JOURNAL OF

CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER

CONTENTS

ห้องสมุดกรมวิทยาศาสตร์บริการ

Contents (continued)

CHROM. 18 442

RETENTION AND RESOLUTION IN DENSITY-PROGRAMMED SUPERCRITICAL FLUID CHROMATOGRAPHY

I. THEORY AND SELECTED RESULTS

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(Received December 2nd, 1985)

SUMMARY

A theoretical treatment of the linear velocities of the eluent and a sample band in the column is given for density-programmed supercritical fluid chromatography (SFC). It is shown that during a density programme the linear velocity of the eluent drops from column inlet to column outlet unless there is a considerable density gradient along the column. A numerical integration method for the calculation of retention times in density-programmed SFC is proposed. Peak compression effects and a reduced resolution can be expected when large decreases in mobile phase density or linear velocity occur along the column.

INTRODUCTION

In recent years there has been a growing interest in supercritical fluid chromatography (SFC) as a supercritical mobile phase may combine favourable transport properties (*e.g.*, low viscosity and high diffusivity) with considerable solvent power for substances of low volatility. The retention of substances in SFC depends strongly on pressure and temperature[1] and, in a more straightforward way, on the density of the eluent[2,3]. Therefore, the retention of different sample components can easily be adjusted by changes in pressure and/or temperature, pressure-programmed SFC thus being comparable to temperature-programmed gas chromatography (GC) and gradient elution in high-performance liquid chromatography (HPLC). For many eluents used in SFC equations of state are known, and with computer-controlled pressure programming it is also possible to create any density programme[4,5].

Much experimental work has been carried out on pressure-programmed SFC[6–9] and the advantages of density programming have been demonstrated[10]. However, few papers have treated density-programmed SFC theoretically that help to explain the experimental findings[11,12]. As density programming not only affects the capacity ratios of sample substances but also the flow of the mobile phase through the column, the prediction of retention and resolution in density-programmed SFC is more complicated than in the related techniques in GC and HPLC. A theoretical treatment

seems worthwhile because the number of experiments that have to be performed during an optimization procedure can be reduced drastically if the dependence of retention and resolution on the applied programme and on other operating parameters is known.

In this paper, the effect of a density programme on the linear velocities of the eluent and sample substances is considered for a model system and a method for the calculation of retention times in density-programmed SFC is presented. The influences of operating parameters such as density programming rate and eluent flow-rate on the resolution are considered, and the so-called peak compression effect[8,13] is evaluated.

THEORY

Model system

A scheme of the chromatographic system to be considered here is shown in Fig. 1. A pump delivers the eluent into the system with the mass flow-rate $\dot{m}(0)$. The eluent streams in the z-direction through a column with a uniform cross-sectional area A and length L, with the z-coordinate being 0 at the column inlet and L at the column outlet. The mass flow-rates of the eluent at the column inlet and outlet are denoted by $\dot{m}(0)$ and $\dot{m}(L)$, respectively. After having passed through the column the eluent is expanded by a reducing valve RV and leaves the system with a mass flow-rate $\dot{m}_0$. The volume between the column outlet and the reducing valve is V_2.
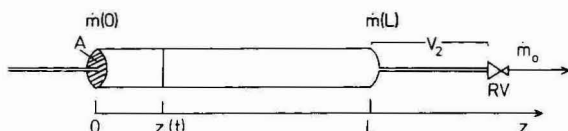
Fig. 1. Scheme of the model system.

Now, for the calculation of retention times the linear velocities of the eluent and sample in the column are of interest.

Linear velocity of the eluent

The mean linear velocity of the eluent in the z-direction is denoted by u_{mob}; in the column it is

$$u_{mob} = \frac{\dot{V}}{A} \tag{1}$$

where $\dot{V}$ is the volume flow-rate of the eluent, which can be replaced according to

$$\dot{V} = \frac{\dot{m}}{\rho} \tag{2}$$

by the mass flow-rate $\dot{m}$ and the eluent density ρ. Combination of eqns. 1 and 2 results in

$$u_{mob} = \frac{\dot{m}}{A\rho} \tag{3}$$

For the simple case when the density in the column is constant with respect to time, the mass flow into the column, $\dot{m}(0)$, is equal to the mass flow out of the column, $\dot{m}(L)$. If the density is constant throughout the column, u_{mob} does not depend on z. Especially in columns packed with small particles, however, there may be a significant pressure drop from the column inlet to the outlet, which results in a density drop in the z-direction that is greater the higher the compressibility of the eluent is. The linear velocity of the eluent then increases from the column inlet to the outlet.

In density-programmed SFC the density of the eluent is increased with time. This requires the mass flow into the system to be higher than the mass flow out of the system. The mass flow through the column then becomes a function of z, and it will now be divided into a part which does not depend on z and equals $\dot{m}_0$, and a z-dependent part $\dot{m}(z)$:

$$\dot{m} = \dot{m}_0 + \dot{m}(z) \tag{4}$$

Here $\dot{m}(z)$ is the mass flow that is necessary to increase the density in the volume V_z between z and the reducing valve RV by $d\rho$ in the time interval dt; it is given by

$$\dot{m}(z) = V_z \cdot \frac{d\rho}{dt} \tag{5}$$

The volume V_z can be written as the sum of the volume between z and the column outlet plus the volume between the column outlet and reducing valve V_2:

$$V_z = (L - z)A + V_2 \tag{6}$$

Using eqns. 4–6, eqn. 3 becomes

$$u_{mob} = \frac{\dot{m}_0 + [(L - z)A + V_2]d\rho/dt}{A\rho} \tag{7}$$

With eqn. 7 the momentary values of u_{mob} during a density programme can be calculated for any point in the column. Here A, L and V_2 are constants; $\dot{m}_0$ does not depend on z but may depend on time. For a density programme, of course, ρ depends on time, as is the case for $d\rho/dt$ if the programme is not linear. If there is a significant density drop along the column, both ρ and $d\rho/dt$ also depend on z. Several important features of eluent flow in density-programmed SFC can be deduced from eqn. 7:

(a) u_{mob} decreases linearly from the column inlet to the outlet if the density drop along the column can be neglected. With a finite density drop, however, the decrease in u_{mob} becomes less pronounced.

(b) u_{mob} is higher for a given system the more rapidly the density is increased.

(c) u_{mob} is higher for a given density programme the larger is the volume V_2.

(d) u_{mob} decreases with time for a linear density programme if $\dot{m}_0$ is held constant.

Linear velocity of the sample

The mean linear velocity of a sample i in the z-direction, denoted by u_i, is related to the linear velocity of the eluent as follows:

$$u_i = \frac{1}{1 + k_i'} \cdot u_{mob} \tag{8}$$

where k_i' is the capacity ratio of the sample, and the factor $1/(1 + k_i')$ gives the fraction of the time a sample spends in the mobile phase during its passage through the column. In SFC the capacity ratio depends strongly on the density of the mobile phase, and it is the aim of density programming to decrease the capacity ratios of the sample components one after another, because usually k' decreases with increasing density. Often a linear relationship between log k' and density is assumed[11,12]. For the supercritical region, however, we prefer the two-parameter exponential form[4,14]

$$k_i' = a(\rho/\rho^0)^{-b} \tag{9}$$

where ρ^0 is a unit density of 1 g cm^{-3}, and a and b are fitting parameters that can easily be determined from a few measurements at different densities. The parameter a gives the value of k_i' at the unit density ρ^0, and the parameter b can be related to the size ratio of the molecules of the sample and the eluent[4].

The combination of eqns. 7 and 9 with eqn. 8 yields the following expression for the linear velocity of a sample:

$$u_i = \frac{1}{1 + a(\rho/\rho^0)^{-b}} \cdot \frac{\dot{m}_0 + [(L - z)A + V_2]d\rho/dt}{A\rho} \tag{10}$$

Therefore, in density-programmed SFC u_i is a function of time and also of the distance z the sample has already travelled in the column. For a linear density programme and constant $\dot{m}_0$, u_i will usually increase with time because the effect of the decrease in the capacity ratio is mostly more pronounced than the effect of decreasing mobile phase velocity caused by the increased density.

Calculation of retention times

At the retention time $t_{R,i}$ a sample travelling through the column with a linear velocity u_i has reached the column outlet. The equation

$$\int_0^{t_{R,i}} u_i \, dt = L \tag{11}$$

generally holds. If the linear velocity of the sample is constant with respect to time and position in the column, $t_{R,i}$ can easily be calculated from

$$t_{R,i} = \frac{L}{u_i} \qquad (12)$$

This way of determining $t_{R,i}$ is no longer possible when u_i changes during the elution of the sample, as in density-programmed SFC. From eqn. 10 it becomes obvious that the exact solution of the integral in eqn. 11 for the conditions given by density programming may often not be feasible. However, a simple and universal approach for the calculation of retention times is the computer-aided numerical integration of eqn. 11. In this procedure the time is increased by small intervals Δt and the linear velocity of the sample for time t and position z is calculated. From the product of Δt and u_i the distance, Δz, the sample travels during the time interval Δt is determined. The increments of time and distance are summed, and when the sum of all increments Δz equals the column length L the retention time $t_{R,i}$ is given by the sum of all time intervals Δt. A similar calculation method has recently been proposed for the determination of retention time in gradient HPLC[15].

An important advantage of this numerical integration method is that any time and z dependence of the variables $\dot{m}_0$, ρ and $d\rho/dt$ in eqn. 10, which can be expressed mathematically, can be accounted for by the calculation. So, e.g., a comparison of the effects of different density programmes on retention times can be made without additional experiments, once the fitting parameters for the density dependence of k' and the system parameters A, L and V_2 are known.

Peak compression and resolution

In a chromatogram that is taken at a constant mobile phase density and velocity the substance peaks mostly become broader the longer the retention time of a substance. If the density of the mobile phase is increased during an analysis, peaks eluted later, however, can be of the same width or even be narrower than preceding peaks.

The width of a peak on the recorder trace is characterized by σ_t, which is half the width (in time units) of a Gaussian peak at 67% of the maximum height. In density-programmed SFC, therefore, a decrease in σ_t can be observed.

It has been stated that a "peak compression effect" should play an important role in pressure- and density-programmed SFC[8,13]. This effect has been explained as follows[8]. During a density programme there is a density drop from the column inlet to the outlet leading to an increase in the capacity ratio of a sample inside the sample zone in the column from the column inlet to the outlet. Hence the parts of the sample zone that are nearest to the column inlet are moving faster in the z-direction than the parts further down the column, resulting in a compression of the sample zone in the column.

However, if there is no density drop, peak compression should also occur according to eqn. 10 because of the gradient of the eluent velocity along the column. This gradient also causes the parts of a sample zone to move faster the nearer they are to the column inlet. Generally, the peak compression should increase with increasing decrease in the sample velocity along the column.

The dimension of a sample zone in the column is characterized by σ_z, the standard deviation of the peak in length units. At the column outlet, the standard deviation in length units is σ_L. The standard deviation σ_t that can be observed on the recorder is related to σ_L by

$$\sigma_t = \frac{\sigma_L}{u_i(L, t_{R,i})} \tag{13}$$

where $u_i(L, t_{R,i})$ is the linear velocity of the sample as it is eluted from the column at the retention time $t_{R,i}$.

As it can be seen from eqn. 13, the narrowing of peaks by density programming can either be a result of a decrease in σ_L (which itself could be caused by the peak compression effect) or could be caused by an increase in $u_i(L, t_{R,i})$ for substances eluted one after another. This second factor certainly plays a very important role, and as the application of eqn. 10 allows the calculation of $u_i(L, t_{R,i})$ it is also possible to determine σ_L from experimental values of σ_t and to evaluate the extent of the compression of the sample zones in the column quantitatively. We shall report experimental work concerned with this aspect elsewhere[5].

Up to now only the factors that lead to a decrease in σ_L as a consequence of a density programme have been considered. However, increasing the density also can result in an increase in σ_L. The standard deviation in length units is closely related to the plate height, H, which is a measure of the efficiency of a column, by

$$H \equiv \frac{\sigma_L^2}{L} \tag{14}$$

From theory it follows that H increases with increasing viscosity of the mobile phase[16]; in addition, in the region of higher linear velocities of the eluent, H increases with increasing u_{mob}. Because of the favourable transport properties of supercritical mobile phases, this increase in H is less pronounced in SFC than in HPLC, especially when columns packed with small particles[17] or small-diameter open-tubular capillary columns[18] are used. To compensate for the decrease in efficiency caused by an increased eluent viscosity, it is advisable to decrease the linear velocity of the eluent during the density programme. This can easily be done be holding constant the mass flow of eluent that leaves the system[16].

In chromatographic practice, it is not the width of single substance peaks that is of primary interest, but the resolution of several peaks. For one pair of peaks the resolution R is given by

$$R = \frac{t_{R,2} - t_{R,1}}{1/2(t_{B,1} + t_{B,2})} \tag{15}$$

where $t_{R,1}$ and $t_{R,2}$ are the retention times of the substances 1 and 2, respectively (with $t_{R,2} > t_{R,1}$), and $t_{B,1}$ and $t_{B,2}$ are the baseline widths of the peaks. The baseline width t_B is given by the baseline distance of the tangents at the points of inflection of the peak; for a Gaussian peak, t_B is equal to $4\sigma_t$.

Here, the influence on the resolution of the factors that lead to peak com-

pression will be considered qualitatively. As has been stated above, the peak compression effect is more pronounced the higher are the gradients of ρ and u_{mob} along the column. However, the same mechanisms that cause a single sample zone to become narrower also result in a greater increase in the velocity of a whole sample zone that is nearer to the column inlet than a sample zone farther down in the column. Therefore, the distance between the sample zones and hence the selectivity of the column are decreased by high density and eluent velocity gradients. Usually, the distance between two sample zones will be larger than each sample zone width. Therefore, the effect of the decrease in distance (that is, in selectivity) will be more pronounced than the peak compression effect, which leads to increased efficiency, the net result being a decrease in resolution.

     To achieve optimal resolution in density-programmed SFC, both the relative density drop along the column and the relative decrease in u_{mob} should be kept small. A small density drop is favoured by low eluent flow-rates and the use of open-tubular capillary columns or columns packed with larger particles. Large particles, on the other hand, yield columns with low efficiencies. Hence an optimal value exists for the particle size in packed columns, as has been reported for one application[8].

     From eqn. 7, it can be deduced that the relative decrease in the eluent velocity becomes smaller with a decreased density-programming rate at a constant mass flow out of the system or with an increased mass flow $\dot{m}_0$ at constant $d\rho/dt$. Also for $\dot{m}_0$ there may be an optimal value above which the resolution decreases again because of the increase in the density drop connected with higher flow-rates. Hence it results from theoretical considerations that high eluent flow-rates (without a considerable density drop) and a slow increase in the density should lead to improved resolution. These relationships have also been found experimentally; Klesper and Hartmann[9] reported that the resolution of styrene oligomers in pressure-programmed SFC was the better the more slowly the pressure (and thereby the density) was increased. They used relatively high pressure drops along the column and observed an increased resolution with decrease in the eluent flow-rate.

     Graham and Rogers[8] used columns packed with larger particles, where the pressure drop along the column was not much affected by the eluent flow-rate. Under these conditions, the resolution was increased by lowering the ratio of the pressure programming rate to the eluent flow-rate. They proposed that this increase in resolution might be caused in part by the peak compression effect. From the theoretical approach given here, it seems more reasonable to assume that the resolution was increased because of the decrease in the relative drop in u_{mob} along the column, which is connected with a decrease in $d\rho/dt$ and/or an increase in $\dot{m}_0$.

