Silica Gel G plates impregnated with mineral oil. The plates were given a single development with dioxane–water (65:35, v/v). Under their conditions, they observed that the migration rate varied inversely with the number of carbon atoms.

Finally, we have found TLC very useful for separation of methanolyzed tissue lipids into methyl esters of fatty acids and DMAs. The presence of free aldehydes in these lipid mixtures is also easily detected by this technique. Detection of aldehyde impurities in DMAs by GLC is not always feasible. We have found that in EGS columns, for example, both the aldehydes and the dimethyl acetals have the same retention times.

GLC alone may not resolve completely complex mixtures of dimethyl acetals. As in the case of methyl esters of fatty acids, dimethyl acetals of saturated normal and branched-chain aldehydes may overlap with those of unsaturated aldehydes of lower chain lengths. In this respect TLC is useful as an adjunct to GLC. Combination of TLC with GLC should lead to a complete resolution and identification of aldehydes present in naturally occurring plasmalogens.

ACKNOWLEDGEMENT

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SUMMARY

A method is described for the separation of fatty aldehydes, aldehyde dimethyl acetals and methyl esters of fatty acids into classes by two-dimensional thin-layer chromatography.

Separations of individual members of long-chain even-numbered saturated fatty aldehydes, their dimethyl acetals and 2,4-dinitrophenylhydrazones and the C_{18} unsaturated aldehydes (oleyl, linoleyl, and linolenyl) and their corresponding derivatives were achieved by reversed-phase partition thin-layer chromatography. The critical pairs having identical R_F values in the reversed-phase system were separated by adsorption chromatography using silver nitrate-impregnated Silica Gel G plates. With this technique the *cis–trans* isomers also could be separated as shown by 2,4-dinitrophenylhydrazine derivatives.

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PAPIER- UND DÜNNSCHICHTCHROMATOGRAPHISCHE CHARAKTERISIERUNG VON ALKALOIDEN DER GATTUNG PAPAVER

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EINLEITUNG

Während aus dem Schlafmohn, *Papaver somniferum* L., vor allem aus dem daraus gewonnenen Opium, bereits im 19. Jahrhundert mehr als 20 Alkaloide isoliert wurden, und dieses reiche Spektrum in letzter Zeit durch das Auffinden einer Anzahl weiterer, ausgesprochener Nebenalkaloide noch erheblich ergänzt werden konnte, war über die Alkaloide der übrigen weit über 100 Papaverarten und -unterarten mit Ausnahme einiger weit verbreiteter Vertreter bis vor einigen Jahren nur wenig bekannt. Durch intensive Bemühungen verschiedener Arbeitskreise sind jedoch in letzter Zeit zahlreiche Arten einer eingehenden Überprüfung unterzogen worden, wobei sich die Zahl der in der Gattung vorkommenden Basen mehr als verdoppelte. Neben der Isolierung mehrerer neuer Alkaloide konnte dabei einerseits gezeigt werden, dass weit mehr Alkaloide anderer Papaveraceengattungen als bisher angenommen auch in Papaver anwesend sind (z.B. Isocorydin, Sinactin, α-Allocryptopin, Sanguinarin, Coptisin) und sogar Basen ziemlich entfernt stehender Pflanzenfamilien angetroffen werden (z.B. Pronuciferin, Nuciferin). Andererseits scheint die Gattung Papaver aber auch über Stoffe zu verfügen, die in anderen Familien oder Gattungen der Papaveraceen offenbar nicht angetroffen werden (Papaverrubine, Rhoeadin und verwandte Basen). Die Bedeutung solcher Befunde für die botanische Systematik sind evident. Neben den mehr als 50 bisher aufgefundenen Basen mit bekannter Struktur sind zahlreiche weitere isoliert, die der Strukturaufklärung noch harren. Es besteht darüber hinaus kein Zweifel, dass sich die Zahl in der nächsten Zeit noch erheblich vergrössern wird, zumal viele Arten vorerst nur oberflächlich oder noch gar nicht überprüft worden sind.

Für die weiteren Arbeiten dürfte es daher nützlich sein, über das papier- und dünnschichtchromatographische Verhalten der bisher bekannten Basen unterrichtet zu sein. Da viele der angeführten Alkaloide auch in anderen Familien oder Papaveraceengattungen auftreten, können die Charakteristika ferner über das engere Gebiet der Papaverforschung hinaus ausgewertet werden.

In biosynthetischer Hinsicht dürften die bisher aufgefundenen Basen ausnahmslos der Tyrosinfamilie angehören. Sie können verschiedenen Strukturtypen zugeordnet werden, die einen Isochinolin- oder Tetrahydroisochinolinkern enthalten oder sich leicht von solchen Basen ableiten lassen (z.B. Narcein oder Alkaloide der Protopingruppe) bzw. in solche übergehen können (z.B. Proaporphine). Zwischen

den einzelnen Strukturen bestehen enge biogenetische Beziehungen; manche Alkaloide sind Vor- oder Zwischenstufen für andere[1]. Lediglich die in der Gattung weit verbreiteten, erst kürzlich isolierten Papaverrubine und das ebenfalls häufig anzutreffende Rhoeadin sowie einige verwandte Basen dieses Strukturtyps leiten sich nach neuesten Untersuchungen von einem zum 7-Ring ausgeweiteten Tetrahydroisochinolinskelett ab[2-8]. Sie stellen somit substituierte Benzoazacycloheptene dar. Es ist sehr wahrscheinlich, dass auch diese Basen in enger biogenetischer Relation zu anderen Strukturen der Gattung stehen.

METHODEN UND ERGEBNISSE

In die papier- und dünnschichtchromatographischen Überprüfungen wurden mit Ausnahme von Oripavin, 10-Hydroxykodein und Oxynarcotin, die uns nicht zur Verfügung standen, alle strukturell bekannten Basen einbezogen, die bisher in Papaverarten nachgewiesen wurden. Die beiden zuletzt genannten Stoffe dürften wie verschiedene andere Basen (z.B. Hydrocotarnin, Papaveraldin, Pseudomorphin) sowieso Artefakte der Drogenaufarbeitung sein. Die Formeln der Alkaloide können Handbüchern[9], Übersichten[1] oder Originalarbeiten[2,3,5-8] entnommen werden. Zur Charakterisierung der Stoffe dienten die in unserem Arbeitskreis in den letzten Jahren mit Erfolg eingesetzten Methoden. Für die Dünnschichtchromatographie wurden Kieselgel G Merck (Verfahren A) mit dem Fliessmittelsystem Benzol–Aceton–Methanol (7:2:1) und Aluminiumoxid G Merck (B) mit dem Fliessmittelsystem Heptan–Chloroform–Äther (4:5:1) verwendet, zur Papierchromatographie gepufferte Papiere (pH 5.5 und 6.5) und wassergesättigtes *n*-Butanol (C und D) sowie wassergesättigter Äther (E und F) als Laufmittel bzw. ungepufferte Papiere (G) mit dem System *n*-Butanol–Eisessig–Wasser (10:1:3).

Die R_F-Werte der Dünnschichtchromatographie (A und B) sind als R_X-Werte berechnet und auf Rhoeadin bezogen (Kieselgel G: R_F 0.57 $\pm$ 0.03; Aluminiumoxid: R_F 0.37 $\pm$ 0.03).

Die Auswertung der Tabelle I, die auch das Vorkommen der Alkaloide in den einzelnen Papaverarten berücksichtigt, lässt erkennen, dass fast alle Alkaloide ausreichend charakterisiert werden können, und dass sich nur wenige Basen in allen 7 Systemen gleichartig verhalten. Durch Anwendung der von uns schon früher beschriebenen Reagentien zum Nachweis der Opiumalkaloide[73] kann eine weitere Differenzierung getroffen werden. Während Kieselgel als Adsorbens allgemeiner anwendbar ist, werden zahlreiche Basen auf Aluminiumoxid nur wenig oder gar nicht transportiert. Zur Unterscheidung verschiedener Alkaloide (z.B. Aporphine und Basen der Rhoeadingruppe) erweist sich jedoch die Kombination beider Methoden als sehr geeignet.

Für die Auftrennung von Substanzen mit kleinen R_F-Werten auf Kieselgel-Platten seien ferner alkalische Fliessmittelsysteme empfohlen, wie Benzol–Aceton–Äther–10 proz. Ammoniaklösung (4:6:1:0.3) oder Benzol–Aceton–Äther–Isopropanol–3 proz. Ammoniaklösung (20:15:10:7.5:2.5), die zur teilweisen Desaktivierung des Kieselgels führen und somit einen besseren Transport der Basen gewährleisten. Es sei schliesslich noch erwähnt, dass sich zusätzliche Trennungen durch andere Pufferungen der Papiere (pH $<$ 5.5) insbesondere bei Verwendung von wassergesättigtem Äther erreichen lassen.

TABELLE I

Alkaloid	*Vorkommen in Papaverarten* und Literatur*	R_X-*Werte*		R_F-*Werte*				
		A	*B*	*C*	*D*	*E*	*F*	*G*
Tetrahydroisochinolin-Alkaloide								
Hydrocotarnin	S_2[10]	0.69	0.95	0.44	0.67	0.14	0.58	0.55
Benzylisochinolin- und Benzyltetrahydro-isochinolin-Alkaloide								
Papaverin	C_3[11], S_1[12], S_2[13]	1.08	0.93	0.89	0.92	0.81	0.88	0.69
Papaveraldin	S_2[14]	1.16	0.96	**	**	**	**	0.88
Laudanin/Laudanidin	S_1[15], S_2[16], S_2[17]	0.32	0.05	0.47	0.62	0	0.17	0.62
Laudanosin	S_1[15], S_2[10]	0.40	0.56	0.55	0.76	0.04	0.27	0.66
Reticulin	S_2[18]	0.35	0	0.44	0.50	0	0.06	0.60
Armepavin	A_9[19], C_2[20], F_2[21],F_3[22], P_2[23], P_4[23], T[23]	0.27	0	0.59	0.71	0.06	0.23	0.40
Codamin	S_2[16]	0.49	0.17	0.45	0.59	0.04	0.20	0.61
Phthalidisochinolin-Alkaloide und verwandte Basen								
Narcotin	S_1[12], S_2[24]	1.23	0.92	0.91	0.93	0.93	0.94	0.71
Narcotolin	S_1[15], S_2[25]	1.06	0	0.91	0.92	0.93	0.94	0.60
Gnoskopin	S_2[26]	1.21	0.70	**	**	0.93	0.94	0.76
Oxynarcotin	S_2[27]	—	—	—	—	—	—	—
Narcein	S_1[15], S_2[28]	0	0	0.57	0.62	0	0	0.05
Morphinan-Alkaloide								
Morphin	S_1[29], S_2[30]	0.10	0	0.14	0.22	0	0	0.38
Kodein	S_1[12], S_2[31]	0.19	0.15	0.21	0.38	0	0.04	0.47
Neopin	S_2[32]	0.10	0.15	0.16	0.26	0	0	0.39
10-Hydroxykodein	S_2[33]	—	—	—	—	—	—	—
Thebain	B[34], I[35], O_2[36], S_1[12], S_2[37], S_3[35]	0.45	0.52	0.53	0.70	0.03	0.29	0.68
Oripavin	B[38], O_2[19]	—	—	—	—	—	—	—
Amurin	F_1[5], N_2[39], N_3[40], N_4[40], N_6[41], R_1[42]	0.65	0.22	0.54	0.83	0.06	0.28	0.51
Nudaurin	N_3[40]	0.10	0	0.35	0.51	0	0.05	0.59
Salutaridin	B[44],S_2[43]	0.48	0.22	0.63	0.89	0.04	0.28	0.61
Pseudomorphin	S_2[37]	0	0	0	0	0	0	0.05
Alkaloide der Protopingruppe								
Protopin	A_5[41], A_8[35], A_{10}[35], C_1[35], C_2[20], D_1[45], F_3[23], L_1[42], L_2[46], M_1[47], M_2[47], N_4[40], N_5[41], N_6[41], O_1[48], O_2[49], P_1[50], P_3[47], R_1[42], R_3[51], S_1[15], S_2[52], T[23]	0.40	0.19	0.44	0.49	0.08	0.20	0.65
Cryptopin	A_5[41], S_1[15], S_2[53]	0.20	0.37	0.44	0.52	0	0.11	0.65
α-Allocryptopin	D_2[54], D_3[51], P_1[50]	0.20	0	0.48	0.53	0	0.06	0.72
Muramin	N_2[40], N_1[56], N_5[41], N_6[41], P_3[47]	0.10	0.14	0.53	0.57	0	0	0.66

Aporphin- und Proaporphin-Alkaloide								
Isothebain	B^{38}, O_2^{36}	0.80	0.71	0.49	0.75	0.10	0.43	0.53
Isocorydin	O_1^{48}	0.82	0.93	0.62	0.88	0.19	0.66	0.65
Nuciferin	C_2^{57}, F_3^{23}, P_2^{23}, P_4^{23}, T^{23}	0.85	1.32	0.87	0.94	0.75	0.95	0.54
Nuciferolin	C_2^{57}	0.69	0.20	0.53	0.79	0.15	0.56	0.56
Mecambrolin	C_2^{57}	0.76	0.25	0.25	0.54	0.11	0.41	0.45
Roemerin	C_2^{57}, D_1^{55}, F_1^{5}, F_3^{22}, P_2^{23}, P_4^{23}, T^{23}	0.96	1.28	0.64	0.93	0.56	0.88	0.64
Glaucin	F_1^{5}	0.75	1.00	0.62	0.89	0.15	0.67	0.61
Corytuberin	S_2^{58}	0	0	0.10	0.12	0	0	0.43
Magnoflorin	S_2^{59}	0	0	0.06	0.07	0	0	0.40
Mecambrin (= Fugapavin)	C_2^{20}, D_1^{55}, F_3^{22}, P_2^{23}, P_3^{23}, T^{23}	0.77	0.64	0.63	0.87	0.23	0.68	0.58
Pronuciferin	C_2^{57}, F_3^{23}, P_2^{23}, P_3^{23}, T^{23}	0.66	0.46	0.64	0.88	0.13	0.54	0.64
Orientalinon	B^{44}	0.33	0.13	0.33	0.51	0	0.07	0.51
Protoberberin- und Tetrahydroprotoberberin-Alkaloide								
Berberin	D_1^{60}, D_2^{54}, D_3^{54}, H_2^{60}, L_1^{42}, S_2^{61}	0	0	0.30	0.37	0	0	0.61
Coptisin	A_6^{55}, A_8^{55}, A_{10}^{60}, B^{60}, C_2^{20}, D_1^{60}, D_2^{54}, D_3^{54}, G_1^{60}, M_2^{60}, N_1^{60}, O_2^{60}, P_1^{60}, P_3^{60}, R_3^{51}, S_1^{60}, S_2^{60}, T^{30}	0	0	0.12	0.17	0	0	0.48
Palmatin	C_2^{20}, P_4^{23}, T^{23}	0	0	0.28	0.30	0	0	0.59
Sinactin	R_3^{62}	1.23	1.34	0.86	0.90	0.90	0.95	0.66
Benzophenanthridin-Alkaloide								
Sanguinarin	A_1^{60}, A_{10}^{60}, C_2^{20}, C_3^{60}, D_1^{60}, F_2^{23}, G_1^{60}, H_1^{60}, H_2^{60}, M_2^{60}, N_1^{60}, O_1^{48}, O_2^{60}, P_2^{23}, P_3^{60}, P_4^{23}, R_1^{42}, R_3^{60}, S_2^{60}, T^{60}	1.39	1.38	0.77	0.94	0.05	**	0.66
Chelerythrin	F_3^{23}, R_3^{60}	1.16	1.40	0.50	0.77	0.03	**	0.56
Alkaloide der Rhoeadingruppe								
Rhoeadin	A_5^{41}, A_7^{47}, A_8^{35}, A_{10}^{35}, C_1^{35}, D_1^{45}, D_3^{47}, G_1^{35}, G_2^{47}, I^{35}, L_2^{46}, M_1^{47}, M_2^{47}, N_5^{41}, N_6^{41}, O_1^{48}, P_1^{35}, P_3^{47}, R_3^{63}, S_3^{35}	1.00	1.00	0.72	0.75	0.51	0.66	0.73
Rhoeagenin	A_7^{47}, A_8^{47}, A_{10}^{47}, D_1^{40}, O_1^{48}, P_3^{47}, R_3^{64}	1.08	0.38	0.54	0.74	0.95	0.95	0.76
Isorhoeadin	O_1^{48}, R_3^{65}	1.52	1.40	0.94	0.95	0.95	0.95	0.61
Glaudin	G_1^{4}, R_3^{66}, S_2^{66}	1.27	1.25	0.88	0.93	0.95	0.96	0.59
Glaucamin	G_1^{67}	1.17	0.45	0.80	0.90	0.82	0.95	0.70
Oreodin	O_1^{48}	0.85	0.84	0.69	0.86	0.28	0.72	0.92
Oreogenin	O_1^{48}	1.00	0.44	0.44	0.71	0.93	0.95	0.60
Papaverrubin A	A_{10}^{68}, C_2^{68}, D_1^{68}, H_1^{68}, H_2^{68}, L_2^{68}, O_1^{68}, P_3^{68}, R_3^{68}, R_3^{69}, R_4^{68}, S_3^{68}	1.24	0.96	0.80	0.88	0.83	0.96	0.84
Papaverrubin B	A_2^{68}, A_3^{63}, A_4^{68}, A_{10}^{68}, B^{68}, C_2^{68}, F_1^{63}, G_1^{68}, G_1^{70}, H_1^{68}, L_2^{68}, N_1^{63}, O_1^{68}, O_2^{68}, P_3^{68}, P_5^{68}, R_1^{68}, R_2^{68}, R_3^{68}, R_4^{68}, S_1^{68}, S_2^{66}, S_3^{68}	1.13	0.85	0.66	0.84	0.45	0.81	0.80

(*Fortsetzung S. 368*)

TABELLE I (*Fortsetzung*)

Alkaloid	*Vorkommen in Papaverarten und Literatur*	R_X-*Werte*		R_F-*Werte*				
		A	*B*	*C*	*D*	*E*	*F*	*G*
Papaverrubin D	A_2^{68}, A_3^{68}, A_4^{68}, A_8^{68}, A_{10}^{68}, B^{68}, C_2^{68}, D_1^{68}, F_1^{68}, F_3^{68}, G_1^{68}, H_1^{68}, H_2^{68}, L_2^{68}, M_1^{68}, N_3^{68}, O_1^{68}, O_2^{68}, P_1^{68}*, P_2^{68}, P_3^{68}, P_5^{68}, R_1^{68}, R_2^{68}, R_3^{68}, R_4^{68}, S_1^{68}, S_2^{71}, S_3^{68}	0.98	0.30	0.51	0.76	0.24	0.67	0.64
Papaverrubin E	A_2^{68}, A_3^{68}, A_4^{68}, A_8^{68}, A_{10}^{68}, B^{68}, C_2^{68}, D_1^{68}, F_1^{68}, F_3^{68}, H_1^{68}, H_2^{68}, L_2^{68}, M_1^{68}, O_1^{68}, O_2^{68}, P_1^{68}, P_3^{68}, P_5^{68}, R_2^{68}, R_3^{68}, R_3^{69}, R_4^{68}, S_1^{68}, S_2^{72}, S_3^{68}.	0.80	0.40	0.71	0.80	0.19	0.64	0.82

* Abkürzungen der Arten:

A_1 = *P. aculeatum* Thunb.
A_2 = *P. alpinum* ssp. *kerneri* (Hay.) Fedde
A_3 = *P. alpinum* ssp. *rhaeticum* (Ler.) Mgf.
A_4 = *P. alpinum* ssp. *sendtneri* Schinz et Keller
A_5 = *P. anomalum* Fedde
A_6 = *P. apulum* Ten.
A_7 = *P. arenarium* Marsch.-Bieb.
A_8 = *P. argemone* L.
A_9 = *P. armeniacum* (L.) DC.
A_{10} = *P. atlanticum* Ball
B = *P. bracteatum* Lindl.
C_1 = *P. californicum* A. Gray
C_2 = *P. caucasicum* Marsch.-Bieb.
C_3 = *P. commutatum* Fisch. et Mey.
D_1 = *P. dubium* L.
D_2 = *P. dubium* ssp. *albiflorum* (Boiss.) Dost
D_3 = *P. dubium* ssp. *lecoquii* (Lamotte) Fedde
F_1 = *P. feddei* Schwz.
F_2 = *P. floribundum* Desf.
F_3 = *P. fugax* Poir.
G_1 = *P. glaucum* Boiss. et Hauskn.
G_2 = *P. gracile* Auch.
H_1 = *P. heldreichii* Boiss.
H_2 = *P. hybridum* L.
I = *P. intermedium* (Becker) O. Ktze.
L_1 = *P. lapponicum* Nordh.
L_2 = *P. lateritium* C. Koch
M_1 = *P. macrostomum* Boiss. et Huet.
M_2 = *P. monanthum* Trautv.
N_1 = *P. nudicaule* L.
N_2 = *P. nudicaule* var. *amurense* N. Busch
N_3 = *P. nudicaule* var. *aurantiacum* Loisel.
N_4 = *P. nudicaule* var. *croceum* Ledeb.
N_5 = *P. nudicaule* L. ssp. *rubroaurantiacum* Fedde
N_6 = *P. nudicaule* L. ssp. *xanthopetalum* (Trautv.) Fedde
O_1 = *P. oreophilum* Rupr.
O_2 = *P. orientale* L.
P_1 = *P. pavoninum* Fisch. et Mey.
P_2 = *P. persicum* Lindl.
P_3 = *P. pilosum* Sibth. et Smith
P_4 = *P. polychaetum* Schott et Kotschy
P_5 = *P. pseudocanescens* M. Pop.
R_1 = *P. radicatum* Rottb.
R_2 = *P. radicatum* ssp. *hyperboreum* Nordh.
R_3 = *P. rhoeas* L.
R_4 = *P. rupifragum* Boiss. et Reut.
S_1 = *P. setigerum* DC.
S_2 = *P. somniferum* L.
S_3 = *P. strigosum* Schur.
T = *P. triniaefolium* Boiss.

** Streifenbildung; nicht auswertbar.

EXPERIMENTELLES

Dünnschichtchromatographie

Sorbentien: Je 5 g Kieselgel G Merck (A) bzw. Aluminiumoxid G Merck (B) auf Platten 17,× 24 cm; Trocknung der beschichteten Platten 30 min bei 105°.

Aufgetragene Mengen: 10–20 μg Alkaloidbase.

Laufmittel: Benzol–Aceton–Methanol (7:2:1) für A und Heptan–Chloroform*–Äther (4:5:1) für B.

Laufstrecke: *ca.* 19 cm.

Laufzeiten: *ca.* 55 min (A); *ca.* 75 min (B).

Temperatur: 16–17°.

Detektion: Modifiziertes Dragendorff-Reagens[74].

Papierchromatographie

Papier: Schleicher & Schüll 2043 bm, 30 × 3 cm.

Puffer: Citronensäure–Phosphat-Puffer nach McIlvaine, pH 5.5 (C und E) und pH 6.5 (D und F).

Aufgetragene Mengen: *ca.* 50 μg Alkaloidbase.

Laufmittel: Wassergesättigtes *n*-Butanol (C und D) und wassergesättigter Äther (E und F) auf gepufferten Streifen; *n*-Butanol–Eisessig–Wasser (10:1:3) auf ungepufferten Streifen (G); alle aufsteigend.

Laufstrecke: *ca.* 22 cm.

Laufzeiten: *ca.* 8 h (C und D), 1 h (E und F), 7 h (G).

Temperatur: 16–17°.

Detektion: Modifiziertes Dragendorff-Reagens[75], daneben Eisen(III)-chlorid–Schwefelsäure[73] und Phenolreagentien[73].

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ZUSAMMENFASSUNG

Es wird das papier- und dünnschichtchromatographische Verhalten von mehr als 50 strukturell bekannten Alkaloiden der Gattung Papaver in 7 Systemen beschrieben. Gleichzeitig wird das Vorkommen dieser Basen in den einzelnen Arten der Gattung zusammengefasst.

SUMMARY

Data are presented for the paper and thin-layer chromatography of more than 50 alkaloids of the genus Papaver of which the structure is known. The presence of these bases in the species of the genus is summarized in tabular form.

* Es wurde Handelsware (DAB 7) mit einem Äthanolgehalt von etwa 1 % verwendet.

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DÜNNSCHICHTELEKTROPHORETISCHE TRENNUNG QUATERNÄRER AMMONIUMVERBINDUNGEN

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In letzter Zeit erschien eine Reihe von Arbeiten über die Dünnschichtchromatographie aliphatischer, quaternärer Ammoniumverbindungen[1-6]. Darin wurden als Vorteile dieser Methode gegenüber der Papierchromatographie übereinstimmend die grössere Nachweisempfindlichkeit und die schärfere Auftrennung der Substanzen hervorgehoben. Im Laufe unserer eigenen Untersuchungen über die Möglichkeiten zur Auftrennung und Identifizierung kleinster Mengen von physiologisch wichtigen quaternären Ammoniumverbindungen beschäftigten wir uns neben der Dünnschichtchromatographie auch mit der Dünnschichtelektrophorese dieser Verbindungen.

EXPERIMENTELLES

Für unsere Untersuchungen verwendeten wir eine Elektrophoreseapparatur nach WIELAND und PFLEIDERER[7], doch kommt dafür prinzipiell jedes Elektrophoresegerät in Frage, das eine kühlbare Auflagefläche für das Trägermaterial besitzt und das Anlegen von Spannungen bis etwa 1000 V gestattet. Die Dünnschichtplatten vom Format 20 × 20 cm oder 20 × 10 cm wurden mit einem Streichgerät auf die übliche Art und Weise beschichtet. Wir überprüften Kieselgel G, Kieselgur G, Aluminiumoxid G und Cellulose auf ihre Brauchbarkeit als Trägermaterial und versuchten die Trennung der quaternären Ammoniumverbindungen in einer Reihe von verschiedenen Puffersystemen.

Nach Besprühen der Dünnschichtplatte mit dem Puffer, in dem die Elektrophorese durchgeführt wird, trägt man, um die Trennstrecke voll ausnützen zu können, das Substanzgemisch etwa 3 cm vom Plattenrand entfernt auf. Die Platte wird dann auf die gekühlte Auflagefläche der Elektrophoreseapparatur gelegt, durch Filterpapier- oder Leinenstreifen, die an den Enden, die auf der Trägerschicht aufliegen, in Cellophanfolien eingeschlagen sind, mit den puffergefüllten Elektrodengefässen verbunden und mit einer Glasplatte gleicher Grösse überdeckt. Die quaternären Ammoniumverbindungen wandern im elektrischen Feld zur Kathode. Die Trenndauer ist von der Art der Trägerschicht, der Pufferlösung und der Höhe der angelegten Spannung abhängig.

Der Nachweis der quaternären Ammoniumverbindungen erfolgte auf Kieselgel-, Kieselgur- oder Aluminiumschichten mit einem modifizierten Dragendorff-Reagens, dem eine alkoholische Jodlösung zugesetzt wurde[4], auf den Celluloseschichten mit dem Dragendorff-Reagens nach THIES UND REUTHER[8]. Wie bei der Dünnschicht-

chromatographie liegt auch bei der Dünnschichtelektrophorese die untere Nachweisgrenze für die einzelnen Verbindungen bei 0.5–1.0 μg.

ERGEBNISSE

Die besten Trennergebnisse erzielten wir bei Verwendung von Kieselgel G der Firma Merck, Darmstadt, als Sorptionsmittel. Mit einer Pufferlösung pH 3.6, die sich aus Pyridin–Eisessig–Wasser (1:10:89) zusammensetzt, gelingt in einem Spannungsgefälle von 30–40 V/cm innerhalb von etwa zwei Stunden die vollständige Auftrennung eines Gemisches der fünf in Tabelle I genannten quaternären Ammonium-

TABELLE I

DÜNNSCHICHTELEKTROPHORETISCHE WANDERUNGSWEGE VON QUATERNÄREN AMMONIUMVERBINDUNGEN

	R_{Ch}-Werte*	
	Kieselgel pH 3.6	*Cellulose pH 6.5*
Tetramethylammoniumchlorid	0.91	1.16
Cholinchlorid, (2-Hydroxyäthyl)-trimethylammoniumchlorid	1.00	1.00
Chlorcholinchlorid, (2-Chloräthyl)-trimethylammoniumchlorid	0.67	0.94
Acetylcholinchlorid, (2-Acetoxyäthyl)-trimethylammoniumchlorid	0.52	0.86
Glykokollbetain	0.10	0.19

* $R_{Ch} = \frac{\text{Wanderungsstrecke der Analysensubstanz}}{\text{Wanderungsstrecke von Cholin}}$

verbindungen (Fig. 1a). Mit einem Pyridinacetatpuffer pH 6.5 ist auf Kieselgelschichten keine zufriedenstellende Auftrennung von Cholinchlorid und Tetramethylammoniumchlorid zu erreichen, während die Wanderungswege der übrigen quaternären Ammoniumverbindungen etwa gleich wie bei pH 3.6 sind.

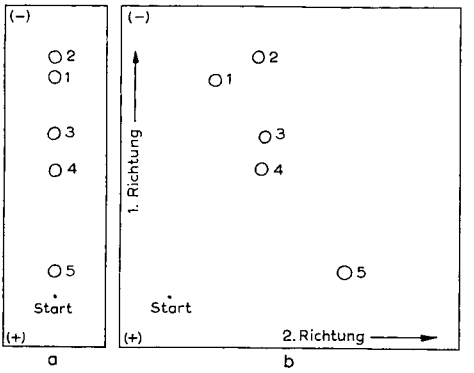

Fig. 1. Trennung quaternärer Ammoniumverbindungen auf Kieselgelschichten. (a) Dünnschichtelektrophorese, Pyridin–Eisessig–Wasser (1:10:89), pH 3.6, 40 V/cm; (b) 1. Richtung: Dünnschichtelektrophorese wie unter a; 2. Richtung: Dünnschichtchromatographie, aufsteigend in Methanol–Aceton–Salzsäure (90:10:4) (Lit. 5). 1 = Tetramethylammoniumchlorid; 2 = Cholinchlorid; 3 = Chlorcholinchlorid; 4 = Acetylcholinchlorid; 5 = Glykokollbetain.

Auch auf Dünnschichtplatten, die mit Cellulose MN 300, ohne Gipszusatz, der Firma Macherey, Nagel & Co., Düren, beschichtet sind, ist eine Auftrennung der quaternären Ammoniumverbindungen möglich, wenn man mit einem Puffergemisch aus Pyridin–Eisessig–Wasser (10:1:89), pH 6.5, arbeitet (Fig. 2a). In einem Spannungsgefälle von 30–40 V/cm beträgt die Trenndauer nur etwa 40 Min., doch sind die Unterschiede der Wanderungsgeschwindigkeiten von Cholinchlorid, Chlorcholinchlorid und Acetylcholinchlorid viel geringer als auf Kieselgelschichten bei Verwendung des Pyridinacetatpuffers pH 3.6 (Tabelle I). Auf Celluloseschichten ist daher nur dann eine zufriedenstellende elektrophoretische Auftrennung der drei genannten Substanzen zu erwarten, wenn sie in der Mischung in etwa gleichen Mengen vorhanden sind und die Beladung der Platte mit dem Substanzgemisch nicht zu gross ist.

Auf Kieselgur- und Aluminiumoxidschichten sind bei Verwendung der Pyridinacetatpuffer pH 3.6 oder pH 6.5 keine grossen Unterschiede zwischen den Wanderungsgeschwindigkeiten von Tetramethylammoniumchlorid, Cholinchlorid, Chlorcholinchlorid und Acetylcholinchlorid festzustellen. Cholinchlorid und Chlorcholinchlorid lassen sich unter diesen Bedingungen überhaupt nicht trennen.

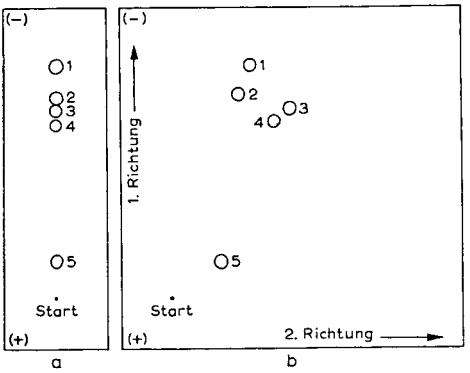

Fig. 2. Trennung quaternärer Ammoniumverbindungen auf Celluloseschichten. (a) Dünnschichtelektrophorese, Pyridin–Eisessig–Wasser (10:1:89), pH 6.5, 40 V/cm; (b) 1. Richtung: Dünnschichtelektrophorese wie unter a; 2. Richtung: Dünnschichtchromatographie, aufsteigend in *n*-Butanol–Eisessig–Wasser (4:1:5) (Lit. 2). 1 = Tetramethylammoniumchlorid; 2 = Cholinchlorid; 3 = Chlorcholinchlorid; 4 = Acetylcholinchlorid; 5 = Glykokollbetain.

DISKUSSION

Ein Vorteil der Dünnschichtelektrophorese besteht darin, dass sie im Gegensatz zur Dünnschichtchromatographie auch bei relativ stark verunreinigten Extrakten aus biologischem Material noch eine gute Auftrennung der quaternären Ammoniumverbindungen gibt, da anorganische Ionen sehr rasch aus dem Trennbereich auswandern und nach unseren Erfahrungen etliche andere Verunreinigungen nur sehr langsam wandern oder am Start hängen bleiben.

Die sichersten Aussagen über die Zusammensetzung eines Substanzgemisches erhält man auch bei den quaternären Ammoniumverbindungen, ähnlich wie bei Aminosäuren und Aminen, durch eine zweidimensionale dünnschichtelektrophoretische und chromatographische Auftrennung auf einer quadratischen Dünnschichtplatte. Es ist dabei vorteilhaft, zuerst die elektrophoretische Auftrennung durchzu-

führen, um gleichzeitig Verunreinigungen abzutrennen und nach einer Zwischentrocknung der Platte in der zweiten Richtung mit einem geeigneten Laufmittel aufsteigend chromatographisch zu entwickeln (Fig. 1b und 2b). Die Dünnschichtelektrophorese stellt somit bei der Auftrennung von Gemischen quaternärer Ammoniumverbindungen eine wertvolle Ergänzung der Dünnschichtchromatographie dar.

ZUSAMMENFASSUNG

Es wird die Auftrennung aliphatischer, quaternärer Ammoniumverbindungen mit Hilfe der Dünnschichtelektrophorese beschrieben. Die besten Trennergebnisse erzielt man auf Kieselgelschichten mit einem Pyridinacetatpuffer pH 3.6 und auf Celluloseschichten mit einem Pyridinacetatpuffer pH 6.5. Besonders vorteilhaft erweist sich auch bei den quaternären Ammoniumverbindungen eine zweidimensionale dünnschichtelektrophoretische und chromatographische Auftrennung.

SUMMARY

Separation of aliphatic, quaternary ammonium compounds by thin-layer electrophoresis is described. Use of silica gel layers and pyridine acetate buffer pH 3.6 or cellulose layers and pyridine acetate buffer pH 6.5 is recommended. Two-dimensional separation by combination of thin-layer electrophoresis and chromatography has proved very useful.

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REVERSED-PHASE PARTITION CHROMATOGRAPHY OF THE RARE EARTHS USING METHYLENEBIS[DI(2-ETHYLHEXYL)-PHOSPHINE OXIDE] AS THE STATIONARY PHASE*

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INTRODUCTION

The separation of the rare-earth elements by reversed-phase partition chromatography using the neutral, bifunctional compound, methylenebis(di-*n*-hexylphosphine oxide) (MHDPO), was recently reported[1]. In view of the rather large effects which variations in the organic groups of monoacidic esters of phosphoric and phosphonic acids have on the partition coefficients and separation factors in the extraction of several trivalent lanthanide and actinide cations[2], it seemed of interest to investigate the use of bifunctional phosphine oxides analogous to MDHPO, but with branched alkyl side chains, as stationary phases for the partition chromatography of the rare-earth cations. The extraction of samarium, thorium and uranium(VI) by methylenebis[di(2-ethylhexyl)phosphine oxide], $(2\text{-ethylhexyl})_2P(O)CH_2P(O)(2\text{-ethylhexyl})_2$ (MEHDPO), and methylenebis[di(2-ethylbutyl)phosphine oxide] (MEBDPO) had been previously studied by PARKER AND BANKS[3] and it was concluded that: (a) methylenebis(dialkylphosphine oxide) extractants with straight chain alkyl groups were better extractants than compounds with branched side chains; (b) the compounds with branched alkyl side chains were relatively better extractants for uranium(VI) than for thorium, and the converse was true for compounds with unbranched alkyl groups; (c) the partition coefficient of samarium nitrate was much smaller than for thorium or uranium(VI) nitrate.

The possibility that the partition coefficients of the rare earths into MEHDPO might vary more rapidly with ionic radius than they would with less sterically hindered extractants, such as MHDPO, seemed likely; and the movement of the rare-earth nitrates, chlorides and perchlorates on paper treated with MEHDPO was studied. The partition behavior of the rare earth salts into MEHDPO is considerably different from that reported for MHDPO and significantly larger separation factors are obtained in some cases.

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EXPERIMENTAL

Methylenebis[di(2-ethylbutyl)phosphine oxide] (MEBDPO) and methylenebis[di(2-ethylhexyl)phosphine oxide] (MEHDPO) were synthesized by a procedure previously reported[4]. Some cleavage of the compounds, presumably at the methylene bridge, invariably resulted during the distillation step (215°/0.1 mm Hg, and 225°/1 mm Hg for the ethylbutyl and ethylhexyl compounds, respectively). After the distillation step, any acidic compounds were removed by extraction with a 5 % aqueous solution of potassium hydroxide. Unlike MHDPO, it was not possible to recrystallize either MEBDPO or MEHDPO from diethyl ether, quite possibly because the introduction of the ethyl group in the side chain results in an asymmetric carbon and, hence, a number of stereoisomers are possible.

Paper chromatography of the distilled product (after extraction of acidic impurities) indicated substantial amounts of some neutral impurity. The paper was developed with a 40 % ethanol solution and then sprayed with a titanium–thiocyanate reagent[5]. Two bright yellow bands appeared, one near the solvent front and the other near the origin. It was assumed that the more water-soluble band (near the solvent front) was caused by a monofunctional impurity, possibly methyldialkylphosphine oxide. The ethylhexyl compound, MEHDPO, was further purified by column chromatography on cellulose columns, again using 40 % ethanol, to remove the more water-soluble impurity. The final product showed only one band when chromatographed on paper. It was recently observed that the chromatographic procedure was not necessary if a molecular distillation step at 10^{-4} mm/Hg was used for the initial purification rather than normal distillation procedure.

The papers were treated and developed in exactly the same manner as with MHDPO[1,6]. Because of the apparently greater solubility of the ethylbutyl compound in water and the very similar behavior of the rare earths on papers treated with MEBDPO and MEHDPO in some preliminary studies with these compounds, only the latter, MEHDPO, was purified and studied in the present work.

RESULTS AND DISCUSSION

R_F values for the movement of the rare-earth nitrates on paper impregnated with MEHDPO are given in Table I. The paper was soaked in a 0.2 M solution of MEHDPO in carbon tetrachloride and the latter allowed to evaporate at room temperature. Plots of $1/R_F - 1$ for several of the rare-earth nitrates as a function of the nitric acid concentration are shown in Fig. 1. Minimum values for $1/R_F - 1$ were obtained with 9 M nitric acid as the mobile phase. This is also the acid concentration at which maximum values for the separation factor, β, were obtained for most rare-earth couples (β is here defined as the ratio of the two $1/R_F - 1$ values).

Plots of log $(1/R_F - 1)$ as a function of the log of the concentration of MEHDPO on the paper are shown in Fig. 2 for three rare earths at both 3 M and 9 M nitric acid. The solid lines are drawn with a slope of unity and fit the data fairly well, except at low values of $1/R_F - 1$ (R_F values over 0.8). This indicates a value of one for the solvation number[1,7].

R_F values for the rare-earth chlorides on paper treated with 0.2 M MEHDPO were 1.0 (moved with solvent front) at all acid concentrations of the mobile phase

TABLE I

R_F VALUES FOR RARE-EARTH NITRATES ON MEHDPO-TREATED PAPER

Element	*Nitric acid concentration*						
	0.1 M	*1.0 M*	*3.0 M*	*6.0 M*	*9.0 M*	*12.0 M*	*16.0 M*
La	0.25	0.61	0.88	0.93	0.99	1.0	1.0
Ce	0.13	0.25	—	—	—	—	—
Pr	0.14	0.27	0.70	0.80	0.96	0.95	1.0
Nd	0.13	0.26	0.67	0.75	0.93	0.91	1.0
Sm	0.10	0.17	0.44	0.58	0.82	0.73	0.87
Eu	0.10	0.16	0.37	0.50	0.76	0.64	0.78
Gd	0.12	0.15	0.36	0.49	0.75	0.62	0.74
Tb	0.07	0.08	0.18	0.30	0.47	0.36	0.48
Dy	0.06	0.06	0.13	0.23	0.37	0.27	0.33
Ho	0.05	0.05	0.09	0.17	0.29	0.19	0.21
Er	0.05	0.04	0.06	0.11	0.18	0.13	0.13
Tm	0.03	0.03	0.05	0.08	0.12	0.07	0.09
Yb	0.03	0.03	0.04	0.06	0.08	0.05	0.06
Lu	0.03	0.03	0.04	0.06	0.07	0.06	0.05
Y	0.08	0.08	0.12	0.20	0.29	0.19	0.24

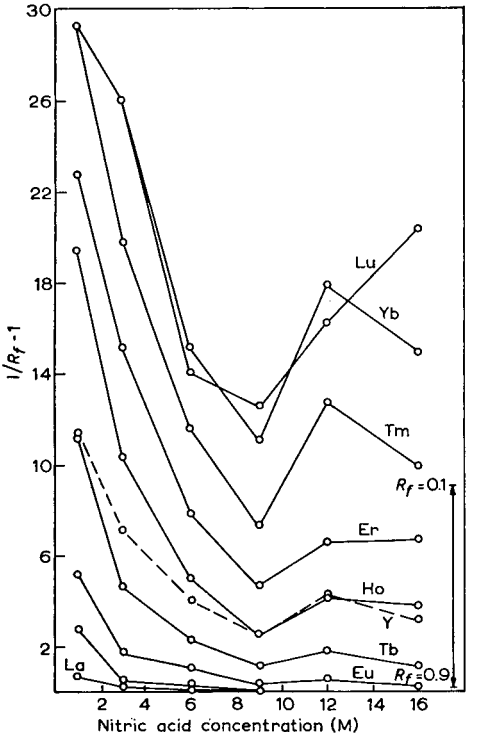
except 9.0 M and 12.0 M. In this case the heavy rare earths were slightly retained with an R_F value for lutetium of approximately 0.80.

R_F values for the rare-earth perchlorates on MEHDPO-treated paper are given in Table II for 0.5 M, 1 M, and 3 M perchloric acid. The R_F values appear to be relatively insensitive to the acid concentration of the mobile phase or the amount of MEHDPO on the paper (compare R_F values for 0.2 M $HClO_4$ and 0.5 M $HClO_4$). Furthermore, there is little change in R_F with atomic number.

Log $(1/R_F - 1)$ values for the rare-earth chlorides, nitrates and perchlorates on papers treated with 0.2 M MHDPO, MEHDPO and TOPO (tri-*n*-octylphosphine oxide) are plotted as a function of atomic number in Fig. 3. The R_F data for MHDPO were taken from an earlier paper[1]. Several differences in the partition behavior of the rare-earth salts into these three extractants are clearly evident.

Fig. 3 and previously published liquid–liquid extraction data[8] show that the partition coefficients for the rare–earths salts into MHDPO from dilute acid decrease in the order perchlorates > nitrates > chlorides. The order in the case of MEHDPO is nitrates > perchlorates > chlorides. The decrease in log $(1/R_F - 1)$ as a function of decreasing atomic number in 9 M nitric is also considerably greater with MEHDPO than with MHDPO. Except in the case of MHDPO when dilute nitric or hydrochloric acids are used as the mobile phases, which results in an inversion of the usual order[6], the partition coefficients increase with atomic number. Certain characteristic variations from a linear increase are clearly evident, however. The most striking of these are the similarity in the partition behavior of europium and gadolinium and the sudden increase in the partition coefficient from gadolinium to terbium.

The log $(1/R_F - 1)$ values (if the assumptions previously discussed concerning the linear relationship of the overall equilibrium constant, K, and $1/R_F - 1$ hold) are linear functions of the overall change in free energy for the extraction process. The latter can be viewed as the sum of a number of partial changes corresponding to the solvation (or complex formation) energies of both cation and anion in the



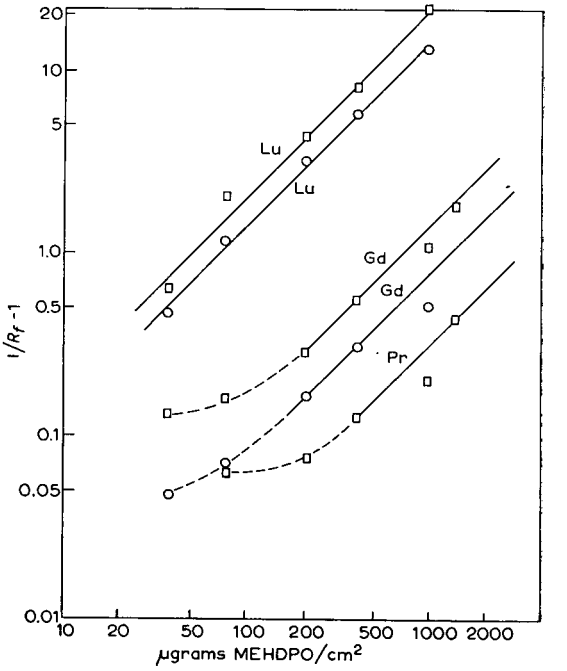


Fig. 1. $1/R_F - 1$ values for the rare earth nitrates on MEHDPO-treated paper. Mobile phase: nitric acid.

Fig. 2. Log $(1/R_F - 1)$ values for several rare earth nitrates as a function of MEHDPO-concentration on paper. (○), 9 *M* HNO_3; (□), 3 *M* HNO_3.

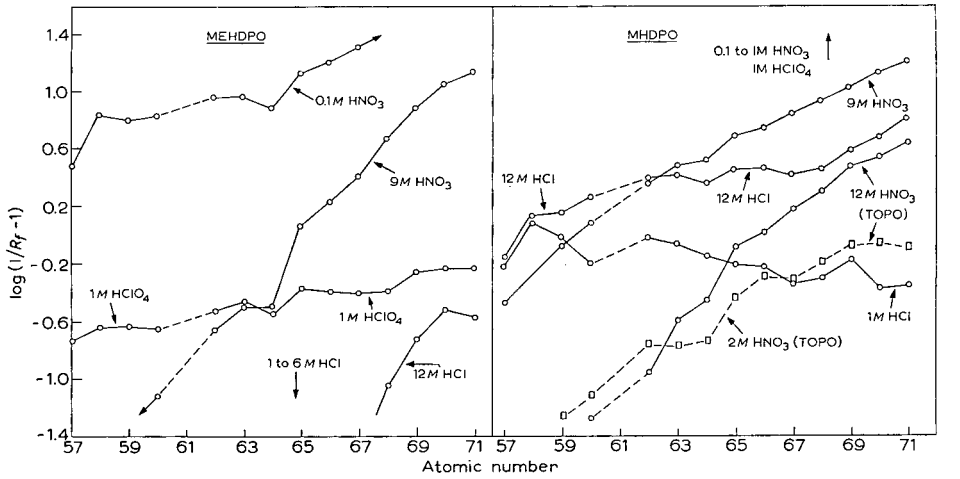


Fig. 3. Log $(1/R_F - 1)$ data for the rare earths in nitric, hydrochloric and perchloric acid media on paper treated with TOPO, MHDPO and MEHDPO.

TABLE II

R_F VALUES FOR RARE-EARTH PERCHLORATES ON MEHDPO-TREATED PAPER*

Element	*Perchloric acid concentration*			
	0.2 M	*0.5 M*	*1.0 M*	*3.0 M*
La	0.87	0.85	0.85	0.89
Ce	0.80	0.82	0.81	0.86
Pr	0.75	0.81	0.81	0.84
Nb	0.79	0.81	0.82	0.85
Sm	0.67	0.76	0.77	0.83
Eu	0.69	0.75	0.75	0.81
Gd	0.78	0.75	0.78	0.84
Tb	0.79	0.71	0.70	0.82
Dy	0.78	0.72	0.71	0.77
Ho	0.79	0.73	0.72	0.78
Er	0.76	0.72	0.71	0.79
Tm	0.75	0.71	0.65	0.74
Yb	0.71	0.70	0.63	0.74
Lu	0.72	0.70	0.63	0.75
Y	0.80	0.73	0.76	0.80

* Paper treated with 0.1 *M* solution of MEHDPO in carbon tetrachloride except in the case of data for 0.2 *M* acid where paper was treated with 0.2 *M* MEHDPO in carbon tetrachloride.

aqueous and the organic phases[9,10]. Because the change in free energy of each partial process is relatively large and the overall change is given by the difference of the sums of these partial processes in the two phases, it would be difficult to predict the magnitude of the partition coefficient even if reasonably accurate data for the partial processes were available. Conversely, it is rather risky to attribute differences in extraction behavior to any one of the partial processes involved in the overall extraction mechanism. It does seem probable, however, that the decreasing extraction order of the rare-earth salts into MHDPO in the order perchlorates > nitrates > chlorides is related to the solvation energy terms for these three anions[1].

The much poorer extraction of the rare-earth perchlorates into MEHDPO in comparison to their extraction into MHDPO can be rationalized on the basis that MHDPO can more effectively solvate the rare-earth cation in the organic phase than can the more sterically hindered MEHDPO. This steric effect in the two extractants should be most evident in the extraction of metal perchlorates; because the perchlorate ion would not be expected to coordinate very well, if at all, with the cation. The coordination positions of the cation would then have to be filled either by the phosphoryl oxygens of the extractant or water molecules. The former would be consistent with high partition coefficients into the organic phase but would seem to require a solvation number (number of extractant molecules for each cation) of two or more.

The very low or high R_F values for the rare-earth perchlorates on paper treated with MHDPO or MEHDPO, respectively, prevented the determination of the solvation number by paper techniques. Although partition data using liquid–liquid extraction techniques should give this information, no such data are available at the present time for the above two extractants. It seems likely, however, that the solvation number of the rare-earth perchlorates is two or more with MHDPO in view

of their relatively good extraction. Both MHDPO and MEHDPO form 1:1 adducts with the rare earths in nitric acid media (Fig. 2 and ref. 1) and apparently act as bidentate donors. The latter assumption is based on the much better extraction of rare-earth nitrates into MHDPO and MEHDPO than into TOPO, and the fact that MHDPO acts as a bidentate donor with the uranyl ion[8,11]. The relatively poor extraction of the perchlorates into MEHDPO can then hardly be attributed to the inability of MEHDPO to act as a bidentate donor. The more likely alternative is that there is a steric problem in fitting more than one molecule of MEHDPO around the rare-earth cation so that the phosphoryl oxygens can effectively fill the coordination positions.

The extraction of the rare-earth nitrates appears to follow the same mechanism with both MHDPO and MEHDPO. It is believed the relatively good extraction of the 1:1 adduct of the rare-earth nitrates with both MHDPO and MEHDPO occurs because nitrate ions are capable of filling the remaining coordination positions of the cation in the organic phase. The inverted order for the extraction of the rare-earth nitrates previously reported for extraction from very dilute nitric acid into MHDPO[1,6] could well reflect a tendency in this case toward a higher solvation number. This inversion at low acid concentrations was not observed in the case of MEHDPO.

The sharper rate of decrease in $1/R_F - 1$ with decreasing atomic number with MEHDPO as compared with MHDPO at the same acid concentration (9 M nitric) is likely to be caused by steric factors. The lighter rare earths with larger ionic radii presumably form less stable adducts with the bulky MEHDPO than with MHDPO.

The increase in $1/R_F - 1$ for the heavier rare earths at nitric acid concentrations greater than 9 M and the smaller separation factors were not expected on the basis of the MHDPO data[1]. With the latter extractant, the $1/R_F - 1$ values continued to decrease and the separation factors to increase up to 16 M nitric. Some recent work[12] on the solubilities of these extractants in nitric acid indicates that there is considerable chemical attack on MEHDPO at acid concentrations higher than 9 M. The possible formation of acidic degradation products could explain the higher $1/R_F - 1$ values and lower separation factors observed with MEHDPO at high acid concentrations of the mobile phase. Although the solubility of MHDPO increased at high acid concentrations, there was no evidence of chemical attack.

The poor extraction of the rare-earth chlorides from dilute hydrochloric acid would be expected on the basis of the unfavorable electrostatic term for the extraction of three chloride ions into the organic phase. This would also be true for the extraction of the rare-earth nitrates from dilute nitric acid, but the nitrate ion, which could act as a bidentate donor, might be more effective in solvating the rare-earth cation in the organic phase than the chloride ion.

The small individual variations in partition behavior with atomic number, such as the generally sharp increase with partition coefficients from lanthanum to cerium and from gadolinium to terbium, might reflect splitting of the 4 f energy levels. YATSIMIRSKII AND KOSTROMINA[13] discuss the "extrastabilization energy" caused by the ligand field effect for rare earths with different symmetries. The gross deviations from linearity in the plots shown in Fig. 3, such as the similarity in the partition coefficients of europium and gadolinium and the rather large difference between gadolinium and terbium, seem consistent with this type of reasoning. The

agreement is not too good in many other cases, which, in view of the complexity of the system, is not too surprising. Other factors such as the possibility of a sudden change in coordination number might well be more important and could either obscure or account for these effects ascribed to ligand field splitting of energy levels[14].

SUMMARY

R_F values are given for the rare-earth nitrates, chlorides, and perchlorates on paper impregnated with methylenebis[di(2-ethylhexyl)phosphine oxide] (MEHDPO). The much lower $1/R_F - 1$ values for the rare-earth perchlorates on MEHDPO-treated paper than on paper treated with the analogous extractant without the ethyl groups in the 2-position (MHDPO) are rationalized on the basis of steric factors. The greater separation factors for the rare earths observed in the nitric acid system with MEHDPO are explained on the same basis.

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REVERSED-PHASE PARTITION CHROMATOGRAPHY ON PAPER TREATED WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID: A SYSTEMATIC STUDY OF 67 CATIONS IN HYDROCHLORIC ACID

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INTRODUCTION

After PEPPARD *et al.*[1] first showed, in 1957, that di-(2-ethylhexyl) orthophosphoric acid (HDEHP) was a very promising extractant for the fractionation of the trivalent lanthanides, a great deal of work was performed in many laboratories on the use of this compound in the separation of various cations.

The high degree of selectivity attainable with HDEHP when separating elements which show very similar chemical properties, made this compound a very effective tool for reversed-phase partition chromatography in the field of inorganic chemistry. Supports treated with a suitable organic solution of HDEHP have been used, in the form of a bed for column applications or in the form of a paper sheet for paper chromatography. It is not possible to list here all the examples given in the literature of the use of HDEHP-treated supports, since, due to their number, a special review would be required.

Many chemical elements have already been investigated in our laboratory as to their behaviour on reversed-phase chromatography with HDEHP-treated papers. With the purpose of completing a list of the behaviour of as many as 67 cations, our efforts were devoted to investigating the HDEHP–HCl system on paper.

This work, starting from our recent results, and extending further their development, is aimed at collecting all the experimental data obtained and correlating such data with some special aspects of liquid–liquid extraction phenomena.

EXPERIMENTAL

Reagents and equipment

The di-(2-ethylhexyl) orthophosphoric acid (HDEHP) was a Virginia-Carolina Chemical Co. (Richmond, U.S.A.) product supplied by Soc. Eigenmann and Veronelli (Milan). It was used as supplied, since no differences in experimental results were noticed[2] when HDEHP samples had been previously purified by a method derived from that described by STEWART AND CRANDALL[3]. All the reagents were analytical grade. A list of cations, not considered in our previous work, and the relative compounds used, is shown in Table I, together with the suppliers and final solutions obtained.

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TABLE I

SOLUTIONS USED TO APPLY THE CATIONS ON THE CHROMATOGRAMS

Cation	*Compound used*	*Final solvent*	*Final ion conc., equiv./l*	*Supplier*
Li^+	LiCl	H_2O	0.005	C. Erba
Na^+	NaCl	H_2O	0.005	C. Erba
K^+	KCl	H_2O	0.005	C. Erba
Rb^+	RbCl	H_2O	0.005	Fluka
Cs^+	CsCl	H_2O	0.005	B.D.H.
Y^{3+}	$YCl_3 \cdot 6\ H_2O$	H_2O	0.08	Merck
Nd^{3+}	Nd_2O_3	0.2 *M* HCl	0.075	B.D.H.
Tb^{3+}	Tb_2O_3	0.1 *M* HCl	0.02	Fluka
Dy^{3+}	Dy_2O_3	0.2 *M* HCl	0.002	Fluka
Ho^{3+}	Ho_2O_3	0.1 *M* HCl	0.035	Fluka
Tm^{3+}	Tm_2O_3	0.2 *M* HCl	0.02	Light
Lu^{3+}	Lu_2O_3	0.1 *M* HCl	0.02	Light
UO_2^{2+}	$UO_2(NO_3)_2 \cdot 6\ H_2O$	5 *M* HCl	0.005	Merck
Ti^{4+}	$TiCl_4$	0.1 *M* HCl	0.005	C. Erba
Hf^{4+}	metal	6 *M* HCl	0.02	Fluka
V^{3+}	V_2O_3	conc. HCl	0.10	C. Erba
V^{4+}	$VOSO_4 \cdot 5\ H_2O$	H_2O	0.10	Merck
Nb^{5+}	metal	conc. H_2SO_4	0.10	B.D.H.
Cr^{3+}	$CrCl_3 \cdot 6\ H_2O$	H_2O	0.10	C. Erba
Mo^{6+}	MoO_3	0.1 *M* NaOH	0.10	C. Erba
W^{6+}	$Na_2WO_4 \cdot 2\ H_2O$	H_2O	0.10	C. Erba
Mn^{2+}	$MnCl_2 \cdot 4\ H_2O$	H_2O	0.005	C. Erba
Re^{7+}	metal	6 *M* HCl	0.01	B.D.H.
Ru^{3+}	$RuCl_3$	1 *M* HCl	0.05	B.D.H.
Rh^{3+}	metal	conc. HCl	0.10	B.D.H.
Pd^{2+}	$PdCl_2$	1 *M* HCl	0.05	C. Erba
Ir^{4+}	$IrCl_3$	conc. HCl	0.10	C. Erba
Pt^{4+}	$H_2PtCl_6 \cdot 6\ H_2O$	conc. HCl	0.005	C. Erba
Cu^{2+}	$CuCl_2 \cdot 2\ H_2O$	H_2O	0.005	C. Erba
Ag^+	$AgNO_3$	H_2O	0.10	C. Erba
Au^{3+}	$HAuCl_4 \cdot 4\ H_2O$	0.1 *M* HCl	0.005	C. Erba
Zn^{2+}	$ZnCl_2$	0.1 *M* HCl	0.005	C. Erba
Cd^{2+}	$CdCl_2 \cdot 2.5\ H_2O$	H_2O	0.012	C. Erba
Hg^{2+}	$HgCl_2$	H_2O	0.005	C. Erba
B^{3+}	$Na_2B_4O_7 \cdot 10\ H_2O$	H_2O	0.10	C. Erba
Ge^{4+}	$GeCl_4$	conc. HCl	0.10	B.D.H.
Sn^{2+}	$SnCl_2 \cdot 2\ H_2O$	95% ethanol	0.10	C. Erba
Pb^{2+}	$Pb(NO_3)_2$	0.1 *M* HCl	0.005	C. Erba
As^{3+}	As_2O_3	conc. HCl	0.10	C. Erba
As^{5+}	As_2O_5	conc. HCl	0.10	C. Erba
Sb^{5+}	Sb_2O_5	conc. HCl	0.10	C. Erba
Bi^{3+}	$Bi(NO_3)_3 \cdot 5\ H_2O$	0.3 *M* HCl	0.10	C. Erba
Se^{4+}	H_2SeO_3	H_2O	0.10	C. Erba
Te^{4+}	$TeCl_4$	conc. HCl	0.10	B.D.H.

The chromatographic paper was Whatman No. 1 (CRL/1 type). It was treated with the appropriate cyclohexane solution of HDEHP, which had been previously equilibrated with hydrochloric acid by shaking 10 min with twice its volume of a 2.5 *M* HCl solution. The paper was immersed for about 30 sec in the equilibrated organic solution and then removed and allowed to drip. It was finally dried in a current of warm air.

The behaviour of the various cations on varying the HCl concentration in the eluent was mostly determined with paper treated with 0.100 *M* HDEHP.

In order to obtain experimental data on the role of HDEHP in the partition mechanism of the ions, simultaneous elutions[4] were carried out on papers treated with cyclohexane solutions of HDEHP at different concentrations, *viz.* 0.100, 0.080, 0.060, 0.040, 0.025, 0.015 and 0.010 *M*; the HCl concentration in the eluent being chosen for each ion from the results obtained with 0.100 *M* HDEHP treated papers.

In the case of yttrium and uranium, an extensive investigation was carried out to determine the dependence of their R_F values on the hydrochloric acid concentration not only with paper treated with 0.100 *M* HDEHP, but also with paper treated with 0.075, 0.050, 0.025 and 0.010 *M* HDEHP, as has already been done for the alkaline earths[4] and aluminium, gallium, indium, thallium and the transition metals of the iron group[2].

The equipment used has already been described in previous work[5].

Chromatographic procedure

Spots with a volume of about 0.02 ml were applied on the paper, which corresponds approximately to 1×10^{-7} equivalents of the cation with a 0.005 *N* solution and to 2×10^{-6} equivalents with a 0.1 *N* solution. After development, the paper was dried in a current of warm air, and then sprayed with a solution suitable for the detection of the spots. The reagent solutions used for this purpose are listed in Table II.

TABLE II

SPRAY REAGENTS FOR DETECTION OF THE SPOTS

Compound	*Reagent solution*	*Cations developed**
Dithizone	0.1 % soln. in chloroform	V^{3+}; V^{4+}; Nb^{5+}; Re^{7+}; Ru^{3+}; Rh^{3+}; Pd^{2+}; Ir^{4+}; Ag^{+}; Au^{3+}; Zn^{2+}; Cd^{2+}; Hg^{2+}; Ge^{4+}; Pb^{2+}; As^{3+}; As^{5+}; Sb^{5+}; Se^{4+}; Te^{4+}
Quercetin	0.1 % soln. in ethanol	Sc^{3+}; Ti^{4+}; *Zr^{4+}*; *Hf^{4+}*; *Mn^{2+}*; *Pt^{4+}*; Cu^{2+}; Sn^{2+}
8-Hydroxyquinoline	0.1 % soln. in 50 % ethanol	*Y^{3+}*; UO_2^{2+}; *rare earths*
Morin	0.1 % soln. in ethanol	*Mo^{6+}*; *W^{6+}*
Diphenylcarbazide	0.1 % soln. in ethanol	Cr^{3+}
Sodium alizarinsulphonate	0.1 % soln. in ethanol	B^{3+}
Ammonium sulphide	20 % soln. in water	Bi^{3+}

* Cations in italics can be detected only under U.V. light.

Except in the case of bismuth, which was sprayed with ammonium sulphide, the sprayed paper had to be exposed to ammonia fumes to make the spots visible.

Further details of the chromatographic technique are described in a previous paper[6].

The elutions, all ascending, were made with hydrochloric acid (supplied by C. Erba, Milan) at different concentrations, ranging from $1 \cdot 10^{-4}$ to 10 *M*.

Each result was checked at least once under the same conditions, except for a few cases in which the relative position of results at various concentrations left no doubt as to their reliability. Reproducibility of the R_F values within $\pm$ 3 % was

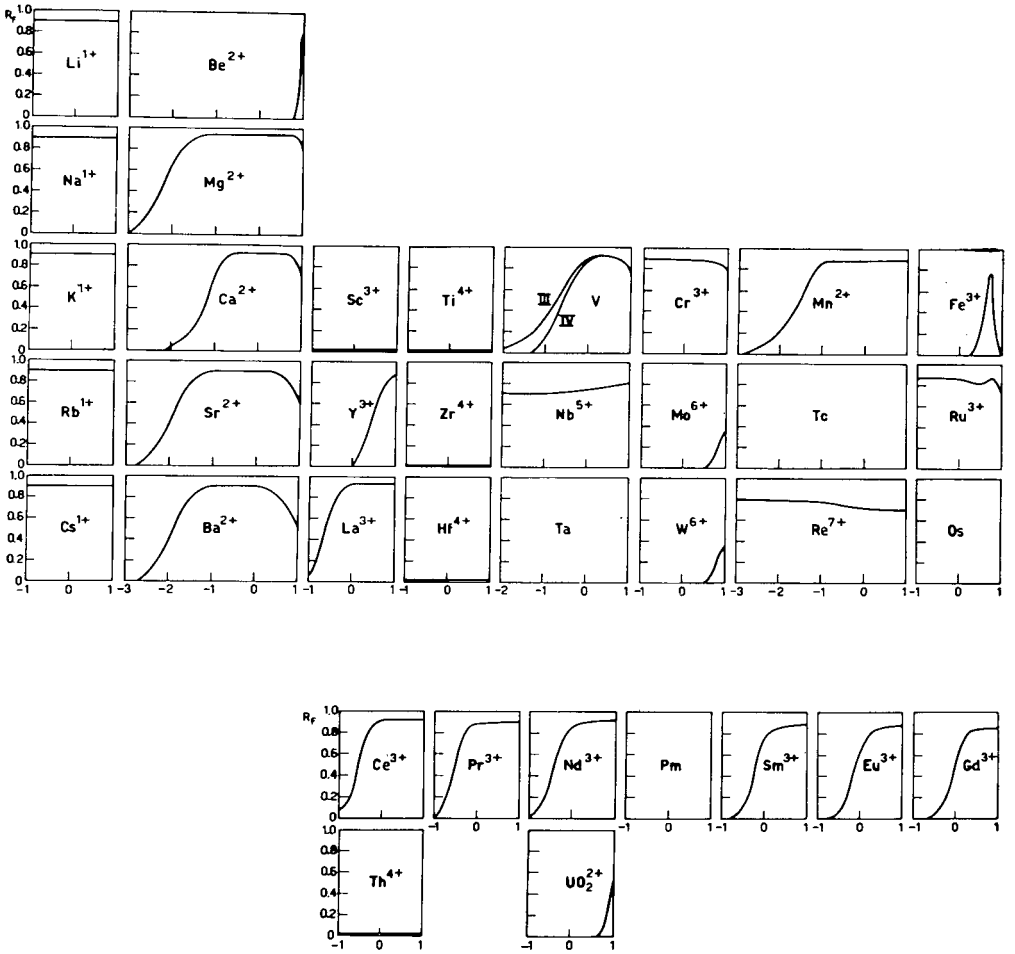


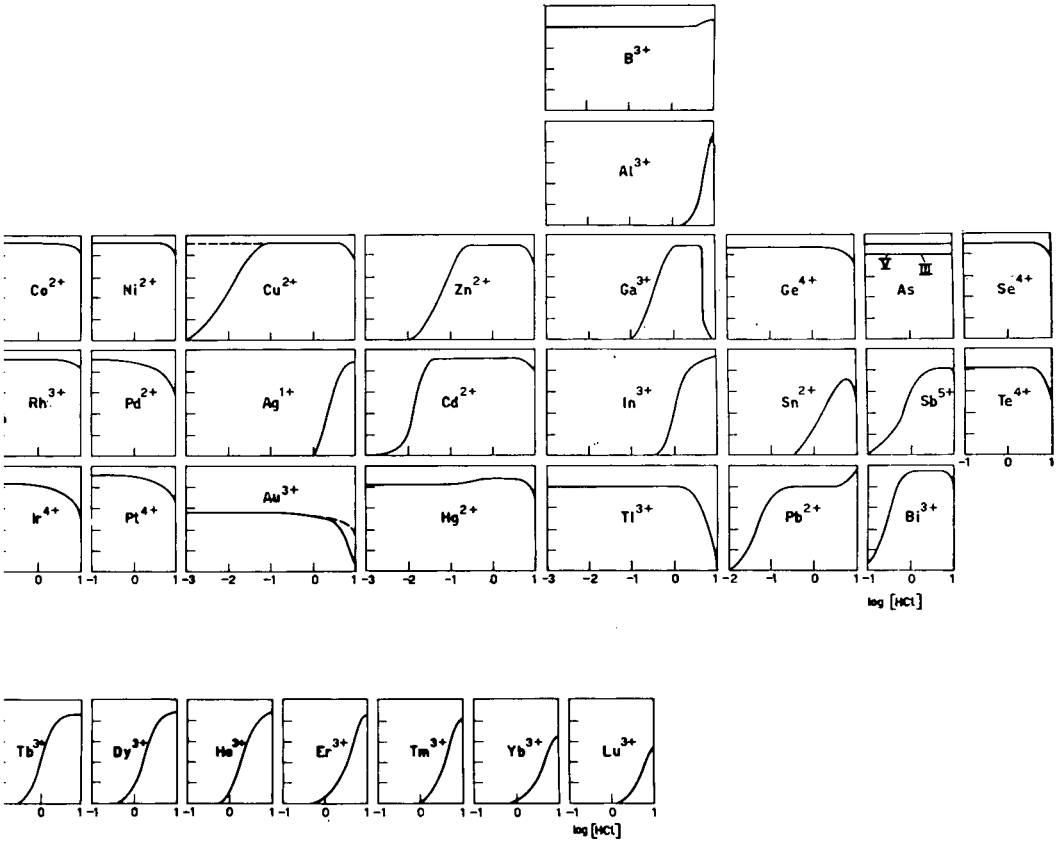
Fig. 1. Chromatography on paper treated with 0.1 M di-(2-ethylhexyl) orthophosphoric acid in cyclohexane; plot of the R_F values of the elements as a function of the logarithm of the molarity of the hydrochloric acid used as the eluent.

considered satisfactory, because of the shortness (about 9 cm) of the chromatograms. All the experiments were carried out at a room temperature of 23° ± 1°.

RESULTS

In Fig. 1, R_F values for all the cations studied both in the present and in previous work are plotted against the molarity of the HCl used as eluent, the papers being treated with an 0.100 M solution of HDEHP in cyclohexane. The cations are arranged according to their order in the Periodic Table.

Data on osmium are necessarily missing, since there were no methods of detection suitable for the low amounts of this cation which had to be used in order that the intrinsically low capacity of the treated paper was not overloaded. The other cations missing were those not available at the moment. Although the experiments



were carried out over a range of acidity from 10 M HCl to $1 \cdot 10^{-4}$ M for all cations, in drawing Fig. 1 the lower concentrations were often ignored, since the relative results showed no interesting features.

The general behaviour of the ions, as it appears in Fig. 1, compares well with the data in the literature on liquid–liquid extraction.

A good survey of the behaviour of elements in the liquid–liquid extraction with HDEHP from hydrochloric acid solutions was carried out by KIMURA[7]. By comparing our R_F results with the distribution coefficient values K reported in KIMURA's paper, a certain generalization can be made about the correspondence between these two variables. Roughly speaking, when in our experiments an element just leaves the start point, a value for K of about unity is found in KIMURA's table; and when an element reaches the front of the eluent, its extraction coefficient is of the order of $1 \cdot 10^{-3}$. It was found that the only disagreement between the two tables

occurs for mercury, which should, according to KIMURA's data, be partially retained on the paper, while in our experiments it always runs with the eluent.

Further comments on the results shown in Fig. 1 can be found in the discussions on the single groups of cations, reported below.

Fig. 2 reports the data obtained in the investigation of the behaviour of yttrium and uranium(VI) on papers treated with HDEHP at various concentrations in cyclohexane. As has been already mentioned, 0.100, 0.075, 0.050, 0.025 and 0.010 HDEHP molarities were studied.

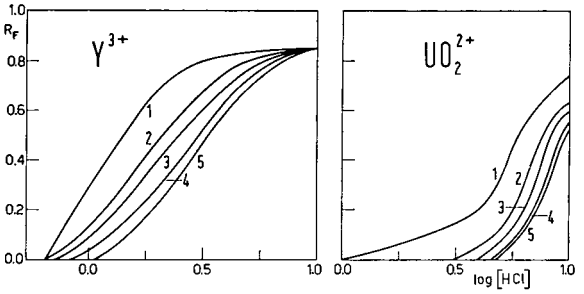


Fig. 2. R_F values for yttrium and uranium plotted *vs.* log M HCl. Paper treated with HDEHP solutions 0.010 M (curve 1), 0.025 M (curve 2), 0.050 M (curve 3), 0.075 M (curve 4), and 0.100 M (curve 5).

DISCUSSION

General

It is well-known that the behaviour of an ion in reversed-phase partition chromatography on supports impregnated with a given compound is closely related to its behaviour in liquid–liquid extraction by the same compound dissolved in a suitable solvent. The R_F values for a given ion on paper treated with a liquid extractant and eluted with a given aqueous solution are related to the extraction coefficients E_a° of the same ion in the analogous liquid–liquid system, through the well-known relationship:

$$\log\left(\frac{1}{R_F} - 1\right) = \log E_a^\circ + \log k \qquad (1)$$

where k is a constant depending on the chromatographic conditions.

As already pointed out[4], the equilibrium accepted as representing the extraction mechanism of a cation with HDEHP may be written as:

$$M^{b+} + c(HDEHP)_2 \rightleftharpoons M(DEHP)_b \cdot b\ HDEHP \cdot (c - b)\ (HDEHP)_2 + bH^+ \qquad (2)$$

The equilibrium constant K of this reaction, written in terms of activities, is:

$$K = \frac{\gamma_A\ [M(DEHP)_b \cdot b\ HDEHP \cdot (c - b)\ (HDEHP)_2]\ a_{H^+}^b}{\gamma_C\ [M^{b+}]\ [MDEHP]_{eff}^c} \qquad (3)$$

where γ_A and γ_C are the activity coefficients of the adduct in the organic phase and of the metallic cation in the aqueous phase, respectively, and $[HDEHP]_{eff}$ is what

we called[5] the effective HDEHP concentration and which is a measure of the activity of the HDEHP present on the paper sheet.

The extraction coefficient E_a° of the metallic ion is defined as:

$$E_a^\circ = \frac{[\mathrm{M(DEHP)}_b \cdot b\ \mathrm{HDEHP} \cdot (c - b)\ (\mathrm{HDEHP})_2]}{[\mathrm{M}^{b+}]}$$

By substituting the left and right terms in this latter equation with their expressions obtainable from the eqns. (1) and (3) respectively, the following relationship is obtained:

$$\log\left(\frac{1}{R_F} - 1\right) = c \log [\mathrm{HDEHP}]_{\mathrm{eff}} - b \log a_{\mathrm{H}^+} + \mathrm{const.} \qquad (4)$$

where the term "const." in eqn. (4) $= \log K + \log k + \log (\gamma_C/\gamma_A)$; γ_C/γ_A being assumed to be practically constant in the ionic strength range pertaining to the experimental conditions considered for a given cation.

For a given element, plots of $\log (1/R_F - 1)$ against either $\log [\mathrm{HDEHP}]_{\mathrm{eff}}$ or $\log a_{\mathrm{H}^+}$ can lead to the determination of the coefficients c and b of the eqn. (4) when either the HCl concentration in the eluent or the HDEHP concentration in the solution used to treat the paper are respectively kept constant. Thus, information on the extraction mechanism can be obtained.

Figs. 3 to 6 show such plots for the various cations considered in this work and whose R_F values in Fig. 1 increase as the HCl concentration is raised. The analogous plots for cations already considered in previous publications are omitted here, as they can be found in the respective papers[2,4]. Data for rare earths are reported below.

The hydrogen ion activities in plots of Figs. 3 to 5 were calculated from the data reported by ROBINSON AND STOKES[8]. The effective HDEHP concentrations on the paper, used in Fig. 6, were calculated from the formula:

$$k\ [\mathrm{HDEHP}]_{\mathrm{eff}} = 0.14 + 1.5\ [\mathrm{HDEHP}]$$

mentioned in a previous paper[5].

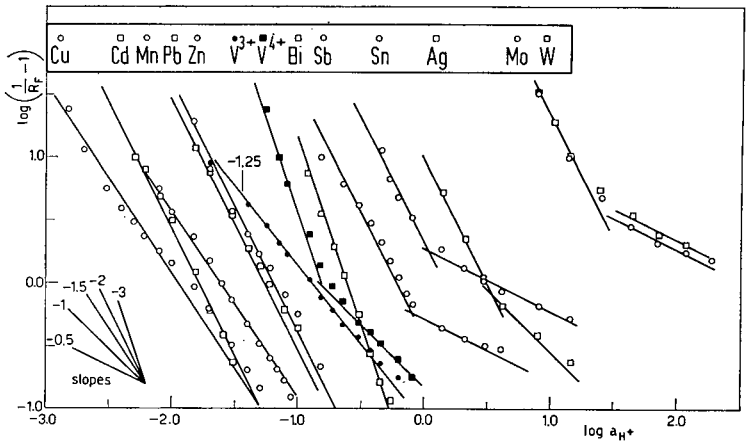


Fig. 3. Plots of $\log (1/R_F - 1)$ *vs.* $\log a_{\mathrm{H}^+}$ for various cations. Paper treated with 0.100 *M* HDEHP.

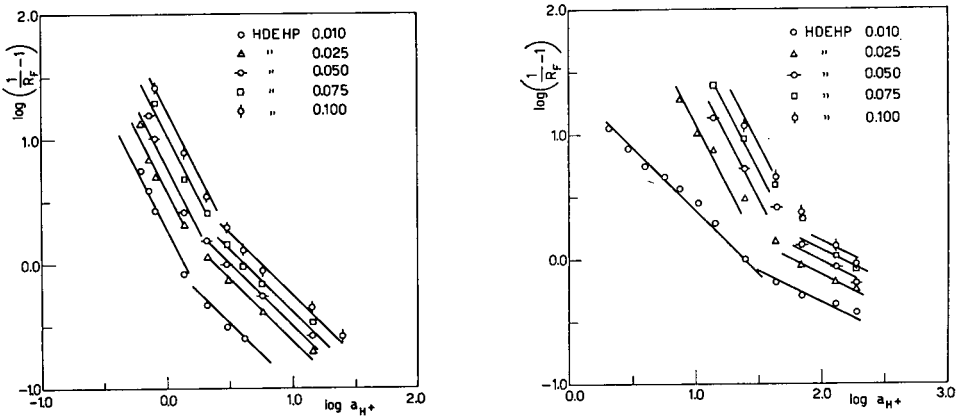


Fig. 4. Plots of log $(1/R_F - 1)$ *vs.* log a_{H+} for yttrium. Paper treated with HDEHP at various concentrations. Slopes —2 and —1.

Fig. 5. Plots of log $(1/R_F - 1)$ *vs.* log a_{H+} for uranium. Paper treated with HDEHP at various concentrations. Slopes —2, —1.5, and —1.

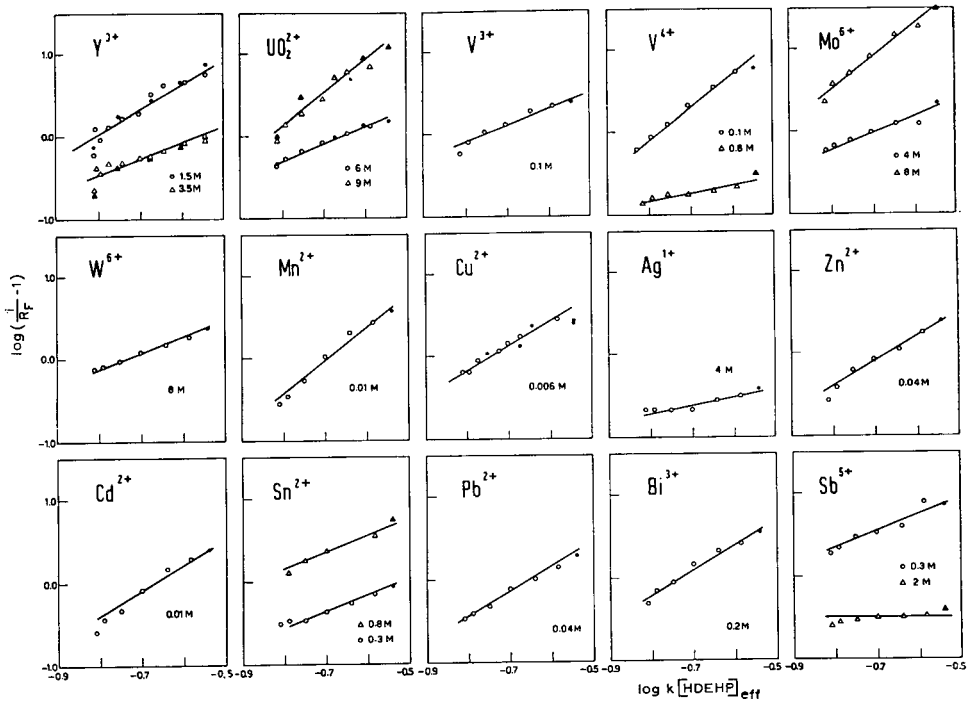


Fig. 6. Plots of log $(1/R_F - 1)$ *vs.* log $k[\text{HDEHP}]_{\text{eff}}$ for various cations. Different HCl concentrations, as quoted in the single plots. Open symbols refer to data obtained with simultaneous elutions with paper treated with HDEHP at different concentrations. Closed symbols refer to data obtained from Figs. 3 to 5. Slopes are found in Table III.

From the slopes of the curves in the Figs. 3–6 the values for the coefficients b and c of eqn. (4) were obtained. These are collected in Table III, together with the similar results obtained for the cations considered in previous publications. From a general standpoint, the results collected in Table III show that although a relationship such as that shown in eqn. (1) holds, the values for coefficient b for a given cation seldom agree with the charge of the cation itself.

The fact that the cations are likely to be partially complexed by the chloride ions present in the eluent solution has to be taken into account. A cation M^{b+} coordinating n Cl^- ions gives rise to a $MCl_n^{(b-n)+}$ complex which actually takes part in the exchange reaction. In this case, the mechanism of exchange may be represented

TABLE III

VALUES OF COEFFICIENTS b AND c OF EQUATION (4) FOR CATIONS ELUTED WITH HCl ON PAPER TREATED WITH 0.1 M HDEHP IN CYCLOHEXANE

Cation	*Coefficient b*		*Coefficient c*	
	Value	*HCl molarity range*	*Value*	*HCl molarity*
Mg^{2+}	1.5	0.001 –0.04	3.0	0.003
Ca^{2+}	1.5	0.008 –0.1	3.0	0.03
Sr^{2+}	2.0	0.002 –0.006	3.0	0.003
	1.5	0.006 –0.04	3.0	0.01
Ba^{2+}	2.0	0.002 –0.006	3.0	0.003
	1.5	0.006 –0.04	3.0	0.01
Y^{3+}	2.0	0.7 –2	3.0	1.5
	1.0	2 –6	2.0	3.5
UO_2^{2+}	2.0	5 –7	4.0	6.0
	1.0	7 –10	2.0	9.0
V^{3+}	1.25	0.01 –1	2.0	0.1
V^{4+}	3.0	0.05 –0.2	4.0	0.1
	1.0	0.2 –1	1.0	0.8
Mo^{6+}	2.0	4 –6	4.0	4.0
	0.5	6 –10	2.0	8.0
W^{6+}	2.0	4 –6	—	—
	0.5	6 –10	2.0	8.0
Mn^{2+}	1.5	0.004 –0.1	4.0	0.01
Fe^{3+}	2.0	2 –6	3.0	2.3
Co^{2+}	(1.5)	0.0001–0.03	—	—
Ni^{2+}	(1.5)	0.0001–0.03	—	—
Cu^{2+}	1.5	0.001 –0.05	3.0	0.006
Ag^{+}	2.0	1 –3	—	—
	1.0	3 –5	1.0	4.0
Zn^{2+}	2.0	0.01 –0.2	3.0	0.04
Cd^{2+}	2.0	0.004 –0.03	3.0	0.01
Al^{3+}	2.0	3.5 –5	3.0	4.0
	1.0	5 –10	3.0	6.0
Ga^{3+}	3.0	0.08 –0.2	3.0	0.08
	2.0	0.2 –1	3.0	4.0
In^{3+}	3.0	0.3 –1	3.0	0.5
	< 3.0	1 –3	< 3.0	—
Sn^{2+}	2.0	0.5 –1	2.0	0.8
	0.5	1 –5	2.0	3.0
Pb^{2+}	2.0	0.01 –0.1	3.0	0.04
Sb^{5+}	2.0	0.1 –1	2.0	0.3
	0.5	1 –3	0.0	2.0
Bi^{3+}	3.0	0.1 –0.7	3.0	0.2

by a reaction similar to reaction (2) in which the element M^{b+} and the coefficient b are substituted by $MCl_n^{(b-n)+}$ and by $(b - n)$, respectively. The slopes of the plots of log $(1/R_F - 1)$ *vs.* log a_{H^+} will then give $(b - n)$ values. Furthermore, the combination of the two equilibria gives a value of b lying between the value of the charge of the cation and the same value minus n.

The same effect is obtained if partial hydrolysis of the cation occurs: the cationic species to be considered will then be $M(OH)_n^{(b-n)+}$, and reaction (2) will be modified in the same way as in the case of the $MCl_n^{(b-n)+}$ species.

In addition to the above, another reason for b coefficients differing from the stoichiometric values may be ascribed to a dehydration effect which takes place at high hydrochloric acid concentrations. This effect may be responsible for the decrease of the R_F values shown to different extent by almost all the elements. A general discussion on this effect which deserves further consideration will be given in a future paper[9].

Except for Y(III) and UO_2(II) and those cations already considered in previous papers on reversed-phase paper chromatography with HDEHP there are not many data on liquid–liquid extraction with HDEHP available in the literature for those cations listed in Table III, though, as already mentioned, a good survey on the behaviour of almost all cations was carried out by KIMURA[7]. However, if the general trend of the different cations, as shown in the previous section, agrees with KIMURA's results, the same cannot be said about the values of coefficients b and c in eqn. (4). The reason for this disagreement may be due to two important factors, which are now considered.

Firstly, in KIMURA's work the determination of the dependence of the extraction coefficients on the concentration of hydrochloric acid was carried out with a 50% HDEHP solution in toluene, this condition being appreciably different from ours. Secondly, consideration also has to be given to the fact that the reversed-phase chromatography gives R_F values differing from zero in a range of conditions in which liquid–liquid extraction gives extraction coefficients which are very low with respect to the ones usually considered: thus, the mechanism of extraction may be appreciably different in the two cases.

Both reasons reported above are quite obvious: nevertheless the discussion on the extraction of uranium(VI) with HDEHP from various acidic media, reported below in the paragraph dealing with uranium, may be a good illustration of them. A feature which further confirms this point is the fact that the disagreement is particularly found with ions which do not follow reaction (2), *i.e.* they are involved in the extraction mechanism as complexes with Cl^- or as partially hydrolyzed ions. It is obvious that ions which undergo these phenomena will behave in quite a different way at two different HCl concentrations.