EXPERIMENTAL

Substances

     In order to determine the accuracy of the retention times calculated with the numerical integration method, experiments with test mixtures were carried out. The test substances were dodecyl phenyl ether, tetradecyl phenyl ether, hexadecyl phenyl ether, octadecyl phenyl ether, phenyl myristate, phenyl palmitate and phenyl stearate, dissolved in heptane. The volume of solution injected was 1 μl, containing about $3 \cdot 10^{-9}$ mol of each test substance.

The mobile phase used was supercritical carbon dioxide. The column (12.5 cm × 5 mm I.D.) was packed with Spherisorb ODS 2 (Phase Separations) with a particle diameter of 5 μm.

Apparatus

The fluid chromatograph used has been described in detail elsewhere[5,19]. It consists of commercial HPLC equipment and some laboratory-made parts and is suitable for operation at temperatures up to 100°C and pressures up to 200 bar.

The mobile phase was delivered by a double-plunger pump, the heads of which were cooled to 0°C. By means of a built-in pressure feedback unit the pump could be used as a manostat. In order to make density programming possible, it was coupled with a CBM 8032 SK microcomputer[4,5]. A conditioning system, the injector and the column were placed in an air thermostat. A high-pressure UV detector with a thermostated flow cell was used. The mobile phase was expanded by a reducing valve after the detector, giving an adjustable constant flow-rate ($\pm 1\%$) that was determined with a soap-bubble flow meter.

The mobile phase pressure was measured before and after the column with two strain gauges. The temperature was determined with a thermocouple mounted in the eluent stream before the injector.

RESULTS AND DISCUSSION

Retention data were first measured at different constant pressures and temperatures in order to determine the density dependence of the capacity ratios of the test substances. Some selected data are presented below.

Fig. 2 shows a chromatogram of the test mixture obtained at a relatively high mobile phase density. Here some of the seven ethers and esters under test exhibit the same retention times and thus only four peaks can be discriminated.

From the chromatogram shown in Fig. 3 the effect of a decrease in pressure at constant temperature becomes obvious. With the same mobile phase flow-rate the retention times of all substances are much higher than in Fig. 2, showing the increase in the capacity ratios with decreasing eluent density. By means of the decrease in pressure, the retention times of the esters are increased more than those of the ethers, being co-eluted at higher pressures; as a result, all components of the test mixture are well separated at the lower density. We shall report our systematic measurements on the density dependence of the capacity ratios in detail elsewhere[14]. The results of the experiments are given in Table I, where the fitting parameters according to eqn. 9 are listed for all test substances at two different temperatures.

It can also be deduced from Fig. 3 that for each homologous series the distances between the peaks and the peak widths increase the longer the retention times become. Hence the analysis takes relatively longer. The analysis time can be decreased, however, by density programming. Fig. 4 shows the effect of a linear density programme on the separation of the test substances. After the injection of the sample the eluent density was first held constant for a delay time t_a of 3.6 min; during t_a the density was low enough to allow the separation of dodecyl phenyl ether and phenyl myristate. The density was then increased linearly at 0.043 g cm^{-3} min^{-1}. All test substances were eluted during the density programme, the peaks being well separated and having almost constant widths.
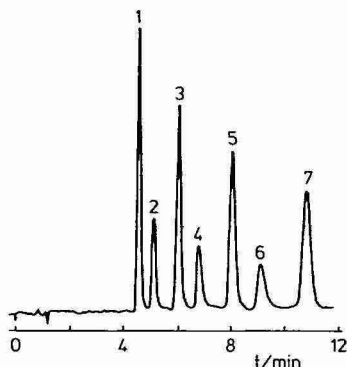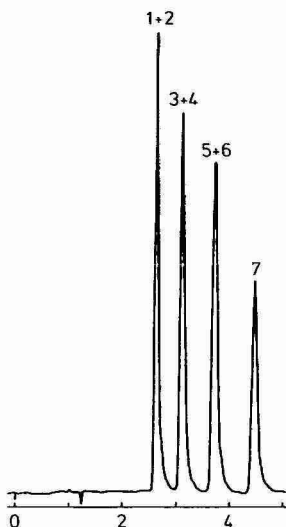
Fig. 2. SFC trace of the test mixture at high constant density of the eluent. Mobile phase, CO_2; $\bar{p} = 181$ bar; $T = 39.5°C$; $\bar{\rho} = 0.82$ g cm^{-3}; $\dot{m}_0 = 1.25$ g min^{-1}. Stationary phase, Spherisorb ODS 2. Substances: 1 = dodecyl phenyl ether; 2 = phenyl myristate; 3 = tetradecyl phenyl ether; 4 = phenyl palmitate; 5 = hexadecyl phenyl ether; 6 = phenyl stearate; 7 = octadecyl phenyl ether.

Fig. 3. SFC trace of the test mixture at low constant density of the eluent. Mobile phase, CO_2; $\bar{p} = 108$ bar; $T = 39.5°C$; $\bar{\rho} = 0.68$ g cm^{-3}; $\dot{m}_0 = 1.25$ g cm^{-3}. Stationary phase and substances as in Fig. 2.

The retention times of the test substances under various density programming conditions were calculated using the numerical integration method. For all density programmes the increase in mobile phase density at the column inlet and the outlet, respectively, within the time of the density increase was determined experimentally from the measured values of pressure and temperature. For the calculation of the densities, pVT data for carbon dioxide taken from the literature[20] were used.

For these calculations it was assumed that during the time before the density programme the eluent density decreased linearly from the value at the column inlet,

TABLE I

FITTING PARAMETERS FOR THE DENSITY DEPENDENCE OF THE CAPACITY RATIOS k' ACCORDING TO EQN. 9

Substance	$T = 39.5°C$		$T = 54.9°C$	
	a	b	a	b
Dodecyl phenyl ether	0.60	5.36	0.38	5.57
Tetradecyl phenyl ether	0.72	5.74	0.44	5.97
Hexadecyl phenyl ether	0.87	6.13	0.49	6.40
Octadecyl phenyl ether	1.02	6.59	0.55	6.84
Phenyl myristate	0.55	6.02	0.32	6.21
Phenyl palmitate	0.66	6.41	0.37	6.64
Phenyl stearate	0.79	6.78	0.42	7.08

TABLE II

MEASURED AND CALCULATED RETENTION TIMES FOR DIFFERENT DENSITY PROGRAMMES

No.	T (°C)	$\rho_A(0)$ (g cm^{-3})	$\rho_A(L)$ (g cm^{-3})	$\rho_E(0)$ (g cm^{-3})	$\rho_E(L)$ (g cm^{-3})	$\dot{m}_0$ (g min^{-1})	t_a (min)	t_g (min)	Substance*	t_R (exp.) (min)	t_R (calc.) (min)
1	55	0.535	0.519	0.697	0.693	0.58	0.1	14.9	C$_{12}$	10.53	10.60
									C$_{14}$	12.91	13.00
									C$_{16}$	15.42	15.53
									C$_{18}$	18.40	18.66
2	55	0.540	0.523	0.697	0.692	0.59	0.2	5.8	C$_{12}$	7.64	7.67
									C$_{14}$	9.37	9.53
									C$_{16}$	11.52	11.83
									C$_{18}$	14.23	14.73
3	55	0.539	0.524	0.697	0.692	0.60	0.2	2.8	C$_{12}$	6.56	6.62
									C$_{14}$	8.19	8.37
									C$_{16}$	10.24	10.53
									C$_{18}$	12.84	13.33
4	39.6	0.676	0.649	0.838	0.832	1.28	3.6	3.8	C$_{12}$	4.48	4.48
									M	4.89	4.93
									C$_{14}$	5.40	5.42
									P	5.81	5.85
									C$_{16}$	6.32	6.37
									S	6.70	6.77
									C$_{18}$	7.23	7.28
5	39.6	0.638	0.589	0.838	0.832	1.30	4.2	1.5	C$_{12}$	4.90	5.07
									M	5.18	5.38
									C$_{14}$	5.43	5.63
									P	5.68	5.95
									C$_{16}$	6.11	6.37
									S	6.42	6.72
									C$_{18}$	6.97	7.25

* C$_{12}$ = dodecyl phenyl ether; C$_{14}$ = tetradecyl phenyl ether; C$_{16}$ = hexadecyl phenyl ether; C$_{18}$ = octadecyl phenyl ether; M = phenyl myristate; P = phenyl palmitate; S = phenyl stearate.
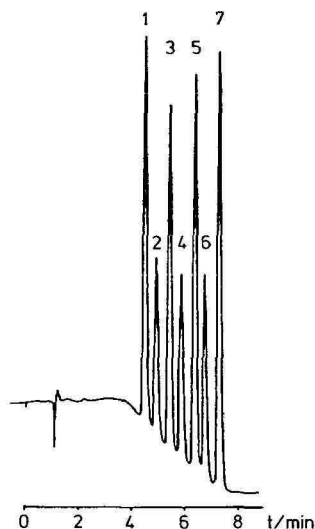
Fig. 4. SFC trace of the test mixture with a linear density programme. Mobile phase, CO_2; $T = 39.6°C$; $\dot{m}_0 = 1.29$ g cm^{-3}. Gradient conditions: $t_a = 3.6$ min; $\bar{p} = 104.3$ bar; $\bar{\rho}_A = 0.63$ g cm^{-3}; $t_g = 3.8$ min; $d\rho/dt = 0.043$ g cm^{-3} min^{-1}. Stationary phase and substances as in Fig. 2.

$\rho_A(0)$, to the value at the column outlet, $\rho_A(L)$. As only linear density programmes have been considered, the density programming rate $d\rho/dt$ was constant; it was calculated according to

$$\frac{d\rho}{dt} = \frac{\rho_E(L) - \rho_A(z)}{t_g} \tag{16}$$

where $\rho_E(L)$ is the eluent density at the column outlet after the programme, $\rho_A(z)$ is the density at position z in the column where the centre of the sample zone is located when the density programme begins, and t_g denotes the duration of the density programme. For the time after the start of the density programme the density drop along the column was no longer taken into account, and the eluent density at the location of the sample zone was calculated from

$$\rho(t,z) = \rho_A(z) + t\,\frac{d\rho}{dt} \tag{17}$$

where t is the time since the start of the gradient and $d\rho/dt$ is given by eqn. 16. In our experimental arrangement, the other parameters needed for the calculation of retention times were $L = 12.5$ cm, $A = 0.112$ cm^2 and $V_2 = 4.7$ cm^3.

In Table II the results of the calculations are compared with the retention times found experimentally. The mean relative deviation between the calculated and the measured values of $t_{R,i}$ is only 2%. The calculation method is equally well suited to slow and fast density programmes. We shall report on further experiments with other packed columns and different mobile phase flow-rates elsewhere[5].

Finally, the effects of the density programming rate, the delay time before the

gradient start and the mobile phase flow-rate on resolution were considered quantitatively. For two model substances 1 and 2 the retention times were calculated using the numerical integration method described above. The density dependence of the capacity ratios of these model substances is characterized by the following set of fitting parameters according to eqn. 9: $a_1 = 0.6$, $a_2 = 0.7$, $b_1 = 5$ and $b_2 = 5.5$. For the parameters L, A and V_2 the values given above that resulted from our experimental arrangement were used. The eluent density after the density programme was assumed to be $\rho_E(L) = 0.8$ g cm^{-3}.

In order to calculate values for the resolution according to eqn. 15, the baseline widths of the substance peaks also had to be determined. This was done by using the equation

$$t_B = \frac{4\sqrt{HL}}{u_i(L,t_{R,i})} \tag{18}$$

For the plate height, a constant value of $H = 15$ μm was used. Thus, no effects of the density programming on σ_L were taken into account, an approximation that is supported for packed columns by our experiments[5]. The linear velocities $u_i(L,t_{R,i})$ needed in eqn. 18 were available from the numerical integration procedure.

Table III gives values of the resolution calculated for different delay times t_a and different density programming rates. The resolution is always much higher than would be necessary in practice, but for the intended comparison of the effect of different operating parameters this is of little importance. It can be seen from Table III that for a constant value of t_a the resolution decreases with decreasing t_g, that is, with a faster increase in the density. For a constant t_g the resolution increases with increasing t_a because the sample substances then spend more time in the column when the eluent density is low and therefore the selectivity for the separation of homologues is high. The well known feature of increasing selectivity with decreasing

TABLE III

EFFECT OF DIFFERENT DENSITY PROGRAMMES ON RESOLUTION

$\dot{m}_0 =$ g min^{-1}; $\rho_A(0) = 0.4$ g cm^{-3}; $\rho_A(L) = 0.35$ g cm^{-3}; $\rho_E = 0.8$ g cm^{-3}.

t_a (min)	t_g (min)	$t_{R,1}$ (min)	$t_{B,1}$ (min)	$t_{R,2}$ (min)	$t_{B,2}$ (min)	R
0	10	7.63	0.170	8.97	0.173	7.82
0	5	4.97	0.107	5.93	0.173	6.86
0	1	2.80	0.144	3.60	0.173	5.05
10	10	16.70	0.203	18.43	0.190	8.80
10	5	14.33	0.126	15.40	0.173	7.16
10	1	11.96	0.144	13.03	0.173	6.75
20	10	25.63	0.259	27.90	0.212	9.64
20	5	23.63	0.158	24.93	0.130	8.72
20	1	21.26	0.144	22.53	0.173	8.01
Conditions as before the density programme		44.2	2.33	88.9	4.67	11.34
Conditions as after the density programme		3.23	0.144	3.87	0.173	4.04

TABLE IV

EFFECT OF ELUENT FLOW-RATE ON RESOLUTION

$t_a = 10$ min; $t_g = 5$ min; $\rho_E(L) = 0.8$ g cm^{-3}.

$\dot{m}_0$ (g min^{-1})	$\rho_A(0)$ (g cm^{-3})	$\rho_A(L)$ (g cm^{-3})	$t_{R,2}$ (min)	R	$(R/t_{R,2})$ (min^{-1})
0.2	0.40	0.39	27.13	5.62	0.21
1	0.40	0.35	15.40	7.16	0.46
2	0.40	0.30	14.06	8.70	0.62

eluent density in SFC also can be recognized from the results given at the bottom of Table III. There the resolutions were calculated for the low density before the programme and the high density after the programme, the densities being constant with respect to time.

Table IV shows the effect of a change in the eluent flow-rate, $\dot{m}_0$, on the resolution calculated according to eqn. 15. Although it was taken into account that the density drop along the column increases strongly with increasing $\dot{m}_0$ for columns packed with small particles, the calculated resolution still increases with increasing eluent flow-rate. Further, the ratio of the resolution to the analysis time, $R/t_{R,2}$, also increases strongly with increasing $\dot{m}_0$.

CONCLUSIONS

From a simple model, equations for the linear velocities of the eluent and sample zones during a density programme in SFC have been derived that are well suited to the determination of retention times by a numerical integration method. The calculated results also agree well qualitatively with the experimental findings of other workers. As the parameters that are necessary for the calculations are readily available from few measurements, it is feasible to apply the calculation method proposed in this paper in mathematical optimization procedures for density-programmed SFC.