Alkali and alkaline earth metals

Alkali metals run generally with the front of the eluent; when eluted with very dilute HCl, they show a slight retention by the paper (R_F values about 0.7), which probably has to be ascribed to a partition phenomenon in which little role is played by HDEHP.

A discussion on the behaviour of alkaline earths has been already reported in previous work[4].

Scandium, yttrium and the lanthanides

Since scandium is strongly retained by HDEHP at any HCl concentrations, no results on the mechanism of its extraction can be derived from reversed-phase chromatographic data.

The behaviour of yttrium in liquid–liquid extraction systems involving HDEHP has been often reported in the literature[1,10,11], where the value 3 is given for both the coefficients b and c in eqn. (4). Its behaviour in reversed-phase chromatography is now discussed together with the behaviour of the rare earths.

Because of their importance in the nuclear field, the rare earths deserve a particular consideration. It has already been demonstrated also in this laboratory that reversed-phase chromatography on supports impregnated with HDEHP is a powerful means of separation of the rare earths. In this work, it is intended to complete the information about their behaviour on paper treated with HDEHP when eluted with HCl, and at the same time to rectify a mistake that unfortunately occurred during the compilation of a previous paper[6].

Fig. 7 shows the behaviour of the rare earths in the form of R_F values plotted against the logarithm of the hydrochloric acid concentration of the eluent. It can be seen how their behaviour (as their atomic weight varies), is consistent with the one expected, and how yttrium falls between holmium and erbium following its usual peculiar behaviour towards rare earths.

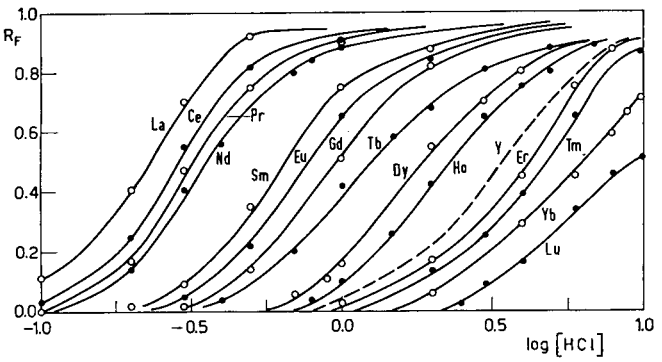


Fig. 7. R_F values for rare earths plotted *vs.* log M HCl. Paper treated with 0.100 M HDEHP. Yttrium behaviour is included.

In plotting these results, it was noticed that the curve referring to dysprosium, as reported in the previous publication[6], was not consistent with the curves referring to the rare earths immediately before and after it. Reinvestigation permitted us to conclude that most probably in the earlier work yttrium instead of dysprosium was used.

Fig. 8 shows the plots of log $(1/R_F - 1)$ against the atomic number Z of the rare earths. According to results in the literature[1,6], parallel straight lines are obtained showing that E_a° is a linear function of the atomic number. From the figure, the average separation factor between two adjacent rare earths is estimated as 1.9.

In order to check the mechanism responsible for their retention, log $(1/R_F - 1)$ has been plotted against the logarithm of the hydrogen ion activity in the eluent,

and results are shown in Fig. 9. Fig. 10 shows some plots of log $(1/R_F - 1)$ against the effective HDEHP concentration on paper; these are from R_F data obtained from simultaneous elution of elements on paper treated with HDEHP at different concentrations in cyclohexane. While all the rare earths are considered in Fig. 9, the plots in Fig. 10 refer only to Sm, Tb, Ho and Yb, the other rare earths behaving in a similar way.

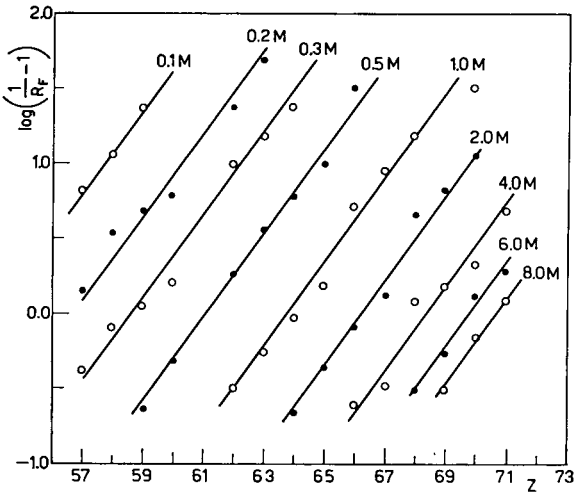


Fig. 8. Plots of log $(1/R_F - 1)$ *vs.* atomic number Z for rare earths. Paper treated with 0.100 M HDEHP, elution with HCl at the indicated molarities. Average separation factor: $(1/R_F-1)_{z+1}/(1/R_F-1)_z = 1.9$.

The main feature arising from Fig. 9 is the fact that log $(1/R_F - 1)$ against log a_{H^+} plots do not follow straight lines of slope —3 as would be expected from the ion exchange mechanism. They tend rather to follow curves which may be approximated to lines of slope —3 only in their upper part. In particular, it will be noticed that this disagreement with the usual ion exchange mechanism predictions is greater the greater the atomic number of the rare earths, that is the higher the hydrochloric acid concentration range involved.

Fig. 10 shows how the slopes of the curves obtained by plotting log $(1/R_F - 1)$ against the logarithm of the effective HDEHP concentration on the paper vary when the eluent concentration is varied. As a general rule, the slope 3 (as expected from the ion exchange mechanism) is only obtained when the hydrogen ion activity of the eluent is kept within the range where the slope is —3 in Fig. 9. When the H^+ ion activity of the eluent is higher, slopes lower than 3 are obtained, reaching values as low as 1 or even less.

These results are in agreement with the results reported in the literature, although this may not become apparent at first sight since the extraction coefficients were usually plotted against the stoichiometric H^+ concentration in the aqueous layer and not against the H^+ activity which we have done, owing to the high HCl concentrations involved.

Liquid–liquid extraction of the rare earths by HDEHP from HCl, HNO_3, H_2SO_4 and $HClO_4$ was first reported by PEPPARD *et al.*[1,10]. Low acid concentrations

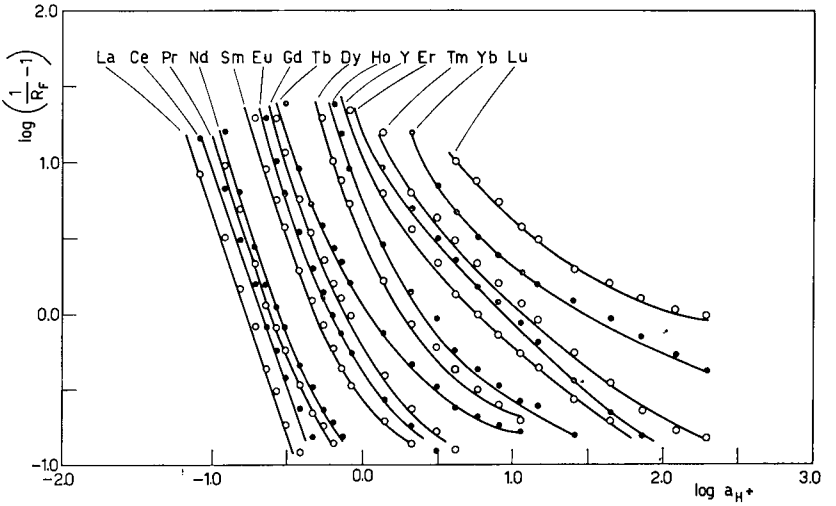


Fig. 9. Plots of log $(1/R_F - 1)$ *vs.* log a_{H+} for rare earths and yttrium. Paper treated with 0.100 *M* HDEHP. Slope of straight lines is −3.

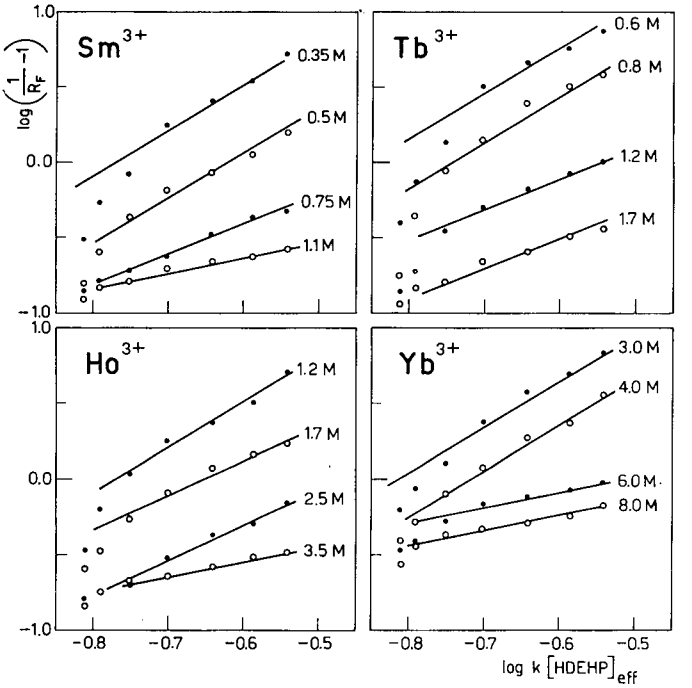


Fig. 10. Plots of log $(1/R_F - 1)$ *vs.* log $k[\text{HDEHP}]_{\text{eff}}$ for samarium, terbium, holmium, and ytterbium at various HCl concentrations of the eluent. Data are obtained from simultaneous elutions with paper treated with HDEHP at different concentrations.

were used and the expected value of 3 for both coefficients b and c in eqn. (4) were found.

On the other hand, PIERCE AND PECK[11] and PIERCE, PECK AND HOBBS[12] report both liquid–liquid extraction and reversed-phase partition chromatography of rare earths on columns with impregnated inert supports and with HDEHP as the extractant; aqueous solutions were in $HClO_4$ or HCl, whose concentrations were rather high. The logarithm of the extraction coefficients in liquid–liquid extraction, and the logarithm of $(1/R_F - 1)$ in reversed-phase chromatography were plotted by these authors against the logarithm of the hydrogen ion concentration of the eluent, and straight lines were obtained. By substituting the H^+ activity for the H^+ concentration, curves similar to those in Fig. 9 can be obtained.

Results of reversed-phase chromatography on paper treated with di-*n*-butyl phosphate (a compound very similar to HDEHP), published by CVJETIČANIN[13], after making the above mentioned substitution, also agree with our results. In this latter work, furthermore, the reported values of coefficients c for some rare earths eluted with 4 M HCl are lower than the values obtained for other rare earths eluted with the less concentrated 0.3 M HCl, the other experimental conditions being the same.

A value of 2.5 has been recently reported by CHENG *et al.*[14] as the dependence of the elution volume on the H^+ activity in elutions of rare earths on HDEHP columns with HCl concentration in the eluent ranging from 0.2 to 0.3 M.

We feel that the disagreement between the values of b and c of eqn. (2) expected from the simple ion exchange mechanism, and the results presented here, can be explained by the dehydration effect already mentioned for the elements whose R_F decreases as the HCl concentration increases in the high concentration range. For a detailed discussion of this phenomenon we defer to future work[9].

Thorium and uranium

No comments can be made on the extraction mechanism of thorium(IV), since it is strongly retained by HDEHP at all the HCl concentrations.

As already mentioned, experiments carried out with uranium (VI) on paper treated with HDEHP solutions having concentrations varying from 0.100 to 0.010 M are shown in Fig. 2, and Fig. 5 shows the plots of log $(1/R_F - 1)$ *vs.* log a_{H^+} from which the coefficient b in eqn. (4) may be obtained.

U(VI) behaves unusually when paper treated with 0.010 M HDEHP is used. This behaviour may be attributed to an insufficient loading of the paper when treated with such dilute HDEHP solutions. Insufficient loading is confirmed also when the results obtained for most cations are correlated with the HDEHP concentration on paper; in Fig. 6, for example, the values of log $(1/R_F - 1)$ for very low HDEHP concentrations are lower than would be expected from the results at higher concentrations of the extractant.

The behaviour of U(VI) in liquid–liquid extraction with HDEHP has been frequently described in the literature: a brief survey of the reported data will confirm the importance of both the hydrochloric acid and the HDEHP concentration ranges in which experiments are carried out, and their effect on the mechanism of extraction for a given ion, thus confirming the reasons we stated above for the discrepancies between KIMURA's extraction data and our results.

Most of the work on the extraction of uranium(VI) by HDEHP has been done with dilute or moderately concentrated acid solutions (see for example the papers by BAES or PEPPARD, and their co-workers[15–17]): all the results obtained led to the conclusion that the reaction responsible for the extraction of the uranium was:

$$UO_2^{2+} + 2\,(HDEHP)_2 \rightleftharpoons UO_2\,(DEHP)_2 \cdot 2\,HDEHP + 2\,H^+ \qquad (5)$$

i.e. coefficients b and c in eqn. (4) are both equal to 2. Perchloric, sulphuric, nitric, and hydrochloric acids have been used, and the reaction just mentioned was true for all of them.

More recently, ASANO *et al.*[18] have published some results obtained with the above mentioned acids used up to very high concentrations in the aqueous phase. It appeared that the reaction represented by eqn. (5) holds only if the H^+ activity is kept below the value 1; with higher H^+ activities, the behaviour of the distribution coefficients is highly dependent on the nature of the acid used: for HCl, in particular, a very much smaller slope than that of —2, which holds at the lower concentrations, is obtained, having a value of about —0.1. Similar abnormal behaviour with concentrated HNO_3 was also reported by GUREEV *et al.*[19]. Very recently, a paper by SATO[20] reported E_a° values for uranium(VI) extracted with HDEHP, at various low concentrations, from HCl solutions which ranged up to high concentrations. By plotting log E_a° *vs.* log a_{H^+}, straight lines can be obtained very similar to those shown in Fig. 5, having slopes —2 and —1, the slope of —2 holding at the lower HCl concentration range for the highest HDEHP concentrations.

With respect to the influence of the HDEHP concentration on the extraction mechanism of U(VI), a recent paper by LASKORIN AND SMIRNOV[21] deals with the liquid–liquid extraction of uranium(VI) from phosphoric acid solutions with HDEHP. In this work, the value 2 for the coefficient c is reported when the HDEHP concentration is about 0.1 M, while a value of 4 for the same coefficient is reported with a more dilute (lower than 0.01 M) HDEHP solution.

Titanium, zirconium and hafnium

R_F values equal to zero were found for these three ions for all HCl concentrations. No information on their extraction mechanism is therefore obtainable.

Vanadium and niobium

Values for coefficients b and c in the case of vanadium(IV) may be obtained from the papers of KIMURA[7] ($b = 4$; $c = 1.5$), BLAKE *et al.*[15] ($b = 4$; $c = 2$), and LASKORIN *et al.*[22] ($b = 2$; $c = 2$). The values $b = 3$ and $c = 4$ were obtained from our data. The different experimental conditions are probably responsible for the discrepancies.

No literature data have been found for vanadium(III). Its behaviour in reversed-phase chromatography is very similar to that shown by vanadium(IV).

The slight retention of niobium(V) can probably be ascribed to the partition phenomena on paper already mentioned for alkali ions.

Chromium, molybdenum and tungsten

Chromium(III) runs with the front of the eluent; only at very high HCl con-

centrations does it show a slight retention due to the already mentioned dehydration phenomena.

It was not possible to find a detection reagent capable of revealing the spots of chromate at such low amounts as those dealt with. Therefore its behaviour was not recorded.

Molybdenum(VI) and tungsten(VI) behave similarly: only highly concentrated HCl solutions were capable of displacing them a little, in accordance with the results reported by LASKORIN and coworkers[23] for molybdenum, in liquid–liquid extraction systems. An attempt was made to obtain the values of the coefficients b and c in eqn. (4) from the R_F values, which never exceed 0.40. The results showed that the same mechanism is responsible for the extraction of both ions, but it is not, however, represented by reaction (2).

Manganese and rhenium

The values of 1.5 and 4 for coefficients b and c are found for manganese(II). Partial hydrolysis may be responsible for this behaviour.

Rhenium(VII) shows high R_F values. Its slight retention could be ascribed to the partition mechanism on paper mentioned for alkali ions.

Iron, cobalt and nickel

A discussion on the behaviour of the transition metals of the iron group has been already published[2]. Worth mentioning here is a peculiarity of nickel and cobalt ions, which sometimes showed a double spot in the HCl concentration range from $1 \cdot 10^{-4}$ to $3 \cdot 10^{-2}$ M, when paper had been treated with 0.1 M HDEHP. One spot, as expected, ran with the front of the eluent, while the second, which appeared in some cases, had a lower R_F value, which decreased the lower the HCl concentration. This spot was only detected in some of the chromatograms out of a series of several similar ones simultaneously eluted under the same conditions. No explanation was found for this behaviour. When using paper treated with more dilute HDEHP solutions (from 0.075 M down), only the spot at the front of the eluent was detected.

A value of 1.5 for the coefficient b was obtained from the retained spots.

The behaviour of cobalt and nickel in liquid–liquid systems has been studied in a sulphate medium by MADIGAN[24], who reported the coefficient b to be lower than 2, in accordance with our results. Only nickel(II) has a value for coefficient b lower than 2 in KIMURA'S data.

The strong dehydration effect on iron(III) is noticeable; another peculiarity of the iron(III) ion is the already reported[2] dependence of its R_F values on the elution time, due to the slow rate to equilibrium of the exchange reaction responsible for its retention.

Ruthenium, rhodium and palladium

Ruthenium(III), rhodium(III) and palladium(II) always run with the front of the eluent: they are partially retained in the high HCl concentration range because of dehydration effects.

Osmium, iridium and platinum

Osmium is not reported since no suitable developing reagent was found to detect it on the paper.

Iridium(IV) and platinum(IV) ions run with the front of the eluent, being somewhat retained in the high HCl concentration range because of the dehydration effect.

Copper, silver and gold

Copper(II) shows a second faint spot at the front of the eluent in the range of HCl concentrations in which it is normally retained by the stationary phase. This spot appears with paper treated not only with 0.1 *M* HDEHP, as for cobalt and nickel, but also with more dilute solutions. The presence of monovalent copper as an impurity may be an explanation of the spot at the front.

The value of coefficient *b* for copper is 1.5, in accordance with a value lower than 2 reported by MADIGAN[24] in the extraction of this ion with HDEHP from sulphate solutions. KIMURA's value is 2.

Silver(I) gave tailed spots when low R_F values were obtained. In this range of acidity a slope of −2 was obtained from the plot log $(1/R_F - 1)$ *vs.* log a_{H^+} in Fig. 3. An explanation of both these facts may be incomplete dissolution of the silver salt at insufficiently high HCl concentrations.

Gold(III) has R_F values of around 0.6 in the whole HCl concentration range explored, up to about 3 *M* HCl. With HCl concentrations higher than 3 *M*, two spots, having different colours, are obtained, the former soon reaching a zero R_F value, the latter being less retained. The retention at low HCl concentrations may be ascribed to the partition phenomena already mentioned, for example, for alkali metals, while the doubling of the spots at the high HCl concentrations will be dealt with in another paper[9], where the peculiar lowering of the R_F values for most elements, when very concentrated hydrochloric acid is used as the eluent, will be discussed.

Zinc, cadmium and mercury

Zinc(II) and cadmium(II) are the first ions considered in this discussion which have values for the coefficient *b* which are in agreement with a mechanism involving the eqn. (2). The same value of 2 is reported for coefficient *b* of both these ions by LEVIN AND SABOLOSKI[25], for extractions from H_2SO_4 solutions.

Mercury(II) runs with the front of the eluent for the whole HCl concentration range explored.

Boron, aluminium, gallium, indium and thallium

Boron(III) is not retained by HDEHP at any concentrations of HCl in the eluent.

The behaviour of aluminium(III), gallium(III), indium(III) and thallium(III) has already been discussed[2]. It is worthwhile pointing out how gallium shows a greater lowering of R_F values, due to the dehydration effect, than all the other cations considered. The R_F value of thallium also considerably decreases in the high HCl concentration range. On the other hand, a partition phenomenon similar to that reported for alkali ions is thought to be responsible for the slight retention ($R_F \simeq 0.8$) shown by the latter ion in the whole HCl concentration range explored.

Although aluminium is reported[15] to have a slow exchange reaction, no dependence of its R_F values on the elution time was noticed.

The peculiar behaviour of the above mentioned ions makes it possible to obtain many interesting separations of them; this has already been shown in a previous paper[26].

Germanium, tin and lead

Germanium(IV) was always found near the front of the eluent.

Tin(II) gave tailed spots in the whole range in which R_F values different from zero were obtained. Nevertheless both tin(II) and lead(II) show values for the coefficient b in eqn. (3) in accordance with the reaction (2).

Arsenic, antimony and bismuth

Both arsenic(III) and arsenic(V) ions run with the front of the eluent, arsenic(III) being slightly retained by a partition phenomenon in which little role is played by the extractant present on the paper.

The behaviour of antimony(V) does not follow reaction (2); furthermore, at the high HCl concentration values, coefficient c becomes zero, *i.e.* it is independent of the HDEHP concentration on the paper. A mechanism differing from ion exchange has to be postulated.

The value of the coefficient b for bismuth(III) is in accordance with the above mentioned reaction. In conclusion, the only ions which can be considered to exhibit such behaviour are zinc(II), cadmium(II), tin(II), lead(II), and bismuth(III), together with the already reported strontium(II) and barium(II).

Selenium and tellurium

Both selenium(IV) and tellurium(IV) run with the front of the eluent. They show, however, a different behaviour in the high HCl concentration range, when tellurium is retained more than selenium. Also a slight retention ($R_F \simeq 0.8$) is shown by tellurium over the whole range considered, probably due to the partition effect already mentioned in the case of alkali metals.

CONCLUSIONS

The R_F data reported in Figs. 1, 2 and 7 are sufficient to suggest many interesting separations of ions by elution with hydrochloric acid of a suitable concentration on paper treated with HDEHP. Many examples of such separations have already been reported[2,4,6,26].

The data are also useful to predict separations of mixtures of ions by eluting them on columns prepared with inert supports treated with HDEHP: the higher capacity of the columns allows the separation of relatively large amounts of substances.

Sometimes, however, especially when very concentrated eluting solutions are used, the results obtained with paper and with these columns do not coincide, since the supports used for the column do not always behave as really inert ones: columns of treated cellulose powder are naturally the ones whose behaviour is closest to that of the paper sheets.

The reversed-phase paper chromatographic technique may also give useful information which will help to elucidate both the mechanism of retention on paper or columns and the mechanism of extraction in the liquid–liquid systems.

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SUMMARY

The behaviour of 67 cations in reversed-phase partition chromatography on paper treated with di-(2-ethylhexyl) orthophosphoric acid was investigated. Hydrochloric acid was used as the eluent in a concentration range of 10 M to $1 \cdot 10^{-4}$ M. For 48 cations the quantity $(1/R_F - 1)$ was related to the hydrogen ions activity in the mobile phase and to the effective HDEHP concentration on the paper.

The chromatographic behaviour was correlated to that in analogous liquid–liquid extraction systems.

Particular attention was devoted to U(VI) and to the rare earths group of metals.

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Notes

Adsorption of polar substances on the solid support of column packings in the gas–liquid chromatographic analysis of air contaminants*

Many air contaminants can be sampled efficiently with the sixfold absorber developed by BERGSHOEFF[1]. If for instance 50 l of air containing 80 mg benzene per m^3 are drawn through 100 ml of liquid the resulting solution contains 40 p.p.m. of benzene.

On analyzing such test solutions gas chromatographically we found that polar compounds practically disappeared due to adsorption on the silanised support coated with a nonpolar liquid phase. We believe this phenomenon may be useful in analyzing air contamination.

Experimental

Test solutions, with propanol-2 (I), hexane (II) and decane (III) as absorption liquids, each containing 600 p.p.m. of benzene, *m*-xylene, isobutyl acetate and 1400 p.p.m. of butanol-2, were made up. These "concentrated" solutions, designated I. 1, II. 1 and III. 1, were also diluted twenty times. The "concentrated" and "diluted" designated I. 2, II. 2 and III. 2 solutions were analyzed gas chromatographically.

For the GLC analysis we used an Aerograph model 1520 with a flame ionization detector and a set of copper columns (length 5 m, I.D. 0.065 in.) packed with 20 % Apiezon L on commercial silanised Chromosorb W, 80–100 mesh.

GLC conditions. Test solutions I. 1 and I. 2: Sample 0.3 μl; R. 1; Att. 16, 8, 4, 2 and 1 (dependent on the tailing of propanol-2); column temp. 101°; injector and detector 180°; carrier gas (N_2) and hydrogen 25 ml/min.

Test solutions II. 1 and II. 2: sample 2.1 μl; R. 1; Att. 8 and 2; column temp. 102°; injector 155° and detector 175°; carrier gas (N_2) and hydrogen 25 ml/min.

Test solutions III.1 and III.2; sample 1.7 μl; R.1; Att. 1; column temp. 15 min at 100°, 15 min programming at a rate of 6°/min, 15 min at 190°; injector and detector 190°; carrier gas (N_2), analytical column 20, reference column 25 ml/min; hydrogen 25 ml/min.

Results

The results of the GLC analysis of the various test solutions are given in Table I.

Discussion

From the results it can been seen that very small quantities of polar components are not detected with the nonpolar columns used. At first we supposed this was caused by decomposition in the injector. However, no peaks on the chromatograms originating from decomposition products (*e.g.* butene) were seen. We now believe that small

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TABLE I

RESULTS OF THE GLC ANALYSIS OF THE TEST RESOLUTIONS ON THE NON-POLAR COLUMN

Test solution		*Response**		
		Aromatics	*Ester*	*Alcohol*
Propanol-2 solutions	I.1	++	+	+
	I.2	++	—	—
Hexane solutions	II.1	++	+	x
	II.2	++	—	x
Decane solutions	III.1	++	+	—
	III.2	++	—	—

* ++ = Positive response; + = positive response, much tailing; — = no response; x = retention time equal to that of hexane.

quantities of polar compounds are adsorbed on the solid support, which is insufficiently deactivated for these traces.

KUSÝ[2] discusses the adsorption of polar substances on the solid support in a study concerning the accuracy of quantitative GLC. We believe that the detection of butanol-2 present in test solution (I.1) is caused by a deactivation of the solid support by the absorption liquid propanol-2. The detection of the ester and not the alcohol in the "concentrated" solutions (*e.g.* III.1) will be caused by a stronger adsorption of the alcohol through hydrogen bonding.

To see if there is any adsorption of small quantities of polar substances on the solid support when using a polar liquid phase, a test solution containing 60 p.p.m. butanol-2 in cyclohexanol was chromatographed on the Aerograph 1520 with a flame ionization detector. The appearance of a butanol-2 peak without tailing indicates that there is no adsorption in this case. (A set of copper columns was used, length 3 m, I.D. 0.065 in., packed with 10% Carbowax 1500 on commercial silanised Chromosorb W, 60–80 mesh. GLC conditions: sample 1 μl; R.1; Att. 4; temperature 10 min at 100°, 20 min programming at a rate of 6°/min; injector and detector 190°; carrier gas (N_2), analytical column 20, reference column 25 ml/min, hydrogen 25 ml/min).

It is concluded that the phenomenon of the adsorption of polar substances on the solid support of nonpolar columns may be used in the qualitative analysis of air contaminants. When this analysis is performed on both polar and nonpolar packed columns, then, very probably, polar substances will not be detected on nonpolar packed columns.

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An improved pyrolytic device suitable for the study of polymer microstructure by pyrolysis gas chromatography

A pyrolytic device for investigation of polymer microstructure and for studying the thermal decomposition mechanism by pyrolysis gas chromatography has to meet very severe requirements with regard to temperature control, the mode of sample heating and the determination of the quantity of the specimen.

In addition it is necessary to remove any residue after pyrolysis in order to keep the pyrolytic system completely clean. This is particularly important for polymers with a whole series of decomposition products such as polyolefins.

In view of the above requirements it is now well known that the pyrolytic furnace has great advantages over the filament type pyrolyzer[1–5]. In order to maintain high reproducibility of experimental conditions in investigation of the microstructure of polyolefins we have designed a pyrolytic furnace with the following features:

samples can be pyrolyzed at any temperature up to 1000°;

the temperature of thermal decomposition can be maintained within the close range of $\pm 0.5°$;

the pyrolysis temperature is measured near to the sample;

the sample is quickly inserted in the hot zone of the pyrolyzer (for 1 sec) and always at exactly the same spot;

the heating-up of the sample is instantaneous;

the pyrolysis time can be controlled accurately;

the samples to be examined are taken in their natural form (granule, film, powder, liquid);

the amount of sample is accurately known;

there is no need to interrupt the carrier gas flow through the system during loading and unloading of the samples;

the fractions of the pyrolyzate can be controlled by selection prior to their admission into the chromatographic column by putting a short precut column between the pyrolytic furnace and the chromatograph;

the residue of the sample after pyrolysis can be measured (by quantity) and easily removed;

the cleansing of the system, from the heavier fractions encountered, is very simple and thorough;

the reproducibility of data is high.

Descriptions of design

The pyrolytic device is shown in Fig. 1. The thermal decomposition of the sample is achieved in the hot zone of a quartz tube (1) which is provided with an electrical heater (2). The sample boat (3) is fixed on to a thin rod on a pusher (5), which enables the sample to be inserted or withdrawn from the furnace. The quartz tube is 200 mm long and 14 mm in diameter, but at the inlet end, in order to allow connections to the metal block (4), the diameter is increased to 20 mm. In addition to the pusher orifice, the block has connections for the carrier gas (6), the thermocouple (7), as well as the teflon tap (8), which is used for opening and closing the furnace.

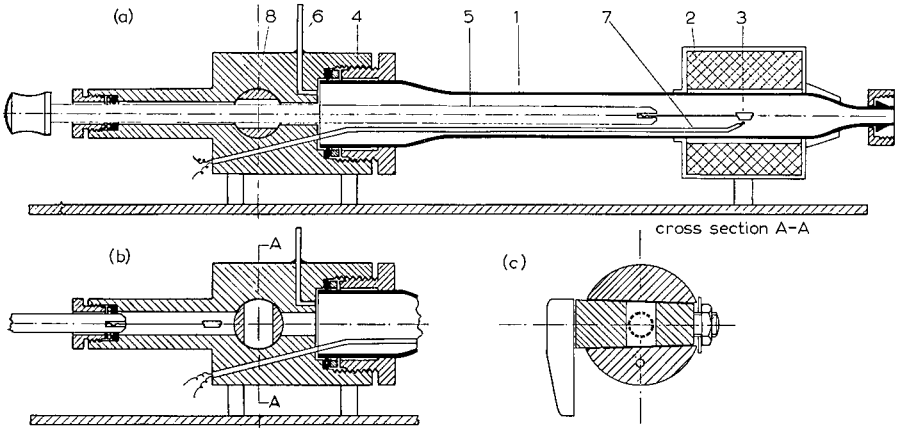

Fig. 1. Diagram of the pyrolytic device. (a) Complete device in operating position; (b) the connecting block with the tap closed; (c) detail of the teflon tap. For explanation of figures, see text.

A heater and ceramic isolator surround the tube along 7 cm of its length The temperature of the furnace is controlled by a 3 A Variac. In order to control the pyrolysis temperature to within such a narrow range as $\pm 0.5°$, the electric current is stabilized and the heater is properly isolated.

It is also possible to connect the pyrolyzer directly to the injection port in order to prevent the heaviest fractions from entering the chromatographic column.

The method of connecting the quartz tube and the metal fittings, as well as other connections, is shown in Figs. 1a and b. The small platinum boat (3×5 mm) is easily attached to the pusher by inserting the platinum wire into the slot on the pusher.

Procedure

The pusher and boat, with a measured amount of sample (1–5 mg), are inserted in the opening of the block, and the pusher is pushed up to the mark on the rod, in other words to the point where the boat reaches the teflon tap. Then the tap is opened and the boat pushed into the cold zone of the quartz tube. After conditioning the sample in the stream of carrier gas, it is pushed into the hot zone of the pyrolyzer. The position of the boat in the furnace is shown in Fig. 1a. The sample can be left in the furnace as long as desirable.

The withdrawal of the boat from the apparatus consists of a single movement. When the boat is in the position as shown in Fig. 1b, the teflon tap is closed and the boat can be taken out from the device. The boat can be weighed and cleaned by burning, if necessary. The tube outlet is cleaned by burning off the residue in an air stream whilst firing the quartz tube with a burner.

Discussion of results and advantages of the improved device

A check on the reproducibility of the pyrolyzer described, connected to a Perkin-Elmer chromatograph Model 800, gave very good results. The samples to be pyrolyzed are weighed on a semimicrobalance with an accuracy of ± 0.02 mg.

Quantitative evaluation of pyrograms by peak height measurements shows a

reproducibility with a standard deviation of 1.7 or, as a percentage of the mean, 2.0 %. The value obtained is well within the accuracy limits gained with the peak height measurement technique[6,7].

The high reproducibility obtained is the result of the operating conditions, such as temperature control, the rate of heating, the method of sample insertion and the maintenance of a thoroughly clean system as described, and thus meets the principal requirements for the determination of polymer microstructure. The results of polymer microstructure investigation will be described in another paper.

Additional advantages of this device, *viz.*, the feasibility of pyrolysis at a desired temperature, accurate measurement of the decomposition temperature, quick transport of pyrolyzate and the possibility of quantitative analysis, fulfil the requirements for the study of a thermal decomposition mechanism.

The continuous flow of carrier gas is ensured in a new, suitable way. The carrier gas passes continuously through the pyrolyzer without any changes in flow rate while the new sample is being inserted. Constant temperature can be maintained over a long period of time without any changes. The four-way valve system which is generally used and is a source of contamination with heavier fractions of the pyrolysate is avoided.

The simple and cheap performance of device, as well as adaptability to practically any chromatograph are additional advantages of the improved pyrolyzer.

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A new method for the gas-chromatographic analysis of the urinary 11-deoxy-17-ketosteroids

The past few years have seen marked progress in the gas-chromatographic analysis of urinary 17-ketosteroids[1–8]. This is probably due to the greater selectivity and speed of this method in comparison with other systems of analysis. Owing to the extreme sensitivity of the method, however, rigorous purification is essential during the extraction from urines, with the result that the time required for the examination is increased.

The object of the present work was to devise a method for the determination of urinary 17-ketosteroids, which would exhibit the advantages of speed and simplicity normally associated with gas chromatography, while at the same time avoiding the need for laborious preliminary purification.

The urinary catabolites examined were confined to androsterone, etiocholanolone and dehydroepiandrosterone; the reasons for this were partly technical, *i.e.* the possibility of obtaining optimum separation of a few steroids in the central part of the chromatogram, and partly clinical, *i.e.* the fact that information about the rates of elimination of these three steroids is sufficient in most cases to act as a guide to diagnosis.

Extraction

50 ml of urine acidified to pH 4.5 with acetic acid and buffered with acetate buffer are subjected to enzymatic hydrolysis by *Helix pomatia* (1,500 U of β-glycuronidases and 12,000 U of sulfatases per ml of urine) for 24 h at 38°. The sample is then extracted with 50 ml and 2 × 25 ml of diethyl ether, and the extract is washed with 2 × 25 ml of *N* NaOH and with 2 × 25 ml of distilled water. The ether extract is dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum. The dry extract is then taken up in 2 × 3 ml of acetone in Teflon containers, and the solvent is evaporated off in a current of nitrogen.

Preparation of trimethylsilyl (TMS) derivatives

1 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane are added to the extract, and the container is closed with a Teflon stopper and kept at 60° for 1 h. The solvents are evaporated off in a current of nitrogen, and the extract is redissolved and centrifuged at 3,000 r.p.m. for 5 min. The supernatant liquid is decanted into another container and evaporated to dryness.

Gas-chromatographic analysis

The gas-chromatographic analysis is carried out by means of a Fractovap Model C* chromatograph with an AID/f analytical unit having a flame-ionization detector.

The chromatographic column is made of glass, and is in the form of a spiral: I.D. 2 mm, length 2 m; packing: 1 % QF-1 on silanized Gas-Chrom P, 100–120 mesh. The conditions for analysis are: column temperature 190°, evaporator temperature 260°, flow rate of N_2 25 ml/min, H_2 0.3 kg/cm^2 and air 1.2 kg/cm^2.

* C. Erba S.p.A.

Injection of the extract

1 μl of a 0.5–2 μg/μl solution of the TMS derivatives in hexane is introduced with a Hamilton microsyringe, the attenuation values being stabilized at 10 $\times$ 8.

Quantitative estimation

The areas of the peaks are compared with those of the corresponding standards, which are introduced at a concentration of 1 μg/μl for each steroid. The values obtained in this way are converted into mg/24 h with the aid of the values for the dilution of the extract and for the diuresis.

Results

The resolution of the three standards introduced is shown in Fig. 1, while Figs. 2 and 3 show an example of the gas-chromatographic analysis of an urinary extract. The retention times listed in Table I are taken from the first line of the solvent peak to the maximum of the peak in question.

TABLE I

RETENTION TIMES (IN MINUTES) OF URINARY 17-KETOSTEROIDS

Steroid	*Retention time (min)*
Andro TMS	7.63
Etio TMS	8.82
DHEA TMS	10.39

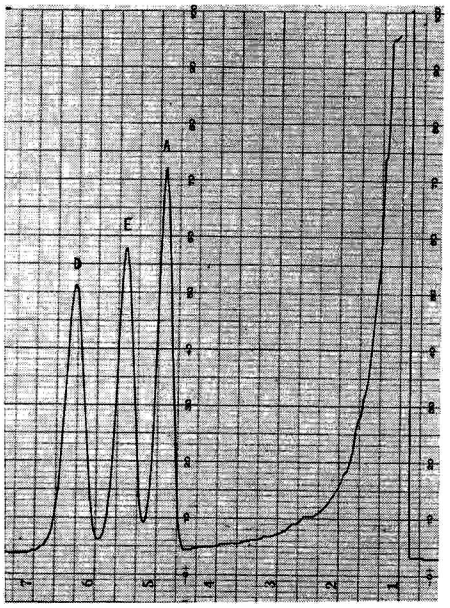
The results of recovery tests carried out after the addition of known quantities of the three steroids to urine were subjected to a statistical analysis, the results of which are shown in Table II.

The time required for the quantitative determination even of several samples from the hydrolysate is about 3 h.

Discussion

It should first be pointed out that for various reasons, considerable difficulty has been encountered in the use of gas-chromatographic methods for the analysis of the urinary 17-ketosteroids. In the first place, it was necessary to eliminate the non-steroid lipids before the extract was introduced into the apparatus; in the best-known of these methods[6], this was achieved by a preliminary separation by thin-layer chromatography, with the result that the time required for the examination was considerably increased. If, on the other hand, this preliminary purification is omitted, a satisfactory separation of the three principal 17-ketosteroids is impossible owing to the presence of interfering peaks[1]. Moreover, it is only recently that the difficulty of obtaining good discrimination between the three 17-ketosteroids by gas chromatography has been partially overcome by the conversion of the steroids into their trimethylsilyl ethers and by the use of highly polar columns.

In our method, the enzymatic hydrolysis gives a less pigmented urinary extract in comparison with acid hydrolysis, while the transfer of the urinary extract into



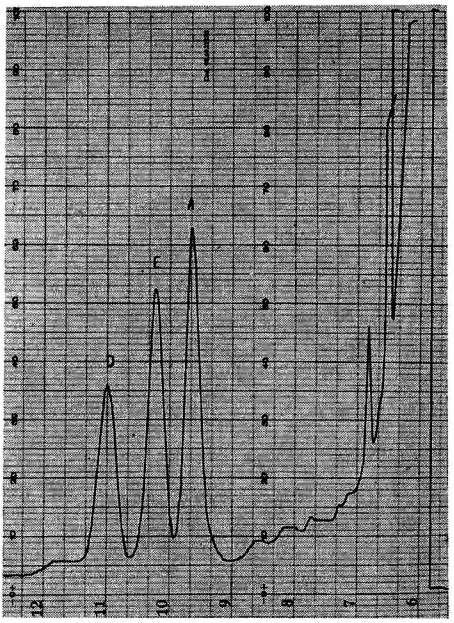


Fig. 1. Analysis of a mixture of androsterone, etiocholanolone, and dehydroepiandrosterone as their TMS derivatives at a concentration of 1 $\mu g/\mu l$. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

Fig. 2. Analysis of a sample of male urine. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

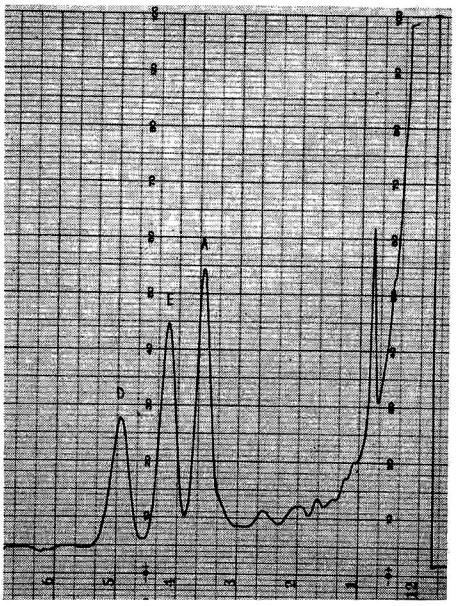


Fig. 3. Analysis of a sample of male urine. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

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TABLE II

RECOVERY OF THE URINARY 17-KETOSTEROIDS

A = androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

Steroids μg/50 ml of urine							*Mean*	σ	*Amount recovered*	%
Basic	A	127.15	125.98	129.67	130.29	135.47	129.71	3.60	—	—
	E	96.69	104.08	94.19	126.73	120.09	108.35	14.38	—	—
	D	41.20	48.03	45.21	54.00	61.53	49.99	7.93	—	—
+ 50 μg of each	A	169.18	169.85	176.89	175.19	176.73	173.56	3.76	43.85	87.70 ± 14
	E	161.64	159.89	157.06	174.72	171.31	164.92	7.66	56.57	113.14 ± 30
	D	94.19	94.02	84.77	97.95	105.49	95.28	7.48	45.29	90.58 ± 29
+ 100 μg of each	A	228.57	218.52	224.48	244.89	230.61	229.41	9.79	99.70	99.70 ± 19
	E	200.00	210.98	189.01	215.38	191.20	201.31	11.66	92.96	92.96 ± 23
	D	149.76	150.70	142.46	149.45	149.45	148.36	3.31	98.37	98.37 ± 7
+ 200 μg of each	A	365.38	366.95	343.79	329.67	334.37	348.03	17.32	218.31	109.15 ± 17
	E	304.94	291.99	326.92	298.90	307.69	306.08	13.11	197.73	98.86 ± 13
	D	222.91	241.83	251.17	238.61	236.81	238.26	10.19	188.27	94.13 ± 10

the Teflon containers by means of acetone before the formation of the TMS derivative subsequently eliminates many of the pigments without interfering with the recovery of the 17-ketosteroids.

Still in connection with technical expedients, it should be noted that the fact that the detector system is kept at high sensitivity permits the use of very small quantities of solvent, which would otherwise interfere with the detection of the steroid peaks.

The results of the recovery tests reported in Table II provide sufficient evidence of the accuracy of the method, even when compared with the results obtained by much more time-consuming methods involving preliminary purification on a thin layer[6] (Table III), and they appear better than the results obtained by other methods that omit this preliminary treatment of the extract.