ACKNOWLEDGEMENT

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CHROM. 18 435

GAS CHROMATOGRAPHY OF HOMOLOGOUS ESTERS

XXXIII*. CAPILLARY CHROMATOGRAPHY OF MONOCHLORINATED C_1–C_8 n-ALKYL ACETATES, CHLOROACETATES, DICHLOROACETATES AND TRICHLOROACETATES

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(Received December 23rd, 1985)

SUMMARY

The effect on retention of the pattern of chlorination in n-C_1–C_8 monochlorinated acetates, chloroacetates, dichloroacetates and trichloroacetates has been studied at temperatures between 100 and 200°C on a low-polarity (SE-30) and a polar (OV-351) capillary column. The work extends earlier studies and shows the effect of the degree and position of chlorination in both the acyl and alkyl chains.

INTRODUCTION

The relative incremental effects of the chain parameters, *i.e.*, length and some substituents, have been studied with a wide variety of alkanoic esters and it has been established that a particular effect of a variable on retention is greater in the alkyl rather than in the acyl chain[1,2]. The retention behaviour and incremental effect of the methyl esters of the isomeric monochlorinated C_5–C_{18} monocarboxylic acids[3] has shown the influence on retention of the position of a chlorine substituent in the acyl chain, and the influence of the same substituent in the alkyl chain was shown by a study of the isomeric monochlorinated C_1–C_{18} n-alkyl acetates[4]. The elution of C_1–C_8 n-alkyl acetates with mono-, di- and trichlorination on SE-30 and OV-351 capillary columns has been reported[5]. The C_1–C_5 and C_6–C_{16} esters have also been separated at 80°C and 200°C, respectively, on an OV-101 capillary column[6].

In this work we studied the C_1–C_8 n-alkyl acetates, chloroacetates, dichloroacetates and trichloroacetates with monochlorination at each position along the alkyl chain on SE-30 and OV-351 capillary columns at temperatures between 80 and 200°C, extending the data obtained previously with temperature programming[7]. The effect

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* For Part XXXII, see ref. 4.

TABLE I

RETENTION INDICES OF C_1–C_8 MONOCHLORINATED n-ALKYL ACETATES ON SE-30 AND OV-351

Isomeric acetate	SE-30								OV-351						
	60°C	80°C	100°C	120°C	140°C	160°C	180°C	200°C	60°C	80°C	100°C	120°C	140°C	160°C	180°C
Methyl	509	505	505						823	839	844	877			
Chloro	691	680	674						1181	1180	1164	1174			
Ethyl	613	577	607						881	880	875	908			
1-Chloro	744	724	726						1150	1153	1123	1130	1202		
2-Chloro	817	801	796						1315	1319	1297	1299	1354		
Propyl	711	685	676	696					981	978	943	992	1075		
1-Chloro	838	821	806	805					1217	1222	1198	1207	1278		
2-Chloro	864	848	835	831					1296	1304	1283	1289	1348		
3-Chloro	924	916	902	895					1418	1425	1414	1419	1470		
Butyl		810	786	774	781				1080	1079	1053	1064	1154		
1-Chloro		930	906	895	912				1292	1298	1278	1279	1342		
2-Chloro		964	944	930	951				1377	1384	1370	1375	1425		
3-Chloro		981	961	949	972				1424	1432	1422	1427	1478		
4-Chloro		1038	1021	1008	1033				1523	1539	1533	1540	1585		
Pentyl		912	885	867	879						1195	1182	1212	1232	
1-Chloro		1026	1006	990	1011						1401	1387	1419	1408	
2-Chloro		1051	1033	1017	1041						1469	1460	1492	1497	
3-Chloro		1078	1062	1048	1072						1528	1525	1558	1562	
4-Chloro		1089	1073	1058	1084						1563	1564	1594	1594	
5-Chloro		1143	1129	1116	1142						1652	1657	1687	1691	

Hexyl	1008	972	993	1000			1277	1255	1286	1295	1277
1-Chloro	1117	1086	1108	1114			1472	1457	1492	1500	1485
2-Chloro	1147	1121	1143	1151			1540	1532	1576	1574	1575
3-Chloro	1166	1143	1166	1173			1581	1578	1627	1629	1628
4-Chloro	1186	1163	1187	1194			1626	1627	1679	1679	1686
5-Chloro	1194	1171	1194	1202			1647	1650	1700	1703	1711
6-Chloro	1247	1227	1249	1257			1747	1737	1789	1796	1802
Heptyl	1095	1070	1086	1090	1093		1385	1372	1401	1400	1385
1-Chloro	1211	1191	1211	1215	1212		1582	1578	1602	1607	1615
2-Chloro	1235	1217	1239	1244	1242		1647	1647	1673	1673	1675
3-Chloro	1254	1238	1260	1266	1262		1686	1691	1718	1720	1725
4-Chloro	1266	1251	1273	1278	1276		1717	1723	1753	1757	1759
5-Chloro	1284	1269	1292	1297	1295		1749	1756	1787	1791	1797
6-Chloro	1291	1276	1298	1303	1299		1767	1775	1806	1809	1818
7-Chloro	1342	1329	1350	1354	1354		1846	1857	1889	1894	1904
Octyl	1194	1165	1188	1189	1190	1178	1468	1454	1490	1486	1485
1-Chloro	1306	1287	1312	1314	1315	1310	1659	1658	1702	1704	1711
2-Chloro	1328	1312	1338	1343	1344	1340	1722	1724	1771	1771	1775
3-Chloro	1345	1331	1358	1362	1364	1364	1758	1764	1814	1819	1824
4-Chloro	1355	1342	1369	1373	1373	1375	1787	1794	1845	1850	1856
5-Chloro	1365	1352	1379	1384	1385	1384	1802	1811	1862	1867	1874
6-Chloro	1382	1370	1396	1402	1405	1404	1830	1840	1893	1899	1909
7-Chloro	1385	1372	1399	1403	1405	1404	1842	1852	1905	1912	1922
8-Chloro	1435	1425	1451	1456	1458	1459	1918	1932	1987	1994	2006

TABLE II

RETENTION INDICES OF MONOCHLORINATED C_1–C_8 n-ALKYL CHLOROACETATES ON SE-30 AND OV-351

Chloro-acetate	SE-30							OV-351					
	80°C	100°C	120°C	140°C	160°C	180°C	200°C	100°C	120°C	140°C	160°C	180°C	200°C
Methyl													
Chloro	705	708	691	679				1270	1264	1314	1345	1318	1368
Ethyl													
1-Chloro	914	903	873	869				1619	1614	1637	1637	1637	1664
2-Chloro	802	791	776	774				1302	1292	1337	1355	1336	1380
Propyl													
1-Chloro	926	911	895	910				1548	1539	1570	1570	1571	1600
2-Chloro	1016	1002	990	1007				1759	1755	1789	1787	1792	1805
3-Chloro	918	907	878	872	870			1381	1370	1410	1419	1420	1423
Butyl													
1-Chloro	1032	1027	1001	1011	1006			1606	1599	1634	1630	1631	1649
2-Chloro	1069	1066	1044	1054	1053			1716	1714	1749	1752	1755	1759
3-Chloro	1134	1138	1118	1130	1128			1848	1851	1890	1896	1902	1909
4-Chloro		1007	996	986	986			1472	1466	1500	1508	1510	1534
Pentyl													
1-Chloro		1113	1103	1102	1106	1096		1674	1669	1701	1701	1701	1709
2-Chloro		1162	1152	1156	1163	1203		1793	1792	1829	1830	1840	1844
3-Chloro		1182	1174	1178	1181	1250		1840	1844	1884	1890	1898	1907
4-Chloro		1247	1241	1247	1251	1283		1960	1968	2010	2017	2028	2036
5-Chloro			1346	1353	1358	1362				2115	2120	2131	2138

Hexyl								
1-Chloro	1176	1188	1192	1184	1178	1690	1686	1687
2-Chloro	1281	1297	1302	1300	1298	1884	1887	1886
3-Chloro	1319	1337	1344	1344	1344	1982	1987	1982
4-Chloro	1341	1361	1369	1368	1370	2033	2042	2042
5-Chloro	1366	1386	1394	1395	1398	2091	2102	2106
6-Chloro	1379	1399	1407	1406	1411	2120	2132	2134
	1436	1455	1464	1465	1470	2211	2226	2232
Heptyl								
1-Chloro		1297	1293	1276	1275	1785	1788	1797
2-Chloro		1400	1399	1393	1395	1967	1974	1976
3-Chloro		1438	1440	1433	1435	2074	2082	2086
4-Chloro		1460	1462	1458	1460	2122	2134	2140
5-Chloro		1476	1479	1476	1479	2162	2176	2181
6-Chloro		1500	1503	1499	1503	2203	2218	2225
7-Chloro		1506	1509	1505	1508	2220	2237	2244
		1559	1563	1563	1564	2308	2325	2333
Octyl								
1-Chloro		1395	1391	1386	1365	1895	1888	1895
2-Chloro		1499	1497	1494	1491	2074	2075	2088
3-Chloro		1536	1537	1537	1526	2179	2184	2187
4-Chloro		1554	1558	1559	1551	2221	2231	2239
5-Chloro		1570	1572	1575	1569	2258	2270	2278
6-Chloro		1586	1588	1591	1586	2282	2295	2305
7-Chloro		1603	1606	1610	1604	2312	2326	2337
8-Chloro		1606	1609	1611	1607	2324	2339	2349
		1659	1663	1668	1663	2408	2425	2438

TABLE III

RETENTION INDICES OF MONOCHLORINATED C_1–C_8 n-ALKYL DICHLOROACETATES ON SE-30 AND OV-351

Dichloro-acetate	SE-30							OV-351				
	80°C	100°C	120°C	140°C	160°C	180°C	200°C	120°C	140°C	160°C	180°C	200°C
Methyl	821	808	791	785				1361	1387	1365	1362	1391
Chloro	976	960	926	962				1687	1705	1695	1706	1700
Ethyl	887	871	872	869				1384	1404	1396	1400	1423
1-Chloro	994	980	979	985				1594	1614	1602	1601	1611
2-Chloro	1094	1083	1083	1094				1846	1870	1866	1873	1876
Propyl	979	964	934	969	969			1454	1478	1471	1469	1488
1-Chloro	1081	1071	1040	1084	1084			1647	1667	1660	1661	1673
2-Chloro	1127	1117	1089	1133	1132			1786	1812	1810	1819	1821
3-Chloro	1197	1190	1163	1209	1212			1923	1954	1959	1971	1978
Butyl		1061	1061	1072	1070			1542	1562	1554	1559	1575
1-Chloro		1156	1159	1172	1173			1709	1729	1723	1734	1733
2-Chloro		1211	1214	1229	1235			1858	1885	1885	1896	1898
3-Chloro		1231	1236	1252	1257			1906	1939	1942	1955	1961
4-Chloro		1302	1308	1325	1332			2044	2076	2083	2097	2106
Pentyl			1129	1167	1173	1172			1658	1651	1658	1664
1-Chloro			1222	1263	1272	1271			1815	1810	1813	1815
2-Chloro			1268	1313	1322	1324			1952	1950	1958	1961
3-Chloro			1300	1347	1356	1360			2014	2017	2031	2036
4-Chloro			1318	1367	1374	1378			2070	2074	2088	2097
5-Chloro			1385	1432	1440	1446			2182	2189	2205	2215

Hexyl	1260	1270	1270	1270	1273	1749	1751	1759
1-Chloro	1360	1362	1364	1367	1372	1900	1901	1900
2-Chloro	1396	1409	1412	1418	1425	2037	2044	2047
3-Chloro	1416	1433	1438	1445	1451	2085	2098	2105
4-Chloro	1442	1468	1468	1475	1485	2153	2169	2175
5-Chloro	1457	1482	1483	1490	1502	2189	2205	2214
6-Chloro	1509	1535	1541	1550	1557	2281	2300	2312
Heptyl		1372	1376	1374	1372	1848	1856	1860
1-Chloro		1462	1468	1469	1471	1999	2004	2021
2-Chloro		1505	1513	1517	1521	2130	2139	2144
3-Chloro		1527	1537	1541	1548	2175	2188	2196
4-Chloro		1548	1558	1563	1569	2224	2239	2249
5-Chloro		1576	1586	1591	1599	2273	2290	2300
6-Chloro		1582	1592	1597	1604	2289	2306	2317
7-Chloro		1636	1646	1654	1661	2377	2395	2409
Octyl		1476	1473	1476	1482	1950	1956	1961
1-Chloro		1560	1571	1567	1573	2101	2115	2124
2-Chloro		1602	1608	1614	1621	2224	2235	2239
3-Chloro		1623	1629	1637	1645	2266	2281	2291
4-Chloro		1641	1648	1657	1665	2311	2328	2338
5-Chloro		1661	1667	1677	1685	2343	2361	2371
6-Chloro		1678	1685	1695	1704	2372	2391	2403
7-Chloro		1682	1689	1699	1706	2384	2404	2416
8-Chloro		1736	1774	1754	1761	2470	2491	2506

TABLE IV

RETENTION INDICES OF MONOCHLORINATED C_1–C_8 n-ALKYL TRICHLOROACETATES ON SE-30 AND OV-351

Trichloro-acetate	SE-30							OV-351				
	80°C	100°C	120°C	140°C	160°C	180°C	200°C	120°C	140°C	160°C	180°C	200°C
Methyl	884	875	867	876				1387	1378	1387	1392	1443
Chloro	1031	1021	1012	1038				1654	1653	1657	1661	1678
Ethyl	955	944	930	949				1400	1390	1396	1392	1443
1-Chloro	1048	1036	1023	1050				1547	1545	1550	1554	1587
2-Chloro	1152	1148	1139	1172				1836	1849	1852	1859	1873
Propyl	1044	1034	1026	1057	1066			1466	1459	1471	1463	1512
1-Chloro	1129	1123	1117	1154	1163			1590	1591	1598	1611	1628
2-Chloro	1180	1176	1172	1210	1217			1765	1778	1784	1794	1809
3-Chloro	1252	1251	1249	1288	1294			1895	1921	1931	1944	1961
Butyl		1127	1117	1148	1156			1542	1543	1550	1554	1587
1-Chloro		1208	1201	1237	1244			1647	1650	1657	1661	1678
2-Chloro		1268	1264	1301	1311			1828	1846	1851	1859	1873
3-Chloro		1291	1289	1326	1337			1875	1900	1911	1923	1941
4-Chloro		1364	1365	1403	1413			2023	2050	2061	2079	2093
Pentyl			1216	1252	1256	1256			1634	1630	1631	1668
1-Chloro			1296	1333	1338	1338			1733	1729	1736	1756
2-Chloro			1348	1387	1393	1398			1905	1908	1920	1935
3-Chloro			1382	1423	1430	1437			1969	1978	1993	2013
4-Chloro			1404	1444	1451	1459			2034	2046	2061	2078
5-Chloro			1474	1513	1523	1530			2155	2170	2188	2205