TABLE III

COMPARISON OF THE CONCENTRATIONS FOUND BY US IN A POOL OF MALE URINE (A) AND THOSE REPORTED BY KIRSCHNER AND LIPSETT[6] (B)

	A	*B*
Andro	2.59 mg/l	2.40 mg/24 h
Etio	2.16 mg/l	2.18 mg/24 h
DHEA	0.99 mg/l	0.50 mg/24 h

In conclusion, owing to the decrease in the time required, the satisfactory recovery, and the clarity of the gas chromatogram reading, our method confirms the possibility of developing and using gas chromatography for the analysis of steroids, and at the same time exhibits the characteristics of simplicity, accuracy, and specificity that are demanded for clinical examinations.

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* Director: Prof. A. BOMPIANI.

Anomalous response of a helium ionization detector

It is generally accepted that the correct functioning of a helium ionization detector, as used in gas chromatography, is dependent upon a reduction of the impurity content of the helium carrier gas to a very low figure[1,2] and furthermore, that a criterion of optimum performance is the lowest attainable standing current when the detector shows an increase in conductivity in the presence of a trace of an impurity.

Recently, a gas chromatograph, with a helium ionization detector and using purified helium as the carrier gas, was used for the analysis of samples of high purity helium and it was noted that instead of obtaining positive peaks, corresponding to an increase in cell conductivity, for hydrogen, oxygen plus argon, and nitrogen, peaks were obtained which were either wholly or partly negative. Methane, carbon monoxide and nitrous oxide always gave positive peaks. Previously this gas chromatograph had only been used for the determination of carbon monoxide and nitrous oxide in hydrogen when in each case normal, positive peaks were obtained.

The gas chromatograph analysis system comprises a cylinder of helium which feeds a recirculation system containing an activated charcoal purifier (77°K), a simple gas sampling system, a column (1 m × 5 mm I.D. stainless steel tube packed with 30–60 mesh molecular sieve type 5A and activated *in situ*), a helium ionization detector[3] and a diaphragm pump. During analysis the column–detector outlet is not connected in the recirculation system to avoid pressure effects associated with the diaphragm pump. Neither renewal of part or all of the system nor extended reactivation of the molecular sieve material, resulted in the formation of positive peaks although it was often found that immediately after a change had been made that the peaks were positive for a short time and then became increasingly negative. It was also found that if the helium cylinder was connected to the sample system, *via* a gas regulator only, positive peaks were always observed.

A series of tests was therefore carried out in which impurities were continuously introduced into the purified carrier gas stream, the molecular sieve material having been previously reactivated at 300°C for 24 h with a continuous helium purge of 20 ml/min. Operation of the column at ambient temperature with a flowrate of 100 ml/min of purified helium containing from 0–20 v.p.m., in turn, of each of hydrogen, argon, nitrogen and methane showed that:

1. With the addition of each of the impurities to the carrier gas the standing current fell initially with increasing impurity content and then rose with further increase in the impurity content, except in the case of methane where a continuous rise was observed, Fig. 1.

2. As the concentration of each of the added impurities in the carrier gas was increased the height of the negative peaks from an injected sample, decreased, and in some cases, finally became positive. An example of this change, where nitrogen is added in increasing amounts to a helium carrier gas containing 4.5 v.p.m. Ne, is shown in Fig. 2.

It is interesting to note that of the gases tested only those having an ionization potential greater than about 15 eV show this phenomenon of inversion.

The purified helium used in these experiments was analysed mass spectrometrically and compared with a corresponding analysis of 'Specpure' helium, Table I.

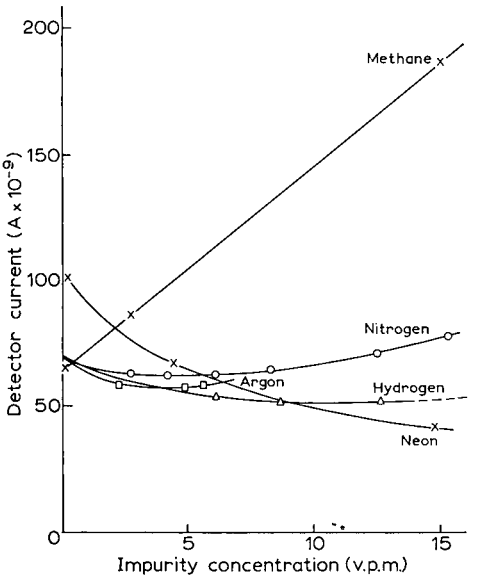

Fig. 1. Effect of carrier gas purity on the detector standing current.

The only significant difference between the two sets of figures is the level of neon. When the cylinder of commercial helium which supplied the purifier, was replaced by 'neon free' helium a much higher standing current was obtained which fell as the neon impurity content was increased, Fig. 1. Insufficient neon could be added, however, to explore fully the change in standing current with neon concentration, and therefore to determine if neon had the same effect as the other impurities. Preliminary experiments indicate that, for hydrogen at least, the use of high purity helium giving negative peaks offers an improvement in sensitivity by a factor of at least 10, although it is probable that a method for the determination of hydrogen

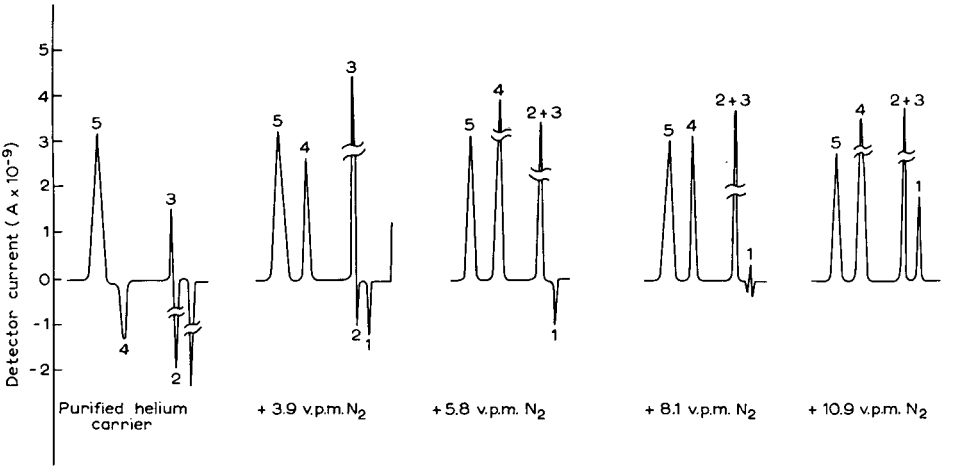

Fig. 2. The effect of the nitrogen concentration in the carrier gas on the sample impurity peak shape. 1 = Hydrogen; 2 = argon; 3 = oxygen; 4 = nitrogen; 5 = methane.

TABLE I

IMPURITY CONCENTRATION IN HELIUM (v.p.m.)

	H_2	N_2	O_2	A	CO_2	Ne
Purified	<0.5	0.3	0.1	0.1	<0.1	4
Specpure	0.8	0.3	0.1	<0.1	0.2	0.5

based upon this effect will cover a very limited range. Experiments are being carried out in which it is hoped to develop methods for each of the gases capable of giving negative peaks.

We would like to acknowledge the cooperation of Mr. J. A. J. WALKER (U.K.A.E.A. Culcheth), who was able to confirm that negative peaks could be obtained using his gas chromatograph and our purifier.

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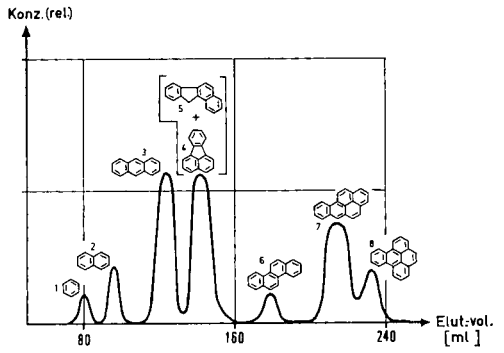
Säulenchromatographie von polycyclischen aromatischen Kohlenwasserstoffen an lipophilem Sephadex LH-20*

Hydrophobierte Adsorbentien eignen sich gut zur Trennung polycyclischer Aromaten ([1–4]). Vor Kurzem hatten wir auf anderem Weg festgestellt, dass Sephadex (G-10 und LH-20) ein starkes Adsorptionsvermögen für Kohlenwasserstoffe höherer Ringzahlen besitzt[5]. Es war daher interessant zu prüfen, in welchem Mass sich die Adsorption bei der Säulenchromatographie bemerkbar macht. Zu diesem Zweck wurden an einer Säule von in Isopropanol gequollenem Sephadex ein Gemisch mehrerer Kohlenwasserstoffe chromatographiert. Das Ergebnis (Fig. 1) war eine mit steigendem Molekulargewicht abnehmende Eluierbarkeit und stand somit im Gegensatz zur normalen Sephadex-Chromatographie. Wie der Fig. 1 zu entnehmen ist, lassen sich sehr gute Trennungen durchführen, z.B. von 1,2-Benzofluoren–Chrysen, Chrysen–Benzpyrene etc. Ja selbst die isomeren 1,2- und 3,4-Benzpyrene sind hinreichend gut trennbar. Die Reproduzierbarkeit der Versuche ist ausgezeichnet.

* Vgl. Firmenschrift: *Sephadex LH-20 zur Gelfiltration in organischen Lösungsmitteln*, Pharmacia Fine Chemicals, Uppsala, Schweden.



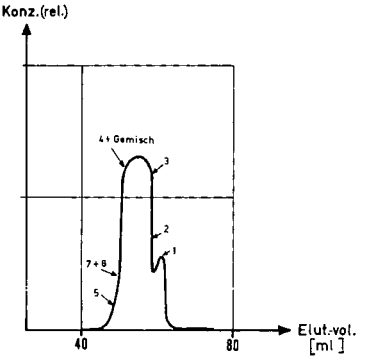


Fig. 1. Trennung eines Kohlenwasserstoff-Gemisches an einer Sephadex-LH-20-Säule in Propanol-2. Säule: 1.1 × 112.0 cm (28 g Sephadex LH-20); 96 ml. Probelösung: 0.25 ml (Konz.: gesättigte Lösungen der Benzpyrene). Elutionsgeschwindigkeit: 2.5 ml/h.

Fig. 2. Trennung des gleichen Gemisches (Fig. 1) in Chloroform. Säule: 1.1 × 117.5 cm (28 g Sephadex LH-20); 102 ml. Probevolumen: 0.2 ml (Konz.: 0.4 mg Kohlenwasserstoff/ml). Elutionsgeschwindigkeit: 6.4 ml/h (vgl. Text).

Bei der Säulenchromatographie des gleichen Kohlenwasserstoff-Gemisches in Chloroform konnte selbst bei der kleinstmöglichen Eluiergeschwindigkeit (6.4 ml/h) keinerlei Trennung der Komponenten erzielt werden. Jedoch zeigte sich bei einem Fraktionsvolumen von 1.1 ml absorptionsspektroskopisch deutlich, dass in den ersten Fraktionen Benzofluoren und die Benzpyrene eluiert wurden. Dagegen waren die Aromaten mit kleinerem Molekulargewicht in der Reihenfolge Fluoranthen, Anthracen, Naphthalin und Benzol in den letzten Fraktionen des gemeinsamen Peaks zu finden (Fig. 2).

In dem stärker hydrophoben Chloroform findet also eine normale Molekularsiebtrennung statt. Man muss annehmen, dass auch in diesem Lösungsmittel die Adsorptionskräfte des Sephadex wirksam werden, jedoch nicht unterstützt von Sol-

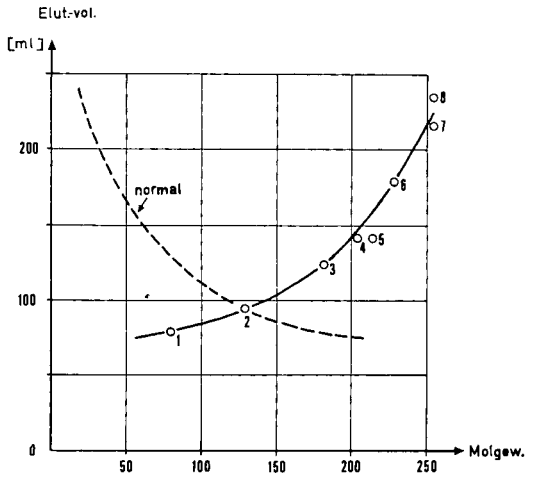


Fig. 3. Inverse Abhängigkeit des Molekulargewichts vom Elutionsvolumen bei Anwendung von Sephadex LH-20 in Propanol-2 (vgl. Fig. 1).

vatationskräften des Lösungsmittels. Wegen der geringeren Löslichkeit der Kohlenwasserstoffe in Isopropanol ist anzunehmen, dass die Solvatationskräfte in diesem Lösungsmittel geringer sind als in Chloroform. So kommt zwischen den Sephadex-Strukturen und den polycyclischen Kohlenwasserstoffen eine hydrophobe Wechselwirkung zustande, ganz ähnlich wie sie bei aromatischen Kohlenwasserstoffen und Desoxycholsäure[6,7], Detergentien[7,8] und Proteinen[9–12] gefunden wurde. Die Fig. 3 zeigt die hieraus resultierende inverse Abhängigkeit des Elutionsvolumens vom Molekulargewicht der Komponenten.

Experimentelles

Sephadex LH-20 wurde nach dreistündigem Quellen in Propanol-2 bzw. Chloroform p.a. (Merck) in dünner Suspension auf einmal in die senkrecht eingespannte Säule eingegossen und die Säule bei geöffnetem Auslauf gepackt. Als Auslauffilter diente ein abgeflammter Bausch Glaswatte. Da das Gel in Chloroform schwimmt, muss unter hoher Strömungsgeschwindigkeit gepackt werden. Setzt man jedoch der Chloroformsuspension 20 Vol.-% Cyclohexan zu, dann lässt sich die Säule auch in normaler Weise füllen. Das Gel schrumpft dabei nur geringfügig; seine Trenneigenschaften sind nach Auswaschen des Cyclohexans unverändert.

Bei der in Chloroform gepackten Säule ist es notwendig, das Gelbett in seiner Lage zu fixieren. Dazu wurde die Geloberfläche mit einer Glasfrittenscheibe abgedeckt, die in einer seitlichen Einkerbung mit einem Teflonband als Dichtungsring versehen war und sich so im Rohr verschieben liess.

Während des Trennungsvorgangs wurde die Säule sorgfältig vor Lichteinfall geschützt, weil die Komponenten stark zur Photooxydation neigen. Die Niveaudifferenz betrug bei Propanol-2 75 cm, bei Chloroform 50 cm. Die Registrierung erfolgte durch Absorption bei 254 nm (Uvicord LKB).

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U-shaped columns for adsorption and partition chromatography

We have during the development of a multi-column chromatographic system (refs. 1–3) constructed U-shaped glass columns that were found for some applications to offer distinct advantages over conventional straight columns, also when they were used for single column chromatography.

Our interest in the U-shaped columns evolved from attemps to construct long, straight, capillary columns. Such columns were found impractical and very inconvenient in use since they approached or exceeded in length the height of the room we worked in. It was decided to bend the tubing into a U to make the columns easier to handle. The capillary columns of this shape were found so convenient in use that the design was adapted also for columns of more conventional length and diameters.

Construction of the U-columns

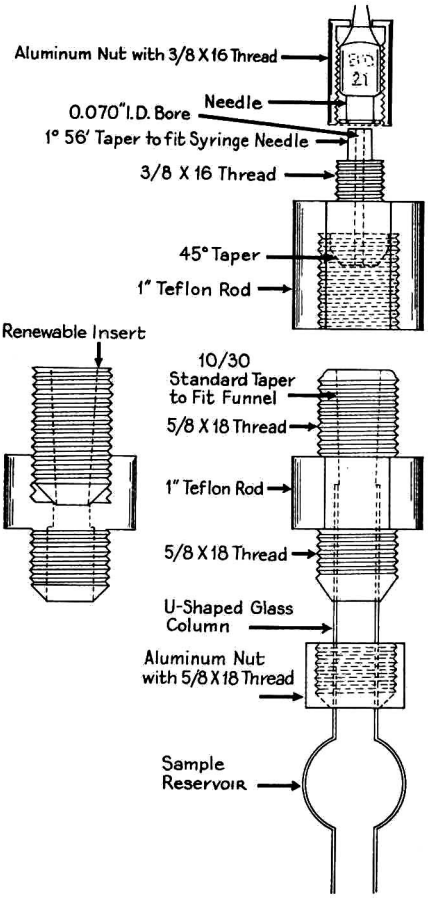
The columns have been made simply by bending a piece of glass tubing of appropriate diameter into a U-shape. A small sample reservoir can if needed be made with simple glass-blowing techniques at one end of the columns to facilitate the application of sample to the column. This has routinely been done in the case of the capillary columns, that otherwise may present difficulties in sample application. Heavy-walled glass tubing is preferable for column construction to give a column of greater mechanical strength and to make it possible to blow a sample reservoir in the column if needed.

The diameter of the glass tubing used in the construction can vary within rather wide limits if the teflon adapter is used on the columns. A set of, for example, three such adapters with increasing inside diameter will cover a wide range of outside diameters in glass tubing. However, tolerances are quite narrow for the swagelok adapter and tubing must either be selected to closely fit available swagelok fittings or a piece of glass tubing with the right O. D. must be annealed on each side to the top of the U-column, if swagelok connections are used for the columns.

Connections between the feed pump or eluant container and the U-column

Teflon adapter. This is the preferred adapter for columns exposed to moderate pressure only and for columns that are packed by a simple sedimentation technique. The bottom part of the adapter (Fig. 1) slides over the top of the chromatographic column. It is squeezed around the column by tightening the aluminum nut at the bottom to slightly more than finger tightness. This part of the adapter is left on permanently if the column is in continuous routine use. The top part of the adapter is screwed to the bottom part after the column has been filled. A removable insert (shown to the left on Fig. 1) has been made for the part of the adapter that is most exposed to wear. This modification is used if the adapter is in very frequent use. A needle is held by another aluminum nut to the top portion of the upper part of the adapter. Teflon tubing leads from here to pump or solvent container on the intake side and to the fraction collector on the outlet side.

Swagelok connection. If pressures are expected to be higher than 100 p.s.i. or if active packing of the column is necessary before chromatography a swagelok (Crawford Fitting Co., Solon, Ohio) connection is preferable. The swagelok fitting consists of a front and a rear ferrule that are squeezed around the tubing by a nut. The front



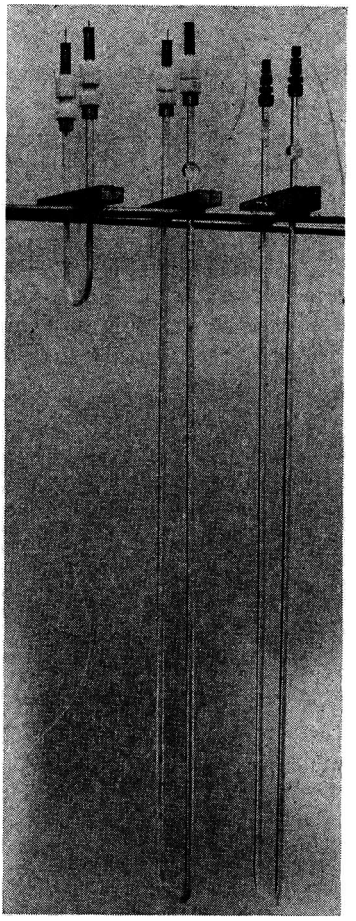


Fig. 1. Teflon adapter used to connect U-shaped column to eluant and fraction collector.

Fig. 2. A small 20-in. U-shaped column and two 8-ft. capillary U-shaped columns. The two columns to the left are equipped with teflon adapters. The column to the right with swagelok fittings.

must be teflon to reduce breakage on tightening the swagelok connection. A swagelok reducing unit is used to make the connection from the glass tubing on the column to the teflon tubing (Fig. 2, right).

Filling the U-columns

Using the teflon adapter. The bottom part of this adapter has an inside female 10/30 standard tapered joint. Glass funnels with corresponding male 10/30 joints are inserted into these joints on both sides of the column. Half the amount of column material to be used is after filling of the columns and partially filling of the funnels with solvent added in suspension to each of the two funnels. The columns are allowed to form by sedimentation. A glass fiber or cotton plug is put on top of the column on the low-pressure side and the column material is compressed and pushed against the

plug by applying appropriate pressure on the high-pressure side. The column is now ready for application of the sample. This can be done with a small syringe equipped with a long needle. After the sample has been washed down on the column with a few portions of eluant under appropriate pressure the adapter is filled up with eluant and connected to pump or container and the column is ready for chromatography.

Using the swagelok connection. Gravity filling of the columns is in this case performed by connecting the swagelok on the column through a straight union of the same diameter to a small piece of wide diameter teflon tubing swaged to the other end of this union. A glass funnel with a stem of appropriate diameter is inserted into the teflon tubing and the columns are filled as above. With this type of connection a perforated disk packer can also be used for partition column materials like Celite where active packing is necessary. The rod in the packer has to be flexible in the small section going into the horizontal part of the U. A stainless steel spring inside teflon tubing performs well for this purpose. The column is stamped from both sides of the U's after filling the horizontal part first. We have not been able to pack capillary columns adequately with hard to pack material like cellulose powder but larger sized diameter tubing packs with little more difficulty than straight columns. After packing the sample is applied to the top of the column with a syringe equipped with a long needle as above.

Emptying the columns

The columns empty easily after use if they are washed out with tap water under pressure. Thin polyethylene tubing connected through an adapter to a cold water faucet is pushed inside the column after removal of the fiberglass plug. The column material is rapidly washed away as the polyethylene tubing is gradually pushed through the column.

Performance

The types of columns and connections used in our work are shown in Fig. 2. The smaller 20-in. U-column with teflon adapters shown to the left in the figure is now used in routine gradient elution chromatography of steroids[1–3] instead of the water-jacketed straight columns of similar length used previously. The 8-ft. long capillary columns with teflon adapters or swagelok connections (Fig. 2, middle and right) have been used to speed up the chromatography of the steroids.

To illustrate the performance of the U-shaped columns some chromatograms of seven 17-ketosteroids separated by gradient elution chromatography on alumina columns with the technique described previously[1–3] are shown in Fig. 3. The chromatograms were recorded with our automatic read out system[3]. The top chromatogram was obtained using a straight column, the middle chromatogram by using a U-shaped column of similar length. The differences in resolution is not greater than is observed between individual chromatograms in a multi-column run using conventional straight columns. These two chromatograms were from 36-h runs. The bottom chromatogram shows a 12-h run on an 8-ft. long capillary column. A resolution essentially similar to that obtained in the three times as slow short column run is obtained on these columns in the shorter time.

Both the capillary and ordinary U-shaped column have besides for adsorption chromatography on alumina also been used with good results for partition chromato-

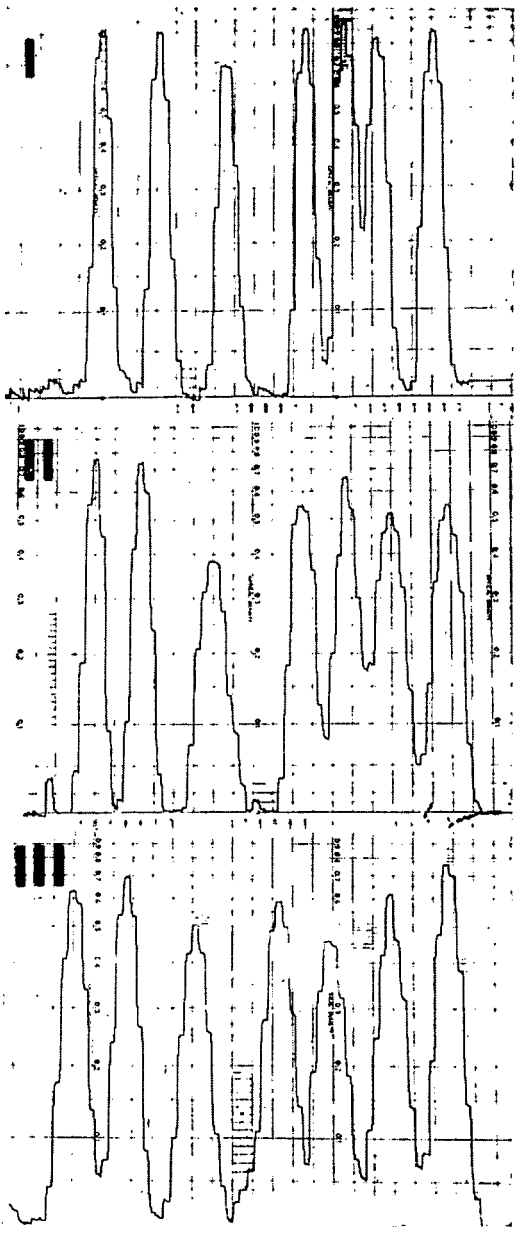


Fig. 3. Chromatograms of the seven major 17-ketosteroids present in human urine obtained and recorded with the technique described previously[3]. The top chromatogram is from a straight column run, the middle chromatogram was done on a U-shaped column of similar length. Both these chromatograms were run for 36 h. The bottom chromatogram is from a 12 h run of the same substances on an 8-ft. capillary column.

graphy of steroids on silica gel with water as the stationary phase. Columns of this material can as the alumina columns be packed by gravity. Partition chromatography on cellulose powder with propanediol as the stationary phase has been performed on U-shaped columns of standard dimensions only.

Advantages of the U-shaped columns

Conventional sized columns. These columns are less expensive and simpler to construct than the usual straight columns. No special glass-blowing skills are needed for the construction since the column is made simply by bending a piece of glass tubing into a U. No glass disk or other column support is needed in the columns. They are much easier to thermostat than conventional columns since the column can be simply dipped into a waterbath for thermostatting and no elaborate system with water-jackets connected to a system carrying circulating water is necessary. An advantage deriving from the low cost of the columns and the U-shape is that it is easy to prepare the columns in quantity before use and store them under solvent, packed and ready for chromatography at a later time. We have stored alumina columns this way for over a month under benzene and found them fully effective at the end of this period. The columns are more compact and take up less space. This is particularly valuable in multi-column systems where thermostating is needed. A small waterbath can easily accommodate 10–20 U-shaped columns.

Capillary columns. These columns are not as convenient in use as the smaller U-columns. They are, however, necessary to perform capillary column chromatography. This type of chromatography will in many applications give better resolution than shorter columns with larger diameter. The capillary columns may also be used to give a considerable reduction in the time necessary to run a chromatogram. Very long capillary columns may bring the running time in column chromatography down drastically if the technical problems involved can be solved.

Acknowledgement

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Capillary teflon columns for adsorption and partition chromatography

We have previously[1] described U-shaped standard sized and capillary columns made of glass. In extension of work started with the U-shaped capillary columns we became interested in the possibility of constructing very long capillary columns.

Glass could not be used for these columns because of leakage through the junction of the swagelok connector and the glass tubing at very high pressures. There was also the danger of breakage of the glass at the high pressures necessary to force the fluid through very long capillary columns.

We turned in our search for a suitable material to teflon tubing because of its chemical inertness, semi-transparency and relatively high mechanical strength.

Construction of the capillary teflon columns

Teflon tubing. Unless special connectors are custom made in the machine shop for use in the column construction the commercial availability of the necessary connectors in increments of 1/16 in. only starting with a connector for 1/16 in. tubing limits the selection of tubing available for the construction of columns to sizes that are within the tolerances specified for the outside diameter of tubing to be used with the connectors.

We have in our work used two types of teflon tubing both with an outside diameter of between 0.121 and 0.126 in. to fit the tolerances of the 1/8 Gyrolok connector. One type has been the standard size flexible teflon tubing with an 1/16 in. (approximately 1.5 mm I.D.). The other is the thinner walled AWG size 10 with an outside diameter of 0.121–0.126 in. and an inside diameter of 0.103–0.107 in. (2.5 mm; both types of tubing available from Pennsylvania Fluorocarbon Company, Inc., Clifton Heights, Pa.).

Gyrolok connectors. We used in our work with the U-shaped glass columns swagelok (Crawford Fitting Company, Solon, Ohio) connectors. We found, however, that these connectors constricted the inside diameter of the capillary teflon columns somewhat and that this slowed the filling of the column. We changed therefore to a similar fitting the Gyrolok (Hoke, Inc., Cresskill, N. J.) tube fitting that does not constrict the inside of the tubing to a degree that slows the filling of the column.

Column construction. The columns were made simply by attaching gyrolok fittings to both ends of a piece of capillary teflon tubing of appropriate length. This is done by inserting the tubing through the fitting and turning the nut of the fitting 1¼ turn thereby swaging the fitting to the tubing. The tubing coming from the feed pump and going to the fraction collector is similarly equipped with gyrolok fittings.

Filling the capillary columns

Filling by gravity. It is possible with some column materials (alumina, silica gel) to fill the columns with the larger inside diameter by gravity alone. The technique for this is illustrated in Fig. 1 that shows to the left in the foreground a column ready to be filled to which a glass funnel has been attached at the top through a reducing union and a piece of thin walled teflon tubing. The columns are plugged at one end with glasswool or cotton. The columns are completely filled up, the funnels partially filled up with mobile phase. The column material is added in a slurry to the funnel and left to settle by gravity in the columns. The column to the right in the foreground of Fig. 1 is an almost filled column filled by this technique.

Filling by pump feeding. This technique is used for the capillary columns with small inside diameters and also for columns with larger inside diameter if the material used for the column preparation cannot be packed by gravity.

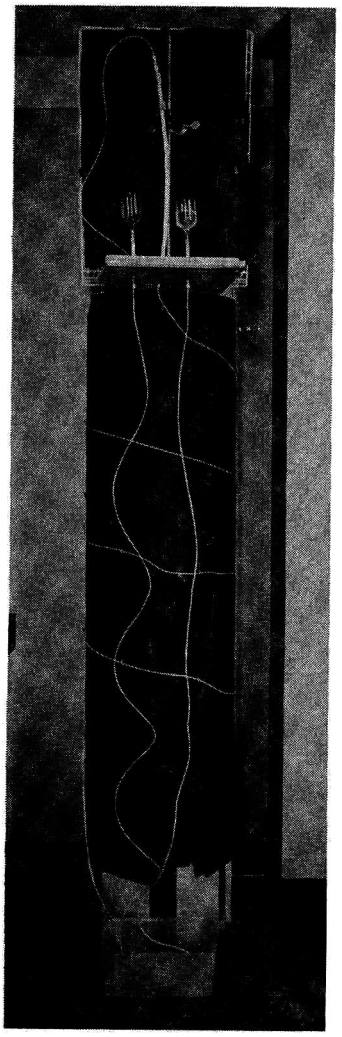

Fig. 1. Filling of capillary teflon columns. The columns with larger I.D. (2.5 mm) are filled by gravity in the foreground. In the background is an 8 m long capillary column with an I.D. of 1.5 mm being packed by pump feeding from the large diameter teflon tubing reservoir connected to the top of the capillary column through a Gyrolok union.

To fill the capillary column by pump action a piece of wide diameter (½ in. O.D.) teflon tubing is attached through a reducing union to the top of the capillary column (Fig. 1 background). This wider section of teflon tubing is as the capillary filled up with mobile phase. The column material is then poured into the wider teflon tubing through a glass funnel attached to the top of the tubing. The top end of the

teflon tubing is now connected through a reducing union to the chromatographic pump and the column material is then pumped into the capillary section when the pump is started up.

The pump feed technique can also be used to fill the previously described[1] U-shaped glass capillary tubing with material that could not without this technique be packed into that type of columns.

We have with the pump feed technique filled columns with a variety of hard to pack partition column materials including cellulose powder and celite.

Application of sample to columns

We have in our work transferred the sample in mobile phase to the columns using a tuberculine syringe equipped with a 4 in. long 22 gauge needle or with a needle to which very thin teflon tubing has been attached. The sample solutions have after introduction into the top part of the column been pushed into the column with compressed air or nitrogen applied through a reducing valve so that the pressure can be regulated at will. Syringe and sample container has been washed through with mobile phase a few times and the rinses washed down into the column with compressed air. The columns are now filled up with mobile phase and connected to the pump to start the chromatographic run.

Emptying the columns

The columns are very easy to empty. The glass wool or cotton plug at one end of the column is removed. The tubing leading to the pump is attached to the other end. The column material empties out of the column when the pump is started up.

Performance

The great savings in time obtainable through the use of the capillary teflon columns are demonstrated in Fig. 2. This figure compares a 36-h chromatogram of a steroid mixture performed on a conventional alumina column with the technique described previously[2,3,4] with 5- and 2.5-h capillary chromatograms of the same mixture on the same column material. All chromatograms were recorded with the automatic read-out system described earlier[4].

It can be seen that the 5-h capillary chromatogram is if anything slightly better in resolution than a 36-h chromatogram on a conventional column and that a 2.5-h capillary chromatogram although somewhat poorer in resolution than both a 5-h capillary chromatogram and a 36-h conventional chromatogram still would be acceptable for example for a clinical analysis for these steroids in biological fluids since the maximal overlap between individual steroids is of the order of 3% of the total amounts applied to the column.

The resolution of a complex steroid mixture of this nature in such a short time period makes column chromatography for such a mixture slower only than gas chromatography of the major chromatographic systems in analytical use. Separation of this steroid mixture by thin layer chromatography has in our hands been possible only by horizontal chromatography or special overrun techniques. Both these procedures take 4–6 h to perform. Fast paper chromatographic systems take 4–5 h for a steroid mixture of this nature.

Further development of the capillary technique might conceivably make capil-

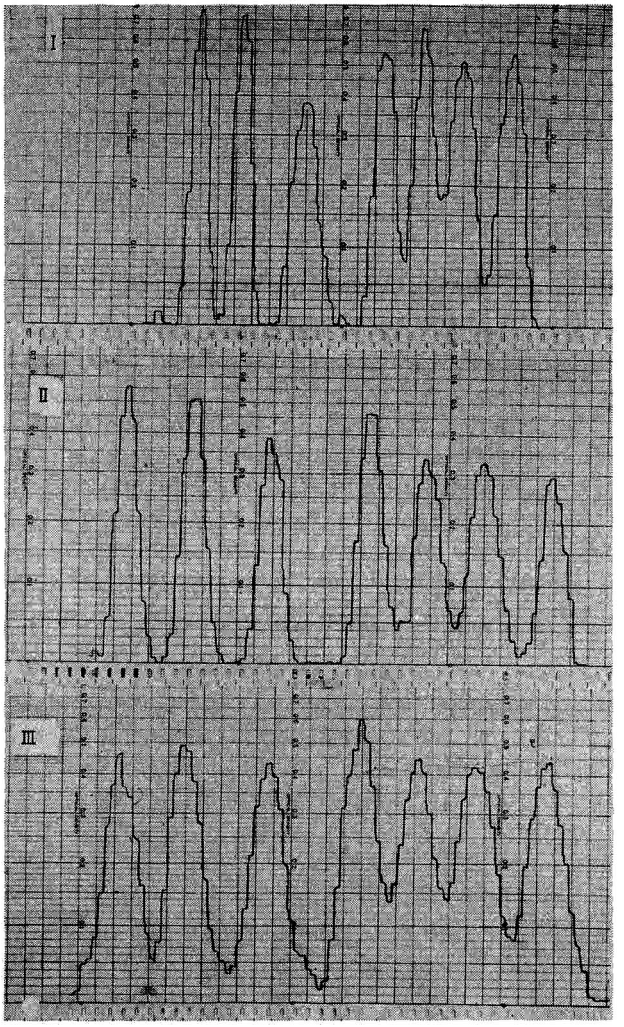

Fig. 2. Comparison of a conventional 36-h chromatogram (I) of the seven common urinary 17-ketosteroids obtained using a straight 40 cm long alumina column (I.D. 6 mm) with chromatograms of the same steroid mixture obtained using 8 m long capillary teflon columns (I.D. 1.5 mm). (II) shows a 5-h capillary chromatogram, (III) a 2.5-h capillary chromatogram. Gradient elution chromatography was used[2,3,4] and the chromatograms were recorded with our automatic read-out system[4]. The 17-ketosteroids separated are in order of elution: dehydroisoandrosterone, androsterone, etiocholanolone, 11-ketoandrosterone, 11-ketoetiocholanolone, 11-hydroxyandrosterone and 11-hydroxyetiocholanolone. Approximately 50 μg of each steroid was chromatographed.

lary column separations the fastest chromatographic method available for complex steroid mixtures.

Advantages of capillary teflon columns

The columns are non-breakable and will outlast glass columns by a wide margin in routine use. Making them involves only cutting off an appropriate length of teflon tubing and swaging on the Gyrolok fittings, and this is done in a few minutes. They

are very convenient to thermostat. We have for our 12 column multi-column runs arranged twelve such 8 ft. long columns in coils in a small waterbath. The columns are easy to fill and empty. They are easy to store and can as the U-shaped glass columns[1] be stored for later use once made up. They must, however, be kept in closed containers over mobile phase when stored since teflon is slightly porous to many organic solvents.

Advantages of the filling technique using pump feeding of column material

This technique is necessary for the filling of capillary columns, since it would be close to impossible to develop mechanical devices that could pack columns with such small inside diameters. The technique can however be used to advantage also for filling conventional sized chromatographic columns including the U-shaped glass columns described earlier[1]. The pump feeding technique is in our experience much faster than techniques using packing rods or other mechanical devices. A semi-automatic technique of this nature makes rapid batch filling of partition columns with material that is hard to pack with other methods possible.

Further developments of the capillary column technique

The 24 ft. long capillary columns we have used for the chromatograms in Fig. 1 has presented the limits of the technique for the capillary teflon columns in our experiments. The pressure obtained was 900 lb. per sq. in. The chromatographic minipumps used (Milton Roy Company, Philadelphia, Pa.) are rated for work to 1000 lb./sq. in. The bursting pressure of the narrow diameter teflon capillary tubing was found to be 1200 lb./sq. in. To get further with the development of the capillary column technique, it will therefore be necessary to turn to other pump types with higher pressure ratings and to metal tubing that can resist the high pressures involved.

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Identification of straight-chain unsaturated aldehydes by thin-layer partition chromatography

Aerial oxidation of oils and fats induces changes in flavour of these products, which are mainly caused by the formation of minute (1 mg/kg or less) amounts of aldehydes[1,2]. For the analysis of these generally strong-flavoured, oxidation products[3,4] various systems of thin-layer chromatography are eminently suited[5–9]. In these techniques the carbonyls, in the form of their 2,4-dinitrophenylhydrazones (DNPH), are separated.

BADINGS AND WASSINK[6] recently developed a method for the separation of C_2–C_{11} saturated aldehydes on Kieselguhr G chromatoplates impregnated with Carbowax 400. We have utilized this system for the study of the chromatographic

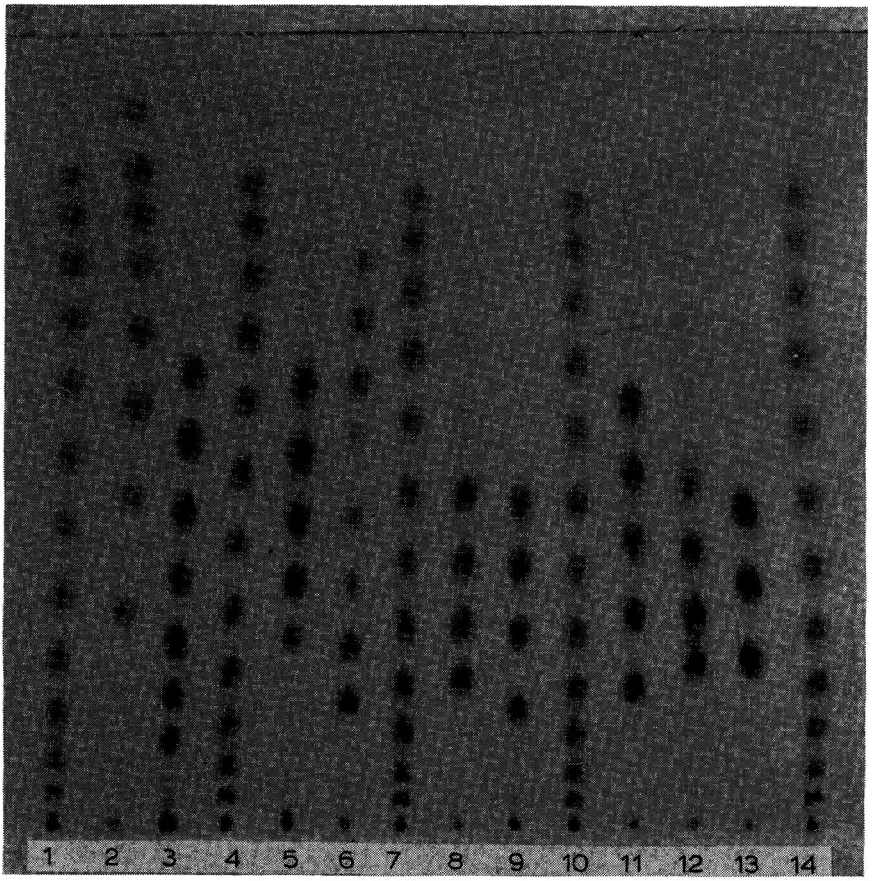

Fig. 1. Separation of aldehyde-DNPHs by thin-layer partition chromatography; $33^1/_3$% Carbowax 400 on Kieselguhr G, solvent: 100% light petroleum b.p. 100–120°. 1, 4, 7, 10, 14 = C_1–C_{12} alkanals; 2 = C_3–C_9 2-alkanones and 2-undecanone; 3 = C_6–C_{12} *trans*-2-, *trans*-4-alkadienals; 5 = *trans*-3-hexenal, *trans*-4-heptenal, *trans*-5-octenal, *trans*-6-nonenal and *trans*-7-decenal; 6 = C_5–C_{12} *trans*-2-alkenals; 8 = C_6–C_9 ω-alkenals; 9 = *trans*-2-, *trans*-4-heptadienal, *trans*-2-, *trans*-5-octadienal, *trans*-2-, *trans*-6-nonadienal and *trans*-2-, *trans*-7-decadienal; 11 = C_6-C_{10} *trans*-5-alkenals; 12 = *trans*-4-hexenal, *trans*-5-heptenal, *trans*-6-octenal and *trans*-7-nonenal; 13 = *trans*-2-, *trans*-7-octadienal, *trans*-2-, *trans*-7-nonadienal and *trans*-2-, *trans*-7-decadienal.

behaviour of a number of unsaturated aldehyde-DNPH's, special attention being paid to the effect of number and location of double bonds and to *cis–trans* isomerism.

Method and results

The chromatoplates (20 × 20 cm), consisting of Kieselguhr G (layer thickness 0.35 mm) impregnated with Carbowax 400 were prepared along the lines indicated by BADINGS AND WASSINK[6], except that 33.3 instead of 25 % Carbowax was used for impregnation. With this modification improved separation according to chain length

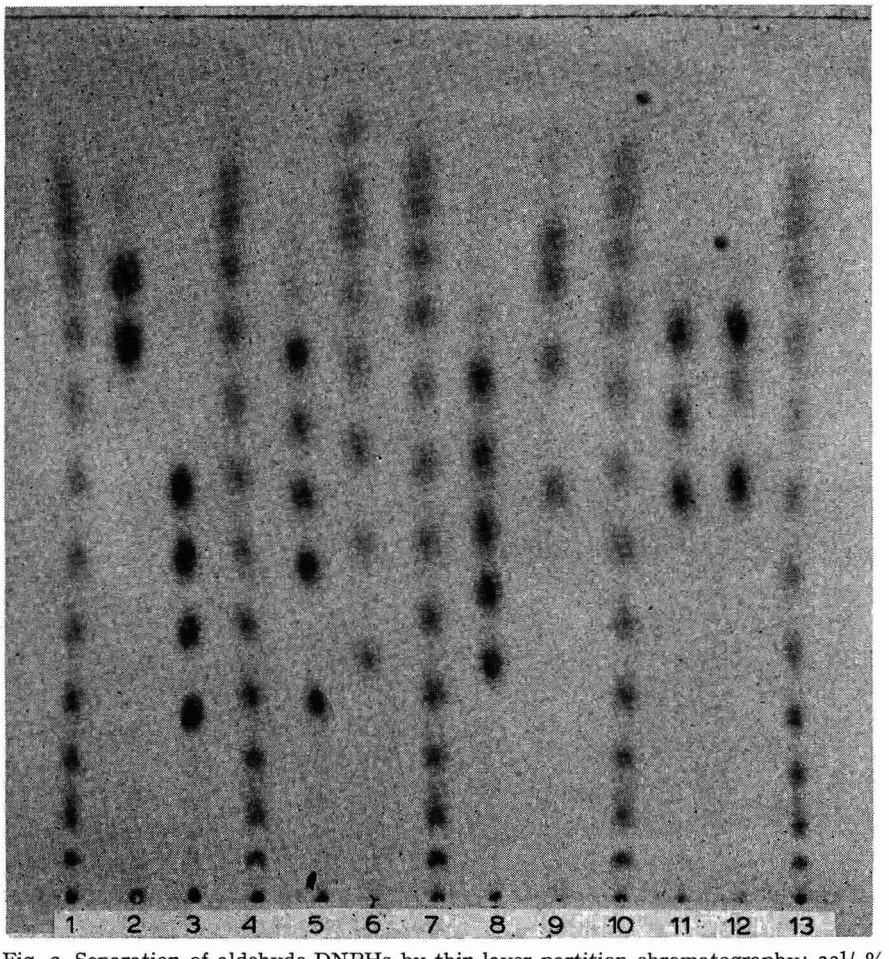

Fig. 2. Separation of aldehyde-DNPHs by thin-layer partition chromatography; $33^1/_3$% Carbowax 400 on Kieselguhr G; solvent: 100% light petroleum b.p. 100–120°. 1, 4, 7, 10, 13 = C_1–C_{12} alkanals; 2 = *trans*-2-, *cis*-5-, *cis*-8-tetradecatrienal and *trans*-2-, *cis*-6-, *cis*-9-pentadecatrienal; 3 = *trans*-2-, *cis*-4-heptadienal, *trans*-2-, *cis*-5-octadienal, *trans*-2-, *cis*-6-nonadienal and *trans*-2-, *cis*-7-decadienal; 5 = *trans*-2-, *cis*-4-heptadienal and C_9–C_{12} *trans*-2-, *cis*-4-alkadienals; 6 = C_3–C_9 2-alkanones and 2-undecanone; 8 = *cis*-3-hexenal, *cis*-4-heptenal, *cis*-5-octenal, *cis*-6-nonenal and *cis*-7-decenal; 9 = *cis*-4-, *cis*-7-decadienal, *cis*-6-, *cis*-9-dodecadienal, *cis*-4-, *cis*-7-tridecadienal and *cis*-5-, *cis*-8-tetradecadienal; 11 = *trans*-2-, *cis*-7-decadienal, *trans*-2-, *cis*-6-dodecadienal and *trans*-2-, *cis*-5-undecadienal; 12 = *trans*-2-, *trans*-7-decadienal, *trans*-2-, *trans*-6-dodecadienal and *cis*-6-, *cis*-9-dodecadienal.

was achieved for the DNPHs of saturated C_{10}–C_{12} aldehydes and C_8, C_9 and C_{11} methyl ketones.

Mixtures of 2 μg of the available DNPHs (methyl ketones, alkanals, monoenals, dienals and trienals) in 10 μl chloroform were spotted on to the plate, at distances of 1 cm, with a 10 μl micropipette. Subsequently, ascending chromatography was applied for 25–30 min at 20°, using light petroleum (b.p. 100–120°) as eluant.

From Figs. 1 and 2 it appears that the components of the various homologous series can be clearly separated by this technique. The accompanying, regularly distanced spots are not attributable to impurities, but most probably to *syn*-isomers. This phenomenon was not observed when the DNPHs had been stored dry.

After spraying the plate with dilute alcoholic alkali, the DNPHs undergo their characteristic colour change, as described by LAPPIN AND CLARK[10], which could serve as an aid in the analysis and identification of carbonyl compounds.

Discussion

The relative migration (R_H) of the DNPHs with respect to hexanal-DNPH has been established. The relevant data, which show little scattering, are collected in Table I.

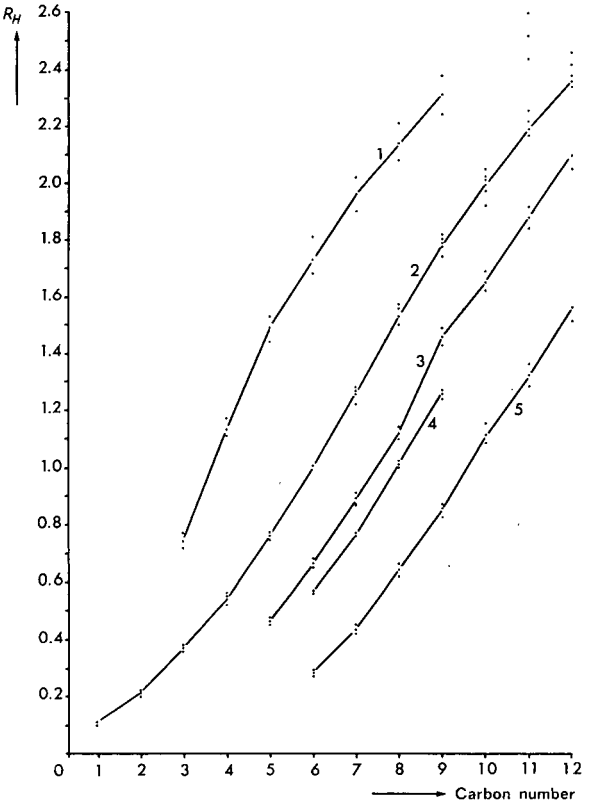

Fig. 3. Migration of aldehyde-DNPHs, R_H = distance moved by hydrazone/distance moved by hexanal-DNPH. 1 = 2-Alkanones; 2 = alkanals; 3 = *trans*-2-alkenals; 4 = ω-alkenals; 5 = *trans*-2-, *trans*-4-alkadienals.

TABLE I

R_H VALUES OF ALDEHYDE-DNPHs

Type of aldehyde	*Double bond at*	*Chain length*														
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
Alkanals		0.11	0.21	0.38	0.54	0.76	1.00	1.25	1.53	1.79	2.05	2.27	2.47			
trans-Alkenals	2					0.43	0.61	0.85	1.09	1.41	1.63	1.89	2.16			
	3						0.67	0.93								
	4						0.62	0.80								
	5						0.55	0.82	1.09	1.40	1.70					
	6							0.75	1.05	1.36						
	7								1.00	1.31	1.65					
	8									1.26						
cis-Alkenals	3						0.60	0.83								
	4						0.57	0.85								
	5							0.80	1.04							
	6									1.28						
	7										1.56					
trans,trans-Alkadienals	2, 4						0.27	0.43	0.62	0.81	1.05	1.32	1.56			
	2, 5								0.70							
	2, 6									0.97			1.76			
	2, 7								0.62	0.92	1.22					
trans,cis-Alkadienals	2, 4							0.45		0.85	1.10	1.34	1.55			
	2, 5								0.68			1.35				
	2, 6									0.93			1.66			
	2, 7									0.88	1.15					
cis,cis-Alkadienals	4, 7										1.07			1.87		
	5, 8														2.08	
	6, 9												1.57			
trans,cis,cis-Alkatrienals	2, 5, 8														1.66	
	2, 6, 9															1.92

On plotting the R_H values as a function of the chain length for the components of various homologous series, smooth curves are generally obtained. An exception is the curve for *trans*-2-monoenals, which shows a distinct bend at C_9 (Fig. 3). A similar phenomenon was observed by NONAKA, PIPPEN AND BAILEY[11] for the series of saturated aldehydes.

With the exception of the monoenals having a terminal double bond approximately equal R_H values were established for the homologues ($> C_3$) of C_{n-3}-methyl ketones, C_{n+1}-monoenals, C_{n+3}-dienals, and C_{n+5}-trienals (see Table I). These R_H values only differ from those of the homologues of the saturated aldehydes in that they are invariably in between two successive members (C_n — C_{n-1}) of that series. However, the R_H values of the members belonging to the series of saturated aldehydes C_n equal those of the ω-alkenals having a chain length C_{n+2}. Similar rules were formulated in the past from the results of column and paper chromatographic methods of analysis[11–14].

Consequently, now that it has appeared that the behaviour of unsaturated aldehyde-DNPHs on chromatoplates is less dependent on their stereochemical configuration (see Table I), but rather on the number and location of their double bonds, the analysis of carbonyls from oils and fats can be largely simplified. On applying the above-mentioned technique to a complex DNPH mixture an orienting identification can be obtained chromatographically in about 30 min, after which a detailed analysis can be carried out more rapidly. Work on this aspect is in hand and will be published in the near future.

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Einsatz des N-Bromsuccinimids zur Fleckenfärbung bei der Dünnschichtchromatographie von Lipiden

Bei der Dünnschichtchromatographie auf Silicagel verschwinden die Flecken nach deren Anfärbung mit Joddämpfen zu schnell. Auch wird das Besprühen mit Schwefelsäure bzw. einem Bichromatgemisch oft als lästig empfunden. Unsere Untersuchungen ergaben, dass diese Nachteile zu beheben sind, wenn N-Bromsuccinimid als Reagens eingesetzt wird. Es wurde bereits im Verein mit Fluorescein (als Fluoreszenzindikator) zur Fleckenanfärbung bei der papierchromatographischen Trennung von Vulkanisations-Beschleunigern mit gutem Erfolg eingesetzt[1].

Das Reagens wurde auf Dünnschicht-Chromatogrammen von Ölsäure, Methyloleat, Stearinsäure, Monostearat, Methylricinoleat, Triglyceriden des Oliven- und des Baumwollsamenöls, Cholesterol, Lecithin, Oleylalkohol, Nonylaldehyd, Tokopherol und Tokopherolacetat geprüft. Chromatographiert wurde in der üblichen Weise[2,3] auf Chromatographie-Platten aus Silicagel. Das entwickelte und getrocknete Chromatogramm wurde mit 5%iger N-Bromsuccinimidlösung in Essigsäure (andere Lösungsmittel wie Methanol, Chloroform und Tetrachlorkohlenstoff erwiesen sich als weniger geeignet) und gleich darauf mit 0.01%iger alkohol. Fluoresceinlösung besprüht: es erschienen weisse Flecken auf blass-rosenrotem Grund. Im U.V.-Licht schimmern alle Flecken gelblich-grün mit Ausnahme der Methyloleat-, Tokopherol- und Tokopherolacetatflecken, die dunkel bleiben. Es erwies sich, dass die üblichen zum Chromatographieren von Lipiden verwendeten beweglichen Phasen (Chloroform–Methanol–Wasser; Hexan–Benzol u.a.) Intensität und Art der Anfärbung nicht beeinflussen: sie bilden keinen die Fleckenentwicklung störenden Untergrund. Es erscheinen stets scharfumrissene Flecken, die selbst nach längerem Aufbewahren der Chromatogramme nicht verschwinden. Das Reagens ist sehr empfindlich: es färbt noch Substanzmengen von etwa 5γ an. Doch ist es für Lipide nicht spezifisch und kann daher zur Anfärbung auch anderer Verbindungen wie Vulkanisationsbeschleuniger[1], Alkaloide u.a. Verwendung finden.

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Separation by thin-layer chromatography of the mercaptoacetic acid addition products of long-chain monounsaturated compounds

Mercaptoacetic acid reacts with monounsaturated fatty acids and esters to form the corresponding carboxymethylthio derivatives[1-3]. Not much information is available regarding their preparation and structural identification. The addition product of oleic acid with mercaptoacetic acid was postulated, without experimental proof, to be a mixture of the 9- and 10-(carboxymethylthio)-stearic acids[1]. In this note, evidence is adduced through TLC separation of isomeric carboxymethylthio derivatives of a number of monounsaturated fatty acids, esters and alcohols. In an earlier publication from this laboratory[4] a similar separation by TLC of isomeric 9(10)-hydroxy-10(9)-mercaptooctadecanoic acids was reported.

Materials and methods

Compounds used for the TLC separation were prepared by reacting the appropriate unsaturated compound with mercaptoacetic acid following the directions of KOENIG AND SWERN[1]. The crude reaction product was purified by urea adduction and repeated low-temperature crystallisation. Desulphurisation studies of these compounds will be reported separately.

The direct TLC procedure in use in this laboratory[5], using Silica Gel G, development with appropriate solvent systems and charring with sulphuric acid, was employed to separate the compounds. For reversed-phase TLC, the Silica Gel G-coated glass plates were first impregnated by ascending development with 5 % silicone oil in ether as described earlier[6].

Results and discussion

TLC separation of mercaptoacetic acid addition products of monounsaturated fatty acids, esters and alcohols by direct (DTLC) and reversed-phase (RPTLC) systems are shown in Table I. Separation was achieved according to chain length by DTLC. Addition products from C_{22} unsaturated acids moved ahead and were resolved from those of the C_{18} and C_{11} acids. Esters have relatively higher R_F values than the corresponding acids, which in turn move faster than the alcohols.

The presence of two components in the addition product, revealed by TLC, lends experimental support to the earlier postulation of KOENIG AND SWERN[1] that both isomers are formed from methyl oleate and mercaptoacetic acid. The 6- and 7-(carboxylmethylthio)-octadecanoic acids are also separable, and had lower R_F values than the respective 9- and 10-isomers. The addition product of erucic acid with mercaptoacetic acid also gave two spots by DTLC. Carboxymethylthioundecanoic acid, apart from having the lowest mobility, gave only a single spot, showing the absence of isomeric forms. This is most probably an anti-Markownikoff addition, whereby 11-carboxymethylthioundecanoic acid is obtained, as observed earlier for terminally-unsaturated short-chain fatty acids[1].

RPTLC gave similar separation patterns except that the polarity was reversed. Separation of 6- and 7- from 9- and 10-isomers was, however, less satisfactory than by DTLC.

Both in DTLC and RPTLC, the respective monounsaturated fatty acids used

TABLE I

TLC SEPARATION OF MERCAPTOACETIC ACID ADDITION PRODUCTS OF MONOUNSATURATED FATTY ACIDS, ESTERS AND ALCOHOLS
(All values as $R_F \times 100$)

Compound	*Acids from which prepared*	*Direct TLC**			*Reversed-phase TLC***		
		Acids	*Esters*	*Alcohols*	*Acids*	*Esters*	*Alcohols*
11-(Carboxymethylthio)-undecanoic acid	Undecenoic	23	45	12	88	86	93
6(7)-(Carboxymethylthio)-octadecanoic acid	Petroselinic	33, 36	52, 55	22, 25	72, 75	53, 56	81, 83
6(7)-(Carboxymethylthio)-octadecanoic acid	Petroselaidic	33, 36	—	—	72, 75	—	—
9(10)-(Carboxymethylthio)-octadecanoic acid	Oleic	42, 45	61, 64	23, 26	74, 77	51, 54	80, 83
9(10)-(Carboxymethylthio)-octadecanoic acid	Elaidic	42, 45	61, 64	23, 26	74, 77	51, 54	80, 83
13(14)-(Carboxymethylthio)-docosanoic acid	Erucic	46, 49	64, 67	31, 34	61, 64	46, 48	75, 77
13(14)-(Carboxymethylthio)-docosanoic acid	Brassidic	46, 49	—	—	61, 64	—	—

* 40% Ether–light petroleum.
** Acetonitrile–acetic acid–water (70:10:20).

as starting material generally moved to the solvent front, while the unsaturated alcohols, being more polar, had lower R_F values.

Apart from providing useful TLC separation procedures, these results also demonstrate that addition of mercaptoacetic acid to monoene fatty acids, esters and alcohols yields both isomers, with the exception of undecenoic acid which gives rise to a single product probably the 11-carboxymethylthioundecanoic acid.

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Separation and quantitative determination of ^{32}P-labelled lipids from brain particulates by thin-layer chromatography

The separation and isolation of phospholipids by thin-layer chromatography (TLC) on silica gel has been reported by several investigators[1–5]. Although a distinct separation of some phospholipids was achieved by these techniques, they yield separations with overlap among such phospholipids as phosphatidic acid and neutral lipids or phosphatidyl inositol and phosphatidyl serine. During our work on the effect of neurohormones and metabolic inhibitors of phosphate metabolism in nerve-ending particulates of rat brain, it was found that phospholipids were highly labelled when incubated in an oxidative phosphorylation medium containing ^{32}P-orthophosphate[6]. By incorporating certain features of previously described techniques[1,4], nerve endings or mitochondrial ^{32}P-labelled phospholipids, including the highly labelled phosphatidic acid and phosphatidyl inositol, were effectively separated on Silica Gel G by means of two-dimensional TLC, and the radioactive specific activities of the individual phospholipids were determined.

Experimental

Lipid extracts. Total lipids were extracted from rat brain nerve-ending particles or purified mitochondria with chloroform–methanol (2:1) after incubation in an oxidative phosphorylation medium containing 100–150 μc of ^{32}P-orthophosphate for 1 h at 37° as described previously[7]. The extract was filtered, concentrated *in vacuo*

and the lipid concentrate taken up in chloroform. Solutions of lipid in chloroform were adjusted so that 1 ml solution contained 27 μmoles of lipid phosphorus.

Phospholipid standards were products of Pierce Chemical Company, Rockford, Ill.

Preparation of chromatographic plates and solvents. To 40 g of Silica Gel G (Brinkmann Instruments), 80 ml of deionized water were added, and the resultant slurry was mixed with constant stirring for 4 min. Five glass plates, 20 × 20 cm, were coated with this slurry to a depth of 0.25 mm using the Desaga adjustable applicator. The plates were air-dried and activated for 1 h at 110° just before use.

Two different solvent systems were used for the development of the two-dimensional thin-layer chromatograms:

Solvent A: chloroform–methanol–acetic acid–water (25:15:4:2)[1].

Solvent B: butanol–pyridine–water (45:5:20)[4].

Spotting and development. For one-dimensional thin-layer chromatograms, 40–60 μl of lipid extract were applied to the TLC plates with micropipettes, and allowed to develop in equilibrated jars containing Solvent A. The solvent was allowed to rise within 2 cm of the top of the adsorbent. Average running time was 1.1 h. The plates were removed from the chromatography jars, dried for 30 min and placed in Solvent B after clockwise rotation through 90°. Average running time was 3 h.

Detection of spots. Lipids were detected on dried chromatograms in the following manner:

The plates were exposed to iodine vapor[8] and the spots were immediately outlined with the point of a needle. For the detection of phosphatidyl serine, phosphatidyl ethanolamine, and plasmalogens, the plates were sprayed with ninhydrin. Phosphatidic acid was identified, after elution from the thin-layer chromatogram, by deacylation according to the procedure of DAWSON[9]. The radioactive product and standard α-glycerophosphate were run in paper electrophoresis at pH 10.8 as described previously[7]. Other phospholipids of nerve-ending particles or purified mitochondria were identified by chromatographing standard phospholipids and comparison with previously published data using the same solvent system[1]. For the detection of radioactive lipids the chromatoplates were wrapped in Saferan film, and then exposed to Kodak No-Screen X-ray film for 3 days.

Analysis of the ^{32}P-labelled phospholipids. The radioactive phospholipid spots detected by autoradiography, and the non-radioactive lipids detected by iodine vapor, were scraped off with a razor blade and the radioactivity was measured with a gas-flow counter and scaler. After counting, the silica gel from each planchette was poured into test tubes and 0.25 ml of concentrated sulfuric acid was added to all tubes including blank silica gel, reagent blanks and inorganic phosphate standards. The tubes were placed in a heating block and digested according to the procedure of PARKER AND PETERSON[10].

Phospholipid phosphorus was determined by a modification of the methods of KING[11] and BARTLETT[12]. To all the tubes, 4.05 ml of water, 0.5 ml of 5 % ammonium molybdate solution and 0.2 ml of 1-amino-2-naphthol-4-sulfonic acid reagent were added. The latter reagent was prepared according to the method of KING[11]. After heating in a boiling water bath for 10 min, the tubes containing silica gel were centrifuged at 300 × g for 40 min. The color was read at wavelength of 620 mμ (see Fig. 2) on a Beckman Model B Spectrophotometer using glass cells with a 1 cm light path.

Results and discussion

The resolution of mixtures of ^{32}P-labelled phospholipids, extracted either from brain mitochondria or nerve-ending particles was found to be incomplete after running in one-dimensional TLC (Fig. 1A). However these mixtures were separated into 16 components by two-dimensional TLC (Fig. 1B), and furthermore a clear

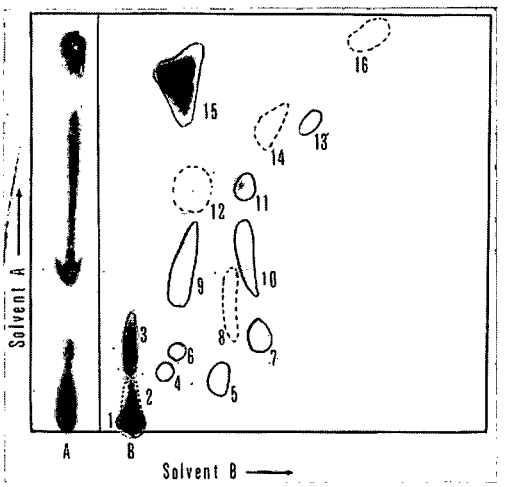

Fig. 1. An autoradiograph showing the separation of rat brain mitochondrial lipids by means of one-dimensional TLC (A) and two-dimensional TLC (B). Compounds (dotted lines) 8, 12, 14 and 16 were detected with iodine. Numbers refer to those given in Table I.

separation of phosphatidyl inositol and phosphatidic acid was achieved (Fig. 1B, Compounds 11 and 15 respectively).

Since these separations were clear and highly reproducible, the present method could be employed in studies on the turnover of the individual phospholipids in brain particulates (Table I).

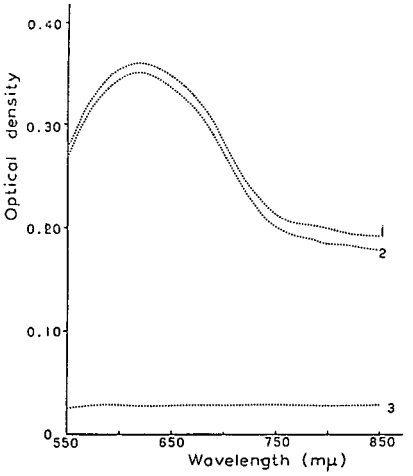

Fig. 2. Spectra of color given by phosphorus assay procedure (see text). (1) 0.2 μmole phosphorus + 100 mg Silica Gel G; (2) 0.2 μmole phosphorus; (3) 100 mg Silica Gel G.

TABLE I

DETERMINATION OF RADIOACTIVE SPECIFIC ACTIVITIES OF MITOCHONDRIAL AND NERVE-ENDING PHOSPHOLIPIDS AFTER SEPARATION BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

Spot No. (Fig. 1B)	*Compound*	*R_F × 100 in solvents*[a]		*R_F × 100 in solvents*[b]		*Counts/min/spot*		*mμmoles P/spot*		*Specific radioactivity c.p.m./mμmole P*	
		A	*B*	*A*	*B*						
1	Proteolipids + ^{32}P + ganglioside	0	0	0	0	4,460[c]	(900)[d]	14	(—)[e]	319	(—)
2	^{32}P ?	9.2	6.3	—	—	1,974	(400)	0	(0)[f]	—	
3	Unknown	34.8	6.3	—	—	5,370	(2,470)	36	(192)	149	(12.8)
4	Lysolecithin	16.4	14.7	17.8	14.0	67	(29)	71	(4)	0.95	(7.3)
5	Unknown	17.0	28.6	—	—	302	(154)	29.2	(9.6)	10.4	(16)
6	Sphingomyelin	34.1	17.5	31.2	16.0	87	(31)	14	(27)	6.2	(1.2)
7	Unknown	27.8	39	—	—	359		18.7		19.2	
8	Plasmalogen ?	39.2	31.5	—	—	123	(20)	110	(43)	11.2	(0.47)
9	Lecithin	50.0	20.3	48.0	23.0	2,000	(210)	484	(216)	4.2	(0.97)
10	Unknown	50.5	35	—	—	1,974		140		14.1	
11	Phosphatidyl inositol	61.2	36	59.0	33.4	1,026	(2,290)	14	(9)	73.1	(254.3)
12	Phosphatidyl serine	63.2	24.4	65.2	24.0	138	(8)	39	(24)	3.5	(0.33)
13	Unknown	78	54.5	—	—	548		0		—	
14	Phosphatidyl ethanolamine	78	44	77.0	43.1	10	(75)	358	(89)	0.03	(0.84)
15	Phosphatidic acid	90.5	24.5	—	—	7,360	(1,985)	43	(16)	172	(124)
16	Cerebroside	97.5	71.5	96.1	76.2	0	(0)	0	(0)	0	(0)

[a] R_F values of mitochondrial lipids; $R_F = \frac{\text{distance of center of spot from starting-point}}{\text{distance of solvent from starting-point}}$.

[b] R_F values of standard lipids.

[c] Mitochondrial lipids.

[d] Nerve-ending lipids.

[e] Was not determined.

[f] There was no detectable phosphorus in this spot. The radioactivity could have been due to contamination from the ^{32}P-orthophosphate.

As can be seen from Table I, it has not been possible to identify all the lipid components on the thin-layer chromatogram. Compounds 3, 5 and 13 did not stain with iodine but contained a considerable amount of activity. Compounds 3 and 5 could be the deacylated derivative of phosphatidic acid (lysophosphatidic acid) and the phosphorylated derivative of phosphatidyl inositol (diphosphoinositide) respectively. In agreement with the work of other investigators[13,14] phosphatidic acid and phosphatidyl inositol were found to be highly labelled in both nerve-ending particles and mitochondria (Table I). On the other hand structural lipids such as phosphatidyl serine and phosphatidyl ethanolamine had little activity.

Unlike Silica Gel H[10], washed Silica Gel G gave poor resolution of phospholipids. This resulted in higher readings of the Silica Gel G blanks (Fig. 2). However, the blank readings could not have been due to the phosphorus-molybdate complex as can be seen from the silica gel spectrum (Fig. 2). The modification which was made in the developing reagents resulted in a shift of the spectral peak from a maximum at 830 mμ[12] to a maximum at 620 mμ (Fig. 2). The separation and determination of the radioactive specific activities of lipids reported in this communication could be applied to the whole brain as well as particulates from other tissues.

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A new method for hexosamine detection using 5-dimethylaminonaphthalene-sulfo-derivatives and separation by thin-layer chromatography

Several methods have been developed for the detection and quantitative estimation of glucosamine and galactosamine. With some of them it is possible to determine the total hexosamine content in natural products[1-5]. The existing methods of separate determination are not completely satisfactory and do not always yield reproducible results[6]. Paper or ion-exchange chromatography have been used for the separation either of free amino sugars[7,8] or their DNP*-derivatives[9,10]. Gas chromatography[11-13] has also been described, and quite recently chromatography of free amino sugars on a thin layer of powdered cellulose[14].

In view of the fact that natural products contain either only glucosamine or only galactosamine, their preliminary qualitative estimation would make the existing methods more precise. We have attempted here to develop a simple and substantially more sensitive method for determining glucosamine and galactosamine in a mixture by use of their DNS-derivatives. The R_F value of DNS-glucosamine is already known[15].

Materials and methods

α-D-Glucosamine hydrochloride (Koch-Light Laboratories, Ltd., England) and D-galactosamine hydrochloride (LOBA-Chemie, Austria), homogeneous in paper chromatography, were used. An apyrogenic preparation of glucose (SPOFA, Č.S.S.R.), DNS-chloride (Calbiochem, U.S.A.), and Kieselgel G für Dünnschichtchromatographie nach Stahl (Merck A.G., Germany) were also used.

The standard thin-layer chromatographic technique according to STAHL[16] with ascending development was used.

The DANSYLation was carried out according to GRAY AND HARTLEY[17] with several modifications: The hexosamine solution (GLA and GAA) was evaporated in a small tube, containing between 0.003 and 0.005 μmole of each substance. The residue was mixed with 20 μl 0.8% $NaHCO_3$ and 25 μl acetone solution of DANSYL-chloride (1 mg/ml). The tube was closed and left for 3 h at 4° or for 2 h at 15–20° in the dark. The reaction mixture was evaporated in a desiccator over NaOH and the DNS-derivatives were dissolved in acetone before placing on the thin-layer plate.

The chromatographic layer had the following composition: 20 ml 0.2 *M* $Na_2B_4O_7$, 45 ml water, 30 g Kieselgel G, with a thickness of 0.2 mm. The plates were dried in air for 10–12 h and then for 3 h at 130°. Before use they were heated for 10 min to 105°. The following solvent systems were found to be the most satisfactory: (I) ethyl acetate–pyridine–water (61:31:8, v/v), (II) petroleum ether–glacial acetic acid–*tert.*-butanol (75:15:15, v/v)[18], and (III) chloroform–methanol–glacial acetic acid (75:25:5, v/v)[19]. When a two-dimensional technique was used, the plates were dried after development in the first direction for 10 min at 105°. The DNS-derivatives were detected by examining the plates under transmitted U.V. light while still wet, when they appear as yellow fluorescent spots.

Results and discussion

One-dimensional chromatography in system (I) resolves DNS-GLA, DNS-GAA

* Abbreviations used: DNP- = dinitrophenyl-; DNS- (DANSYL-) = 5-dimethylaminonaphthalenesulfonyl-; GLA = glucosamine; GAA = galactosamine; GL = glucose.

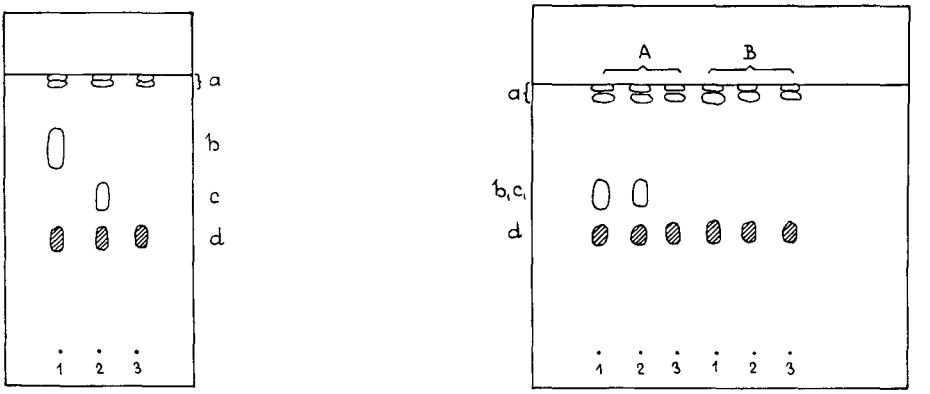

Fig. 1. Chromatogram of DNS-derivatives in system (I) using Kieselgel G with borate. DANSYLated samples: 1 = GLA; 2 = GAA; 3 = GL; a = DNS-NH_2 and artifacts; b = DNS-GLA; c = DNS-GAA; d = DNS-OH.

Fig. 2. Chromatogram of DNS-derivatives in system (III) using Kieselgel G with borate. A = sample DANSYLated by the standard procedure; B = samples incubated in the DANSYLation mixture at 105° for 1 h. Other symbols as in Fig. 1.

and DNS-GL within 40–50 min, the front travelling about 14–16 cm (Fig. 1). The sensitivity of the method is $3–5 \cdot 10^{-3}$ μmole hexosamines. DNS-hexosamines show some properties whereby they differ from the DNS-derivatives of amino acids and these can be made use of for their differentiation.

Thus, *e.g.* DNS-GLA and DNS-GAA in aqueous solution are highly temperature-sensitive. After incubation in the basic medium of the DANSYLation mixture at 37° for 1 h the spot intensity diminishes markedly. After 1 h at 105° the spots disappear completely (Fig. 2). Similarly, after hydrolysis in 6 *N* HCl at 105° the hexosamine

TABLE I

R_F VALUES OF DNS-DERIVATIVES OF GLA, GAA AND SOME AMINO ACIDS DURING THIN-LAYER CHROMATOGRAPHY USING KIESELGEL G WITH SODIUM BORATE

DNS-derivative	*Solvent system* *	
	I	*II*
-GLA	0.65	0.00
-GAA	0.50	0.00
-Val, -Lys, -Tyr, -Phe, -Leu, -Ile	0.45–0.65	0.15–0.60
-Cys,-Pro,-Ser, -Thr,-Glu,-Gln, -Asp,-Asn,-Gly	0.40	0.10
-OH	0.40	0.00
-NH_2	0.92–0.96	0.09
-GL	1.00	0.90

* Composition given in the text.

spots disappear. On the other hand, the DNS-amino acids under identical conditions are quite stable.

The solvent system (III) was used for identifying artifacts and for following the stability of DNS-hexosamines. It was shown that DNS-GL as an O-DNS-derivative gives a weak spot in the front of system (I).

The separation of DNS-hexosamines from DNS-amino acids was attempted. During two-dimensional chromatography, when system (II) was used in the first direction, it separated DNS-GLA and DNS-GAA from most amino acids. In the second direction (system I) the hexosamines are distinguished from each other. The R_F values of DNS-hexosamines and DNS-amino acids in the two systems are shown in Table I where it can be seen that the R_F values of most DNS-amino acids differ from those of DNS-hexosamines. Experiments along these lines are being continued since the present separation is not considered as fully satisfactory.

The use of DNS-derivatives of GLA and GAA for the chromatographic separation described permits a very sensitive and simple detection, as well as a separation of both hexosamines within 3.5 h. Some properties of DNS-hexosamines were established which make it possible to distinguish them from DNS-amino acids.

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Thin-layer chromatographic study of *p*-toluenesulphonates of some amines

Separation of amines by TLC has been carried out by several workers[1–9]. YASUDA[10] has reported on the identification of N-nitroso- and nitrodiphenylamines by this technique. TEICHERT and coworkers[11] have reported on a study of their 3,5-dinitrobenzamides, whereas SEILER AND WEICHMANN[12] investigated their 1-dimethylaminonaphthalene-5-sulphonates.

The present paper describes the resolution and identification of some amines in the form of their *p*-toluenesulphonates. This was achieved by directly converting a mixture of amines on thin-layer plates into their tosylates and subsequently running the chromatograms. Thus, in a mixture of six to eight amines it was possible to characterize distinctly 1–2 μg of each amine in the form of its *p*-toluenesulphonate.

Experimental

All the solvents used were freshly dried and distilled. Kodak photographic glass plates (20 × 28 cm) were used. An ascending irrigation technique was employed.

Preparation of p-toluenesulphonates. The individual amine (1 mole) was dissolved in pyridine (10 moles) and to this *p*-toluenesulphonyl chloride (1.1 mole except in the case of ethylenediamine 2.1 moles) in pyridine (5 moles) added. The clear solution was heated for half an hour on the water bath under anhydrous conditions. After the reaction, the contents were poured into an excess of cold water, and the resultant crystalline precipitate filtered, washed with aqueous sodium carbonate followed by dilute hydrochloric acid and water. The product was dried and repeatedly crystallized from hot ethanol. Their melting points are as follows:

aniline-*p*-toluenesulphonate, m.p. 103°; *o*-toluidine-*p*-toluenesulphonate, m.p. 108°; *m*-toluidine-*p*-toluenesulphonate, m.p. 114°; *p*-toluidine-*p*-toluenesulphonate, m.p. 118°; *o*-nitroaniline-*p*-toluenesulphonate, m.p. 110°; *m*-nitroaniline-*p*-toluenesulphonate, m.p. 138°; *p*-nitroaniline-*p*-toluenesulphonate, m.p. 190°; α-naphthylamine-*p*-toluenesulphonate, m.p. 157°; β-naphthylamine-*p*-toluenesulphonate, m.p. 133°; ethylenediamine-N,N′-di-*p*-toluenesulphonate, m.p. 160°; piperidine-*p*-toluene-

TABLE I

DETAILS OF THIN-LAYER COATINGS OF VARIOUS ADSORBENTS

*Adsorbent**	*Quantity of adsorbent used for slurry (g)*	*Solvent used for slurry*	*Quantity of adsorbent per cm² (mg)*
A	30	Chloroform (55 cc.)–methanol (28 cc.)	7.5
B	30	0.5 *N* oxalic acid (20 cc.)–methanol (70 cc.)	6.8
C	30		
D	30	Water (60 cc.)	6.2
E	30		

* A–D, see text.

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TABLE II

R_F VALUES OF VAROUS AMINE *p*-TOLUENESULPHONATES

Solvent systems: (I) chloroform; (II) chloroform–xylene (80:20); (III) chloroform–xylene (95:5); (IV) petroleum ether–ether (50:50).

No.	*p-Toluenesulphonates of the following amines*	*Silica gel G*			*Buffered silica gel G with N/2 oxalic acid*			*Neutral alumina*			*Basic alumina*			*Acidic alumina*		
		I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*IV*	*II*	*III*	*IV*	*II*	*III*	*IV*
1	Aniline	0.8	0.36	0.49	0.63	0.38	0.52	0.77	0.26	0.48	0.23	0.33	0.26	0.17	0.27	0.28
2	*o*-Toluidine	0.93	0.43	0.52	0.74	0.44	0.62	0.86	0.47	0.74	0.36	0.48	0.33	0.26	0.38	0.50
3	*m*-Toluidine	0.76	0.34	0.41	0.47	0.40	0.53	0.85	0.30	0.56	0.27	0.31	0.30	0.10	0.20	0.34
4	*p*-Toluidine	0.53	0.33	0.35	0.43	0.35	0.50	0.30	0.68	0.53	0.32	0.40	0.28	0.20	0.31	0.40
5	*o*-Nitroaniline	0.98	0.58	0.64	0.50	0.60	0.72	0.57	0.17	0.26	0.50	0.60	0.38	0.06	0.15	0.09
6	*m*-Nitroaniline	0.38	0.22	0.24	0.27	0.24	0.34	0.20	0.08	0.21	0.14	0.23	0.14	0.03	0.10	0.06
7	*p*-Nitroaniline	0.41	0.18	0.21	0.22	0.20	0.30	0.10	0.03	0.07	0.09	0.14	0.10	0.02	0.08	0.03
8	α-Naphthylamine	0.53	0.36	0.57	0.45	0.41	0.54	0.50	0.22	0.42	0.31	0.43	0.24	0.14	0.23	0.20
9	β-Napthylamine	0.60	0.30	0.47	0.40	0.30	0.55	0.54	0.16	0.39	0.21	0.28	0.21	0.10	0.16	0.23
10	Ethylenediamine	0.58	0.33	0.42	0.42	0.34	0.50	0.70	0.25	0.50	0.29	0.38	0.25	0.16	0.25	0.32
11	Piperidine	0.62	0.50	0.58	0.55	0.52	0.66	0.58	0.61	0.94	0.64	0.86	0.23	0.63	0.70	0.87
12	*m*-Chloroaniline	0.55	0.63	0.38	0.43	0.38	0.47	0.42	0.66	0.33	0.23	0.33	0.53	0.18	0.30	0.16
13	Diphenylamine	0.70	0.32	0.70	0.70	0.66	0.73	0.50	0.33	0.70	0.40	0.45	0.43	0.62	0.51	0.17
14	*p*-Anisidine	0.62	0.38	0.39	0.39	0.34	0.57	0.80	0.51	0.40	0.20	0.30	0.15	0.12	0.21	0.36

sulphonate, m.p. 96°; *m*-chloroaniline-*p*-toluenesulphonate, m.p. 138°; diphenylamine-*p*-toluenesulphonate, m.p. 141°; *p*-anisidine-*p*-toluenesulphonate, m.p. 114°.

Adsorbents. The following adsorbents were employed:

(A) Silica gel G (E. Merck)
(B) Silica gel G (E. Merck), buffered with 0.5 *N* oxalic acid
(C) Alumina, neutral (M. Woelm)
(D) Alumina, basic (M. Woelm)
(E) Alumina, acidic (M. Woelm).

Preparation, spotting and irrigation of plates. The plates were coated by pouring a homogeneous slurry of the adsorbant in a suitable solvent on to them and tilting

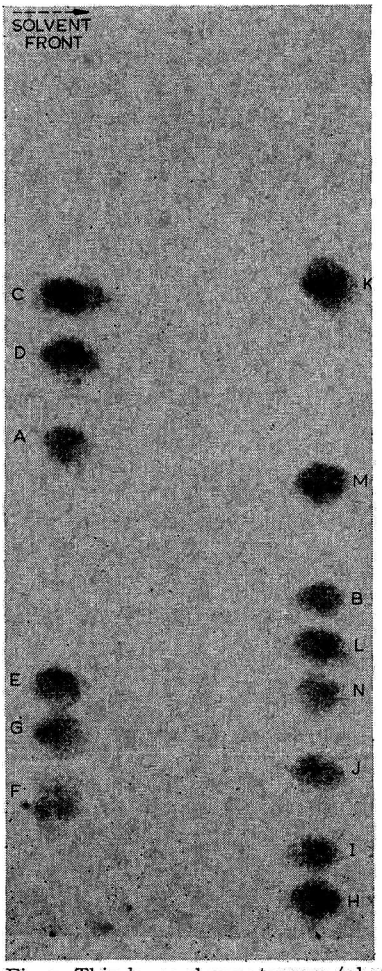

Fig. 1. Thin-layer chromatogram (photograph taken under U.V. light) showing the resolution of mixtures of the following amines as their *p*-toluenesulphonates: A = *p*-anisidine; B = diphenylamine; C = *m*-chloroaniline; D = piperidine; E = ethylenediamine; F = β-naphthylamine; G = α-naphthylamine; H = *p*-nitroaniline; I = *m*-nitroaniline; J = *o*-nitroaniline; K = *p*-toluidine; L = *m*-toluidine; M = *o*-toluidine; N = aniline. Adsorbent: neutral alumina (Woelm). Irrigating solvent: chloroform–xylene (80:20). System: ascending.

them from side to side. The plates were left overnight at room temperature and were activated at 110° for 1 h. They were then weighed and the quantity of adsorbent per cm^2 was noted down. Details are given in Table I. The amine solutions (each containing 1–2 μg) in pyridine–petroleum ether (1:1) were spotted on the plate with a micro-capillary, and on the same spots a solution of *p*-toluenesulphonyl chloride (equivalent to 1.1 mole of amine, or when it was a mixture of amines to 1.5 moles) in pyridine was applied. The authentic *p*-toluenesulphonates were also spotted alongside. The plates were left for 4 h in an oven at 60°, after which they were irrigated with suitable solvents. The spots were observed under U.V. light using a "chromatolite" lamp. Table II gives the R_F values of various amine-*p*-toluenesulphonates.

Discussion and results

It was observed that when the amines were spotted as mixtures or individually and were then converted on the plates into their *p*-toluenesulphonates on subsequent irrigation, they resolved distinctly. Thus, a mixture of six to eight amines, in the form of their *p*-toluenesulphonates, was readily resolved (Fig. 1). The R_F values of these compounds corresponded to the authentic *p*-toluenesulphonates of the amines investigated. The individual compounds proved to be single spots on two-dimensional thin-layer chromatography.

It was found that cellulose–calcium sulphate, Kieselgel G, and 5–10 % olive oil impregnated cellulose were not good adsorbents for these compounds. There was profuse tailing with the majority of these compounds.

The tosylates could be directly observed under U.V. light and there was no necessity for a spray reagent as in the case of amines.

Polar solvents when employed for irrigation of the plates either produced long tailing or highly diffused spots. Non-polar solvents and their mixtures produced excellent resolution of the compounds.

Thin coatings of the adsorbents produced very good resolutions. Silica gel G (unbuffered), silica gel G (buffered with *N*/2 oxalic acid), and all the three forms of alumina gave good results. In general, the mobility of the compounds on acidic alumina in chloroform–xylene solvent mixtures was reduced when compared to basic and neutral alumina. On buffered silica gel G, chloroform gave lower R_F values than on unbuffered silica gel G.