Hexyl	1315	1350	1358	1361	1367	1726	1734	1766
1-Chloro	1393	1429	1436	1442	1447	1821	1829	1844
2-Chloro	1442	1480	1488	1498	1503	1992	2006	2026
3-Chloro	1467	1505	1516	1525	1532	2042	2061	2083
4-Chloro	1498	1537	1549	1558	1566	2119	2141	2159
5-Chloro	1518	1557	1568	1575	1585	2164	2186	2205
6-Chloro	1576	1614	1625	1638	1645	2258	2281	2300
Heptyl		1453	1455	1459	1463	1826	1831	1850
1-Chloro		1527	1533	1538	1546	1915	1923	1935
2-Chloro		1575	1583	1592	1600	2085	2095	2108
3-Chloro		1599	1609	1618	1628	2132	2148	2162
4-Chloro		1622	1632	1642	1652	2191	2209	2223
5-Chloro		1652	1664	1673	1681	2249	2267	2284
6-Chloro		1659	1669	1679	1688	2266	2285	2301
7-Chloro		1714	1726	1736	1745	2354	2375	2393
Octyl		1549	1557	1562	1564	1922	1934	1949
1-Chloro		1624	1632	1637	1645	2023	2042	2057
2-Chloro		1670	1681	1690	1698	2176	2193	2218
3-Chloro		1693	1705	1714	1724	2219	2241	2256
4-Chloro		1713	1726	1736	1746	2275	2296	2313
5-Chloro		1736	1748	1760	1770	2315	2338	2355
6-Chloro		1753	1766	1778	1789	2345	2369	2387
7-Chloro		1757	1770	1782	1793	2358	2382	2401
8-Chloro		1812	1826	1838	1845	2446	2472	2491

TABLE V

INCREMENTAL EFFECT OF MONOCHLORINATION AT EACH POSITION ALONG THE ALKYL CHAIN OF ACETATES, CHLOROACETATES, DICHLOROACETATES AND TRICHLOROACETATES ON SE-30

Temperature (°C)	Substituent	Acetates								Chloroacetates							
		C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
80	1-Chloro	175	147	136	120	114				209	124	114					
	2-Chloro		224	163	154	139					214	151					
	3-Chloro			231	171	166						216					
	4-Chloro				228	177											
	5-Chloro					231											
100	1-Chloro	169	119	130	120	121	109	116	112	195	120	120	106				
	2-Chloro		189	159	158	148	139	140	134		211	159	155				
	3-Chloro			226	175	177	158	159	151			231	175				
	4-Chloro				235	188	178	171	161				240				
	5-Chloro					244	186	189	171								
	6-Chloro						239	196	188								
	7-Chloro							247	191								
	8-Chloro								241								
120	1-Chloro			109	121	123	114	121	122	182	119	123	107	108	105		
	2-Chloro			135	156	150	149	147	147		214	166	156	148	143		
	3-Chloro			199	175	181	171	168	166			240	178	181	165		
	4-Chloro				234	191	191	181	177				245	196	190		
	5-Chloro					249	199	199	187					260	203		
	6-Chloro						255	206	205						260		
	7-Chloro							259	207								
	8-Chloro								260								
140	1-Chloro				131	132	115	125	124	190	136	139	116	112	109	103	104
	2-Chloro				170	162	150	153	150		233	182	170	155	149	141	141
	3-Chloro				191	193	173	174	170			258	192	188	173	163	159
	4-Chloro				252	205	194	187	181				261	203	198	179	175
	5-Chloro					263	201	206	191					267	211	203	191
	6-Chloro						256	212	208						267	209	208
	7-Chloro							264	211							262	211
	8-Chloro								263								264
160	1-Chloro						114	125	125		136	120	113	110	106	106	
	2-Chloro						151	154	154		183	177	157	152	147	146	
	3-Chloro						173	176	173			258	195	192	177	169	167
	4-Chloro						194	188	184				265	206	202	186	181
	5-Chloro						202	207	195					269	215	210	197
	6-Chloro						257	213	213						272	216	215
	7-Chloro							264	214							270	218
	8-Chloro								267								272
180	1-Chloro							119	125					107	116	117	108
	2-Chloro							149	154					154	160	157	151
	3-Chloro							169	174					187	184	182	173
	4-Chloro							173	183					201	211	200	189
	5-Chloro							202	195					266	222	223	205
	6-Chloro							206	215						281	229	224
	7-Chloro							261	215							287	225
	8-Chloro								268								282

Dichloroacetates								Trichloroacetates							
C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
155	107	102						147	93	85					
	207	148							197	136					
		218								208					
152	109	107	95					146	92	89	81				
	212	153	150						204	142	141				
		226	170							217	164				
			241								237				
135	107	106	98	93	100			145	93	91	84	80	78		
	211	155	153	139	136				209	146	147	132	127		
		229	175	171	156					223	172	166	152		
			247	189	182						248	188	183		
				256	197							258	203		
					249								261		
177	116	115	100	96	92	90	84	162	101	97	89	81		74	75
	225	164	157	146	139	133	126		223	153	153	135	130	122	121
		240	180	180	163	155	147			231	178	171		146	144
			253	200	198	176	165				255	192		169	164
				265	212	204	185					261		199	187
					265	210	202							206	204
						264	206							261	
							260								263
	115	103	99	94	92	98				97	88	82	78	78	75
	163	165	149	142	137	135				151	155	137	130	128	124
	243	187	183	168	161	156				228	181	174	158	154	148
		262	201	198	182	175					257	195	191	177	169
			267	213	210	194						267	210	209	191
				271	216	212							267	214	209
					270	216								271	213
						301									269
			99	97	95	91						82	81	79	75
			152	148	143	138						142	137	133	128
			188	175	167	161						181	164	159	152
			206	205	189	181						203	197	183	184
			274	220	217	201						274	214	214	198
				280	223	219							277	220	216
					289	223								277	220
						278									276

TABLE V (continued)

Temperature (°C)	Substituent	Acetates								Chloroacetates							
		C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
200	1-Chloro								132						120	120	126
	2-Chloro								162						166	160	161
	3-Chloro								186						192	185	186
	4-Chloro								197						220	204	204
	5-Chloro								206						233	228	221
	6-Chloro								226						292	233	239
	7-Chloro								226							289	242
	8-Chloro								281								298

on retention of the degree of acyl chlorination and the position of the alkyl chlorine substituent is discussed.

EXPERIMENTAL

The aliphatic C_1–C_5 n-alkyl acetates were commercial products (Fluka, Buchs, Switzerland). The C_6–C_8 n-alkyl acetates were prepared from the corresponding alcohols and acetyl chloride[8], as were the chloroacetates, dichloroacetates and trichloroacetates[8]. The monochlorinated derivatives were prepared by liquid-phase chlorination of the parent esters[9].

The chromatography was carried out on a Perkin-Elmer Sigma 3 instrument. The injector and detector temperatures were 275°C, with nitrogen as the carrier gas at a flow-rate of 1 ml min^{-1} and a splitting ratio of 1:50. The two columns used were an SE-30 vitreous silica wall-coated open-tubular (WCOT) column (25 m × 0.33 mm I.D.), supplied by Scientific Glass Engineering (North Melbourne, Australia) and an OV-351 fused-silica WCOT column (25 m × 0.32 mm I.D.) supplied by Orion Analytica (Espoo, Finland). The chromatographic data were recorded with a Hewlett-Packard Model 3390A reporting integrator. Retention times were measured from the time of injection and the retention indices were determined off-line using a Vector MZ microprocessor system. The dead volume was determined by regression analysis using a series of n-alkanes by the procedure of Grobler and Balizs[10]. The crude chlorination mixtures were used for the determinations.

RESULTS AND DISCUSSION

Retention indices of the isomeric monochlorinated C_1–C_8 n-alkyl acetates are shown in Table I and corresponding data for the chloroacetates, dichloroacetates and trichloroacetates are shown in Tables II–IV, respectively, each determined at several temperatures.

The incremental effect of a chlorine substituent at each position along the chain for all of the acetates and chlorinated acetate series is shown in Tables V and VI. On SE-30, in common with other chlorinated esters studied previously[3,5,11,12], a considerable increase in retention occurs at the 1- or α-position with a further gradual increase in retention as the substituent moves towards the end of the

hloroacetates								*Trichloroacetates*								
C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8		
				99	99	91						80	83	81		
				152	149	139						136	137	134		
				178	176	163						165	165	160		
				212	197	183						199	189	182		
				229	227	203						218	218	206		
				284	232	222						278	225	225		
					289	224							282	229		
						279								281		

chain. At the terminal or ω-position, a considerable increase in retention again occurs. With the chloroacetates the effect of chlorination in the α-position is greater than with the acetates at 80–120°C, whereas at higher temperatures the position is reversed. Chlorination at the ω-position has little effect with the acetate or chloroacetate esters; the values for the former are higher up to 120, at 140°C the values are essentially identical and at higher temperatures the values for the chloroacetates continue to increase such that at 200°C the difference in values is much greater than at any other temperature. The increment $\omega - \alpha$ is influenced by the high values for the α-isomer at low temperatures, which give low $\omega - \alpha$ values; at higher temperatures the retention of the α-isomer decreases slightly but that for the ω-isomer increases more significantly, resulting in higher $\omega - \alpha$ values at higher temperatures.

At all temperatures it is evident that the incremental values for both the α- and ω-isomers decrease with substitution in the chloro-, dichloro- and trichloroacetates. In all instances the absolute values of the increments due to α-substitution decrease with all the esters as the temperature is increased, while the absolute values of the ω-increments increase and accordingly the $\omega - \alpha$ values increase with increasing temperature at a greater rate. The values for the ω- and α-isomers and the $\omega - \alpha$ values are given in Table VII.

The corresponding values showing the effect of a chlorine substituent in the alkyl chain on a polar stationary phase are shown in Table VI. With retention on the polar column, considerably increased retention is shown by all series with substitution in the α-position, with a further gradual increased retention until the ω-position, where a further considerable enhancement occurs. With the three chlorinated acetate series the greatest increase in retention with substitution in the α-position occurs with the chloroacetates, with a smaller increase for the dichloroacetates and the smallest for the trichloroacetates. The values tend to decrease progressively with increasing temperature whereas the values for the ω-isomers tend to show a slight increase.

The trend with the ω-substituted esters varies with temperature. At the lowest temperature the highest value is observed with the chloroacetates, with a progressive decrease to the dichloro- to trichloroacetates. With increasing temperature the dichloroacetates show the highest retention, the values for the trichloroacetates generally being slightly different to those for the dichloroacetates.

With all three series the highest $\omega - \alpha$ values occur with the trichloroacetates,

TABLE VI

INCREMENTAL EFFECT OF MONOCHLORINATION AT EACH POSITION ALONG THE ALKYL CHAIN OF ACETATES, CHLOROACETATES, DICHLOROACETATES AND TRICHLOROACETATES ON OV-351

Temperature (°C)	Substituent	Acetates								Chloroacetates							
		C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
100	1-Chloro	320	248	255	225					349	246	225	202				
	2-Chloro		422	340	317						457	335	321				
	3-Chloro			471	369							467	368				
	4-Chloro				480								488				
120	1-Chloro	297	222	215	215					350	247	229	203				
	2-Chloro		391	297	311						463	344	326				
	3-Chloro			427	363							481	378				
	4-Chloro				476								502				
140	1-Chloro			203	188	207				323	233	224	201	184			
	2-Chloro			273	271	280					452	339	329	292			
	3-Chloro			395	324	346						480	384	358			
	4-Chloro				431	382							510	404			
	5-Chloro					475								506			
160	1-Chloro					176	205	207	218	292	215	211	193	191	194	182	179
	2-Chloro					265	279	273	285		432	333	322	305	292	289	284
	3-Chloro					330	334	320	333			477	382	372	343	337	326
	4-Chloro					362	384	357	364				509	417	401	377	363
	5-Chloro					459	408	391	381					522	430	418	387
	6-Chloro						501	409	413						521	435	417
	7-Chloro							494	426							523	429
	8-Chloro								508								513
180	1-Chloro						208	230	226	319	235	211	191	198	201	186	187
	2-Chloro						298	290	290		456	335	330	316	301	294	296
	3-Chloro						351	340	339			482	388	391	356	346	343
	4-Chloro						409	374	371				518	439	416	388	382
	5-Chloro						434	412	389					548	446	430	407
	6-Chloro						525	433	424						540	449	438
	7-Chloro							519	437							537	451
	8-Chloro								521								537
200	1-Chloro									296	220	226	175	173	199	179	193
	2-Chloro										423	336	310	299	295	289	292
	3-Chloro											486	373	366	355	343	344
	4-Chloro												502	413	419	384	383
	5-Chloro													521	447	428	410
	6-Chloro														545	447	442
	7-Chloro															536	454
	8-Chloro																543

with progressively lower values for the dichloro- and monochloroacetates owing, as indicated previously, to the pattern of variation of the retention increments of the α- and ω-isomers.

The methylene increments on the polar column have little significance owing, as shown previously, to the lack of linearity of some comparable series of acyl chain

Dichloroacetates								Trichloroacetates							
C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
326	210	193	167					267	147	124	105				
	462	332	316						436	299	286				
		469	364							429	333				
			502								481				
318	210	189	167	157				275	155	132	107	99			
	466	334	323	294					459	319	303	271			
		476	377	356						462	357	335			
			514	412							507	400			
				524								521			
330	206	189	169	159	151	151	151	270	154	127	107	99	95	89	101
	470	339	331	299	288	282	274		456	313	301	278	266	259	254
		488	388	366	336	327	316			460	361	348	316	306	297
			529	423	404	376	361				511	416	393	365	353
				538	440	425	393					540	438	423	393
					532	411	422						532	440	423
						529	434							528	436
							520								524
344	201	192	175	155	150	148	159	269	162	148	107	105	95	92	108
	473	350	337	300	293	283	279		467	331	305	289	272	264	259
		502	396	373	347	332	325			481	369	362	327	317	307
			538	430	418	383	372				525	430	407	378	362
				547	454	434	405					557	452	436	404
					549	450	435						547	454	435
						539	448							544	448
							535								538
309	188	185	158	151	141	161	163	235	144	116	91	88	78	85	108
	453	333	323	297	288	284	278		430	297	286	267	260	258	269
		490	386	372	346	336	330			449	354	345	317	312	307
			531	433	416	389	377				506	410	393	373	364
				551	455	440	410					537	439	434	406
					553	457	442						534	451	438
						549	455							543	452
							545								542

esters[2], where markedly curved plots resulted. The most that can be indicated for the various chloroacetates is that the ΔCH_2 values at a particular temperature are generally very similar for the mono-, di- and trichloroacetates.