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Thin-layer chromatography of ε-caprolactam and its cyclic oligomers

After polycondensation polycapramide contains, at equilibrium, approximately 10 % of monomers and oligomers, most of which are cyclic. The paper chromatography of the cycloamide homologue was first reported by ZAHN AND REXROTH[1], who detected it by chlorination and subsequent dipping in *o*-tolidine and potassium iodide mixture. CZEREPKO[2,3] reported the use of chromogenic reagents for alkaloids, such as potassium bismuth iodide, potassium iodoplatinate and potassium antimony iodide, for the detection of ε-caprolactam in paper chromatography. *m*-Dinitrobenzene was also reported to be useful as a detection reagent[4]. The sensitivities of the chromogenic reagents mentioned above were at most about 5 to 10 μg of ε-caprolactam.

It has now been found that the sensitivity of potassium bismuth iodide toward ε-caprolactam and its cyclic oligomers was improved approximately ten times and 1 μg or less of the cycloamides could be detected on thin-layer chromatograms if they are sprayed with diluted sulfuric acid subsequent to Dragendorff's reagent.

Experimental

Apparatus. Standard TLC plates of Tôyô Kagaku Sangyô Co., Ltd., Model HC-20, were used. Glass plates used were 20 × 20 cm.

Materials. Cyclic oligomers (monomer to tetramer) of polycapramide were obtained by fractional vacuum sublimation of an aqueous extract from polycapramide[5].

Higher cycloamide homologues than the tetramer were synthesized by the procedures described by ZAHN AND DETERMAN[6].

The adsorbents used were alumina (Aluminiumoxid-G, Merck) and silica gel (Kieselgel-G, Merck, nach STAHL).

Dragendorff's reagent was prepared as follows:

(A) 1.0 g of bismuth subnitrate was dissolved in a small amount of concentrated hydrochloric acid and precipitated with aqueous ammonia. The precipitate formed after filtering was dissolved again in a small amount of concentrated hydrochloric acid and then 3.0 g of potassium iodide were added. The whole was diluted to 50 ml with distilled water.

(B) 25 g of potassium iodide were dissolved in 100 ml of distilled water.

(C) 70 % aqueous acetic acid.

A, B and C were mixed in the volume ratio of 5:5:40.

TABLE I

R_F VALUES OF ε-CAPROLACTAM AND ITS CYCLIC OLIGOMERS

Solvent systems: A = Isopropanol–methanol–water (1:1:1); B = tetrahydrofuran–petroleum ether (b.p. 100–120°)–water (186:14:10); C = *n*-butanol–acetic acid–water (10:2:5); D = *sec.*-butanol–formic acid–water (75:15:10); E = *n*-butanol–concentrated hydrochloric acid–water (100:20:38); F = *n*-butanol saturated with water.

$[HN(CH_2)CO]_n$	*Kieselgel*					*Alumina*				
	A	*B*	*C*	*D*	*E*	*B*	*C*	*D*	*E*	*F*
n = 1	0.81	0.75–0.79	0.69–0.75	0.69–0.72	0.70	0.92	0.90	0.91–0.95	0.88	0.87
2	0.77	0.70–0.78	0.64–0.52	0.56–0.60	0.61	0.86–0.88	0.90	0.94–0.93	0.88	—
3	—	0.61–0.65	0.55	0.47–0.49	—	0.83	—	0.92	—	—
4	0.74	0.49–0.57	0.39–0.40	0.39–0.43	0.48	0.76–0.77	0.90	0.91–0.92	—	—
5	—	0.35–0.48		0.33–0.38	0.39	0.70–0.71	0.85	0.87	—	—
6	—	0.27	0.24–0.25	0.26–0.35	0.30	0.65–0.66	0.71	0.77–0.80	—	—
7	—	—	—	0.25–0.32	0.21	0.54–0.58	—	0.61	—	—
8	—	—	—	—	—	0.57	—	—	—	—

Procedure. The adsorbent layer was prepared by STAHL's method. The adsorbent slurry was coated on the plates with a standard thickness (0.25 mm), dried in air for 30 min and then for 2 to 3 h at 120°.

The chromatoplates were developed by the ascending technique, and 0.1–2 μl of a 1 % cycloamide methanol solution was applied to the plates by means of a microsyringe.

The time required for development (10 cm) was about 1–2 h. After removing the solvent in a stream of hot air, the plates were sprayed with Dragendorff's reagent, followed by 10 % sulfuric acid.

Results and discussion

The results obtained are tabulated in Table I.

When Kieselgel containing thymol was used as adsorbent, the dependence of the R_F value on the content of thymol is shown in Fig. 1.

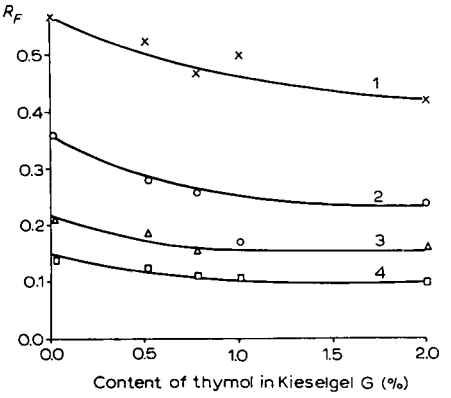

Fig. 1. Dependence of R_F value on the content of thymol in Kieselgel G. 1 = ε-Caprolactam; 2 = cyclic dimer; 3 = cyclic trimer; 4 = cyclic tetramer.

Chromogenic reagents. The complex of ε-caprolactam with potassium bismuth iodide was reported to be precipitated with hydrochloric acid[7].

It has now been found that the precipitation behavior of the complex differs depending on the acids used. Acetic acid does not permit formation of the precipitate. In the case of hydrochloric acid, only suitable amounts would precipitate the complex and excess readily redissolve the precipitate. The suitable range of hydrochloric acid concentration was narrow. Sulfuric acid accelerated the precipitation of the complex but only redissolved it with decomposition when a large excess of concentrated sulfuric acid was used.

One of the reasons for the increased sensitivity, we suppose, was the easy formation of insoluble dark red bismuth complexes with cycloamides. Acids which have no function in the formation of the precipitate when used together with Dragendorff's reagent, such as acetic acid, do not show the improvement of sensitivity. A further reason for increased sensitivity may be due to the disappearance of the yellow back-ground formed with Dragendorff's reagent when sulfuric acid is used.

Developing reagents. ε-Caprolactam and its cyclic homologues are not separated by non-polar solvents, for example, a chloroform and cyclohexane mixture.

When heterocyclic compounds containing nitrogen, such as pyridine and water, were used as developing reagents, trace amounts of the reagents remained after drying at 100° for several hours and the detection of spots was interfered with.

The system tetrahydrofuran–petroleum ether–water gave good separation of the cyclic oligomers, but the separation of ε-caprolactam and its dimer was not sufficient.

The system *n*-butanol–acetic acid–water gave good separation of monomer and dimer, although separation of tetramer and pentamer was incomplete.

sec.-Butanol, formic acid and water or *n*-butanol saturated with water did not show good separations of higher members of the series than the tetramer.

In paper chromatography of cycloamides, CZEREPKO[8] used paper strips impregnated with thymol. The author utilised this information in the present work. The dependence of the R_F value on the content of thymol in the Kieselgel is shown in Fig. 1, which indicates that the variation in R_F value is small and the separation of members higher than the trimer is extremely poor. Alumina did not give as good a separability as Kieselgel G.

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Thin-layer chromatography of 2,4-dinitrophenylhydrazones of α-alkyl substituted carbonyl compounds

The carbonyls isolated from edible products are generally of a very complicated mixture. On converting them into their 2,4-dinitrophenylhydrazones (DNPHs) almost complete separation of such mixtures is possible by a combination of partition and adsorption thin-layer chromatography:

(i) according to chain length, *e.g.* with Kieselguhr G plates impregnated with Carbowax[1],

(ii) according to number and position of double bonds, *e.g.* with silica gel or alumina plates impregnated with silver nitrate[2–4].

On using TLC plates impregnated with silver nitrate we have now found that DNPHs of some branched, saturated carbonyls can be separated from other DNPHs having the same chain length.

The plates (20 × 20 cm) were impregnated with 44 wt. % $AgNO_3$ (layer thickness: 0.25 mm), as described earlier[2,5,6].

An amount of 2–4 μg of the DNPHs, dissolved in a minimum quantity of chloroform, was spotted on to the plates, which were then developed in a mixture of light petroleum (b.p. 40–60°) and diethyl ether (85:15, v/v), until the liquid front had travelled 13 cm.

The separation of a number of DNPHs of straight-chain, as well as branched, saturated aldehydes and ketones on an alumina plate impregnated with silver nitrate is shown in Fig. 1.

On both silica gel and alumina plates, impregnated with silver nitrate, the

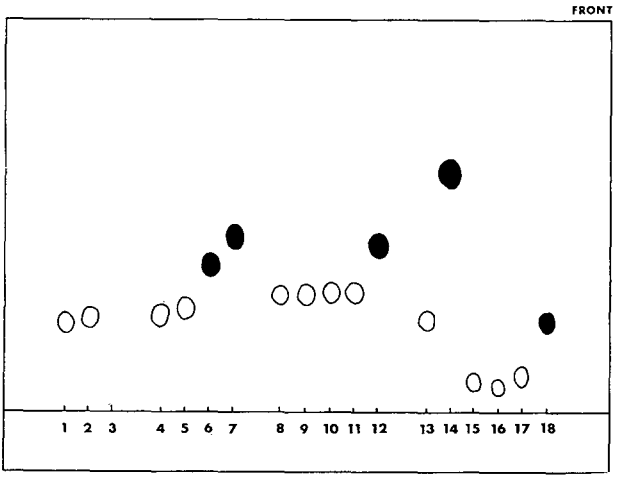

Fig. 1. Separation of carbonyl-DNPHs by TLC on alumina impregnated with silver nitrate. Mobile phase: light petroleum (b.p. 40–60°)–ether (85:15, v/v). Plate: 20 × 20 cm. The DNPHs of α-alkyl substituted carbonyls are shown as black spots. The R_F values of the compounds are given in brackets. 1 = pentanal (0.22); 2 = 3-methyl butanal (0.23); 3 = 2-methyl butanal (0.32); 4 = heptanal (0.24); 5 = 3-ethyl pentanal (0.26); 6 = 2-ethyl butanal (0.37); 7 = 2-ethyl hexanal (0.45); 8 = dodecanal (0.29); 9 = 10-methyl dodecanal (0.30); 10 = tetradecanal (0.29); 11 = 12-methyl tridecanal (0.30); 12 = 2-methyl undecanal (0.42); 13 = methyl butyl ketone (0.23); 14 = 3,3-dimethyl-2-butanone (0.61); 15 = cyclopentanone (0.08); 16 = cyclohexanone (0.06); 17 = 3-methyl cyclopentanone (0.09); 18 = 2-methyl cyclopentanone (0.23).

DNPHs of α-alkyl substituted saturated carbonyls showed higher R_F values than other DNPHs having the same chain lengths. The results obtained with the two types of plate did not show any marked differences. Separation was also possible on non-impregnated plates, but here the R_F value differences were smaller than with the impregnated plates.

It is immaterial whether the α-alkyl substituent is a methyl or an ethyl group. At the time, corresponding aldehyde-DNPHs with an even longer side chain at the α-position, and comparable ketone-DNPHs, were not available. A ketone-DNPH having two substituents at the α-position (3,3-dimethyl 2-butanone) had an R_F value which differed appreciably from that of the normal ketone-DNPH. DNPHs of saturated ketones with one substituent at the α-position can probably also be separated on silver nitrate plates from other ketone-DNPHs having the same chain length.

We have also compared 2-methyl cyclopentanone-DNPH with the DNPHs of 3-methyl cyclopentanone, cyclopentanone, and cyclohexanone. Surprisingly, the DNPHs of cyclic ketones were absorbed much more strongly on silver nitrate plates than DNPHs of aliphatic ketones. 2-Methyl cyclopentanone-DNPH, however, migrated much faster than the other cyclic ketone-DNPHs. This difference was less pronounced on plates not impregnated with silver nitrate.

We suppose that the bond between the C=N bond and the silver nitrate is weakened by the α-alkyl substituent, as a result of which separation becomes possible.

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Thin-layer chromatography of cyanocobalamin, hydroxocobalamin and B_{12} coenzymes

Vitamin B_{12} vitamers and their coenzyme forms can be separated by paper partition chromatography and paper electrophoresis. Paper partition chromatography takes a long time for the development and a shortcoming is that spots tail. On the other hand, paper electrophoresis is performed within a comparatively short time and has a good resolution. However, with the latter method not many samples can be examined at the same time a factor which is very desirable during the preparation and purification of such coenzyme forms of the vitamin.

Application of thin-layer chromatography for the separation of cyanocobalamin, hydroxocobalamin etc. was reported by CIMA AND MANTOVAN[1] and KAMIKUBO AND HAYASHI[2].

The present communication reports the separation by thin-layer chromatography of cyanocobalamin (CN-B_{12}), hydroxocobalamin (OH-B_{12}), adeninylcobamide coenzyme (ACC), benzimidazolylcobamide coenzyme (BCC) and dimethylbenzimidazolylcobamine coenzyme (DBCC), which can be done within a very short time with a good resolution.

CN-B_{12} and OH-B_{12} were obtained from Kaken Chemicals Co. and Roussel Co., respectively. DBCC was prepared by the following procedure: *Propionibacterium shermanii* IAM 1725 was grown in a medium containing the following ingredients per liter: glucose, 20 g; meat extract, 35 g; yeast extract, 5 g; $Na_2HPO_4 \cdot 12H_2O$, 1.75 g; K_2HPO_4, 1.75 g; $MgSO_4 \cdot 7H_2O$, 400 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CoCl_2 \cdot 6H_2O$, 5 mg; calcium pantothenate, 4 mg; biotin, 0.3 mg. Incubation was carried out, stationary, at 30°. After the maximum growth was attained, 12.5 mg of CN-B_{12} and 5 g of glucose were added per liter of the medium, and the incubation was continued for a further 5 days. The cells were harvested by centrifugation and washed twice with 0.1 *M* phosphate buffer, pH 7.0. These cells were extracted with 80% ethanol at 82° for 30 min. The cells were filtered off and the orange-red colored filtrate was evaporated to dryness on a water bath at 50°. The dried residue was dissolved in water, followed by the addition of phenol. The orange-red color was transferred into the heavier phenol phase, which was displaced back into water when the phenol was removed by shaking with ether. Then, the colored solution was passed through a column of DEAE-Sephadex, A-25, equilibrated with 0.01 *M* sodium acetate. The effluents containing the color were combined and buffered with acetate buffer, pH 3.5, to give a concentration of 0.04 *M*, which was subsequently placed on a P-cellulose column equilibrated with 0.04 *M* acetate buffer, pH 3.5. The column was eluted first with the same buffer and next with 0.04 *M* acetate buffer, pH 4.7. The red fractions eluted were combined and extracted by using phenol and water as above. All operations were conducted with the maximal exclusion of light. Finally, red crystals were obtained by lyophilization which showed coenzyme B_{12} activity as measured by the formation of propionaldehyde from propanediol[3] with cell-free extracts of *Aerobacter aerogenes* ATCC 8308. The absorption spectra[4] over the range of 220 to 600 mμ in the absence and presence of 0.1 *M* KCN and electrophoretic behavior[5] agreed entirely with the described characteristics of DBCC. Additional confirmatory evidence of the identity of DBCC was obtained by the exposure of the compound to visible light which led to the degradation to OH-B_{12}. ACC was prepared from *Prop. arabinosum* IAM 1714 cells by a similar procedure to that described above except that no CN-B_{12} was added as the precursor. Identification of ACC was made by a similar method to that employed for DBCC. BCC was a gift from Prof. H. A. BARKER.

The CM-cellulose plates were prepared with MN300 and MN300CM (products of Macherey, Nagel & Co.). The developing solvent system was the lower layer of the mixture of *sec.*-butanol, 0.1 *M* acetate buffer, pH 3.5, and methanol (4:12:1) which gave a most satisfactory separation. Fig. 1 shows the result. The compounds could be sharply separated by this system, the R_F value of ACC differing particularly greatly from those of DBCC and BCC. Fig. 2 also shows the result of the exposure of DBCC to visible light by which it was degraded to OH-B_{12}.

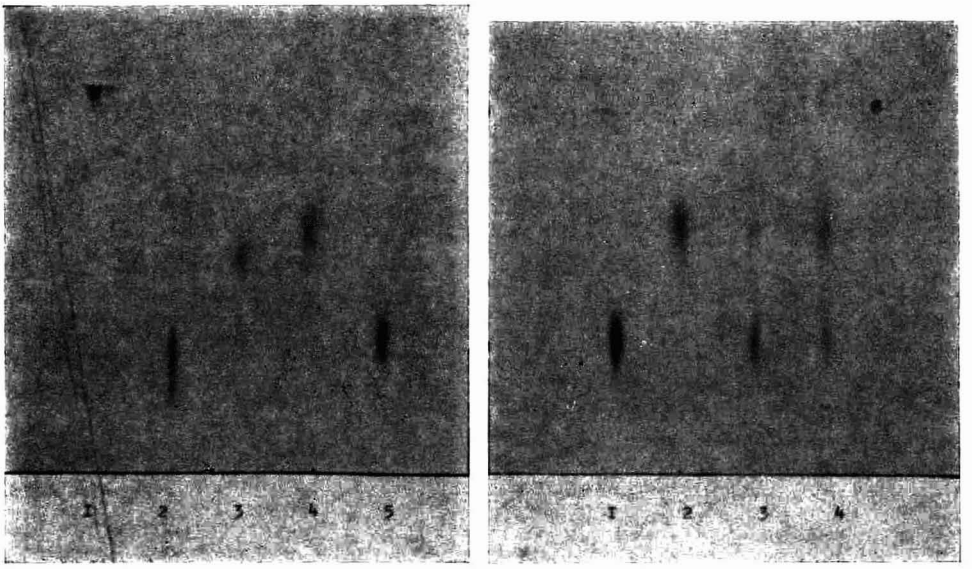

Fig. 1. Thin-layer chromatogram of B_{12} vitamers and coenzymes. Length of run was about 10 cm. 1 = CN-B_{12}; 2 = ACC; 3 = BCC; 4 = DBCC; 5 = OH-B_{12}.

Fig. 2. Thin-layer chromatogram of DBCC with and without exposure to visible light. Aliquots of a DBCC solution were exposed for 30 min to a 500-watt tungsten filament lamp at a distance of 30 cm. The exposure was made in a normal and an amber glass tube kept at 0°. Length of run was about 10 cm. 1 = OH-B_{12}; 2 = DBCC; 3 = DBCC exposed to light in a normal tube; 4 = DBCC exposed to light in an amber tube.

It should be added that in this chromatographic method it is essential that the inorganic ions are removed from the samples in order to obtain a good resolution, since CM-cellulose is a cation-exchanger. The removal can be easily accomplished by washing the phenol phase with water.

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Separation and detection of oral hypoglycaemic agents by thin-layer chromatography

The use of orally administered drugs to replace insulin for the control of certain conditions of diabetes mellitus has gained popularity during the past few years. Four such compounds are listed in the recent edition of "New Drugs"[1] as being of general availability in North America. They include the three sulphonylurea derivatives, acetohexamide [1-(*p*-acetylphenylsulphonyl)-3-cyclohexylurea], chlorpropamide [1-(*p*-chlorophenylsulphonyl)-3-propylurea], and tolbutamide [1-butyl-3-(*p*-tolylsulphonyl) urea]; and the biguanide, phenformin hydrochloride [N^1-β-phenethylbiguanide hydrochloride]. The increased use of these drugs and a recent indication that combinations of hypoglycaemic agents can be more effective than the individual drugs alone in controlling diabetes[2] infer that a rapid method for their detection would be of value.

The three sulphonylurea derivatives may be classified with the general sulphonamides, the paper and thin-layer chromatography of which has been widely investigated. However, there are few references to the chromatography of the above sulphonylureas and visual detection has had limitations due to their lack of a chemical grouping suitable for colour reactions. CHAKRABARTI[3] achieved a slight difference in R_F values for the separation of chlorpropamide and tolbutamide together with carbutamide on paper using phenylhydrazine and nickel sulphate spray solutions for detection (limit 10 μg). HENTRICH[4] included tolbutamide in a series of sulphonamides separated on paper and was able to detect the hypoglycaemic agent by hydrolysis and reaction of the liberated butylamine with either ninhydrin or Folin reagent. The application of thin-layer chromatography to the separation of chlorpropamide and tolbutamide was investigated by REISCH *et al.*[5], and to the separation of tolbutamide from other sulphonamides by NEIDLEIN *et al.*[6]. SMITH *et al.*[7] separated acetohexamide from its metabolite by this technique. Apart from the use of ninhydrin in one instance[5], detection of the drugs has been by absorbance on fluorescent plates under short-wave U.V. light.

Since the chromatography of the sulphonylureas has been limited and no rapid and sensitive microchemical characterisation or specific assay is available, a thin-layer chromatographic method is described here whereby microgram quantities of the four commercially available oral hypoglycaemic agents can be conveniently separated and detected.

Experimental

Solvent systems

S_1 = Acetone–benzene–water (65:30:5),
S_2 = Acetone–butanol–water (20:50:30),
S_3 = Butanol saturated with water,
S_4 = Butanol-formamide–water (50:10:50) (upper phase used),
S_5 = Dioxane–ammonia (0.88 sp.gr.)–water (100:3:10).

Chromatographic plates

Plates (20 × 20 cm) were coated with a uniform thickness of 250 μ of silica gel G.F. (Merck) according to STAHL's method[8]. After air drying, they were activated at

TABLE I

AVERAGE $R_F \times 100$ VALUES FOR HYPOGLYCAEMIC AGENTS

Hypoglycaemic agent	*Solvent system*									
	S_1		S_2		S_3		S_4		S_5	
	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*	*Range**	*Av.value*
Acetohexamide	47–57	52	66–71	67	46–53	49	48–53	50	40–45	42
Chlorpropamide	56–63	59	68–72	70	49–55	53	49–57	52	32–38	35
Phenformin HCl	—	03	49–55	52	21–27	24	29–35	32	02–03	03
Tolbutamide	72–79	75	73–79	76	62–69	65	63–70	66	31–36	33

* Experimental range representing fifty measurements.

100–110° for 30 min and left at room temperature for at least 30 min before use. The plates were used on the same day of activation.

Spray reagents

1 Ninhydrin (0.3 g) in *n*-butanol (100 ml), mixed with glacial acetic acid (3 ml)[9].

2 Vanillin (5 %) in conc. sulphuric acid.

3 Sodium nitroprusside–potassium ferricyanide spray[9]. One volume each of sodium hydroxide (10 %), sodium nitroprusside (10 %) and potassium ferricyanide (10 %) solutions was mixed with three volumes of water and the mixture left to stand for 20 min prior to use.

Method

R_F values for individual compounds were determined by the application of 5 μg quantities of an ethanolic solution of the drug to the plates and by allowing the solvent front to advance 15 cm in a previously saturated, paper lined tank. The approximate development times were: for S_1, 25 min; S_2, 110 min; S_3, 130 min; S_4, 140 min; S_5, 45 min. The method of drying and visualisation of the plates is described below. An average R_F value based on approximately fifty applications run on five plates was obtained for each compound in each solvent system. Detection limits for each compound were determined by spotting varying sample amounts with a Burroughs Wellcome "Agla" syringe.

The following scheme was found successful for identifying any of the four compounds. Two spots of 5 μg each of the material to be examined were applied to a plate and the solvent was allowed to advance 15 cm. After air evaporation of the solvent, the plate was observed under short-wave U.V. light and each hypoglycaemic agent detected by its absorbance. The plate was then heated for 10 min at 150–160° and one half of the plate (*i.e.* one spot) was sprayed with ninhydrin reagent and heated at the same temperature for a further ten minutes. Chlorpropamide and tolbutamide appeared as pink spots. The same half of the plate was sprayed further with the vanillin–sulphuric acid reagent and warmed slightly for 1–2 min. Acetohexamide was thus detected by the almost immediate appearance of a red spot. The remaining half of the plate was sprayed with the sodium nitroprusside–potassium ferricyanide reagent; a red spot appeared immediately to indicate the presence of phenformin hydrochloride.

Results and discussion

Average R_F values and their experimental range based on fifty applications of the hypoglycaemic agents in the five solvent systems are given in Table I. The limits of detection were found to be about 1 μg for the U.V. absorbance of chlorpropamide and tolbutamide, and less than 1 μg for the U.V. absorbance of acetohexamide and phenformin hydrochloride as well as for the colour reactions of all the compounds studied. The pink coloration produced by ninhydrin with chlorpropamide or tolbutamide was found to be permanent but faded on spraying with the vanillin–sulphuric acid reagent. The red colour produced by phenformin hydrochloride on treatment with the nitroprusside–ferricyanide spray was found to fade after a few minutes while that of acetohexamide with vanillin–sulphuric acid changed to a permanent orange-

brown after about 15 min, thus enhancing the identification of this drug. Phenformin hydrochloride cannot be detected by the nitroprusside–ferricyanide spray when solvent system S_4 is used. This may be attributed to the interference of residual formamide on the plate.

A mixture of all four compounds can be separated by solvent system S_1. Systems S_2 to S_4 will separate tolbutamide from phenformin hydrochloride and either acetohexamide or chlorpropamide and are intended as alternatives to system S_1. A mixture of acetohexamide and chlorpropamide will not separate in systems S_2 to S_4 and should a spot be detected which may correspond to either of these compounds alone or in combination, system S_5 will separate a mixture of the two efficiently and in conjunction with the differing colour reactions will confirm which substance is present.

This chromatographic method presents a rapid and convenient method of differentiating between the four hypoglycaemic agents and may find application in pharmaceutical or forensic work.

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Preparative polyamide layer chromatography

The rapidity of analysis, only slight broadening of the spots and easy handling in diagnostic polyamide layer chromatography[1–6] encouraged us to use preparative polyamide layer chromatography in the purification of dinitrophenyl amino acids from dinitrophenylation products. Satisfactory results were obtained and various advantages over the recrystallization method were observed in our laboratory[7]. In order to test the characteristics of the polyamide layer for preparative scale work, we selected isomeric nitroanilines for this evaluation because these compounds were brightly coloured, quite stable on handling, easily available in pure form and had large differences in R_F value.

As in previous experiments[6], we prepared the polyamide layer by spreading 15 ml of polyamide solution (20 g polycaprolactam in 100 ml of 75% formic acid) on

15 × 15 cm glass plates which were kept horizontally in a chromatographic cabinet (50 × 45 × 45 cm), saturated in advance with water vapour, where the slow evaporation of the formic acid was allowed to proceed. The resulting layer had a thickness of 0.07 cm. In order to increase the loading capacity, we tried to make thicker layers but this often resulted in cracked layers. The upper limit seemed to be 0.10 cm (30 ml polyamide solution on a 15 × 15 cm plate). For quantitative work, as well as for the recovery of separated substances, the layer had to be prewashed with a polar solvent, usually the solvent used in the later extraction of the substances.

Because of the durability of the polyamide layer, the application of sample solution was so easy that no special equipment was necessary. With the help of a tapered capillary, several ml of a solution of a mixture of nitroanilines could be applied on a 15 × 15 cm layer in less than 30 min. The applied mixture formed a band less than 0.5 cm in width. The start line was set 1.5 cm from the bottom edge and was 13 cm long in order to avoid the edge effects.

The loading capacity was about 15 mg per substance on a 15 × 15 cm layer of 0.07 cm thickness for nitroanilines whose R_F values were 0.57 (*o*-nitroaniline), 0.35 (*m*-nitroaniline) and 0.16 (*p*-nitroaniline) in a carbon tetrachloride–glacial acetic acid (9:1) system. The ratio of adsorbent to a single substance was 200:1 by weight. Of course, the loading varied greatly with the substances to be separated, but this ratio was distinctly better than with other adsorbents. After development, each separated band which was *ca.* 2 cm in width was scraped into a flask and extracted several times with ethanol until no more of the substance could be detected in the extract. The yields were always above 90%. The purity of the recovered substances was checked by polyamide layer chromatography and melting point.

This experiment shows that polyamide layer chromatography is excellent for the separation and purification of small amounts of substances.

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Polyamide layer chromatography
Further studies on dinitrophenyl amino acids

In the previous paper[1], we have described an excellent separation of seventeen dinitrophenyl (DNP) amino acids together with 2,4-dinitrophenol and 2,4-dinitroaniline by polyamide layer chromatography. Here we want to report on the separation of fourteen additional DNP amino acids* by the same method. Two-dimensional chromatograms of all 31 amino acids are also shown.

Experimental

Preparation of polyamide layer and chromatographic techniques. Details are to be found in the previous paper[1]. The polyamide resin was Amilan CM 1007s (poly-ε-caprolactam) of Toyo Rayon Co., Tokyo, Japan. Ascending methods were used in all developments as before. All solvents were purified to meet chromatographic requirements. U.V. contact photography was used to record the results (light source: a germicide lamp).

Results and discussion

Table I shows the R_F values for one-dimensional developments of the 31 DNP amino acids. The DNP amino acids are numbered according to their R_F values in solvent system II. Figs. 1-5 show the two-dimensional developments of all 31 DNP amino acids on 15 × 15 cm polyamide layers. The solvent systems are the same as those used in the previous paper[1] except solvent I and II. We changed solvent I and II to continuous development in order to separate slower moving spots. 2,4-Dinitrophenol was used as standard and the development was stopped after the 2,4-dinitrophenol had run 10.0 cm from the origin. Overall times required (from the application of sample to recording the chromatograms by U.V. contact photography) are as shown in Table II.

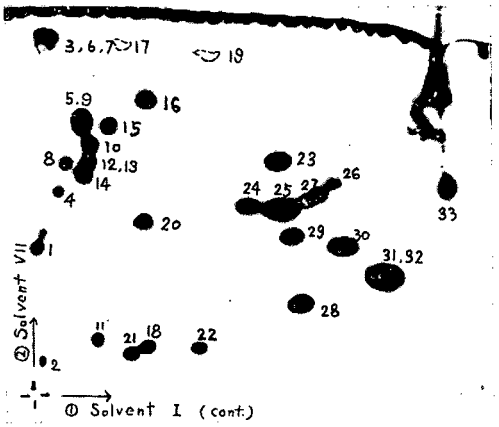

Fig. 1. Two-dimensional chromatogram. Solvent: 1st dimension: I (cont.), 2 h, 10.5 cm; 2nd dimension: VII, 1 h, 10 cm. Layer: ε-polycaprolactam resin CM 1007s. Loading: *ca.* 0.2 μg of each DNP derivative. Numbers: *cf.* Table I.

* We wish to thank Dr. A. TSUGITA, Osaka University, Osaka, Japan, for the gift of these samples.

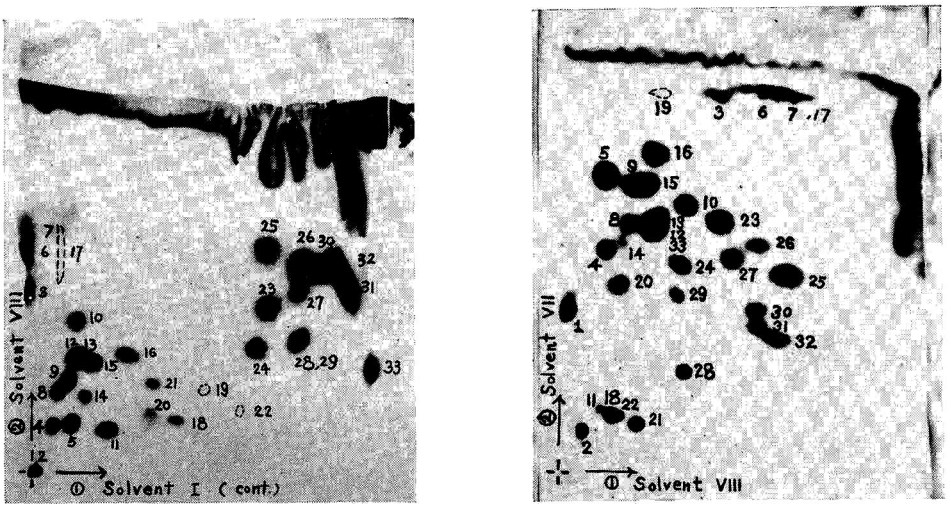

Fig. 2. Two-dimensional chromatogram. Solvent: 1st dimension: I (cont.), 2 h, 9 cm; 2nd dimension: VIII, 3.5 h, 10 cm. Layer: ε-polycaprolactam resin CM 1007s. Loading: *ca.* 0.2 μg of each DNP derivative. Numbers: *cf.* Table I.

Fig. 3. Two-dimensional chromatogram. Solvent: 1st dimension: VIII, 3.5 h, 9 cm; 2nd dimension: VII, 1 h, 11 cm. Layer: ε-polycaprolactam resin CM 1007s. Loading: *ca.* 0.2 μg of each DNP derivative. Numbers: *cf.* Table I.

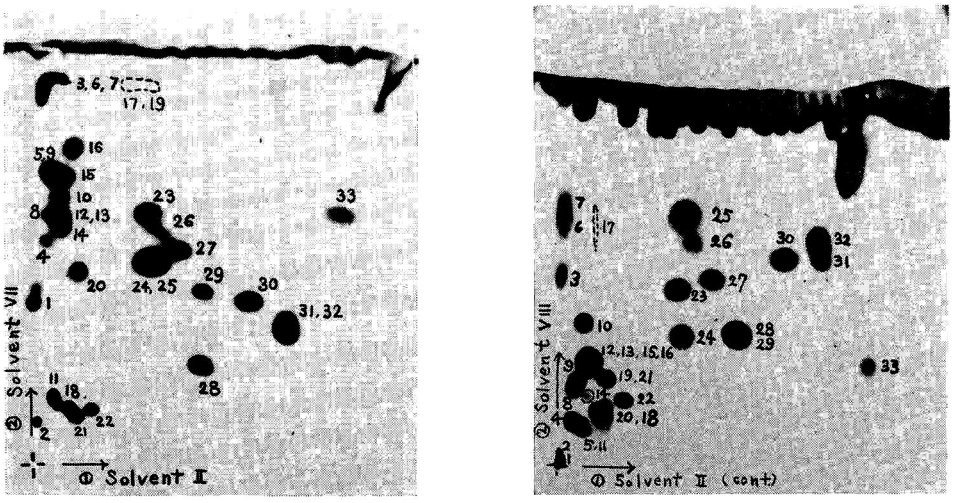

Fig. 4. Two-dimensional chromatogram. Solvent: 1st dimension: II (cont.), 3 h, 8.5 cm; 2nd dimension: VII, 1 h, 11 cm. Layer: ε-polycaprolactam resin CM 1007s. Loading: *ca.* 0.2 μg of each DNP derivative. Numbers: *cf.* Table I.

Fig. 5. Two-dimensional chromatogram. Solvent: 1st dimension: II (cont.), 3 h, 8.5 cm; 2nd dimension: VIII, 3.5 h, 10 cm. Layer: ε-polycaprolactam resin CM 1007s. Loading: *ca.* 0.2 μg of each DNP derivative. Numbers: *cf.* Table I.

TABLE I

R_F VALUES OF DNP AMINO ACIDS ON A POLYAMIDE LAYER

No.	*DNP derivative*	*Solvent system*			
		I	*II*	*VII*	*VIII*
1	DNP-cysteic acid sodium salt	0.00	0.00	0.38	0.01
2	Bis-DNP-L-cystine	0.01	0.01	0.11	0.01
3	α-DNP-L-arginine	0.03	0.01	0.90	0.47
4	DNP-aspartic acid	0.06	0.04	0.55	0.12
5	DNP-DL-methionine sulphone	0.09	0.05	0.72	0.10
6	δ-DNP-L-ornithine	0.09	0.05	0.90	0.56
7	ε-DNP-lysine hydrochloride	0.12	0.05	0.90	0.62
8	DNP-DL-serine	0.08	0.05	0.61	0.20
9	DNP-L-asparagine	0.10	0.05	0.70	0.21
10	DNP-L-hydroxyproline	0.12	0.08	0.64	0.33
11	Bis-DNP-L-ornithine	0.17	0.09	0.17	0.11
12	DNP-DL-allo-threonine	0.13	0.09	0.59	0.27
13	DNP-threonine	0.16	0.10	0.60	0.27
14	DNP-L-glutamic acid	0.15	0.10	0.56	0.18
15	DNP-L-glutamine	0.17	0.10	0.70	0.24
16	DNP-DL-methionine sulphoxide	0.22	0.10	0.75	0.26
17	O-DNP-L-tyrosine	0.20	0.10	0.88	tailing
18	Bis-DNP-lysine	0.35	0.14	0.14	0.15
19	Bis-DNP-L-histidine	0.48	0.15	0.88	0.23
20	DNP-glycine	0.32	0.16	0.48	0.21
21	DNP-tryptophane	0.32	0.17	0.12	0.23
22	Bis-DNP-DL-tyrosine	0.58	0.24	0.12	0.16
23	DNP-sarcosine	0.63	0.41	0.61	0.46
24	DNP-alanine	0.62	0.42	0.49	0.33
25	2,4-Dinitroaniline	0.64	0.42	0.49	0.64
26	DNP-DL-β-alanine	0.75	0.44	0.56	0.60
27	DNP-L-proline	0.75	0.51	0.50	0.46
28	DNP-phenylalanine	0.85	0.62	0.23	0.33
29	DNP-DL-methionine	0.77	0.62	0.41	0.32
30	DNP-DL-valine	0.88	0.76	0.38	0.53
31	DNP-DL-leucine	0.94	0.90	0.32	0.53
32	DNP-isoleucine	0.94	0.90	0.31	0.56
33	2,4-Dinitrophenol	1.00	1.00	0.60	0.26

Solvent I: Benzene–glacial acetic acid (80:20) (continuous flow).
Solvent II: Carbon tetrachloride–glacial acetic acid (80:20) (continuous flow).
Solvent VII: 90% Formic acid–water (50:50).
Solvent VIII: *n*-Butanol–glacial acetic acid (90:10).

TABLE II

TIME REQUIRED FOR TWO-DIMENSIONAL CHROMATOGRAPHY OF DNP AMINO ACIDS ON A POLYAMIDE LAYER

Fig.	*1st dimension*	*2nd dimension*	*Time required (h)*
1	Benzene–HOAc (80:20) (cont.) (Solvent I)	90% HCOOH–H_2O (50:50) (Solvent VII)	3.5
2	Benzene–HOAc (80:20) (cont.) (Solvent I)	*n*-BuOH–HOAc (90:10) (Solvent VIII)	6
3	*n*-BuOH–HOAc (90:10) (Solvent VIII)	90% HCOOH–H_2O (50:50) (Solvent VII)	5
4	CCl_4–HOAc (80:20) (cont.) (Solvent II)	90% HCOOH–H_2O (50:50) (Solvent VII)	4.5
5	CCl_4–HOAc (80:20) (cont.) (Solvent II)	*n*-BuOH–HOAc (90:10) (Solvent VIII)	7

As little as 0.1 μg of DNP amino acids are easily recognizable by U.V. contact photography after two-dimensional development. DNP-proline, DNP-hydroxyproline, DNP-tryptophan and DNP-sarcosine give an orange colour when the chromatograms are dried, while the rest are yellow. Furthermore, DNP-glycine always gives a characteristic shape on development in a *n*-butanol–glacial acetic acid system. These observations greatly help the assignment of spots on the chromatograms of 31 DNP amino acids.

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A solvent system for the thin layer chromatographic separation of hippuric and mandelic acids

During the course of studies on the metabolism of drugs our attention has been focused on thin-layer chromatography of organic acids.

In recent years many paper chromatographic separations of organic acids have been reported. Techniques for the efficient separation of hippuric acid and mandelic acid by paper chromatography[1,2] and thin-layer chromatography do not appear to have been developed.

In the practice of thin-layer chromatography the developing solvent systems are frequently complex. The purpose of this report is to describe a water saturated ether–methanol–87% formic acid system to facilitate the thin-layer chromatography separation of hippuric acid and mandelic acid. Other aromatic organic acids also can be separated by this system.

Experimental

Silica gel HF (E. Merck A.G., Darmstadt), 25 g, was shaken vigorously with 50 ml distilled water for 60 sec, and this suspension was immediately transferred to an open spreader (Shandon Unoplan Spreader) on 23 glass plates (50 × 200 mm). The glass plates were coated to a thickness of 250 μ. The plates were then allowed to stand for 30 min at room temperature and were activated by drying in a oven at 100–110° for 3–4 h. Hippuric acid, mandelic acid, and other organic acids were applied and the plates developed in the solvent systems described. The system having the best solvent combination for the general separation of organic acids was water saturated ether–

TABLE I

R_F VALUES OF ORGANIC ACIDS IN THE DIFFERENT SOLVENT SYSTEMS ON SILICA GEL HF

Organic acids	*R_F in solvent system*			
	S_1	S_2	S_3	S_4
Hippuric	0.46	0.63	0.58	0.47
Mandelic	0.58	0.75	0.74	0.55
Benzoic	0.96	0.98	0.98	0.94
p-Hydroxymandelic	0.30	0.51	0.52	0.32
p-Hydroxyphenyl-pyruvic	0.81	0.91	0.92	0.70
p-Hydroxyphenyl-lactic	0.82	0.89	0.95	0.83
p-Hydroxybenzoic	0.93	0.94	0.97	0.89
p-Aminobenzoic	0.90	0.89	0.91	0.84

The solvent systems used were: S_1 = water saturated ether–methanol–87% formic acid (98:1:1); S_2 = water saturated ether–methanol–87% formic acid (90:5:5); S_3 = water saturated ether–methanol–87% formic acid (95:1:4); S_4 = water saturated ether–methanol–87% formic acid (95:4:1).

methanol–87% formic acid (98:1:1). The best system for differentiating hippuric and mandelic acids was water saturated ether–methanol–87% formic acid (95:1:4). The chromatograms were examined under ultraviolet light for the detection of fluorescent materials. The acids reported were later detected by iodine vapor and by coupling with 0.02% methyl red in ethanol.

Results and discussion

The R_F values obtained in solvent system S_3 were hippuric acid, 0.58, and mandelic acid, 0.74. This degree of separation of hippuric and mandelic acids has not been previously reported. The R_F values for these and other organic acids studies are shown in Table I.

In our experience it has been noted that the R_F values tend to be variable even when determined under as nearly identical conditions as practicable. The migration rate of a compound is related to the amount of formic acid and methanol contained in the water saturated ether. Satisfactory separations can be obtained over a very wide range of solvent combinations. The solvent system R_F values tend to decrease with increasing methanol content and tend to increase with increasing formic acid content. The effect of solvent change on the R_F values of organic acids studied is shown in Table I. The time required for chromatographic development of the plates varied slightly with the solvent system used. In any solvent combination, 30–40 min was sufficient for development. The solvent system was prepared fresh in small amount for each set of chromatograms.

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A thin-layer system for the separation of iodine-containing compounds using binary mixtures of adsorbents

The behavior of chromatographed compounds is dependent upon both the adsorbent and the solvent used. The R_F value of a substance can thus be changed and to a certain extent predicted by changing the solvent according to the eluotropic series; or, on the other hand, the modification of the adsorbent should bring about the same results[1,2]. Up to now, because of the limited number of adsorbents available, there has been no systematic study of the behaviour of organic compounds on binary mixtures of adsorbents.

CARLTON AND BRADBURY[3], studying the behavior of organic compounds on mixtures of adsorbents on a column, concluded that two behavior patterns are possible for mixtures: (1) the adsorption strength is characteristic of one or the other of the two adsorbents in the mixture while the other adsorbent acts as a diluent; (2) because of competition between the two adsorbents, the resultant adsorption is characteristic of the mixture and varies linearly with the percentage composition of the mixture.

Our interest in applying this procedure to the separation of iodine-containing compounds on thin-layer plates stems from the necessity for a maximum and approximately equal separation of iodide, 5-monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT), 3,5,3′-triiodothyronine (T3), and 3,5,3′,5′-tetraiodothyronine (T4), and our failure to attain this goal on either pure cellulose or silica gel. Previous attempts to separate these compounds on thin-layer plates did not satisfy us entirely[4–8].

Material and method

The behavior of iodine-containing compounds on thin-layer chromatoplates in the following solvent systems[4] is studied using mixtures of cellulose and silica gel:

(1) ethanol–methyl ethyl ketone–2 N NH_4OH (1:4:1);
(2) *tert.*-amyl alcohol–dioxane–1 N NH_4OH (2:2:1);
(3) acetone–butanol–1 N NH_4OH (4:1:1);
(4) ethylene dichloride–butanol–1 N NH_4OH (1:8:1).

Cellulose MN 300 and Silica Gel G* are mixed in water in known proportions and applied to the glass plates to a thickness of 0.25 mm with the help of the Desaga–Brinkmann equipment. The plates are dried overnight at room temperature to insure reproducible results. Iodide, MIT, DIT, T3, and T4 are dissolved in a 5 % ammonia in ethanol solution at a concentration of 250 μg/ml. A 2-μl aliquot is applied with a Hamilton microliter syringe at a point 1 cm from the bottom of the plate and then the plates are run for a standard length of 10 cm in a saturated tank.

The tyrosines and thyronines are developed by spraying with a 5 % sodium bicarbonate diazosulfanilic acid solution, while iodide is visualized by spraying with a 0.5 % $PdCl_2$ in HCl solution.

Results

Iodide R_F values change very little with silica gel concentration in the above mentioned solvent systems and therefore proved to be a good internal standard. All the results are quoted in terms of $R_{Iodide} \times 100$.

* Brinkmann Instrument Inc., New York.

TABLE I

R_{Iodide} × 100 VALUES OF IODINE-CONTAINING COMPOUNDS AT DIFFERENT SILICA GEL CONCENTRATIONS IN THE *tert.*-AMYL ALCOHOL–DIOXANE–1 *N* NH_4OH (2:2:1) SOLVENT SYSTEM

	Percent Silica Gel G										
	0	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
MIT	49	72	67	60	56	51	50	49	51	46	47
DIT	12	18	15	19	20	22	24	25	30	31	32
T_3	200	190	176	157	140	130	120	112	108	96	88
T_4	128	140	128	114	102	95	90	85	81	73	71

TABLE II

R_{Iodide} × 100 VALUES OF IODINE-CONTAINING COMPOUNDS AT DIFFERENT SILICA GEL CONCENTRATIONS IN THE ETHANOL–METHYL ETHYL KETONE–2 *N* NH_4OH (1:4:1) SOLVENT SYSTEM

	Percent Silica Gel G										
	0	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
MIT	35	37	39	42	39	40	41	42	43	43	45
DIT	8	14	17	20	22	22	24	26	27	29	30
T_3	142	120	118	113	109	100	95	87	82	80	73
T_4	92	92	89	89	87	83	81	77	73	68	63

TABLE III

R_{Iodide} ×. 100 VALUES OF IODINE-CONTAINING COMPOUNDS AT DIFFERENT SILICA GEL CONCENTRATIONS IN THE ACETONE–BUTANOL–1 *N* NH_4OH (4:1:1) SOLVENT SYSTEM

	Percent Silica Gel G										
	0	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
MIT	36	37	39	40	41	42	45	47	47	47	53
DIT	9	10	13	14	16	20	26	28	35	39	42
T_3	139	113	110	103	99	94	86	80	78	74	71
T_4	82	77	86	85	83	81	76	71	66	67	68

TABLE IV

R_{Iodide} × 100 VALUES OF IODINE-CONTAINING COMPOUNDS AT DIFFERENT SILICA GEL CONCENTRATIONS IN THE ETHYLENE DICHLORIDE–BUTANOL–1 *N* NH_4OH (1:8:1) SOLVENT SYSTEM

	Percent Silica Gel G										
	0	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
MIT	50	48	56	53	52	44	45	42	40	38	29
DIT	15	22	20	20	20	17	21	22	20	26	27
T_3	475	332	280	233	222	178	132	120	102	95	62
T_4	261	200	189	169	141	106	105	96	96	89	61

It can be seen from Tables I–IV that the R_{Iodide} values decrease with increasing concentration of silica gel for the thyronines, while they are relatively constant or slightly increasing for the tyrosines. One can also observe that the R_{Iodide} values for a given adsorbent are decreasing with increasing number of iodine atoms in the compound. It appears that for a given adsorbent, the R_F values increase with the molecular weight and decrease with the number of iodine atoms. On the whole the image observed is one where there is competition between the two adsorbents.

Our objectives are to achieve the best separation of the two duplets MIT–DIT and T3–T4. The ratio of the R_F values of MIT–DIT and T3–T4 were calculated at different concentrations of silica gel. In all solvents the separation of MIT–DIT is independent of the concentration of silica gel (the ratio being constant); as for the T3–T4 ratio, it increases with a decrease in silica gel concentration and reaches, in general, its maximum on pure cellulose. On the other hand, all these compounds show maximum tailing effects on pure cellulose, resulting in poor separation. However, observations have shown that tailing effects decrease considerably with increase in concentration of silica gel. A 20 % silica gel–80 % cellulose composition was found to be ideally suited for our purpose. The best separations are obtained with the *tert.*-amyl alcohol–dioxane–1 *N* NH_4OH (2:2:1) and the ethanol–methyl ethyl ketone–2 *N* NH_4OH (1:4:1) solvent systems.

Discussion

Although no systematic attempt has been made to study the behavior of organic compounds on binary mixtures of adsorbents, sporadic occurrence of the use of mixtures of adsorbents for a special purpose can be found in the literature. Kieselguhr has been added to silica gel (1:1) to decrease the developing time without enlarging the spot[9]. Celite 545 added to Silica Gel G also shortens the developing time and was shown to increase the R_F value of organic compounds because of dilution of the active adsorbent[10]. Furthermore, an equal mixture of Silica Gel G and Aluminum Oxide G designated as Alusil has been used for the separation of sugars, dyes, etc.[11]. Recently an increase in the resolution of amino acid separation was obtained on a mixture of silica gel and cellulose[12]. STAHL[13] was the first to design a spreading device for the preparation of gradient layers. A few combinations of available adsorbents were tried for the separation of dyes and alkaloids. WARREN[14] later developed an improved gradient spreader for thin-layer chromatography.

It should be remembered that the R_F value is not a constant but is influenced by a number of factors several of which have still not been thoroughly studied. The aim of this paper is to show the general effect of changing proportions of silica gel and cellulose on the chromatography and separation of iodine containing compounds of biomedical interest.

The effect of the molecular weight on the R_F values of the chromatographed compounds being greater on cellulose than on silica gel speaks for itself. Molecular weight affects the solubility of the compounds and thus the partition coefficient regulating the behavior of compounds on cellulose substratum.

The effect of the number of iodine atoms can be interpreted as an increase in acidic properties following inclusion of iodine in the β-ring of thyronine[15]. An ion-exchange phenomenon could, at this moment, be taken into consideration to explain the decrease in R_F values. The adsorption should also increase with the presence of

iodide since this atom is very polar and forms hydrogen bonds with the silanol groups of the silica gel.

More theoretical and empirical studies are needed before a complete understanding of the factors affecting the migration of compounds on thin layers can be attained.

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Dünnschichtchromatographische Trennung mehrkerniger Aromaten mit Pikrinsäure als Komplexbildner

Im allgemeinen ist zum Nachweis und zur Identifizierung nicht zu hoch siedender mehrkerniger Kohlenwasserstoffe die "Gas-Chromatographie allen anderen Verfahren weit überlegen"[1]. Im Laufe unserer Arbeiten über die katalytische Homologisierung kondensierter Aromaten[2a] stiessen wir jedoch auf Verbindungen, die thermisch so instabil sind, dass eine Gas-Chromatographie nicht mehr in Frage kommt. Dünnschichtchromatographisch sind die anfallenden Isomerengemische ebenfalls nicht zu trennen. Aus diesem Grund übertrugen wir das Prinzip der Belegung einer Chromatographiesäule[2b] mit einem Komplexbildner auf die Dünnschichtchromatographie.

Kürzlich wurde dieses Verfahren schon von anderer Seite[3,4] auf die Trennung kondensierter Aromaten angewandt. Die Autoren brachten jeweils den Komplexbildner auf die ganze Platte und führten dann die Trennung in üblicher Weise auf der vorbehandelten Platte aus. Bei der Belegung der Dünnschichtplatte mit einer Nitroverbindung verschwindet die Eigenfluoreszenz des Kohlenwasserstoffes beim Betrachten im ultravioletten Licht[4]. Während höher kondensierte Aromaten durch die Dunkelfärbung des Komplexes direkt erkannt werden können[4], sind Verbindungen wie Diphenyl oder Fluoren auf der Platte unsichtbar. Zum Nachweis dieser Verbindungen kann die Platte nach dem Entwickeln mit Antimon-V-chlorid in Tetrachlorkohlenstoff besprüht werden[3]. Damit ist aber die Platte für einen mehrfachen Lauf, der oftmals eine starke Verbesserung des Trenneffektes bewirkt, unbrauchbar geworden.

Will man dieses Verfahren zur präparativen Dünnschichtchromatographie benutzen, so muss der Komplexbildner erst in einer weiteren Operation von den getrennten Substanzen getrennt werden. Diese Nachteile können vermieden werden, wenn man die Platte nur auf der *unteren Hälfte* mit dem Komplexbildner belegt. Gelangen die in der ersten Zone getrennten Substanzen in die unbelegte zweite, so sind sie vom Komplexbildner getrennt und können nach dem Trocknen der Platte direkt im U.V.-Licht nachgewiesen oder präparativ abgetrennt werden (Kieselgel mit Fluoreszenzindikator). Stärker zurückgehaltene Substanzen, wie z.B. das Pyren, werden durch mehrfaches Entwickeln successive aus der eigentlichen Trennschicht herausgetrieben und können nun in üblicher Weise präparativ gewonnen werden.

Experimenteller Teil

Herstellen der Platten. Die Herstellung der Dünnschichtplatten wird in üblicher Manier[5] vorgenommen. Als Sorptionsschicht verwenden wir Kieselgel GF_{254} (mit Fluoreszenzindikator) der Fa. E. Merck, Darmstadt. Nach 24-stündigem Lufttrocknen werden die Platten 30 Min. bei 105° aktiviert.

Als Komplexbildner bevorzugen wir Pikrinsäure, die besonders fest auf der Platte haftet, und die mit Petroläther praktisch nicht wandert.

Zur Belegung wird die Platte einmal in einer gesättigten Lösung von Pikrinsäure in Benzol entwickelt. Dabei werden die unteren 8 cm der Platte mit Pikrinsäure gesättigt. Nach dem Lufttrocknen sind die Platten gebrauchsfertig.

Entwickeln. Die Substanzen werden so aufgetragen, dass sie 6.5 cm auf der belegten Schicht laufen und dann in die unbelegte zweite Zone gelangen. Als Fliessmittel dient Petroläther 50–70° (ohne Kammersätt.).

Sichtbarmachen. Nach dem Lauf sind die Substanzen in der unbelegten zweiten Schicht im U.V.-Licht als dunkle Flecken sichtbar. Höher kondensierte Substanzen, wie Anthracen oder Pyren, waren auch in der unteren belegten Zone durch ihre gefärbten Komplexe sichtbar.

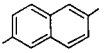
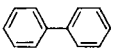
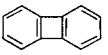
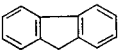
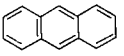
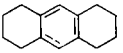
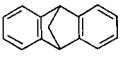
Ergebnisse

In Tabelle I sind die R_F-Werte ($\times$ 100) von 21 Kohlenwasserstoffen bei der Dünnschichtchromatographie auf Kieselgel GF_{254} mit[1] (Spalte I) und ohne Kammersättigung (Spalte II) denjenigen bei der belegten Platte ohne Kammersättigung gegenübergestellt (Spalte III). Die Zahlenangaben haben nur relativen Charakter und sollen die relativen Wanderungsgeschwindigkeiten widerspiegeln.

TABELLE I

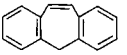
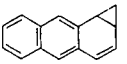
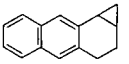
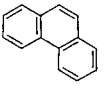
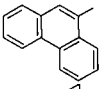
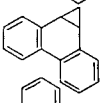
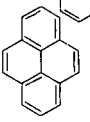
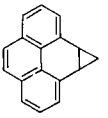
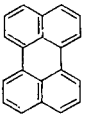
TRENNUNG KONDENSIERTER AROMATEN DURCH DÜNNSCHICHTCHROMATOGRAPHIE

(I) Mit Kammersättigung[1], (II) ohne Kammersättigung, (III) ohne Kammersättigung. (6.5 cm Laufschicht gesättigt mit Pikrinsäure). Fliessmittel Petroläther. R_F-Werte $\times$ 100.

Nr.	*Substanz*	*Formel*	*I*	*II*	*III*
1	Naphthalin		36	74	50
2	1,5-Dimethylnaphthalin		—	72	36
3	2,6-Dimethylnaphthalin		31	71	34
4	Diphenyl		28	61	57
5	Biphenylen		—	63	42
6	Acenaphthen		32	67	36
7	Acenaphthylen		28	63	11
8	Fluoren		24	57	38
9	Azulen		25	59	25
10	Anthracen		27	59	17
11	Octahydroanthracen		33	77	75
12	Dibenzonorbornadien[2d]		—	48	12

(*Fortsetzung S. 471*)

TABELLE I (*Fortsetzung*)

Nr.	*Substanz*	*Formel*	*I*	*II*	*III*
13	1,2,5,6-Dibenzocycloheptatrien[2d]		—	51	37
14	Naphthonorcaradien[2d]		—	55	20
15	Naphthonorcaren[2d]		—	57	24
16	Phenanthren		24	56	20
17	9-Methylphenanthren[2b]		—	55	15
18	Dibenzonorcaradien[2b]		—	48	24
19	Pyren		23	56	6
20	1,2-Dihydro-1,2-methanopyren[2c]		—	47	12
21	Perylen		—	37	2

Um die *Leistungsfähigkeit* dieses Verfahrens zu demonstrieren, seien drei instruktive Beispiele herausgegriffen (vgl. Fig. 1):

(*1*) *Fluoren* (*8*)/*Phenanthren* (*16*)/*9-Methylphenanthren* (*17*)/*Pyren* (*19*). Alle Substanzen haben auf der unbelegten Dünnschichtplatte praktisch die gleiche Wanderungsgeschwindigkeit (vgl. Tabelle I) und sind daher nicht trennbar. Die mit Pikrinsäure zur Hälfte belegte Platte trennt alle Substanzen gut.

Bei diesem Beispiel ist jedoch auch eine gas-chromatographische Trennung möglich[1,2]. Der Vorteil der Dünnschichtchromatographie liegt aber bekanntlich neben dem Einsatz kleinster Substanzmengen in dem geringen apparativen Aufwand. Das folgende Beispiel kann zwar ebenfalls gas-chromatographisch getrennt werden, bei der präparativen Gas-chromatographie kann jedoch nur noch das thermische Folgeprodukt der Cyclopropanverbindung[2b] isoliert werden.

(*2*) *Phenanthren* (*16*)/*Dibenzonorcaradien* (*18*). Das an der unbelegten Platte schneller laufenden Phenanthren wird durch die Pikrinsäure stärker zurückgehalten als die homologe Verbindung 18. Der Trenneffekt ist durch die Imprägnierung grösser geworden.

Bei der Gas-chromatographie lagert sich das Norcaradienderivat 18 teilweise in 9-Methylphenanthren (17) um, da jedoch sowohl 17 als auch 18 grössere gaschromatographische Retentionsvolumina als 16 haben, kann der analytische Nachweis für ein Zweistoffgemisch auf diese Weise indirekt erbracht werden[2b]. Es gibt

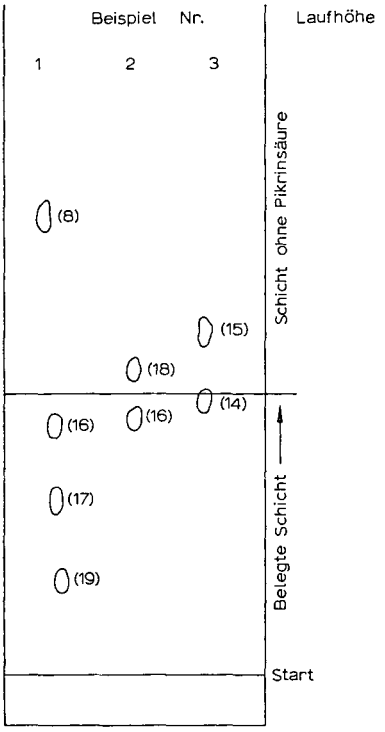


Fig. 1. Dünnschichtchromatographie auf einer mit Pikrinsäure halb belegten Platte. Drei Beispiele (siehe Text) bei zweimaligem Lauf.

jedoch auch Beispiele, bei denen selbst dieser nicht möglich ist. Das folgende Substanzpaar 3 unterscheidet sich nur durch zwei Wasserstoffatome, die gas-chromatographische Retention beider Verbindungen ist gleich. Infolge ihrer thermischen Instabilität wäre diese Art der Trennung sowieso hier nicht empfehlenswert. Auch durch Dünnschichtchromatographie können beide Substanzen nicht getrennt werden (vgl. Tabelle I).

(3) *Naphthonorcaradien/Naphthonorcaren.* Beide Substanzen werden auf der zur Hälfte mit Pikrinsäure belegten Platte gut getrennt. Die Dünnschichtchromatographie mit Komplexbildnern ist daher die einzige Trennmethode.

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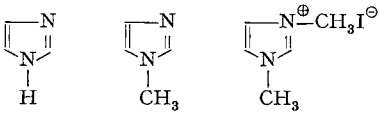
Separation of imidazole and its N-methyl derivatives on polyamide thin layers

Separation of imidazole derivatives by chromatographic techniques has been reported by several investigators[1–3]. In our laboratory a rapid procedure was needed for the identification and the semi-quantitative estimation of the purity of N-methyl imidazole (II) and N,N'-dimethyl imidazolium iodide (III). Compound II was synthesized according to HÄRING[4] as a colourless liquid and III by the method of SARASIN[5] as a yellow coloured crystalline compound. For both compounds imidazole (I) served as the starting material.



(I) (II) (III)

Preliminary paper chromatographic experiments following the directions given by COWGILL[1] gave unsatisfactory results. Thin-layer chromatography, now being applied widely, offers the great advantage of a rapid development and it is possible to choose a stationary phase suited for a special problem. Silica gel shows a great affinity towards III as a consequence of the ionic character of the compound, resulting in low R_F values. These findings are in agreement with the data on similar compounds reported by GRIMMETT AND RICHARDS[3]. We observed that the imidazoles were separated rapidly and efficiently on thin layers of polyamide, using methyl ethyl ketone as the solvent.

Thin layers (20 × 5 and 20 × 20 cm) were prepared according to the procedure described by WOELM[6]. The smaller plates were placed directly into the solvent in cylinders (10 cm diameter, 25 cm height), the walls lined with filter paper soaked in the solvent. In order to obtain a regular front and reproducible R_F values the larger plates were developed after equilibration with solvent vapour for 15 min in chambers (25 × 25 × 10 cm) also lined with solvent soaked filter paper. As a result of this technique no serious variations in R_F values were found.

The liquid was allowed to rise about 10 cm in approximately 10 min. After a drying period of 1 h the spots were visualized with iodine vapour. Only compound I gave a positive reaction with PAULY'S[7] reagent because, according to BURIAN[8], a substituent in the 1-position prevents coupling with a diazonium salt. Dragendorff reagent[9] was used for the specific detection of III. The compounds I and II formed

TABLE I

Compound	R_F	*Standard deviation*	*Number of experiments*
I	0.54	0.05	10
II	0.84	0.03	6
III	0.36	0.06	9

nearly circular spots but III showed some minor tailing probably due to its ionic character.

The median R_F values with their corresponding standard deviations resulting from a small number of experiments are collected in Table I. The data were obtained using the method described by DEAN AND DIXON[10].

The use of polyamide as a stationary phase in column[11] and thin-layer chromatography[12] has been described previously. It is generally assumed that hydrogen bonding plays a predominant role in these separations[11].

In our case, compound I is the only compound having the -NH-group available to form a hydrogen bond with the amide groupings. Compound III possesses an ionic character and shows a strong attraction to polyamide, probably by ion–dipole interactions at the N-atom of the amide groupings, as described by SARDA AND PEACOCK[13] for LiCl. Using water as developing solvent, III showed an elongated spot but after the addition of 2 % of sodium chloride, a nearly circular spot with R_F 0.70 was found. The salt probably functioned as a displacer. Compound II is unable to form a hydrogen bond neither does it have an ionic character under the circumstances of the present investigation.

Thus the three compounds have different attractive forces towards polyamide resulting in a satisfactory separation which gives information about the identity and purity of the N-methyl substituted imidazoles prepared.

I would like to thank Miss R. DE VOS and Mr. W. J. VAATSTRA who carried out the separations and the syntheses respectively.

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Formation and separation of Girard hydrazones on thin-layer chromatography by elatographic techniques

Characterization of a steroid by thin-layer chromatography requires the calculation of R_F values in different systems, utilization of specific colour reactions and formation of derivatives. Derivatives can be formed directly on the chromatoplate by reactions at the starting line[1], or during the development on treated adsorbents[2].

Formation of hydrazones with Girard's reagent T is a very useful technique for separating ketonic from alcoholic steroids, as well as for the purification of ketonic materials[3]. In the present communication, the formation and separation of steroid hydrazones with Girard's reagent T by elatography is described.

Material and methods

The apparatus and techniques have been described in a previous paper[4]. In the present experiments, Silica Gel G (Merck, Darmstadt, Germany) has been used. Unless stated otherwise, all the steroids used were obtained from a commercial source (Steraloids Inc., Pauling, N.Y., U.S.A.).

Reaction on the plate. An 0.1 % solution of Girard's reagent T (trimethylacetohydrazide ammonium chloride) in a solution of 10% (v/v) glacial acetic acid in methanol was deposited by a capillary pipette on the area around the starting line in a band 1.5 cm wide. After 3 min (before this band was completely dry) the steroids

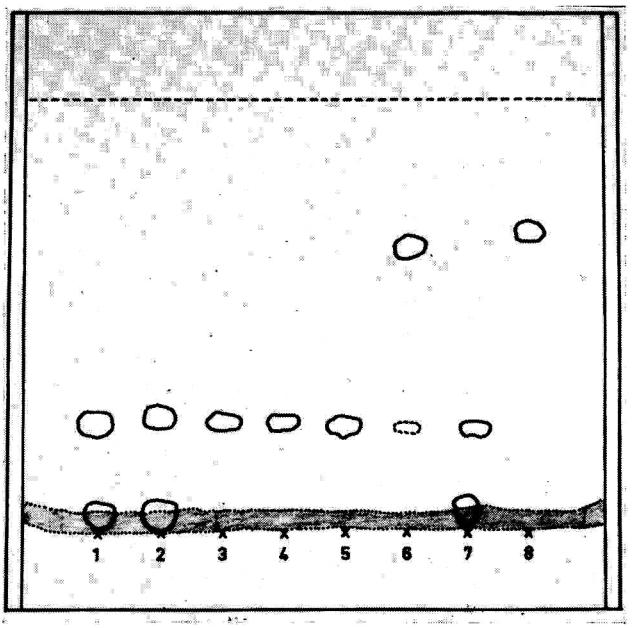

Fig. 1. Chromatography in solvent system J of Girard hydrazones formed by elatography. The shaded zone represents the unreacted Girard's reagent T after development of the plate. Steroids: 1 = androst-4-ene-3,17-dione; 2 = androst-1-ene-3,17-dione; 3 = 17β-hydroxy-androst-4-en-3-one (testosterone); 4 = 3 β-hydroxy-androst-5-en-17-one (dehydro-epi-androsterone); 5 = androsta-1,4,6-triene-3,17-dione; 6 = 17β-hydroxy-androst-1-en-3-one; 7 = pregn-4-ene-3,20-dione (progesterone); and 8 = 5α-pregnane-3β,20α-diol. For particulars, see text.

were deposited on the starting line. The chromatoplate was then allowed to stand for 15 h in a tank saturated with acetic acid vapour. To remove the acetic acid vapour, the plate was heated for 10 min at 80° and, after cooling, submitted to thin-layer chromatography in solvent system J[5]; this consists of the upper phase of the system obtained by shaking a mixture of *n*-butanol, *tert.*-butanol and water (1:1:1).

Results

Fig. 1 shows a thin-layer chromatoplate of one alcoholic and seven ketonic steroids after elatography. A complete separation is shown for free steroids, monohydrazones and dihydrazones of Girard's reagent T. The migration of the dihydrazones in this system is the same as that found for the unreacted Girard's reagent. The R_F values of twelve ketonic and two alcoholic steroids, their monohydrazones formed with Girard's reagent T and the ΔR_{MT} values for these conversions are summarized in Table I.

Under the conditions of the experiment cross-conjugated dienones give only traces of hydrazones, if any.

TABLE I

R_F AND ΔR_{MT} VALUES OBTAINED FOR TWELVE KETONIC STEROIDS AND THEIR MONOHYDRAZONES FORMED WITH GIRARD'S REAGENT T RUN IN SOLVENT SYSTEM J AND THE R_F VALUES OF TWO NON KETONIC STEROIDS

$\Delta R_{MT} = R_{M_1} - R_{M_2}$, where R_{M_1} is the R_M of the hydrazone and R_{M_2} is that of the corresponding free steroid. For particulars, see text.

Steroid	*R_F values*		*ΔR_{MT} values*
	Free steroid	*Mono-hydrazone*	
5α-Androst-1-ene-3,17-dione	0.74	0.25	0.93
Androst-4-ene-3,17-dione	0.73	0.23	0.96
Androsta-1,4,6-triene-3,17-dione	0.65	0.24	0.77
17β-Hydroxy-androst-4-en-3-one	0.71	0.24	0.89
17β-Hydroxy-androsta-1,4-dien-3-one	0.69	0.24*	—
3β-Hydroxy-androst-5-en-17-one	0.73	0.24	0.93
Pregn-4-en-3-one**	0.76	0.26	0.96
Pregn-4-ene-3,20-dione	0.70	0.23	0.89
Pregna-4,16-diene-3,20-dione	0.72	0.24	0.91
3β-Hydroxy-pregn-5-en-20-one	0.74	0.27	0.89
3β-Hydroxy-pregna-5,16-dien-20-one	0.76	0.25	0.98
3β,16α-Dihydroxy-pregn-5-en-20-one	0.69	0.24	0.85
5α-Pregnane-3β,20α-diol	0.72	—	—
5β-Pregnane-3α,11β,17α,20α-tetrol**	0.66	—	—

* Traces.

** The author is greatly indebted to Prof. W. KLYNE for these steroids.

Discussion

The technique described in this communication is similar to the elatographic procedure described by BECKER[6] for paper chromatography. By means of this technique derivatives may be formed and separated on the chromatoplate, thus avoiding the loss of material during extractions or transfers of samples.

A small number of chemical reactions, *e.g.*, oxidation[1], reduction[1], bromination[7]

and epoxide formation[8], have been tried by adding the reagents to the steroid at the starting point but only partial formation of derivatives was achieved. Also the modification of this technique[9], in which the reaction was performed directly in a capillary tube employed to transfer the material to the chromatoplate, could not be performed in a quantitative manner and was not applicable to slow rate reactions.

An advantage of this technique is that only a small band of reagent is required; this allows the use of subsequent staining techniques. This would not be possible with plates in which the adsorbent was completely impregnated with the reagent. If only one part of the starting line is treated with the reagent, it is possible to chromatograph a substance as both the free compound and the derivative on the same plate, thus facilitating the calculation of ΔR_{Mr} values. A further advantage of this technique is that optimum conditions of reactions may be selected so that even slow reactions may be performed.

This technique seem to be a useful procedure for the separation of alcoholic and ketonic steroids on a microscale, and as a quick test for the identification of ketones by the formation of Girard hydrazones.

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Neue Sprühreagenzien für die Dünnschichtchromatographie von einigen Zoosteroiden

In vielen Fällen beinhalten Steroidextrakte die aus biologischem Material gewonnen werden, Fettsäuren, Glyceride und Phospholipide. Ohne der Entfernung dieser Balastsubstanzen ist für die qualitative Charakterisierung von Steroiden aus solchen Extrakten wünnschenswert, solche Farbreagenzien zu gebrauchen, welche ohne Störeffekt der Fettsäuren, Glyceride und Phospholipide die einzelnen Steroide selektiv oder spezifisch färben.

Sprühreagenzien, die dieser Bedingung entsprechen, fanden wir in einer Gruppe von Komplexverbindungen des zweiwertigen Quecksilbers: $K_2[HgJ_4]$, $K_2[Hg(SCN)_4]$ und $K_2[HgBr_4]$. Wir verfolgten die Detektion an diesen Testsubstanzen: Pregnan-3α-20α-diol, Pregnan-3α-20β-diol, Allopregnan-3β-20α-diol, Progesteron, Testosteron, Cholesterin und Oestriol.

Methodik

Nach der Entwicklung verschiedener Mengen einzelner Steroide, Testgemische und Harnextrakte auf basischen Kieselgelschichten (20 × 20 cm) gepuffert mit $Ca(OH)_2$ in dem System: Benzol–Äthylacetat (70:30, V/V) wurde die Detektion wie folgt durchgeführt: Nach der Entfernung des Kieselgels von den Rändern der Träger-

TABELLE I

FARBE UND EMPFINDLICHKEITSMASS DER DETEKTION

Die Anfärbung wurde nicht höher wie 50 μg verfolgt.

Steroiden	*Sprühreagenz*					
	$K_2[HgJ_4]$		$K_2[Hg(SCN)_4]$		$K_2[HgBr_4]$	
	Farbprodukt	*Erfassungsgrenze (μg/0.5 cm²)*	*Farbprodukt*	*Erfassungsgrenze (μg/0.5 cm²)*	*Farbprodukt*	*Erfassungsgrenze (μg/0.5 cm²)*
Pregnan-3α-20α-diol	gelb	1	wird bis 50 μg nicht angefärbt		okker	2
Pregnan-3α-20β-diol	gelb	1	wird bis 50 μg nicht angefärbt		okker	2
Allopregnan-3β,20α-diol	blau grau	1	wird bis 50 μg nicht angefärbt		violett	3
Cholesterin	violett	1	rot	30	violett bis blau	1
Progesteron	gelb	3	gelb	10	wird bis 50 μg nicht angefärbt	
Testosteron	gelb	3	gelb	1	wird bis 50 μg nicht angefärbt	
Oestriol	wird bis 50 μg nicht angefärbt		okker	3	gelb-grün	1

platte in der Breite von 1.5 cm und dem Besprühen des Chromatogrammes bis eben zur Transparenz wurden die Chromatogramme mit der Deckplatte[1] eingedeckt und bei 140° 20 Min. lang erhitzt. Die Chromatogramme wurden dann in eine Salzsäureatmosphäre gebracht wo sich in kurzer Zeit die Steroide charakteristisch anfärbten.

Ergebnisse

Die Ergebnisse sind in Tabelle I dargestellt.

Als praktisches Anwendungsbeispiel seien zwei Versuche von geteilten Steroidextrakten (hergestellt nach WALDI[2]) angeführt. Die Extrakte wurden gewonnen:

I (a) aus dem Normalharn einer nicht graviden Frau;
(b) aus dem Harn einer eindeutig graviden Frau;
II (a) aus dem Normalharn einer nicht graviden Frau;
(b) aus dem Normalharn eines Mannes.

Die Färbung erfolgte mit $K_2[HgJ_4]$ und $K_2[HgBr_4]$. In beiden Fällen zeigten die Chromatogramme im Frauenharn deutlich Pregnandiol und Allopregnandiol (bei I (b) vermehrt), während diese Steroide im Extrakt des männlichen Harnes fehlten.

Zubereitung der Sprühreagenzien

In allen Fällen handelt es sich um Wasserlösungen.

$K_2[HgJ_4]$. Zu 100 ml 10 %-iger $HgCl_2$-Lösung wird unter Mischen (Magnetmischer) eine 10 %-ige KJ-Lösung bis zur Auflösung des vorübergehend Entstandenen HgJ_2 zugesetzt.

$K_2[Hg(SCN)_4]$. Aus 100 ml 0.1 %-iger HNO_3 wird eine gesättigte Lösung von $Hg(NO_3)_2$ hergestellt, zu der unter Mischen eine 5 %-ige KSCN-Lösung bis zur Auflösung des vorübergehend Entstehenden $Hg(SCN)_2$ zugesetzt wird. Als überschuss werden noch 10 ml KSCN-Lösung beigemengt.

$K_2[HgBr_4]$. Das Sprühreagenz wird wie bei $K_2[Hg(SCN)_4]$ aus $Hg(NO_3)_2$ und KBr hergestellt.

Anmerkungen

(a) Ein Lösungsgemisch von gleichen Volumen 5 %-iger KCl-Lösung und 5 %-iger $HgCl_2$-Lösung färbt unter den obengenannten Bedingungen Cholesterin und Allopregnandiol in Mengen von 20 μg an.

(b) Die Sprühreagenzien sind in braunen Glasflaschen längere Zeit haltbar.

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* Mit technischer Mitarbeit von Mr Ph. K. SEKEROVÁ.

A spot test for ketosteroids on thin-layer chromatograms

It is well known that the Zimmermann reaction lacks sensitivity, when used as a spot test for 17-ketosteroids on thin-layer chromatograms[1]. A method whereby the Zimmermann reagent is used in combination with Tetrazolium Blue (BT) reagent has been developed and provides a more general and sensitive color reaction for ketosteroids.

After spraying the dry chromatogram with freshly prepared reagent (2 vol. of 0.01% BT and 0.1% *m*-dinitrobenzene in methanol and 1 vol. of 3.0 *N* aqueous potassium hydroxide) and heating to approx. 60° in a stream of air (using a fan–heater) blue formazane spots appear. While Zimmermann-positive 17-ketosteroids react quite readily, just like reducing steroids, 3- and 20-ketosteroids often require a second spraying and heating. The sensitivity of the spot test approximates that of the BT reaction for reducing steroids, allowing the detection of 0.5 μg steroid/cm^2. It is assumed, that the condensation product between *m*-dinitrobenzene and ketosteroid as the first stage of the Zimmermann reaction[2] serves as reducing agent for the BT.

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Detection of sulphur, organic disulphides and some other compounds on paper chromatograms by means of hydrogen selenide

It has been recently shown[1] that the disulphide bond in proteins can be readily reduced by hydrogen selenide, according to the reaction,

$$RSSR + H_2Se \longrightarrow 2\ RSH + Se$$

The reduction process is indicated by separation of selenium. That hydrogen selenide is a rather strong reducing agent may be concluded from the free energy change accompanying the reaction,

$$S + H_2Se \longrightarrow H_2S + Se,\ \Delta F = -23.2\ \text{Kcal}$$

which may be successfully used for the detection of various reducible compounds on paper chromatograms. According to the amount of selenium that separates, brown, red, orange, or yellow spots are produced, in decreasing order of concentration; the

limit of visual detection being *ca.* 0.5 μg Se/cm^2. By comparison with the colour intensities of spots prepared from solutions of known concentration, a semi-quantitative evaluation is possible.

Reduction of samples on paper by hydrogen selenide is accelerated by moisture and retarded by strong acids, the best conditions being at pH 4–6, using hydrogen selenide saturated with water vapour. Any acids on the paper can be neutralized with ammonia vapour. As oxygen causes slow formation of selenium on the paper, the air must be replaced by an inert gas (H_2,N_2, CO_2) before treatment with hydrogen selenide.

The procedure suggested can be briefly outlined as follows: The dry strips of paper are put into a vertical glass tube and air is removed by a stream of inert gas. Afterwards the tube is filled with hydrogen selenide prepared from aluminium selenide and hydrochloric acid, and saturated with water vapour from a wash bottle at room temperature. The reaction time depends on the compounds being investigated, *e.g.*, simple disulphides such as cystine are reduced quantitatively in 20 min., whereas the reduction of all disulphide bonds in proteins needs *ca.* 2 h. The wet wool is rapidly coloured by hydrogen selenide. After treatment the hydrogen selenide is replaced by an inert gas again and absorbed in sodium hydroxide solution. Because of its poisonous properties care must be taken in order to prevent escape of hydrogen selenide.

The substances which can be readily detected by treatment with hydrogen selenide are: sulphur, sulphite, thiosulphate, nitrite, disulphides (investigated: cystine, dithiodiglycollic acid, di-β-propionic acid disulphide, di-β-propionamide disulphide), disulphide bonds in proteins, picric acid and benzoyl peroxide. The reaction with nitrate and chlorate is very slow, whereas sulphate, perchlorate and thiocyanate do not react at all. As far as could be observed no other group in proteins is capable of reaction with hydrogen selenide, and on this basis the disulphide bonds can be estimated in various fractions of proteins after paper electrophoresis. The influence of previous reduction of disulphide bond on the electrophoretic distribution can be of interest for protein chemistry as well.

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A transistorised solvent front detector

This circuit was designed to assist in the paper chromatography of unstable derivatives of vitamin B_{12} and folic acid, which have to be chromatographed at low temperatures, under an inert gas, in the dark. At very low concentrations of folate and vitamin B_{12} derivatives, the problem is that of locating the solvent front on ascending or descending chromatograms in the dark, or by the light of a very low intensity safelight. It is also useful to locate the solvent front at a prearranged distance from the origin so that the area of chromatography paper covered by the solvent will fit a bioautograph tray without having to cut the chromatogram to fit. The detector described here was produced to trigger an alarm system when the end of a predetermined solvent path-length had been reached.

The detector locates the leading edge of electrically conductive solutions by arranging the transistor (TR1 in Fig. 1) to conduct when the solvent resistance appears between points A and B (Fig. 1). Relay RL1 then closes and fires an alarm or other

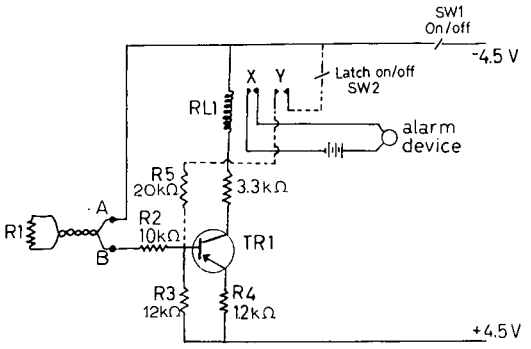

Fig. 1. Circuit diagram of the detector. R1 represents interelectrode solvent resistance. Latching circuit shown in broken lines (– – –).

circuit. The two detector electrodes (one connected to A, and one to B) can be applied to sense either across the width, thickness, or length of the paper (Fig. 2). Suitable electrodes may be made from small paper-clips soldered to thin flexible polyvinyl-chloride-insulated flex, such as is supplied with hearing-aid earpieces. The spring tension in the clips and their connecting leads, and their combined weight should be insufficient to distort the wet chromatography paper. If using papers with low wet-strength, the paper and electrodes may need to be supported in an insulated frame which may be removed when the paper is dry. Whatman No. 1, No. 20 and ion-exchange papers have been used successfully without additional supports. Some ion-exchange papers have low wet-strength and need very careful handling; acid pre-treatment of these papers may improve their wet-strength characteristics. The transistor and alarm circuits are placed outside the chromatography tank, the electrode leads being connected to insulated gas-tight conductors passing through the tank lid or wall.

Construction and circuit details

The circuit has proved to be adequately sensitive for use with electrically con-

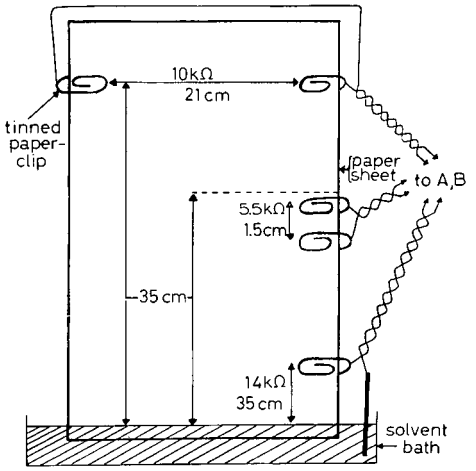

Fig. 2. Some alternative electrode arrangements. Interelectrode resistances shown for Whatman No. 20 paper and 50 mM NaCl at +1°.

ductive solvents. Some solvent resistance values are shown in Fig. 2. When using a Mullard OC76 transistor for TR1 a solvent resistance of 1.25 MΩ or lower caused the relay RL1 to close, the base voltage being —75 mV. The sensitivity could be increased by the use of higher gain transistors or possibly a Darlington pair. Substituting a selected low-gain Mullard OC71 for TR1 the relay closed with a solvent resistance of 330 kΩ or lower. In this case a base voltage of —150 mV was required to trigger the relay. The circuit values in Fig. 1 are those found suitable for a Mullard OC76. The emitter current is limited to 400 μA by R4 at maximum base drive. With a solvent resistance of 100 kΩ a base current of 30 μA was recorded with the circuit in Fig. 1. R2 is included to limit base current when low solvent resistances are present. The current, voltage and resistance values were measured on the 1 mA, 2.5 V d.c., and RX100 ranges of a Taylor Multimeter type 100 A (100 kΩ/Vd.c.). Using silicon or germanium transistors, temperature stability was found to be satisfactory in the range 0° to +37°.

An alarm bell is preferably driven from a separate dry battery. If both the transistor and the alarm bell are driven from the same battery the circuit should be checked for the presence of high-voltage transients. These may occur when the bell operates, possibly leading to irreversible damage to the transistor with subsequent unreliability of the device. The battery terminal voltage should also be checked with the alarm operating to ensure that the transistor is supplied with at least 3.8 V. A crude test for the absence of high-voltage transients is to connect a neon-bulb across the bell; even a feeble glow should not be seen in the neon-bulb with the bell operating. Such transients may usually be suppressed by the use of simple resistance-capacitance filters, although if this fails a thyrector diode may have to be connected across the bell or buzzer to ensure that the transistor is not damaged. The relay RL1 should have a d.c. coil resistance of about 350 Ω, the contacts (X in Fig. 1) being suitably rated for the current and voltage load of the alarm circuit(s). Electrical latching of the relay in the 'on' position when the transistor conducts may be provided by the use of extra contacts (Y in Fig. 1) and a reset switch (SW2), base drive being provided

via R5. Using a uniselector or multi-way switch and electric clock, with electrodes placed at measured intervals down the paper, solvent run times may be recorded. This has been of considerable use when working at 0°.

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Some technical improvements in the paper chromatography of sugars

A method of sample desalting and a sensitive staining reagent

Pyridine extraction procedures for desalting sugar samples are known[1,2]. Hot pyridine, however, is known to cause chemical transformations in some sugars[3]. In the method to be described in this paper, pyridine comes into contact with sugars at room temperature and for a short period.

Aromatic amine–acid mixtures are well known as specific staining reagents for sugars[4], but most of them lack sensitivity[5]. The *p*-aminobenzoic acid–phosphoric acid mixture to be described is more sensitive than most of the commonly recommended reagents of this type. It has the further advantage of being stable at room temperature for a long time.