The effect of chlorine substitution in the acetate group on retention is shown in Figs. 1 and 2. Fig. 1 shows plots for the ω-substituted esters of n-alkyl acetates

TABLE VII

SUMMARY OF AVERAGE INCREMENTAL EFFECT OF POSITION OF MONOCHLORINATION IN THE ALKYL CHAIN OF ACETATES, CHLOROACETATES, DICHLOROACETATES AND TRICHLOROACETATES

Temperature (°C)	Position of substituent*	SE-30				OV-351			
		Acetates	Chloro-acetates	Dichloro-acetates	Trichloro-acetates	Acetates	Chloro-acetates	Dichloro-acetates	Trichloro-acetates
80	α	138	149	121	108				
	ω	218	213	193	184				
	ω−α	99	96	108	114				
	ΔCH₂	116	110	111	111				
100	α	125	135	116	102	262	256		
	ω	224	219	208	201	423	440		
	ω−α	113	112	123	132	242	246		
	ΔCH₂	109	115	114	114	108	114		
120	α	118	124	107	95	237	257	224	161
	ω	243	234	221	224	398	449	440	403
	ω−α	124	131	138	155	249	256	288	323
	ΔCH₂	106	113	117	113	108	118	119	123
140	α	125	126	109	95	199	233	208	154
	ω	260	250	244	240	434	457	460	445
	ω−α	134	111	158	166	246	277	314	364
	ΔCH₂	105	113	111	111	106	120	119	126
160	α	121	115	100	83	202	207	188	130
	ω	263	271	269	260	491	474	492	478
	ω−α	141	153	169	177	289	305	347	397
	ΔCH₂	100	107	112	106	101	110	111	113
180	α	122	112	96	79	221	216	191	136
	ω	265	279	278	276	522	492	503	491
	ω−α	143	167	183	202	300	316	358	406
	ΔCH₂	104	102	103	103	102	113	112	116
200	α	132	122	96	81		208	182	118
	ω	281	293	284	280		482	498	472
	ω−α	149	171	188	199		313	361	404
	ΔCH₂		97	102	100		111	115	116

* $\Delta CH_2 = I_{\omega\text{-}Cl}(CH_2)_{x+1} - I_{\omega Cl}(CH_2)_x$.
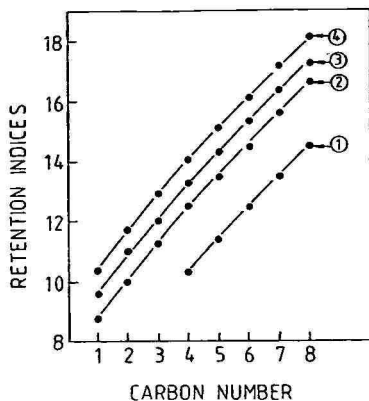
Fig. 1. Retention plots for ω-substituted esters of n-alkyl (1) acetates, (2) chloroacetates, (3) dichloroacetates and (4) trichloroacetates on SE-30 at 140°C.
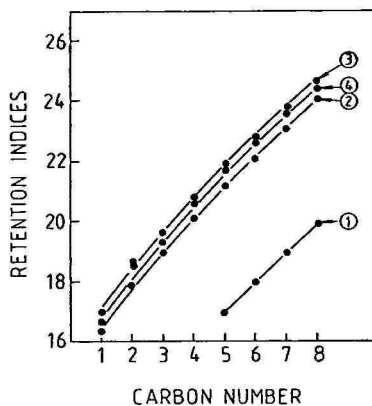
Fig. 2. Retention plots for ω-substituted esters of n-alkyl (1) acetates, (2) chloroacetates, (3) dichloroacetates and (4) trichloroacetates on OV-351 at 140°C.

and chloro-, dichloro- and trichloroacetates on SE-30 at 140°C, where an increased retention occurs with increasing chlorination of the acetate group.

Fig. 2 shows a comparable plot for the same esters on OV-351 at 160°C, where it is evident that the retention is increased with the mono- and dichloroacetates but is reduced with the trichloroacetates. It is apparent with both plots that the greatest increase occurs with the monochloroacetates but that the effect is considerably greater on the polar stationary phase. The incremental changes that occur are shown in Table VIII, where it is evident that monochlorination of the acetate group leads to an enhancement of retention of 206–214 retention index units (i.u.) on the non-polar stationary phase whereas dichlorination leads to a further increase of 77–93 i.u. and trichlorination to a further increase of 76–81 i.u. With the polar stationary phase monochlorination results in an incremental increase of 414–429 i.u. with dichlorination showing a further increase of 58–79 i.u. and trichlorination a decrease of 19–38 i.u. compared with the dichloroacetates.

Table VIII also shows corresponding values for α-substitution of the same ester series and the same effects are evident, although the values are more variable. On SE-30 monochlorination results in a retention increase of 187–190 i.u., increasing by 61–93 and 64–76 i.u. with di- and trichloroacetates. On the polar stationary phase the variations are 360–384 i.u. for monochlorination, with an increase of 16–58 i.u. on dichlorination and a reduction of 38–84 i.u. on trichlorination.

Ratios of the retentions on the two stationary phases are shown in Table VIII. With ω-chlorination the values decrease both with increasing chain length and with increasing degree of chlorination, showing the increasing effect of steric considerations[13,14]. With the α-substituted esters the same trend in the results occurs but with the added consideration that the values are all reduced as compared with the values for the ω-substituted esters. This result is to be expected, as greater steric effects would be expected with the series with the alkyl chlorine substituent adjacent to the chlorinated acetate group.

TABLE VIII

INFLUENCE OF CHLORINATION OF ACETATE GROUP ON SE-30 AND OV-351 WITH α- AND ω-CHLORINATION OF THE ALKYL CHAIN

Monochloro n-alkyl*	OV-351 (160°C)							
	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
ω-Chlorination								
Acetate					1691	1796	1894	1994
Chloroacetate	1637	1787	1896	2017	2120	2211	2308	2408
Dichloroacetate	1695	1866	1959	2083	2189	2281	2377	2470
Trichloroacetate	1657	1852	1931	2061	2170	2258	2354	2446
ClA − A					429	415	414	414
2ClA − A						485	483	476
3ClA − A						462	460	452
ΔCl					429	415	414	414
Δ2Cl − ΔCl	58	79	63	66	69	70	69	62
Δ3Cl − Δ2Cl	−38	−14	−28	−22	−19	−23	−23	−24
α-Chlorination								
Acetate					1408	1500	1607	1704
Chloroacetate	1637	1570	1630	1701	1789	1884	1967	2074
Dichloroacetate	1695	1602	1660	1723	1810	1900	1999	2101
Trichloroacetate	1657	1550	1598	1657	1729	1821	1915	2023
ClA − A					381	384	360	370
2ClA − A					402	400	392	397
3ClA − A					321	321	308	319
ΔCl					381	384	360	370
Δ2Cl − ΔCl	58	32	30	22	21	16	32	27
Δ3Cl − Δ2Cl	−38	−52	−62	−66	−81	−79	−84	−78

* ClA − A = chloroacetate − acetate; 2ClA − A = dichloroacetate − acetate; 3ClA − A = trichloroacetate − acetate; Δ2Cl − ΔCl = dichloroacetate − chloroacetate; Δ3Cl − Δ2Cl = trichloroacetate − dichloroacetate.

SE-30 (140°C)								Ratio OV-351/SE-30							
C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
			1033	1142	1249	1350	1451					1.48	1.44	1.40	1.37
869	1007	1130	1247	1353	1455	1559	1659	1.88	1.77	1.68	1.62	1.57	1.52	1.48	1.45
962	1094	1209	1325	1432	1535	1636	1736	1.76	1.71	1.62	1.57	1.53	1.49	1.45	1.42
1038	1172	1288	1403	1513	1614	1714	1812	1.60	1.58	1.50	1.47	1.43	1.40	1.37	1.35
			214	211	206	209	208								
			292	290	286	286	285								
			370	371	365	364	361								
			214	211	206	209	208								
93	87	79	78	79	80	77	77								
76	78	79	78	81	79	78	76								
			912	1011	1108	1211	1312					1.39	1.35	1.33	1.30
869	910	1011	1102	1198	1297	1400	1499	1.88	1.73	1.61	1.54	1.49	1.45	1.41	1.38
962	985	1084	1172	1263	1362	1462	1560	1.76	1.63	1.53	1.47	1.43	1.40	1.37	1.35
1038	1050	1154	1237	1333	1429	1527	1624	1.60	1.48	1.38	1.34	1.30	1.27	1.25	1.25
			190	187	189	189	187								
			260	252	254	251	248								
			325	322	321	316	312								
			190	187	189	189	187								
93	75	73	70	65	65	62	61								
76	65	70	65	70	67	65	64								

ACKNOWLEDGEMENT

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GLASS CAPILLARY GAS CHROMATOGRAPHY OF HOMOLOGOUS SERIES OF ESTERS

VI. SEPARATION OF HOMOLOGOUS SERIES OF HALOPROPIONATES OF C_1–C_{10} ALIPHATIC ALCOHOLS AND HALOPROPYL ESTERS OF C_2–C_{10} ALIPHATIC ACIDS OF THE SAME MOLECULAR WEIGHT ON AN SP-400 GLASS CAPILLARY COLUMN

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(Received December 12th, 1985)

SUMMARY

The possibility of the gas chromatographic separation and identification of lower and higher halogenated esters of the same molecular weight and empirical formula but differing in their molecular "mirror image", *i.e.*, having the same carbon number and positions of the halogen atoms in the acid chains of halopropionates as in the alcohol chains of halopropyl esters, was studied.

INTRODUCTION

In our previous paper[1] we described the gas chromatographic (GC) separation and identification of halogenated esters and a comparison of the retentions of pairs of halogenated esters with the same molecular weight but differing in their molecular "mirror image", *i.e.*, having the same carbon number and positions of the halogen atoms in the acid chains of haloacetates as in the alcohol chains of 2-haloethyl esters. Because of the imperfect separation of some halogenated esters, capillary GC with temperature programming coupled with mass spectrometry was used[2].

Previously we studied the GC separation of homologous series of halopropionates of C_1–C_{10} aliphatic alcohols and halopropyl esters of C_2–C_{10} aliphatic acids on packed columns under isothermal conditions[3–5].

The systematic GC separation of various types of non-halogenated homologous series of lower esters on packed columns was studied in great detail by Haken and co-workers[6]. More recently, a systematic GC separation of homologous series of various alkyl chloropropionates and chloropropyl esters on glass and silica capillary columns either with temperature programming or under isothermal conditions

was reported by Korhonen[7-10] and Haken and co-workers[6,11-13], who compared the retentions of homologous esters with the same carbon number in the alcohol as in the acid chains.

In this work, model mixtures of homologous series of C_1-C_{10} *n*-alkyl and C_3-C_5 isoalkyl propionates (Pr), 2-chloropropionates (2-ClPr), 2,2-dichloropropionates (2,2-DClPr), 3-chloropropionates (3-ClPr), *n*-butyrates (Bu) and *n*-propyl esters (PrE), 3-chloropropyl esters (3-ClPrE), 2,3-dichloropropyl esters (2,3-DClPrE), 1,3-dichloroisopropyl esters (1,3-DClisoPrE), isopropyl esters (isoPrE) and *n*-butyl esters (ButE) of n-C_2-C_{10} and *iso*-C_4-C_6 aliphatic monocarboxylic acids were separated. Kováts retention indices were used to interpret the results.

EXPERIMENTAL

GC separations were carried out on a Fractovap Model 2150 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector and an SP-400 glass capillary column. This column was made in our laboratory from soft Unigost soda-lime glass etched with methyl 2-chloro-1,1,2-trifluoroethyl ether vapour, statically silylated with dimethyldichlorosilane vapour and dynamically coated with SP-400 silicone stationary phase. The column efficiency was found to be as follows: capacity ratio, $k = 2.94$; number of theoretical plates per metre, $N = 4020$ for *n*-decane; "Trennzahl", $TZ = 40.5$ for *n*-nonane and *n*-decane.

First, all of the homologous series of lower halogenated esters were separated on a 37 m × 0.16 mm I.D. column, then the column was divided into two lengths of 16 and 21 m. All of the homologous series of the higher halogenated esters were separated on the shorter 16-m column. Nitrogen was used as the carrier gas at a flow-rate of 10–100 cm/s with a splitting ratio of 1:100. The column temperature was maintained at 80°C for the separation of the lower C_1-C_6 *n*-alkyl and C_3-C_5 isoalkyl chloropropionates and chloropropyl esters of n-C_2-C_6 monocarboxylic acids, and at 200°C for those of the higher C_6-C_{10} *n*-alkyl chloropropionates and chloropropyl esters of n-C_6-C_{10} monocarboxylic acids. The temperatures of the injector and the detector were 250 and 300°C, respectively, for the separation of the higher halogenated esters. The dead time was determined by the injection of methane.

Model mixtures of the lower and higher halogenated esters were obtained from the individual esters. Non-halogenated esters were prepared by the usual sulphuric acid-catalysed esterification and halogenated esters by azeotropic esterification.

RESULTS AND DISCUSSION

The influence of chlorine atoms situated in the alcohol chain of chloropropyl esters of n-C_2-C_{10} and *iso*-C_4-C_6 carboxylic acids, the increasing length or branching of the acid carbon chain of esters, the influence of these in the acid chain of C_1-C_{10} *n*-alkyl and C_3-C_5 isoalkyl chloropropionates and the increasing length or branching of the alcohol carbon chain of esters was studied using retention index increments (see Tables I–IV).

Retention index increments, $\Delta I_{\overline{3\text{-Cl}}}$, $\Delta I_{\overline{2,3\text{-Cl,Cl}}}$, $\Delta I_{\overline{1,3\text{-Cl,Cl}}}$*, for chlorine atoms

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* The increments with a bar belong to chlorine atoms and methylene groups of the alcohol chain of the esters.

TABLE I

RETENTION INDICES, I, OF C_1–C_6 ALKYL ESTERS AND INCREMENTS OF RETENTION INDICES FOR METHYLENE AND HALOGEN GROUPS AT 80°C

Ester	I	ΔI_{CH_2}	$\Delta I_{2\text{-}Cl}$	$\Delta I_{2,2\text{-}ClCl}$	$\Delta I_{11\text{-}Cl}$	$\Delta I_{3\text{-}Cl}$
PrC$_1$	626.78	—				
PrC$_2$	704.69	77.91				
PrC$_3$	803.78	99.09				
PrC$_4$	903.37	99.59				
PrC$_5$	1003.05	99.68				
PrisoC$_3$	748.11	—				
PrisoC$_4$	863.89	115.78				
PrisoC$_5$	966.13	102.24				
2-ClPrC$_1$	782.91	—	156.13			
2-ClPrC$_2$	858.84	75.93	154.15			
2-ClPrC$_3$	952.56	93.72	148.78			
2-ClPrC$_4$	1046.11	93.55	142.74			
2-ClPrC$_5$	1143.56	97.45	140.51			
2-ClPrisoC$_3$	896.75	—	148.64			
2-ClPrisoC$_4$	1009.68	112.93	145.79			
2-ClPrisoC$_5$	1110.34	100.66	144.21			
3-ClPrC$_1$	842.95	—	—	—	—	216.17
3-ClPrC$_2$	920.04	77.09				215.35
3-ClPrC$_3$	1018.24	98.20				215.46
3-ClPrC$_4$	1117.08	98.84				213.71
3-ClPrC$_5$	1215.75	98.67				212.70
3-ClPrisoC$_3$	961.77					213.66
3-ClPrisoC$_4$	1077.45	115.68				213.56
3-ClPrisoC$_5$	1178.40	100.95				212.27
2,2-DClPrC$_1$	864.13	—		237.35	81.22	
2,2-DClPrC$_2$	929.53	65.40		224.84	70.69	
2,2-DClPrC$_3$	1014.63	85.10		210.85	62.07	
2,2-DClPrC$_4$	1109.45	92.82		206.08	63.34	
2,2-DClPrC$_5$	1204.33	94.88		201.28	60.77	
ButC$_1$	717.84	—				
ButC$_2$	795.17	77.33				
ButC$_3$	893.23	98.06				
ButC$_4$	992.26	99.03				
ButC$_5$	1090.42	98.16				
ButisoC$_3$	838.43	—				
ButisoC$_4$	953.82	115.39				
ButisoC$_5$	1055.27	101.45				

were calculated as the differences between the retention indices for 3-chloro- or 2,3-dichloropropyl esters or 1,3-dichloroisopropyl esters and those for corresponding non-halogenated n-propyl esters or isopropyl esters, $e.g.$, for the 3-chloropropyl ester of propionic acid, $\Delta I_{\overline{3\text{-}Cl}} = I_{3\text{-}ClPrEC_3} - I_{PrEC_3}$. The retention index increments for chlorine atoms, $\Delta I_{2\text{-}Cl}$ and $\Delta I_{2,2\text{-}Cl,Cl}$, were calculated as the differences between the retention indices for the chloropropionates studied and those for the corresponding