The desalting procedure

2 ml of pyridine (B.D.H., A.R.) is added to a 0.5 ml sample (urine) to precipitate the salts[2]. After mixing and allowing to stand for 10 min., the tube is centrifuged at 2000 r.p.m. for 5 min. Supernatant is spotted over the area CDFGE of a strip of Whatman No. 1 filter paper, chromatographic grade (Fig. 1), pyridine being evaporated off with a blast of cold air. Two washings, each of 0.5 ml pyridine, are similarly spotted. Next, the strip is immersed in ether in a jar, for a period of 10 min, after which the strip is taken out and kept under a fan for another 10 min. This completely removes pyridine from the filter paper strip.

The sugars are washed in 0.3–0.5 ml distilled water by a descending technique, running time 1 h at 70°. The elution is conducted in an incubator 40 × 40 × 60 cm adjusted to the requisite temperature. Rectangular staining jars 8.5 × 6.5 × 4.0 cm, supported on wooden blocks, are used as solvent troughs. A glass rod which fits tightly between the two opposite walls of the staining jar is used to support the paper strip along one wall. The strip is rolled down over a thin glass rod placed on the upper

edge of the trough wall, to prevent capillary siphoning of water down the paper. About 2000 ml water at a temperature of 90–100° is placed inside the incubator in four 1000 ml beakers, just before elution is started. This ensures the requisite satura-

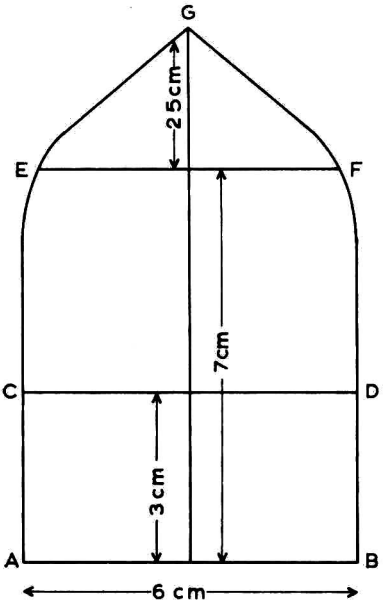

Fig. 1. The paper strip used in the described method of sample desalting.

tion inside the incubator. The sugar solution is received in a graduated tube and adjusted to a definite volume before spotting.

In the recovery experiments 100% recovery was obtained for all sugars tested, *e.g.* fructose, galactose, glucose, lactose, maltose, ribose, sucrose, and xylose.

p-Aminobenzoic acid reagent

3 g *p*-aminobenzoic acid is added gradually, while stirring, to 5.0 ml hot phosphoric acid. When the *p*-aminobenzoic acid has dissolved completely, 300 ml of a mixture of *n*-butanol–acetone–water (10:5:2, v/v) is added to make up the solution. If some residue remains, it is disregarded. This is used as a dip reagent. Colours are seen after the stained chromatograms have been heated at 105–110° for a few minutes. Pentoses give a dark red colour while other sugars are stained dark brown.

Results and discussion

In this desalting process the sample may be preserved on paper for any length of time, the step of washing the sample into water being undertaken only when it is desired to spot it. Similarly easy transport of the samples, if required, is an added advantage.

Fig. 2 compares the staining reagent described with three well known similar reagents. 25 μl of a sugar mixture (containing equal volumes of 0.7% solutions of disaccharides and 0.5% solutions of monosaccharides) was spotted in each case.

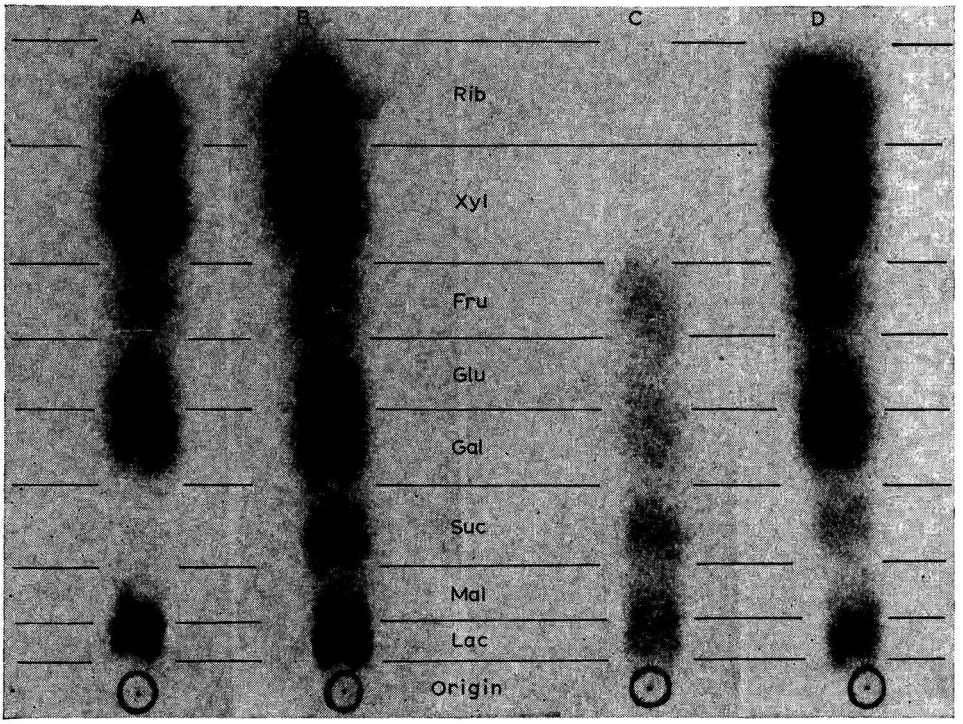

Fig. 2. Comparison of the staining reagent described (B) with aniline reagent[2] (A), aniline diphenyl reagent[2] (C) and aniline oxalate reagent[4] (D).

Separation was effected using isopropyl alcohol–*tert.*-butyl alcohol–*n*-butyl alcohol–water (4:2:2:2) as solvent. The high concentrations of sugars used were necessary to allow comparison of the stain with the aniline–diphenyl reagent.

The reagent described is stable below 25° almost indefinitely. Development of a yellow colour does not affect the usefulness of the reagent, but it should be discarded on development of a deep orange colour.

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Identification of glucose and 2-deoxyglucose with glucose oxidase on chromatograms

When carbohydrate spots are to be detected on chromatograms, an oven is required, as high temperatures, 100–110°, are often necessary. Sometimes, for instance when ammonical silver nitrate is used, it is also advisable to inspect the chromatogram continuously during the colour development. In order to eliminate the heating procedure, the use of glucose oxidase for the detection of glucose and 2-deoxyglucose was investigated in the present study.

MÜLLER[1] showed that glucose was converted into gluconic acid and hydrogen peroxide by means of glucose oxidase. HARTREE AND KEILIN[2] investigated the specificity of glucose oxidase and found that other carbohydrates reacted with the enzyme. The highest reaction rate, however, was obtained with glucose. SOLS AND DE LA FUENTE[3] showed that glucose oxidase reacted with different derivatives of carbohydrates. It can thus be concluded that the glucose oxidase reaction with glucose is not specific.

TELLER[4] developed a quantitative colorimetric method of determining glucose by introducing a reduced aromatic amine into the reaction system. Further, WHITE AND SECOR[5] showed that glucose could be identified on paper chromatograms by first spraying the chromatogram with glucose oxidase preparation followed by developing either the gluconic acid formed by means of an acid iodate–iodine–starch solution or by spraying the chromatogram with a solution of *o*-toluidine in ethanol.

Among the carbohydrates rapidly oxidised by glucose oxidase is 2-deoxyglucose[3]. This substance has very often been used in experimental studies on the permeability of cell membranes because it behaves like glucose. It would thus be of value to be able to separate these two carbohydrates in a simple manner. Paper chromatography and thin-layer chromatography were used in the present study.

A commercial freeze-dried glucose oxidase reagent, suitable for quantitative glucose determination, was used to detect glucose and 2-deoxyglucose on the chromatograms.

Spray reagents

A glucose oxidase reagent (KABI) containing reduced *o*-dianisidine was used according to the directions for glucose determination. The solution can be stored in a refrigator for four weeks.

Paper chromatography

Glucose and 2-deoxyglucose were applied to Whatman No. 1 paper in quantities of 1–10 μg. The chromatogram was run for 6 h in a solvent system of *n*-butanol–pyridine–water (45:25:40, v/v) in a descending direction. The chromatogram was dried at room temperature and then sprayed with the glucose oxidase reagent. Within a minute a green spot, changing to reddish brown appeared in the position to which the glucose had moved. The colour changes are the same for 2-deoxyglucose but occur more slowly. The spots of 2-deoxyglucose are visible within 5 min.

The spots are more clearly demarcated and the colour is more easily discernible if the chromatograms are placed in a moistened chamber after the spraying. The colour is not stable when the chromatograms are stored in air.

Thin-layer chromatography

Spots containing 1–10 μg of glucose and 2-deoxyglucose were applied to thin-layer chromatoplates made of Kieselgel G (Merck) dried at 50°. The chromatogram was run for 30 min in *n*-butanol–acetone–water (40:50:10, v/v), dried at room temperature, and then sprayed with the oxidase reagent. The colour development was somewhat slower than for the paper chromatograms, but both substances were identified within 5 min.

Some other carbohydrates giving colours with glucose oxidase reagent were also run on paper chromatograms and treated as described above. A very faint colour was obtained with the following substances applied at the amounts mentioned: mannose 20 μg, maltose 20 μg, and galactose 5 μg. Glucosamine hydrochloride, lactose, arabinose, and sucrose gave no colours.

Results

The R_F values obtained for glucose and 2-deoxyglucose are given in Table I.

TABLE I

Chromatography system	R_F *values*	
	Glucose	*2-Deoxy-glucose*
Whatman No. 1 paper: *n*-butanol–pyridine–water (45:25:40, v/v)	0.36	0.50
Kieselgel G; *n*-butanol–acetone–water (40:50:10, v/v)	0.59	0.71

Conclusions

This method of detecting glucose and 2-deoxyglucose on chromatograms is very simple and rapid. The chromatograms can be sprayed and developed in an open room, thus excluding the use of an oven. The oxidase reagent is stable for a long time when kept in a refrigerator. Furthermore, quantities as small as 1 μg can be detected.

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Thin-layer chromatography on supports impregnated with neutral organophosphorus compounds

Recently, in this laboratory considerable attention has been paid to the thin-layer chromatography of inorganic ions on silica gel impregnated with high-molecular-weight amines and substituted ammonium salts. In the course of this work, comparisons have been made with literature data[1-3] concerning chromatography on paper impregnated with neutral organophosphorus compounds such as tri-*n*-butylphosphate (TBP), tri-*n*-octylphosphine oxide (TOPO) and bis(di-*n*-hexylphosphynyl)methane. Here, in a limited number of cases—*e.g.*, with Sc and Th (see below)—discrepancies were noted[4] in the HCl system between the behaviour of ions on supports treated with liquid anion-exchangers and with neutral organophosphorus compounds. We are therefore now reporting briefly on thin-layer chromatography using TBP and TOPO.

In preliminary experiments, it was ascertained that with TBP (Fluka) a 0.2 *M* solution sufficed for impregnation of the silica gel (Type DO, Fluka), which contrasts favourably with the 0.8 *M* solution used by O'LAUGHLIN AND BANKS[1,2]. With TOPO (Eastman), a 0.1 *M* solution was generally used, as against 0.2 and 0.025 *M* cited in the literature[1-3]. For all details concerning the preparation of the thin layers, elution and detection, one is referred to refs. 4 and 5; TBP and TOPO were dissolved in chloroform and equilibrated with 3 vol. of 2 *N* HCl before use.

The results of our experiments are summarized in Fig. 1. They show a good over-all correspondence with the literature data, both for TOPO and TBP. However, some relatively large deviations may be noted. This is the case for Ti(IV), where thin-layer chromatography yields lower R_F values in the whole HCl range investigated, both with TOPO and TBP. With Zr(IV) and Hf(IV), the same phenomenon is encountered, though only with TBP and at low HCl strengths. With all three ions, hydrolysis may well be (partly) responsible for the differences found, as is suggested by the good correspondence between the curves found here and those obtained in the presence of liquid anion-exchangers[4]. Lastly, attention is called to Cr(III), the only ion over which the TOPO data of O'LAUGHLIN AND BANKS and CERRAI AND TESTA disagree; our results here confirm the R_F *vs.* *N* HCl plots recorded by the former authors.

Our data on TOPO most resemble those using a 0.2 *M* equilibration solution in work with paper. However, distinctly higher R_F values, showing good agreement with the data of CERRAI AND TESTA[3], are obtained when the TOPO molarity in our experiments is reduced to 0.04 *M*. In this context, it may be remarked that overloading was repeatedly observed in the 0.04 *M* experiments; however, after 3-fold dilution of the cation solutions normally used, this phenomenon disappeared. Concerning the results presented for TBP, these include data on 6 ions not chromatographed by the earlier authors. Here, *viz.* with Sn(IV), Sb(III), Ag(I), Re(VII)O_4^-, Pb(II) and Bi(III), good agreement is found with our results on weakly adsorbing liquid anion-exchangers.

As might be expected from our previous results and discussions[4,5], for most ions a good agreement exists between the thin-layer data concerning TBP and TOPO, and liquid anion-exchangers, respectively. As was outlined above, in some cases

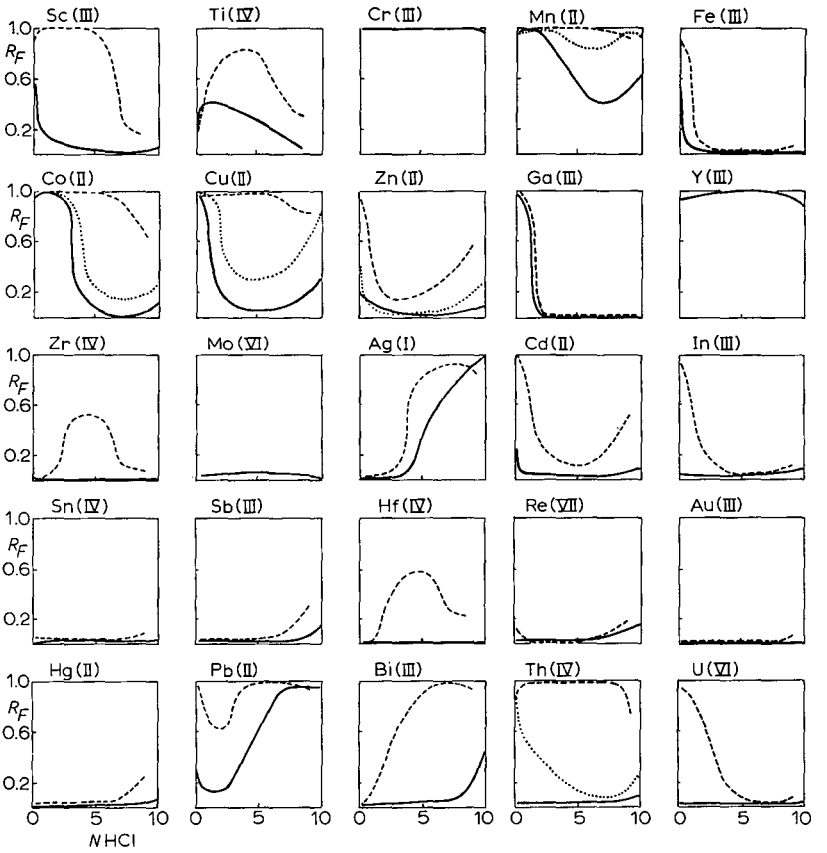

Fig. 1. Behaviour of 25 ions in the HCl system in thin-layer chromatography using silica gel impregnated with neutral organophosphorus compounds. (———), 0.1 *M* TOPO; (· · ·), 0.04 *M* TOPO; (– – –), 0.2 *M* TBP. Tailing of the spots incidentally occurs at steep parts of the curves; with TBP, elongated and/or tailing spots are repeatedly observed, especially with Ti.

however, *viz.* with Zr and Hf in the case of TOPO and with Sc and Th for both organophosphorus compounds, significant discrepancies occur between the paper data on TOPO and TBP, and the thin-layer results on liquid anion-exchangers[4]. It is interesting to note now that in all these cases, very good agreement exists between the paper and thin-layer data of the organophosphorus compounds. This implies that the discrepancies noted before are not due to the influence of the support.

As regards the usefulness of TBP and TOPO in chromatography, we would like to point out that the former compound shows weak adsorption strength only and the elution times increase to over 40 min when using 7 (or higher) *N* HCl as eluant. TBP will thus be of limited importance only, the more so because many ions gave tailing spots in our experiments. On the other hand, TOPO belongs to the strongly adsorbing class and the elution times at high HCl strengths are far less prolonged; further, the quality of the thin layers is satisfactory. TOPO may be valuable, therefore, but when considering all relevant practical aspects together, it still does not surpass the liquid anion-exchangers, even apart from the price difference. In isolated cases,

however, such as separations including Sc (*e.g.* from Y or rare earths) and Th in HCl systems, TOPO chromatography will come in useful. Similar applications may occur with other eluants, notably HNO_3.

The most important aspect of our present study is the indication of the usefulness and reliability of the thin-layer technique, which may be recommended for further studies concerning the neutral organophosphorus compounds because of its rapidity and relative inexpensiveness.

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Separation of square planar complexes by thin layer chromatography

Recently, there have been a number of reported chromatographic separations of octahedral transition metal coordination complexes on silica gel by both column and thin layer methods[3,5,6,7]. A suggested mechanism for these separations has been ion exchange of the cationic complexes between the polar solvents used and the silanol sites (≡Si—O—H) of the silica gel[1,2,3]. In all of these reported systems, neutral or anionic complexes move with the solvent front, and show no retention on the stationary phase.

For means of determining purity, and in order to ascertain the composition of certain reaction mixtures, it has been of interest to determine whether square planar complexes of platinum(II) and palladium(II), particularly the uncharged diacidodiamine complexes, could be separated by thin-layer chromatography. In all of the solvent systems previously reported[3,5,6,7] the diacidodiamineplatinum(II) and -palladium(II) complexes moved with the solvent front as predicted but the charged ions such as tetraammineplatinum(II) and tetrapyridoplatinum(II) showed only limited mobility. By changing the solvent from an acidic polar solvent to a non-polar solvent, however, a separation of these complexes was possible. Since many of the *cis–trans* pairs of the diacidodiammine-platinum(II) have different solubilities in chloroform (a fact used advantageously in the preparation of the pure stereoisomer), this was chosen first as a solvent, and found to be very successful.

In contrast to the polar solvents where an ion exchange mechanism is thought to function, and where such variables as layer thickness, activation time, solvent travel time are not too important, it was found that the above mentioned variables are quite critical in the separation of the square planar complexes in order to achieve reproducible R_F values. Because the spreading apparatus used frequently gave varying silica gel layer thicknesses depending on the glass plates used and the viscosity of the slurry, uniformity of plate thickness was hard to achieve. For this reason, commercially coated plates, Eastman Chromatosheets, Type K301R2, were used. These have a 100 μ coating of silica gel bound by polyvinyl alcohol. Best separations were obtained when the plates were activated for 30 min at 115°. In a standard glass chromatographic chamber, the solvent required 45–50 min to travel 10 cm. Using the Eastman Chromatographic chamber, by virtue of its much smaller free volume, the solvent travel time was only 10 min, but the resolution of the complexes was very poor, and the mobilities were much lower.

The solvent used was reagent grade chloroform to which was added three drops of dimethylsulfoxide (DMSO) per 100 ml to reducing tailing of some of the complexes. The platinum(II) and palladium(II) complexes were prepared by methods outlined in Inorganic Syntheses[4]. Chloroform solutions of these complexes were applied to the plate. The separated species were detected by spraying the developed plate with a 5 % aqueous $SnCl_2$ solution. Several runs were made with plates containing a fluorescent indicator in which case those complexes containing an aromatic ring could be detected under an ultraviolet light with almost the same sensitivity as by the $SnCl_2$ developer. Unfortunately, such complexes as $[Pt(NH_3)_2Cl_2]$ could not be detected in this manner.

Table I lists some typical R_F values obtained for some representative platinum(II) and palladium(II) complexes. These mobilities are qualitatively related to the respective solubilities of these complexes in chloroform. Separation of the *cis*- and *trans*-

TABLE I

R_F VALUES OF SOME PLATINUM(II) AND PALLADIUM(II) COMPLEXES ON SILICA GEL

(Abbreviations: pip = piperidine; py = pyridine; MPYO = *p*-methylpyridine-N-oxide; $P\phi_3$ = triphenylphosphine; P(p-Cl-ϕ)$_3$ = tris(*p*-chlorophenyl)phosphine.)

Complex	R_F
$K_2(PtCl_4)$	0.00
$[Pt(NH_3)_4](PtCl_4)$	1.00
$[Pt(pip)_4]Cl_2$	0.00
$[Pt(py)_4]Cl_2$	0.00
cis-$[Pt(NH_3)_2Cl_2]$	0.00
trans-$[Pt(NH_3)_2Cl_2]$	0.00
cis-$[Pt(py)_2Cl_2]$	0.04
trans-$[Pt(py)_2Cl_2]$	0.78
cis-$[Pt(py)_2Br_2]$	0.08
trans-$[Pt(py)_2Br_2]$	0.92
cis-$[Pt(MPYO)_2Cl_2]$	0.20
cis-$[Pt(P\phi_3)_2Cl_2]$	0.05
cis-$[Pd(P\phi_3)_2Cl_2]$	0.18 comet
cis-$[Pd(P(p\text{-}Cl\text{-}\phi)_3)_2Cl_2]$	1.00 comet
trans-$[Pd(P(p\text{-}Cl\text{-}\phi)_3)_2Cl_2]$	0.25 comet

dibromo- and dichloro-dipyridoplatinum(II) complexes was particularly successful with mixtures of all four species being clearly resolved. Indeed, as a check of stereochemical purity of the preparation of these complexes, the thin-layer method proved more sensitive than the usual spectrophotometric determination of purity. Except for some comets, the corresponding palladium(II) compounds separated as well as did the platinum(II) compounds. The *cis*- and *trans*-dichlorodiammineplatinum(II) complexes could not be separated. Both are reported to be insoluble in most common solvents except N,N-dimethylformamide. This was tried as a solvent for these complexes, but now instead of being immobile, both complexes moved with the solvent front. No attempt was made to separate these two with a mixed solvent system.

The mechanism of separation of these electrically neutral complexes appears to be via surface adsorption of the complex on silica gel as shown by the dependence of mobility on solubility in the solvent. As mentioned previously, layer thickness, activation conditions, and solvent travel time are very much more critical than in the separation of the ionic complexes. Thicker layers of silica gel result in decreased mobilities because of a greater number of adsorbing sites per unit distance of travel time. Longer activation times appear to decrease the number of sites as evidenced by the general increase in mobilities. Also, the solvent travel time is important. In a large chromatographic chamber, even when saturated with chloroform, the solvent required 45–50 min to travel 10 cm while in the Eastman Chromatosheet chamber, with its very much smaller "free" volume, the travel time was only 10–15 min. In the latter case, very low R_F values were observed, and poor separations were obtained as a result of the solvent "outrunning" the solubility equilibrium.

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Inorganic thin-layer chromatography on microcrystalline cellulose*

The rapid growth of thin-layer chromatography (TLC) in the past decade since STAHL first standardized the basic equipment and procedures has been phenomenal[1,9,19,20]. Strangely, although much of the first work done in this field was inorganic in nature, the greatest development has been in the realm of organic chemistry. Recently, however, the number of inorganic publications has begun to increase, with SEILER[10–18] in Switzerland and (the late) Dr. POLLARD[6,7] in England among the leaders. The latter, with MCOMIE, had published the leading book in the field of inorganic paper chromatography[8], but had turned to TLC from paper procedures in the last year or two.

The application of paper chromatographic methods to TLC on cellulose plates appeared to be a logical step, but a number of factors made this only partially possible. The solvents, many of which contained large proportions of water, loosened and even washed off the cellulose layers. Various binders were tried, but most of them affected the results obtained in one way or another. Calcium sulfate could not be used where either of its ions might interfere – or where insoluble compounds might be formed with the ions to be separated. Starch was sometimes successful, but affected both the R_F values and many of the visualization reactions. The fibrous nature of the cellulose layers reduced resolution due to the greater diffusion taking place. In spite of all these disadvantages, however, some work has been done on cellulose plates, but more research has apparently gone into silica gel as an adsorbent. Most of SEILER's work has been done on this medium, but its ion exchange properties have necessitated the development of entirely new solvent systems.

In our laboratory silica gel was first used for inorganic separations, but the availability of so many well detailed paper chromatographic procedures led us to try cellulose layers. Results, for the reasons outlined above, were not as satisfactory as desired. A number of grades of cellulose were tried and finally, following the work of WOLFROM and his co-workers[21,22] microcrystalline cellulose ("Avicel" —a product of the American Viscose Division of FMC) was found to meet the necessary requirements. This material forms a remarkably stable layer, which stands up in water solutions, and can be written upon with a pen or pencil like paper. Initial separations made with this adsorbent, using paper chromatographic solvents and visualization reagents, gave results almost identical with those obtained on paper. Possible application to water analysis was investigated and reported previously[4].

Work with most of the same group of ions (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}, SO_4^{2-}, PO_4^{3-}, and CO_3^{2-} has continued in an effort to learn more about the use of this new adsorbent, and to increase the sensitivity of the visualization and simplicity of the separations.

Experimental

Since this phase of the work was qualitative in nature, solutions of the ions were made up on the basis of 1.00% of the anhydrous salt in water (Table I). In the case of a mixture of equal volumes of all the solutions, however, it was necessary to add a few drops of conc. HCl to keep insoluble compounds from precipitating.

* Paper presented at the Pittsburgh Analytical Conference, February 21, 1966.

TABLE I

TEST SOLUTIONS

(1.00% of anhydrous salt in water (10 μg/μl))

No.	*Salt*	*Quantity (g)*
1	$Al_2(NH_4)_2(SO_4)_4 \cdot 24\ H_2O$	1.91
2	$CaCl_2 \cdot 2\ H_2O$	1.32
3	$Fe(NH_4)_2(SO_4)_2 \cdot 4\ H_2O$	1.25
4	$Mg(NO_3)_2 \cdot 6\ H_2O$	1.73
5	Na_2CO_3	1.00
6	$(NH_4)_2HPO_4$	1.00

All TLC plates used in this work were the pre-coated Uniplates*, and consisted of 250 μ layers of Avicel on 20 × 20 cm glass plates. (WOLFROM, for work with carbohydrates, etc. prefers a 1.00 mm thick layer). The guaranteed uniformity of these pre-coated plates insures the repeatability of all results.

The reagents used in the preliminary work (and some of the final separations) were taken from the work of POLLARD[6–8] and also from ELBEIH *et al.*[2,3]. The solvent systems are listed in Table II, and the visualization reagents in Table III. As the work progressed other solutions were selected from the literature of paper chromatography and our own experience, in an effort to obtain more nearly "universal" reagents, and thus to simplify the procedures. These are also included in the same two tables.

TABLE II

SOLVENT SYSTEMS

A	Cations	4 ml H_2O + 4 ml HNO_3 + 0.01 g EDTA (free acid). Cool, and add 200 ml dioxane + 2 g antipyrine.
B	Cations	0.4 *N* HNO_3 saturated with *s*-collidine (2,4,6-trimethylpyridine) at room temperature.
C	Anions	120 ml ethanol + 40 ml pyridine + 32 ml H_2O + 8 ml ammonium hydroxide, conc.
D	Cations and anions	1-Butanol saturated with 4 *N* HCl. Shake 200 ml 1-BuOH with 100 ml 4 *N* HCl.
E	Cations and anions	Same as (D) with the addition of 1% HF.
F	Cations and anions	140 ml 1-butanol, saturated with 10% HBr, separated and 60 ml HBr added to BuOH layer.
G	Cations	1-Butanol + 1% HCl conc. + 1% TOPO. (TOPO = tri-*n*-octylphosphine oxide).
H	Cations	Methanol + 1% HCl conc. + 1% TOPO.

The plates were used directly from the package, as received. A border 4 mm in width was scraped from the two sides and bottom of the adsorbent layer. (This has been standard procedure in our laboratory on all plates to eliminate edge effects and contamination resulting from handling them by the edges.) The sample solutions were spotted along a line 1.5 cm above the bottom edge of the adsorbent by means of an

* Available from Analtech., Inc., Wilmington, Dela. (U.S.A.).

TABLE III

VISUALIZATION REAGENTS

A	Cations	0.5 g kojic acid + 2.5 g 8-hydroxyquinoline in 500 ml 60 % ethanol. Spray, dry. Expose to NH_3 vapors. Examine visually and with 365 mμ U.V. light. Spray with $(NH_4)_2S$. Dry. Examine as above.
B	Cations	1.5 % solution of violuric acid in water. Heat after spraying for 15–20 min at 100°.
C	Cations	0.25 % PAN [1-(2-pyridylazo-2-naphthol)] in ethanol.
D	Cations	0.25 % PAR [4-(2-pyridylazo) resorcinol] in ethanol.
E	Cations	0.25 % SNAZOXS in ethanol (8-hydroxy-7-[(4-sulfo-1-naphthyl)-azo]-5-quinoline-sulfonic acid).
F	Anions (acids)	0.04 % bromocresol green in ethanol, made just basic with 0.1 *N* NaOH.
G	Anions (halides)	0.1 % bromocresol purple in ethanol, made just basic with dilute NH_4OH.
H	Anions	Mix equal volumes of 3.3 % $AgNO_3$ in water and 0.3 % sodium fluoresceinate in water. Examine in U.V. light.
J	Anions (phosphate)	1 % ammonium molybdate + 1 % $ZnCl_2$ in 10 % HCl.

8 μl micropipet* and the spots dried completely. Development was carried out in covered glass chambers (3 in. × $8^1/_4$ in. × $10^1/_4$ in. approx. I.D.) lined with chromatographic grade paper, and allowed to reach an equilibrium condition for at least 30 min after loading with 200 ml of the developing solvent. (Reagent grade solvents were used wherever possible.) In almost all cases the separation was carried out until the solvent front has reached a line 10 cm above the spotting line, thus making the calculation of R_F values a little simpler.

Following development the plates were removed and dried thoroughly with a warm air current, and then sprayed with the visualization reagents. Drying after the spraying was also with warm air. Examination of the spots was by both visual light and under excitation by a long-wave (365 mμ) U.V. lamp. R_F values were determined by measurement with a millimeter rule or by means of the Desaga spotting template, which is calibrated for this purpose.

Results

The separations obtained with the published paper chromatographic procedures were, in most cases nearly identical with those on paper. Since many of the spots are not compact, but are diffuse, complete separation of each ion in a mixture was not always possible within the 10 cm development distance. The use of smaller sample spot sizes will reduce this condition, but time did not permit repeating the entire series.

The more important results of the work are summarized in Table IV.

Discussion

Many of the difficulties encountered with the use of paper are obviated when microcrystalline cellulose TLC plates are employed. The impurities, usually found in the purest of chromatographic paper, are almost completely removed in the manufacturing process. Ca^{2+}, Mg^{2+}, Fe^{3+}, and Cu^{2+} are not found in measurable quantities in the Avicel.

The greatest difference between the two media is, of course, the matter of the

* Drummond Micro-caps.

TABLE IV

$R_F \times 100$

Solvent system	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Visualization	*A*	*B*	*C*	*A*	*A*	*A*	*A*	*A*
Mg^{2+}	12	63	—	24	48	46	0	D I
Ca^{2+}	17	53	—	18	40	23	0	F F
Fe^{3+}	23	0	—	43	78	100	0	U S
Al^{3+}	16	0	—	20	44	37	0	E
PO_4^{3-}	—	—	4	92	100	97	35–45	S T
SO_4^{2-}	—	—	8	64	74	—	0–60	R E
CO_3^{2-}	—	—	12	—	—	—	35	A K S

time required to make a separation. A paper procedure requiring 16–20 h can usually be completed in 30 min. Very few TLC separations take over an hour under normal conditions.

The double front (demixing or demixion) characteristic of paper chromatography[5] is found with microcrystalline cellulose also. This is a result of the cellulose removing water from the solvent as it rises up the adsorbent layer. In a saturated chamber this is minimized, providing all the solvent constituents are volatile. However, with mineral acids, such as HCl and HNO_3 present for instance in butanol, it cannot be eliminated. In this type of solvent mixture, an area poor in acid content will precede the major part of the solvent, and two fronts are formed. The first is primarily organic in content, while the other more nearly represents the original composition of the solvent system. With Avicel, due to its high purity, the dark line at the secondary front is missing, or is only barely visible. It can be detected, however, by the shape and location of some of the spots. The height of the acid ascent depends on both the concentration and the temperature. The higher its concentration, the closer the second front is to the first one.

Although some people hold to the idea that TLC is not affected by temperature, we have found that this is not true. Changes of more than a few degrees can make marked differences in R_F values. This is particularly important with cellulose as an adsorbent, since the separations are largely a partition process and the coeffcient of partition is temperature dependent. The dehydration of the cellulose must also be considered.

Although rather good separations of many combinations of inorganic ions can be made using butanol–HCl mixtures, there are some variations of this basic combi-

nation that can be used to improve the results obtained under certain conditions. The use of HF or HBr usually gives higher R_F values to the ions that do move away from the starting line. That this is due to complex formation is borne out by the fact that complexing agents such as EDTA (ethylenediaminetetraacetic acid), anti-pyrine, TOPO (tri-*n*-octylphosphine oxide), etc. also improve the separations in many cases.

Conclusions

The use of microcrystaline cellulose (Avicel) as an adsorbent in TLC is useful in the separation of inorganic ions, and has all the advantages of paper chromatography without many of its disadvantages. Paper chromatographic methods are directly applicable to these layers without modification, and will produce almost identical results in all the systems investigated thus far.

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News

VARIAN AEROGRAPH BASIC GAS CHROMATOGRAPHY COURSES

Three-day training courses in gas chromatography are being held every month at the European headquarters in Basel, Switzerland. They offer basic theory and practical laboratory sessions.

Please write for your registration to the course secretary, Varian Aerograph AG., Pelikanweg 2, Basel, Switzerland.

Recent Event

151ST AMERICAN CHEMICAL SOCIETY MEETING, PHOENIX, ARIZ.

The 151st ACS meeting was held in Phoenix, Ariz., and included the following symposia:

1. Symposium honouring Lyman C. Craig (January 18th, 1966)

The lectures given below were delivered:

P. B. HAMILTON: Introductory remarks.
L. KESNER AND E. MUNTWYLER: An automatic organic acid analyzer.
W. KONIGSBERG: The development of peptide separation methods for amino acid sequence determinations.
E. H. AHRENS, Jr.: Isolation, quantification, and identification of neutral and acidic steroids in biologic mixtures.
P. B. HAMILTON: Introduction of LYMAN C. CRAIG.
L. C. CRAIG: (Award address—Fisher award in analytical chemistry). The use of membrane diffusion as an analytical tool.
W. E. COHN AND M. UZIEL: A one-hour quantitation of nucleic acid components at nanomole levels by elution ion-exchange chromatography.
R. TERANISHI: Gas–liquid partition chromatography separations.

2. General Symposium

The General Symposium included the following lectures delivered on January 20th, 1966:

R. C. SHEPARD: A simple, sensitive ionization detector for hydrogen.
H. WIDMER: Gas chromatographic identification of hydrocarbons using retention indices.
W. G. SCRIBNER AND W. J. TREAT: Solvent extraction of metal ions with butanol–trifluoroacetylacetone.
R. W. MOSHIER AND D. R. GERE: Gas chromatography of amine adducts of β-ketoenolate divalent metal chelates.
W. G. GULDNER: The determination of residual gases in thin metal films by means of a flash discharge lamp.
K. SHIMOMURA, L. DICKSON AND H. F. WALTON: Ligand exchange with chelating resins and cellulosic exchangers.
J. L. SIDES AND C. T. KENNER: Effect of pH and ionic strength on the distribution coefficients of the alkaline earth metals and nickel with a chelating resin.

3. Symposium on Gas Chromatography of Carbohydrates (January 18th, 1966)

J. N. BeMiller: Introductory remarks.
C. R. Perisho and J. A. Thoma: The selection of chromatographic conditions.
C. C. Sweeley, N. C. Saha and R. Bentley: Recent developments in gas chromatography of carbohydrates.
H. G. Jones, J. K. N. Jones and M. B. Perry: Applications of gas–liquid chromatography to the structural analysis of glycans.
K. N. Brobst and C. E. Lott, Jr.: Gas chromatographic investigation of (hydroxyethyl) starch hydrolyzates.
M. B. Perry and R. K. Hulyalkar: The analysis of neutral, basic, and acidic glycoses by gas–liquid chromatography.
P. D. Berger and S. E. Borodkin: The application of thin-layer chromatography to the separation of carbohydrates in sugar refining.
J. W. Berry, J. T. Marvel, S. K. Sen and A. J. Deutschman, Jr.: Methyl O-ethyl-α-D-glucopyranosides: their separation and identification utilizing gas chromatography.
S. P. Rowland, V. O. Cirino and A. L. Bullock: Structural components in methyl vinyl sulfone-modified cotton cellulose.
H. H. Sephton: Gas chromatographic examination of the hydrolyzate from a methylated xylan after trimethylsilylation.
J. H. Sloneker and J. S. Sawardeker: Quantitative analysis of complex mixtures of carbohydrates by gas chromatography.
G. G. S. Dutton and A. M. Unrau: Separation by gas–liquid chromatography of Smith degradation products from complex synthetic polysaccharides.
D. W. Clayton and M. E. J. MacMillan: Analysis of wood and pulp components. Part I. Determination of monosaccharides in pulp hydrolyzates by gas–liquid chromatography of their trimethylsilyl derivatives.
K. Brendel, N. O. Roszel, R. W. Wheat and E. A. Davidson: Separation of amino sugars and amino acids on the Technicon amino acid analyzer.
H. H. Sephton and N. K. Richtmyer: Identification of the second nonulose from the Avocado with synthetic D-*erythro*-L-*galacto*-nonulose.

4. Symposium on Application of Chromatography to Cellulose, Wood, and Fiber Chemistry (January 18th, 1966)

M. A. Millett: Introductory remarks.
D. French, J. F. Robyt, M. Abdullah and P. Knock: Separation of maltodextrins by charcoal chromatography.
R. B. Kesler and E. E. Dickey: Anion-exchange chromatography of sugar–borate complex ions.
I. A. Pearl and C. R. Pottenger: Studies on the barks of the family Salicaceae. XI. Studies on the hot water extractives of the green bark of *Populus tacamahaca*.
G. S. Fisher: Variations in composition of turpentines from slash pines.
E. Zavarin: Gas–liquid chromatography of terpenes in service of conifer taxonomy.
M. A. Millett: Introductory remarks.
H. L. Frush: The use of paper chromatography in the separation, purification, and analysis of labeled carbohydrates.
T. A. Bhaskaran and A. von Koeppen: Determination of carbohydrate material in kraft black liquor by paper chromatography.
E. E. Fisher, H. Tai and C. N. Huber: Thin-layer chromatography of mono- and oligosaccharides.
M. L. Wolfrom: Chromatography of water-soluble substances with microcrystalline cellulose.
M. J. Effland and W. E. Moore: Spreader for the preparation of uniform plates for thin-layer chromatography.

Apparatus

Victoreen Instrument Company, 10101 Woodland Avenue, Cleveland, Ohio, U.S.A., experienced in electronics and nuclear instrumentation have introduced a new Series 4000 *modular gas chromatographs*. The unique features that are claimed for this system are: (a) 4 injection ports, (b) 4-column system (all glass or all metal), (c) 4 individually controlled detector ovens, and (d) a vibrating reed electrometer which is standard in all instruments incorporating ionization detectors. Victoreen believe they are the only gas chromatograph manufacturers offering a vibrating reed electrometer as a standard component. Increased sensitivity, stability and response speed are claimed, naturally Victoreen use solid-state electronics and printed circuit techniques.

Other features available are:

(i) A digital log electrometer, basically an analogue-to-digital converter. The log electrometer covers a wide dynamic range, 10^{-11} to 10^{-7} A; range switching or attenuation of peaks is eliminated. A complete chromatographic profile of wide-range mixtures is provided on a single trace. A broad region of application should be in trace analysis for improved identification of small peaks and shoulders. Logarithmic and digital outputs permit the use of log chart paper on standard recorders, or low cost electronic counters for digital peak area readout.

(ii) Automatic cryogenic programming, allowing highly selective stationary phases to be used. The linear programmer uses liquid N_2 or CO_2, is calibrated in increments of 2°, and controls from —195° to 500°.

F. & M. Scientific Division of Hewlett—Packard have announced a new Model 5750 *research gas chromatograph*, which we gather is intended as a successor to the Series 810 instruments.

Chief features claimed are:

(i) Improved T.C. and E.C. detectors. A new spiral flow through design has significantly improved the high temperature base-line stability of the T.C. detector. The 5750 offers a choice of 2 E.C. detectors, standard H^3 cell to 220°, and a nickel cell for up to 400°.

(ii) The linear dynamic range of the dual flame detector is a tenfold improvement on that previously reported for the 810. The detector gives better qualitative and quantitative precision, especially with larger sample sizes.

(iii) The redesigned column oven allows faster response and more even temperature uniformity.

(iv) The 5750 permits simultaneous installation of three detectors.

(v) Further improvements in injection port design are claimed.

Varian Aerograph (formerly Wilkens Instrument and Research) have announced two new *gas chromatographs*, the Models 1200 and 2000. Although available in the U.S.A., the former is expected to be released in Europe in June, and the latter in November. The Model 1200 is a low cost solid-state electronics modular design, and incorporates temperature programmer and a low-drift, low-noise level electrometer. The Model 2000 is designed specifically for pesticide and bio-medical analyses, including steroid, carbohydrate and fatty acid work. The features claimed include U-

shaped columns, tall column oven for 4 columns and 4 detectors, a completely all-glass system and on-column injection. Again the electronics are all solid-state. Dohrmann coulometers can be connected to the apparatus.

Barber-Colman Company have automated their modular Series 5000 Selecta-system *gas chromatographs*. This first automated gas chromatograph for laboratory use was announced recently. The instrument is capable of analyzing as many as 72 successive samples before reloading. Operating conditions are selected for isothermal or programmed temperature separations. Then after starting the apparatus, it will operate until all loaded samples have been run. Successive isothermal analyses are performed on the basis of selected and precisely reproducible times between injections. Control of programmed separations is more sophisticated, but a gearless electronic programmer provides excellent reproducibility of column temperature programming. Provision is made for re-equilibration of column before the next sample is injected. The automated system is available in a variety of options, and can be added to existing Series 5000 units.

Finally, the same company have announced a Model 5077 *column bath*. This permits rapid interchange between glass, metal, capillary and preparative columns. It is hoped that with a large detector capacity, a wide choice of detectors can be accommodated without sacrifice in detector stability.

Microtek Instruments Inc., Baton Rouge announced the availability of the MF-50 *gas chromatographs* which are tailored to suit the customers' requirements.

An essential feature of this chromatograph is the MT-U/60 ultra-sonic detector. The time required for a sound wave to travel from one end of a detection cell to the other passing through a gas mixture is compared directly with the time required for a sound wave to travel the same distance through a pure carrier gas. Time comparison is accomplished by using a continuous sound wave and measuring phase difference between reference and a sample signal. The phase difference, read out as DC signal, is linear with change in gas composition. The simplest to use of all gas chromatographic detectors has high sensitivity, linear and predictable response, wide dynamic range, fast response and is insensitive to flow. Other MicroTek developments include a stacked flame detector for improved phosphorus and sulphur detection, a cryothermal system for column temperature, and a high temperature electron capture detector using Ni^{63}.

Dohrmann Instruments announce that by use of a specially designed cell their *microcoulometric titrating system* can be used for the determination of nitrogen.

Because of the re-interest being shown in liquid column chromatography, both Packard Instruments and Barber-Colman's announce the availability of suitable *detecting systems*. More details will be given when they are available.

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