TABLE II

RETENTION INDICES, *I*, OF HALOPROPYL ESTERS OF LOWER CARBOXYLIC ACIDS AND
INCREMENTS OF RETENTION INDICES FOR METHYLENE AND HALOGEN GROUPS AT
80°C

Ester	*I*	$\Delta I_{\overline{CH_2}}$	$\Delta I_{\overline{3\text{-}Cl}}$	$\Delta I_{\overline{2,3\text{-}Cl,Cl}}$	$\Delta I_{\overline{1,3\text{-}Cl,Cl}}$
PrEC$_2$	708.12	—			
PrEC$_2$	803.78	95.66			
PrEC$_4$	893.21	89.43			
PrEC$_5$	992.95	99.74			
PrEC$_6$	1092.13	99.18			
PrEisoC$_4$	852.09	—			
PrEisoC$_5$	946.59	94.50			
PrEisoC$_6$	1058.13	111.54			
3-ClPrEC$_2$	946.50	—	238.38		
3-ClPrEC$_3$	1039.89	93.39	236.11		
3-ClPrEC$_4$	1127.92	88.03	234.71		
3-ClPrEC$_5$	1225.70	97.78	232.75		
3-ClPrEisoC$_4$	1083.62	—	231.53		
3-ClPrEisoC$_5$	1178.51	94.89	231.92		
3-ClPrEisoC$_6$	1289.78	111.27	231.65		
2,3-DClPrEC$_2$	1068.23	—		360.11	
2,3-DClPrEC$_3$	1159.32	91.09		355.54	
2,3-DClPrEC$_4$	1245.84	96.52		352.63	
2,3-DClPrEC$_5$	1342.85	97.01		349.90	
2,3-DClPrEC$_6$	1439.03	96.18		346.90	
2,3-DClPrEisoC$_4$	1200.98	—		348.84	
2,3-DClPrEisoC$_5$	1296.27	95.29		349.68	
2,3-DClPrEisoC$_6$	1403.94	107.67		345.81	
isoPrEC$_3$	747.78	—			
isoPrEC$_4$	837.19	89.41			
isoPrEC$_5$	935.82	98.63			
isoPrEC$_6$	1034.05	98.23			
isoPrEisoC$_4$	792.52	—			
isoPrEisoC$_5$	851.09	98.57			
isoPrEisoC$_6$	1000.44	109.55			
1,3-DClisoPrEC$_3$	1138.77	—			390.93
1,3-DClisoPrEC$_4$	1225.19	86.42			388.00
1,3-DClisoPrEC$_5$	1321.34	96.15			385.52
1,3-DClisoPrEC$_6$	1417.52	96.18			383.47
1,3-DClisoPrEisoC$_4$	1178.06	—			385.54
1,3-DClisoPrEisoC$_5$	1276.68	98.62			385.59
1,3-DClisoPrEisoC$_6$	1383.13	106.45			382.69
ButEC$_2$	809.04	—			
ButEC$_3$	903.41	94.37			
ButEC$_4$	992.26	88.85			
ButEC$_5$	1088.90	96.64			
ButEC$_6$	1189.00	100.10			

TABLE III

RETENTION INDICES, I, OF C_6–C_{10} ALKYL ESTERS AND INCREMENTS OF RETENTION INDICES FOR METHYLENE AND HALOGEN GROUPS AT 200°C

Ester	I	ΔI_{CH_2}	$\Delta I_{2\text{-}Cl}$	$\Delta I_{2,2\text{-}ClCl}$	$\Delta I_{II\text{-}Cl}$	$\Delta I_{3\text{-}Cl}$
PrC$_6$	1103.35	—				
PrC$_7$	1201.56	98.21				
PrC$_8$	1298.14	96.58				
PrC$_9$	1399.44	101.30				
PrC$_{10}$	1497.70	98.26				
PrC$_{12}$	1699.60	202.00				
PrC$_{14}$	1898.60	199.00				
PrC$_{16}$	2098.09	199.49				
2-ClPrC$_6$	1253.04	—	149.69			
2-ClPrC$_7$	1353.22	100.18	151.66			
2-ClPrC$_8$	1452.09	98.87	153.95			
2-ClPrC$_9$	1559.88	98.79	152.44			
2-ClPrC$_{10}$	1649.76	98.88	152.06			
2,2-DClPrC$_6$	1329.03	—	—	225.68	75.99	
2,2-DClPrC$_7$	1426.56	97.53		225.00	73.33	
2,2-DClPrC$_8$	1524.86	98.30		226.72	72.77	
2,2-DClPrC$_9$	1622.99	98.13		223.55	63.11	
2,2-DClPrC$_{10}$	1722.45	99.46		224.75	72.69	
3-ClPrC$_6$	1324.72					221.47
3-ClPrC$_7$	1424.24	99.52				222.68
3-ClPrC$_8$	1523.35	99.11				225.21
3-ClPrC$_9$	1623.60	100.25				224.16
3-ClPrC$_{10}$	1722.99	99.39				225.29

non-halogenated propionates, *e.g.*, for *n*-propyl 3-chloropropionate, $\Delta I_{3\text{-}Cl} = I_{3\text{-}ClPrC_3} - I_{PrC_3}$.

Retention index increments, $\Delta I_{II2,2\text{-}ClCl}$, for the second chlorine atoms introduced into the 2-chloropropionate molecules were calculated as the differences between retention indices for the 2,2-dichloropropionates studied and those for corresponding 2-chloropropionates, *e.g.*, for ethyl 2,2-dichloropropionate, $\Delta I_{II2,2\text{-}ClCl} = I_{2,2\text{-}DClPrC_2} - I_{2\text{-}ClPrC_2}$.

The retention index increments $\Delta I_{\overline{3\text{-}Cl}}$, $\Delta I_{\overline{2,3\text{-}ClCl}}$, $\Delta I_{\overline{1,3\text{-}Cl,Cl}}$, $\Delta I_{2\text{-}Cl}$, $\Delta I_{3\text{-}Cl}$ and $\Delta I_{2,2\text{-}ClCl}$ decrease with increasing length of the alcohol or acid chains for all the homologous series of the lower halogenated esters. The decreases in these increments are greater and sharper for C_1–C_6 *n*-alkylchloropropionates, whereas those for chloropropyl esters of n-C_2–C_6 carboxylic acids are smaller and more gradual. A small and gradual decrease in retention index increments for chlorine atoms is observed for both types of homologous series of lower halogenated esters with branched carbon chains.

The retention increments $\Delta I_{\overline{3\text{-}Cl}}$, $\Delta I_{\overline{2,3\text{-}Cl,Cl}}$, $\Delta I_{\overline{1,3\text{-}Cl,Cl}}$, $\Delta I_{2\text{-}Cl}$, $\Delta I_{2,2\text{-}ClCl}$ and $\Delta I_{3\text{-}Cl}$ for both types of homologous series of higher halogenated esters do not change very much with increasing length of the alcohol or acid chains.

Special attention was paid to halogenated esters with the same molecular

TABLE IV

RETENTION INDICES, *I*, OF HALOPROPYL ESTERS OF HIGHER CARBOXYLIC ACIDS AND INCREMENTS OF RETENTION INDICES FOR METHYLENE AND HALOGEN GROUPS AT 200°C

Ester	*I*	$\Delta I_{\overline{CH_2}}$	$\Delta I_{\overline{3-Cl}}$	$\Delta I_{\overline{2,3}\text{-}Cl,Cl}$	$\Delta I_{\overline{1,3}\text{-}Cl,Cl}$
PrEC$_6$	1092.98	—			
PrEC$_7$	1191.40	98.52			
PrEC$_8$	1287.80	98.40			
PrEC$_9$	1387.36	99.56			
PrEC$_{10}$	1487.46	100.10			
PrEC$_{12}$	1687.03	199.57			
PrEC$_{14}$	1888.09	201.06			
PrEC$_{16}$	2088.18	200.09			
3-ClPrEC$_6$	1337.77	—	244.79		
3-ClPrEC$_7$	1436.66	98.84	245.26		
3-ClPrEC$_8$	1536.39	99.73	248.52		
3-ClPrEC$_9$	1635.29	98.90	247.93		
3-ClPrEC$_{10}$	1737.21	101.92	249.75		
2,3-DClPrEC$_6$	1464.68	—	—	371.70	
2,3-DClPrEC$_7$	1563.46	98.78	..	372.06	
2,3-DClPrEC$_8$	1662.40	98.94	—	374.60	
2,3-DClPrEC$_9$	1762.16	98.76	—	374.80	
2,3-DClPrEC$_{10}$	1861.09	98.93	—	373.63	
isoPrEC$_6$	1037.56	—			
isoPrEC$_7$	1133.33	95.77			
isoPrEC$_8$	1225.62	92.29			
isoPrEC$_9$	1324.16	98.44			
isoPrEC$_{10}$	1424.13	99.97			
isoPrEC$_{12}$	1622.57	198.44			
isoPrEC$_{14}$	1822.74	200.17			
isoPrEC$_{16}$	2023.31	200.57			
1,3-DClisoPrEC$_6$	1437.17	—			399.61
1,3-DClisoPrEC$_7$	1535.82	98.65			402.49
1,3-DClisoPrEC$_8$	1634.45	99.63			408.73
1,3-DClisoPrEC$_9$	1733.94	99.49			409.78
1,3-DClisoPrEC$_{10}$	1833.40	99.46			409.26
ButEC$_6$	1187.24	—			
ButEC$_7$	1286.02	98.78			
ButEC$_8$	1385.74	99.72			
ButEC$_9$	1484.62	98.88			
ButEC$_{10}$	1584.27	99.65			
ButEC$_{12}$	1783.57	199.30			
ButEC$_{14}$	1984.94	201.37			
ButEC$_{16}$	2185.96	201.02			

weight and empirical formula but differing in their molecular "mirror image" in the carbon number and the positions of chlorine atoms in the alcohol and acid chains. To determine the influence of the number and position of individual chlorine atoms in these types of halogenated esters, we calculated the differences, *D*, between retention indices for pairs of corresponding alkyl chloropropionates and chloropropyl

TABLE V

CALCULATED DIFFERENCES, D, IN RETENTION INDICES OF "MIRROR" PAIRS OF LOWER HALOGENATED ESTERS

"Mirror" pair	Capillary columns		Packed columns			
	OV-101[14,15]	SP-400	Silicone grease[3-5]	QF-1[3-5]	SE-30[6]	Silar 10C[6]
PrC_2–PrEC_2	− 1.8	6.57	− 3	−28	−4.99	−16.11
PrC_3–PrEC_3	0.0	0.00	1	3	0	0
PrC_4–PrEC_4	10.2	10.16	8	12	−3.21	17.73
PrC_5–PrEC_5	10.2	10.10	8	16	−3.15	17.78
PrisoC_4–PrEisoC_4	10.8	11.80	11	42	−	−
PrisoC_5–PrEisoC_5	20.0	19.54	19	40	6.64	− 5.4
3-ClPrC_2–3-ClPrEC_2	−21.9	−26.46	−25	−79		
3-ClPrC_3–3-ClPrEC_3	−18.5	−21.65	−21	−48		
3-ClPrC_4–3-ClPrEC_4	−10.3	−10.84	−10	−34		
3-ClPrC_5–3-ClPrEC_5	− 8.6	− 9.95	−10	−33		
3-ClPrisoC_4–3-ClPrEisoC_4	− 5.6	− 6.17	− 7	−14		
3-ClPrisoC_5–3-ClPrEisoC_5	0.2	− 0.11	1	−15		
ButC_2–ButEC_2	−14.2	−13.87	−12	−60	−7.44	1.11
ButC_3–ButEC_3	−10.2	−10.18	− 8	−24	−3.21	−17.73
ButC_4–ButEC_4	0	0	1	− 5	0	0
ButC_5–ButEC_5	0.4	1.52	0	− 1	−6.2	22.95

TABLE VI

CALCULATED DIFFERENCES, D, IN RETENTION INDICES OF "MIRROR" PAIRS OF HIGHER HALOGENATED ESTERS

"Mirror" pair	Capillary columns		Packed columns	
	OV-101[14,15]	SP-400	Silicone grease[3,5]	QF-1[3–5]
PrC_6–$PrEC_6$	11.2	10.37	4	13
PrC_7–$PrEC_7$	10.2	10.16	9	14
PrC_8–$PrEC_8$	10.1	10.34	8	14
PrC_9–$PrEC_9$	10.0	12.08	11	16
PrC_{10}–$PrEC_{10}$	10.0	10.24	11	11
PrC_{12}–$PrEC_{12}$	–	12.57	10	12
PrC_{14}–$PrEC_{14}$	–	10.51	9	15
PrC_{16}–$PrEC_{16}$	–	9.91	7	10
3-$ClPrC_6$–3-$ClPrEC_6$	– 9.0	–13.05	–16	–38
3-$ClPrC_7$–3-$ClPrEC_7$	– 8.0	–12.42	–13	–42
3-$ClPrC_8$–3-$ClPrEC_8$	– 8.4	–13.04	–12	–44
3-$ClPrC_9$–3-$ClPrEC_9$	– 8.9	–11.63	–12	–42
3-$ClPrC_{10}$–3-$ClPrEC_{10}$	– 8.8	–14.22	–12	–41
$ButC_6$–$ButEC_6$	1.7	0.66	– 5	8
$ButC_7$–$ButEC_7$	0.8	– 0.25	– 4	8
$ButC_8$–$ButEC_8$	1.3	– 0.49	– 4	7
$ButC_9$–$ButEC_9$	1.9	0.53	0	7
$ButC_{10}$–$ButEC_{10}$	2.0	0.90	0	6
$ButC_{12}$–$ButEC_{12}$	–	1.37	– 1	4
$ButC_{14}$–$ButEC_{14}$	–	0.20	– 4	10
$ButC_{16}$–$ButEC_{16}$	–	– 0.30	1	6

esters of carboxylic acids (see Tables V and VI). In these tables, we compare calculated values of D with those calculated from our earlier work on packed columns[3–5], on glass capillary columns with OV-101[14,15] and with those calculated from Haken and Srisukh's paper[6], where the lower esters were separated on packed columns.

Comparisons of the D values showed that those for non-halogenated "mirror" pairs AcC_x–$EtEC_x$ were 11–16 units[1], whereas those for non-halogenated "mirror" pairs of esters $ButC_x$–$ButEC_x$ were only about 0.2–1.4 units. The differences in D for "mirror" pairs of halogenated esters 3-$ClPrC_x$–3-$ClPrEC_x$ are greater then those for "mirror" pairs of non-halogenated esters PrC_x–$PrEC_x$.

These results are in agreement with those of Haken and co-workers[6,12,13] and Korhonen[7,8,10].

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PURIFICATION OF GUANOSINE TRIPHOSPHATE CYCLOHYDROLASE I FROM *ESCHERICHIA COLI*

THE USE OF COMPETITIVE INHIBITORS *VERSUS* SUBSTRATE AS LIGANDS IN AFFINITY CHROMATOGRAPHY

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(Received December 18th, 1985)

SUMMARY

Different affinity chromatography ligands have been compared for the purification of guanosine triphosphate (GTP) cyclohydrolase I, an enzyme that catalyses the transformation of GTP into formate and dihydroneopterin triphosphate, the first metabolite in the biosynthetic pathway of the pterins. When this enzyme is purified by affinity chromatography on GTP-Sepharose a major fraction of the activity is lost and the yield of enzyme decreases as the amount of enzyme applied to the column decreases. The use of nucleotide competitive inhibitors (UTP and ATP) as ligands in the affinity column has shown that the extent of inactivation of the enzyme is related to the affinity of the enzyme for the ligand. Further, the extent of inactivation was reduced by reducing the length of the columns when using the same volume of GTP-Sepharose. Dihydrofolate-Sepharose gave consistently higher yields of GTP cyclohydrolase I regardless of the amount of enzyme applied, but several other proteins were also obtained. For a high purification of GTP cyclohydrolase I the best yield may be obtained with UTP as the affinity ligand and with the shortest length possible of the affinity column, and the purity of enzyme is comparable with that obtained with GTP-Sepharose.

INTRODUCTION

Guanosine triphosphate (GTP) cyclohydrolase I (E.C. 3.5.4.16) catalyses the transformation of GTP into two products, formate and D-*erythro*-7,8-dihydroneo-

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pterin 3'-triphosphate[1]. This is the first enzyme in the biosynthesis of riboflavin. tetra-hydrofolate and tetrahydrobiopterin, the natural cofactor for aromatic amino acid hydroxylases[2]. The lack of the latter pterin leads to a type of phenylketonuria in humans with decreased levels in the neurotransmitters norepinephrine, serotonin and dopamine. At least two cases of GTP cyclohydrolases I deficiency have been reported in humans[3,4]. Because this enzyme catalyses the first step in the biosynthetic pathway of pteridines it may play a key role in the regulation of the biosynthesis of pteridines in most organisms.

GTP cyclohydrolase I has been purified from many sources, including bacteria[5], *Drosophila melanogaster*[6], chicken[7], rat[8] and man[9]. However, the use of affinity chromatography to purify this enzyme has been restricted to GTP cyclohydrolases from *Escherichia coli*[5], *Lactobacillus plantarum*[10] and *Serratia indica*[11] as the enzyme from other sources was either destroyed or not bound by conventional affinity chromatography columns[6,7,9]. Only recently has GTP cyclohydrolase from human liver been purified by affinity chromatography using a GTP analogue as ligand[9].

For several years we have purified GTP cyclohydrolase I of *E. coli* by affinity chromatography[12-14] and regularly found this system to behave in a non-ideal manner but not always for the same reason. At times the enzyme was not retained and at times it was retained but not released, much like the enzyme from other sources[6,7]. This paper evaluates different ligands for the purification of GTP cyclohydrolase I of *E. coli* and offers suggestions for obtaining improved yields.

EXPERIMENTAL

Sephadex G-25 (fine) and Sepharose 4B were obtained from Pharmacia. Agarose–hexane–guanosine 5'-triphosphate (GTP-agarose), agarose–hexane–adenosine 5'-triphosphate (ATP-agarose) and agarose–hexane–uridine 5'-triphosphate (UTP-agarose) (each nucleotide was periodate oxidized) were purchased as Type 4 from P-L Biochemicals, Bio-Gel A-0.5m from Bio-Rad Labs., GTP, ovalbumin, myoglobin, chymotrypsinogen and ammonium sulfate (grade III) from Sigma, ε-aminocaproic acid methyl ester from Vega Chemical, cyanogen bromide from Aldrich, bovine albumin (crystallized twice) from Nutritional Biochemical Corp. and [8-^{14}C]guanosine 5'-triphosphate (58 Ci/mol) from Amersham. Adipic acid dihydrazide was synthesized according to Lamed *et al.*[15]. Folic acid (crystalline) was obtained from Sigma and 1,6-diaminohexane from Aldrich.

Growth of bacteria
E. coli B cells were grown in 200-l batches on a glucose carbon source in a mineral salts media, harvested at late log, and stored at −80°C.

Composition of buffers
Buffer A is composed of 50 mM potassium phosphate–5 mM EDTA (pH 7.0), buffer B of 10 mM potassium phosphate–2.5 mM EDTA (pH 7.0), buffer D of 20 mM potassium phosphate–2.5 mM EDTA (pH 7.0), buffer E of 200 mM Tris–HCl (pH 8.0)–20 mM 2-mercaptoethanol–1 M potassium chloride, and buffer F of 10 mM sodium phosphate–2.5 mM EDTA–20 mM 2-mercaptoethanol (pH 7.0).

Preparation of enzyme for affinity chromatography

Frozen cells (500 g) were allowed to thaw at 4°C for 16 h and suspended in 1 l of buffer A. The cells were disrupted at 11 000 psi in a Gaulin press previously cooled with cold distilled water. The suspension was passed twice through the press to ensure complete breakage of the cells and the long fibers of DNA. Alternatively, the enzyme was extracted from the cells by incubating 300 g of *E. coli* at 30°C with 300 mg of lysozyme dissolved in 600 ml of 0.2 *M* Tris–HCl (pH 8.0) for 15–45 min. Then 30 mg of DNase I were added and incubation was continued for 15 min. The reaction was stopped by adding 300 g of ice. In either procedure for lysis, after chilling the suspension was centrifuged at 6000 *g* for 90 min at 4°C to remove the cell debris. Unless indicated otherwise, all further manipulations of the enzyme were performed at 4°C.

With continuous stirring, the supernatant was adjusted to 35% saturated ammonium sulfate by dropwise addition of saturated ammonium sulfate solution. The mixture was stirred for 20 min and then centrifuged at 6000 *g* for 45 min. The supernatant was treated again to bring it to 50% saturated ammonium sulfate, and stirred for 20 min. It was centrifuged as before (supernatant volume = 1600 ml). The pellet was suspended in buffer A and dialysed for 3 h *vs.* 6 l of buffer A and then for 12 h after replacing the buffer. After dialysis most of the protein was dissolved and the volume of the solution inside the bags was 100–200 ml. Any undissolved protein was removed by centrifugation.

The dialysed fraction was applied to a column (97 × 5 cm I.D.) of Bio-Gel A-0.5m (100–200 mesh) equilibrated with buffer A. The enzyme was eluted with the same buffer. The fractions that contained GTP cyclohydrolase I activity were pooled and either used immediately or stored in 50-ml batches at −20°C or −80°C. This pool was used for subsequent experiments on affinity columns.

Preparation of nucleotide-Sepharose

AMP-, ATP-, UTP- and GTP-Sepharose were synthesized following the procedure of Jackson *et al.*[10] with slight modifications. Sepharose 4B (45 g) was washed on a sintered-glass funnel with 1 l of cold water and suspended in 120 ml of water. The temperature was maintained at <5°C in an ice-bath, the pH was adjusted to 11 with 5 *M* potassium hydroxide solution and 12 g of crushed cyanogen bromide were added. The pH was maintained at *ca.* 11 (± 0.3) with potassium hydroxide solution until the rate of consumption of the latter had decreased significantly (75 min). The cyanogen bromide-activated Sepharose was then washed with 1 l of cold water and 1 l of cold 0.1 *M* sodium hydrogen carbonate buffer (pH 9.5) and suspended in 120 ml of the same buffer. Adipic acid dihydrazide (6.7 g) was added to the suspension and allowed to mix on a rotator for 22 h in the dark at 4°C (unless indicated otherwise, the columns used for this work were synthesized with adipic acid as spacer). Alternatively, ε-aminocaproic acid methyl ester (4 g) was added in place of the adipic acid dihydrazide, together with 70 ml of hydrazine hydrate (98–100%); the mixture was heated at 70°C for 15 min[10]. The gel with the extender arm was washed with 500 ml of cold water and 1 l of 0.1 *M* sodium acetate solution (pH 6.0) and then mixed with periodate-oxidized nucleotide and rotated for 2 h in the dark at 4°C. The oxidized nucleotide-gel was filtered, rinsed with water and stored at −20°C in 50% aqueous glycerol. The oxidation of the nucleotides was carried out as follows: 45 mg

of nucleoside triphosphate or 31 mg of AMP were dissolved in 15 ml of 0.1 M acetate buffer (pH 6.0); 1.5 ml of 0.1 M sodium metaperiodate solution were added and the solution was incubated in the dark on ice for 60 min. Ethylene glycol (45 μl) was added and allowed to react for 30 min to destroy the excess of oxidant. The formaldehyde formed was removed by bubbling with an inert gas [nitrogen or helium–butane (98.7:1.3)] for 5 min.

Measurement of enzyme activity

The procedure of Burg and Brown[1] was slightly modified. The reaction mixture of 200 μl contained 0.1 M Tris–HCl (pH 8.5)–0.1 M sodium chloride–0.01 M EDTA (pH 7.5), 0.1 mM [8-^{14}C]GTP (376 000 cpm/μmole) and enzyme and was incubated at 42°C for 30 min. The reaction was stopped by adding 0.25 ml of 0.5 M formic acid. Activated charcoal (15 mg) suspended in water (Darco G-60, acid and alkali washed) was added and the mixture was transferred into a Pasteur pipet with a cotton plug where the charcoal was retained forming a column about 0.5–1 cm high. The column was washed twice with water (0.5 ml each). The radioactive GTP was retained in the charcoal, and the radioactive formate that had been released in the enzymatic reaction was eluted from the column. The original solution and two washes that passed through the column were combined and mixed with ten volumes of 0.27% 2,5-bis[2-(5-*tert.*-butylbenzoxalyl)]thiophene (BBOT) in toluene–Triton X-100 (2:1, v/v), and analysed for radioactivity in a scintillation counter. The amount of formate released was linear with the enzyme concentration. One unit of enzyme released 1 nmole of formate per minute[5]. Specific activity is defined as units per milligram of protein.

Dihydrofolate-Sepharose affinity chromatography

Folic acid was bound to Sepharose 4B through 1,6-diaminohexane and subsequently reduced to the dihydrofolate state as described by Then[16]. The adsorption of GTP cyclohydrolase and subsequent washing of the column followed the published procedure and the enzyme was eluted with 3.3 mM dihydrofolic acid–1 M potassium chloride–0.2 M Tris–HCl–20 mM 2-mercaptoethanol (pH 8.0). A method to reduce folic acid to the dihydro form was described by Then[16]. A 3-ml aliquot of the eluted enzyme was passed over a Sephadex G-25 column (18 × 2.4 cm I.D.) equilibrated with buffer D to remove dihydrofolate, an inhibitor of *E. coli* GTP cyclohydrolase.

Determination of protein content and purity

Protein was determined by the method of Miller[17] with slight modifications. The samples were dialysed against water to remove EDTA, which interferes in the reaction. Electrophoresis was performed using 5% polyacrylamide in sodium dodecylsulfate-containing buffer as described by Weber and Osborn[18].

RESULTS

Affinity chromatography on GTP-Sepharose and dihydrofolate-Sepharose

When various amounts of enzyme activity were applied to several GTP-Sepharose columns, the recovery decreased as the amount of enzyme decreased, as shown in Table I. The specific activity of each enzyme preparation also showed a

TABLE I

RECOVERY OF GTP CYCLOHYDROLASE I FROM GTP-SEPHAROSE AND DIHYDROFO-LATE-SEPHAROSE

Column	Enzyme activity (units)		Recovery (%)	Specific activity
	Applied	Recovered		
GTP-Sepharose*	190	35	18	465.0
	133	12	11	8.0
	56	3.5	6	3.5
	19	0	0	0
Dihydrofolate-Sepharose**	768	462	60	14.0
	ᵉ563	442	79	24.7
	152	117	77	11.9
	75	64	85	17.0

   * The enzyme was obtained after gel filtration in buffer A. The GTP-Sepharose column (5 ml in the barrel of a 10-ml syringe) was washed with 80 column volumes of buffer B. The enzyme pool from gel filtration passed through the GTP-Sepharose slowly (20 ml/h); then the column was washed with buffer B + 0.3 M KCl (500 ml) and then with buffer B (15 ml). The enzyme was eluted with buffer B plus GTP (0.3 mg/ml) and it emerged in a sharp peak (8–10 ml) with the leading edge of the GTP. To determine the recovery, the GTP was removed by passing 3 ml of the enzyme solution over a Sephadex G-25 column (24 × 1.8 cm I.D.). The recovery from the Sephadex G-25 column was greater than 90%. When a 300-ml sample (700 units of enzyme) was applied to GTP-Sepharose, the recovery was 50–60% of the activity applied.

   ** Conditions for chromatography followed those of Then[16]. The folate column (14 × 0.6 cm I.D.) was converted into the dihydrofolate form by passing sodium dithionite (40 mg/ml)–sodium ascorbate (200 mg/ml) solution through at 25°C. The column was then operated at 4°C. The reducing solution was removed by washing with buffer F. The enzyme was applied and the column then washed with buffer F with 0.1, 0.5 and finally 1.0 M NaCl added. The enzyme was eluted with 3 mM dihydrofolate in buffer E. Recovery was measured after an enzyme sample was passed over Sephadex G-25 to remove the dihydrofolate. Flow-rates were maintained at 0.5 ml/min.

marked decrease as a function of the amount of enzyme loaded. However, when dihydrofolate-Sepharose columns were used, neither the recovery nor the specific activity of the enzyme decreased with decreasing amounts of enzyme applied to the column (Table I). The cause of the diminishing recovery as smaller amounts of enzyme are used with the GTP-Sepharose columns could be irreversible adsorption or inactivation of the enzyme by the column.

Affinity chromatography on GTP-, ATP- and UTP-Sepharose

   Four Sepharose columns were compared: ATP-, UTP- and GTP-Sepharose from P-L Biochemicals and GTP-Sepharose synthesized in this laboratory. The columns were equilibrated in buffer B and eluted at the same flow-rate in each instance. Using a linear GTP gradient with buffer B kept constant, the enzyme eluted from each of the columns in the first 16 fractions, with the maximum around fraction No. 7 (where the concentration of GTP was 70 mg/l). Table II shows the results obtained in the elution of the different columns. The percentage recovery of the initial amount of enzyme was higher in the UTP-Sepharose column than in the ATP-Sepharose or GTP-Sepharose columns, which were comparable to each other. The specific activity

TABLE II

GTP CYCLOHYDROLASE I PURIFIED BY DIFFERENT AFFINITY CHROMATOGRAPHY COLUMNS

The column (3.8 × 1.0 cm I.D.) contained 3 ml of oxidized nucleotide-Sepharose equilibrated in buffer B. The enzyme purified from the Bio-Gel A-0.5m column was applied (50 ml) to the affinity column at a flow-rate of 1 ml/min. The column was washed with 100 ml of 0.3 M KCl in buffer B, and the enzyme eluted with a linear gradient of GTP using 100 ml of 0.3 M KCl in buffer B and 200 mg of GTP in 100 ml of the same solution at a flow-rate of 1.5 ml/min. Fractions (4 ml each) that contained enzyme activity were pooled; to remove the GTP and KCl, 5 ml of the pooled fractions were loaded on to a Sephadex G-25 column (29 × 1.7 cm I.D.) equilibrated with buffer B and eluted with the same buffer. The final volume of fractions that contained enzyme activity after the Sephadex column was 8.2 ml.

Column[*]	Volume after elution (ml)	Protein content (μg/ml)	Enzymatic[**] activity after Sephadex column (units/ml)	Specific activity (units/mg)	Recovery (% of initial activity)
GTP-Sepharose (Lab)	45.0	22.5	$5.76 \cdot 10^{-3}$	$256 \cdot 10^{-3}$	6[***]
GTP-Sepharose (PL)	54.0	33.0	$8.64 \cdot 10^{-3}$	$262 \cdot 10^{-3}$	10
ATP-Sepharose (PL)	65.0	37.0	$10.8 \cdot 10^{-3}$	$292 \cdot 10^{-3}$	15
UTP-Sepharose (PL)	32.5	46.4	$52.1 \cdot 10^{-3}$	$1123 \cdot 10^{-3}$	36

   [*] The source of the oxidized nucleotide-Sepharose is as follows: Lab, prepared by us using adipic acid as the extender arm; PL, purchased from P-L Biochemicals.
   [**] After elution from the affinity column the enzyme was passed through Sephadex G-25 to remove the GTP.
   [***] Calculated as percentage of the enzyme applied to the column that was obtained by elution with GTP. Some of the enzyme was not retained and another portion lost due to inactivation.

of the purified enzyme was also higher in the UTP-Sepharose column than in the others. The experiment was repeated with each of the columns. No change was found between the first and second use.

When a large amount of enzyme was applied to a 1-ml nucleotide column synthesized in our laboratory, the column was overloaded and the excess of enzyme was measured in a pool of the five fractions of the flow-through. The recoveries in the flow-through from the Sepharose columns were as follows: from GTP 14%, from ATP 18% and from UTP 25% of the amount of enzyme applied to the column. These different recoveries may be due to different inactivation of the unadsorbed enzyme. An AMP-Sepharose column did not bind any enzyme and the enzyme concentration in the flow-through was the same as in the applied sample. When GTP-Sepharose was prepared using ε-aminocaproic acid instead of adipic acid as the extender arm, this affinity column performed similarly to the ones described.

This study of the loading of the affinity column is illustrated in a different way in Fig. 1. The column (5.5 ml) was washed in 50 ml of buffer B and the enzyme was added slowly. The capacity of GTP-Sepharose (prepared in our laboratory) was exceeded. The enzyme that appeared in the flow-through during sample application reached a concentration equivalent to 60% of the sample. When the flow-through fractions were pooled and reapplied to the same column, the same pattern as shown in Fig. 1 was again obtained; a significant amount of enzyme was not adsorbed and its concentration in the flow-through was only 50–60% of the concentration added.
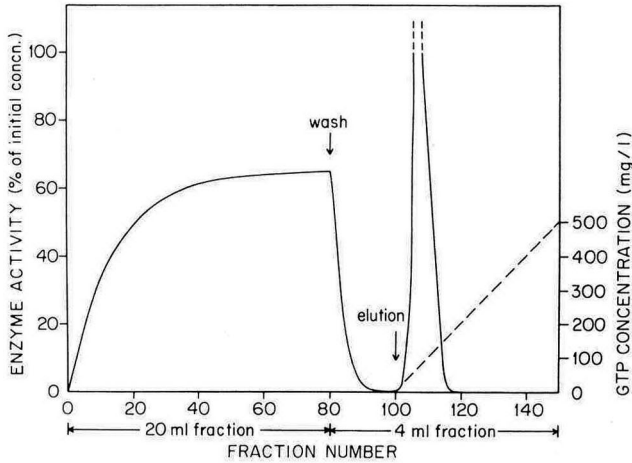
Fig. 1. Behavior of GTP cyclohydrolase I on GTP-Sepharose. The laboratory-prepared GTP-Sepharose (5.5 ml) column was equilibrated with buffer B. The enzyme was recovered in buffer A after the gel filtration column, diluted to 1800 ml in buffer B and added to the GTP-Sepharose at a rate of 2 ml/min. The column was washed with 25 ml of 0.3 M KCl in buffer B before the elution with a linear gradient of GTP in 0.3 M KCl in buffer B.

This pattern has been found with enzyme prepared from different batches of *E. coli* and with different nucleotide-Sepharose columns (either prepared in our laboratory or commercial). The presence of 20% glycerol in the enzyme solution during the
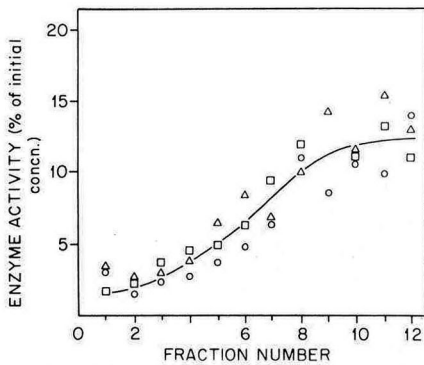
Fig. 2. GTP cyclohydrolase in flow-through from three GTP-Sepharose columns with different lengths. The bed volume was 3.0 ml but the diameter varied (see text). The sample volume was 50 ml and the fractions were 4 ml. (○) Short, (△) medium and (□) long columns. The flow-rate was 1 ml/min.
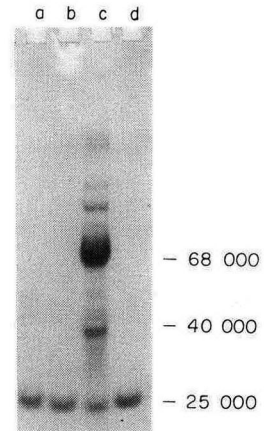
Fig. 3. Electrophoretic analysis of GTP cyclohydrolase I. The enzyme was purified from *E. coli* B on the following affinity columns: (a) GTP-Sepharose; (b) UTP-Sepharose; (c) dihydrofolate-Sepharose; (d) GTP-Sepharose. The enzymes in (a) and (d) were purified from separate batches of cells. The molecular weight markers (not shown) were bovine serum albumin (68 000), ovalbumin (40 000) and chymotrypsinogen (25 000).

TABLE III

COMPARISON OF THE EFFICIENCIES OF THE DIFFERENT SHAPED GTP-SEPHAROSE COLUMNS IN THE RECOVERY OF THE ENZYME

The values are the percentage of the amount of GTP cyclohydrolase applied to the column as measured by enzyme activity. The values were obtained from chromatography experiments from Fig. 2. The flow-rate was 1 ml/min for sample addition, washing and elution.

Shape of GTP-Sepharose column	Enzyme activity (%)	
	Flow-through	Eluted with GTP
Short	8	20
Medium	8	10
Long	8	3

loading step completely eliminated activity in the flow-through but produced no increase in the amount recovered by elution with GTP. Presumably this means that glycerol facilitated the inactivation of the unadsorbed enzyme when passing through the GTP-Sepharose.

Influence of the shape of the column on the recovery of enzyme activity

The GTP-Sepharose prepared in this laboratory was used to prepare three columns, each of 3-ml bed volume but with heights and diameters as follows: short (1.7 × 1.5 cm I.D.), medium (3.8 × 1.0 cm I.D.) and long (7.8 × 0.7 cm I.D.). The amount of GTP cyclohydrolase that failed to bind to each of the three columns was the same for each column (Fig. 2). However, the percentage of enzyme recovered from the shortest column was 6.7 times that from the longest, as shown in Table III.

Purity of the GTP cyclohydrolase I from different affinity columns

The purity of the GTP cyclohydrolase I obtained from GTP-Sepharose, UTP-Sepharose and dihydrofolate-Sepharose was assesed by gel electrophoresis (Fig. 3). The positions of the Coomassie Blue-stained bands were compared with those of bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin. Over 95% of the protein obtained from GTP- and UTP-Sepharose migrated at the same position as chymotrypsinogen (molecular weight, M_r = 25 000), in agreement with earlier results[5]; the minor bands corresponded to molecular weights of 57 000 and 73 000. The protein from the dihydrofolate-Sepharose consisted of four strong bands, one that coincided with chymotrypsinogen, the major band corresponding to a molecular weight of 70 000 and the other two to 40 000 and 160 000; no band was observed at M_r 18 000, the molecular weight of dihydrofolate reductase. We conclude that the M_r 25 000 band represents the GTP cyclohydrolase and that this enzyme is obtained in a high state of purity from affinity chromatography on GTP- and UTP-Sepharose.

DISCUSSION

Affinity chromatography provides the major purification step for GTP cyclohydrolase I from *E. coli*[5] and from *Serratia indica*[11]. In these cases periodate-oxidized

GTP was linked to Sepharose through a six-carbon chain. From the published accounts it is not clear how reproducibly the GTP cyclohydrolase I is bound and released. In one of our laboratories (K.B.J.) we often experienced unpredictable performance with GTP-Sepharose; on the other hand, our other laboratory (J.J.Y.) seldom experienced such problems. Consequently, we examined this step in the purification and we also examined the behavior of GTP cyclohydrolase I on various affinity columns using competitive inhibitors as ligands.

The competitive inhibition of GTP cyclohydrolase I by dihydrofolate has been described by Then, and he subsequently demonstrated the use of this inhibitor in the purification of this enzyme[16]. ATP and UTP have also been reported to be competitive inhibitors for GTP cyclohydrolase I from *E. coli* with K_i values of 0.25 and 2.9 μM, respectively, compared with the K_m value of 0.2 μM for GTP as substrate, and AMP is not an inhibitor[5].

All the data related to the nucleotide-Sepharose columns suggest that part of the enzyme is inactivated while passing through the column. In the experiment shown in Fig. 1, the capacity of the column to remove enzyme from solution was exceeded but the enzyme that appeared in the effluent contained only 60% of the initial activity. As shown in Table III and Fig. 2, the amount of enzyme activity in the flow-through did not vary with the length of the bed but the overall recovery was still low. The amount of enzyme eluted by GTP varied more than six-fold as the column length varied from 1.7 to 7.8 cm. We found that a major fraction of the enzyme is unaccounted for on combining the activities found in the flow-through and the GTP eluate.

These observations can be explained if one assumes that the inactivation is a function of the number of times a molecule collides with the Sepharose-ligand complex. In a steady-state situation like the one found when the sample is passing through a column whose capacity is being exceeded, the extent of the inactivation would depend on the volume of the column (total number of sites where the enzyme can be bound) (Figs. 1 and 2 and Table III). Once the column has been loaded and the enzyme is being eluted, the extent of the inactivation depends on the length of the column, *i.e.*, the number of sites the enzyme is going to bind before it elutes from the column (Table III). This hypothesis was tested in another way with the use of the ATP-Sepharose and UTP-Sepharose columns. As the enzyme has less affinity for ATP and UTP than for GTP, it would be expected that affinity columns made with ATP and UTP as ligands should produce less inactivation because the enzyme would bind fewer times to the matrix while passing through the column. This is what indeed was found (Table II); the UTP-Sepharose gave the greatest recovery and highest specific activity (UTP is the inhibitor with the lowest affinity for the enzyme). A control with AMP-Sepharose gave no inactivation. This seems to exclude the possibility that either the cyanogen bromide-activated sites on Sepharose or the extender molecules, that were unoccupied with the affinity ligand, could be responsible for the inactivation.

Different mechanisms for inactivation could be proposed but only those which imply that the nucleotide ligand behaves in a catalytic way should be considered, as we found that a GTP-Sepharose column never ceased to inactivate the enzyme no matter how much sample was loaded. We suggest that the nucleotide affinity ligand induces a conformational change in the enzyme that results in irreversible inactivation as observed for other proteins[19,20].

Almost no inactivation was found when dihydrofolate-Sepharose was used, although a more poorly purified enzyme was obtained. It should be noted that the dihydrofolate-Sepharose chromatograms were obtained with different salt and buffer conditions than those for the nucleotide-Sepharose. The yield from this affinity column remained high with all amounts of enzyme tested and the specific activities varied no more than two-fold. When a highly pure enzyme preparation is required, the UTP-Sepharose, arranged in as short a column configuration as possible, would give better results. Both GTP-Sepharose and UTP-Sepharose can produce a homogeneous enzyme provided that the column is washed thoroughly with 0.3 M potassium chloride in buffer B after the enzyme is bound. It is also advisable to wash the column with buffer thoroughly before use (80 bed volumes at least), as the glycerol that is used to stabilize the nucleotide-Sepharose during storage or shipping enhances the inactivation of GTP cyclohydrolase I.

ACKNOWLEDGEMENTS

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CHROM. 18 439

DETERMINATION OF GARDENOSIDE, GENIPOSIDE AND RELATED IRIDOID COMPOUNDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received October 29th, 1985; revised manuscript received December 24th, 1985)

SUMMARY

The high-performance liquid chromatographic determination of gardenoside (GAR), geniposide (GEN), deacetylasperulosidic acid methyl ester (DAM), scandoside methyl ester (SSM) and monotropein methyl ester (MTM) is described. GAR, DAM, SSM and MTM were separated on a ODS-Develosil reversed-phase column using acetonitrile–tetrahydrofuran–water (4:1:95, v/v) as the eluent. In the same way, GEN was separated on a ODS-Hypersil reversed-phase column with acetonitrile–tetrahydrofuran–water (8:2:90, v/v) as the eluent. The application of this method to the evaluation of Inchinkoto (traditional Chinese medicine), which contains these compounds, is reported.

INTRODUCTION

Gardenoside (GAR) and geniposide (GEN) are iridoid glycosides found abundantly in Gardeniae Fructus that have choleretic and laxative pharmacological activity[1-5]. Treatment of GAR with hydrochloric acid gives deacetylasperulosidic acid methyl ester (DAM), scandoside methyl ester (SSM) and trace monotropein methyl ester (MTM)[6]. Several high-performance liquid chromatographic (HPLC) methods for iridoid analysis have been described[7-10], but the separation of GAR, DAM, SSM and MTM by HPLC has not so far been reported.

In this paper, the HPLC separation of these compounds and the effects of temperature and pH on their structural transformation are described. In addition, we examined the determination of these compounds in Inchinkoto (traditional Chinese medicine), which includes Gardeniae Fructus as a component.

EXPERIMENTAL

Standards and reagents

GAR and GEN were supplied by Takeda Chemical (Osaka, Japan). DAM, SSM and MTM were obtained by treatment of GAR with hydrochloric acid accord-

ing to the method reported by Ishiguro et al.[6]. Gardeniae Fructus, Artemisiae Capillaris Herba and Rhei Rhizoma were obtained from Daiko Shoyaku (Nagoya, Japan). Specially prepared solvents for HPLC (Kishida Chemical, Osaka, Japan, and Nakarai Chemical, Kyoto, Japan) were used. All other reagents were of special grade.

Apparatus

A Model LC-4A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-2AS variable-wavelength detector was used. Peak heights were measured with a Shimadzu C-R3A computing integrator. A stainless-steel column (250 × 4.6 mm I.D.) packed with ODS-Develosil (5 μm, Nomura Chemical, Aichi, Japan) or ODS-Hypersil (5 μm, Erma Optical Works, Tokyo, Japan) was used. The number of theoretical plates for both columns based on pyrene was 16 000 (flow-rate, 1.0 ml/min; solvent, 60% aqueous acetonitrile).

For the separation of GAR, DAM, SSM and MTM, a column packed with ODS-Develosil and acetonitrile–tetrahydrofuran–water (4:1:95, v/v) as the eluent were used with a flow-rate of 0.6 ml/min and an oven temperature of 40°C. The effluent was monitored at 242 nm and the detector sensitivity was 0.16 a.u.f.s. The chart speed was 2.0 mm/min.

For the separation of GEN, a column packed with ODS-Hypersil was used with acetonitrile tetrahydrofuran–water (8:2:90, v/v) as the eluent at a flow-rate of 0.6 ml/min and an oven temperature of 40°C. These compounds were detected at 237 nm and the detector attenuation was 0.64 a.u.f.s. The chart speed was 2.0 min/min.

Preparation of samples for HPLC

A mixture of Gardeniae Fructus (3 g), Artemisiae Capillaris Herba (4 g) and Rhei Rhizoma (1 g) was added to 480 ml of distilled water and the volume was reduced to 120 ml. After cooling to room temperature, the pH of a decoction of Inchinkoto was measured with a pH meter (Horiba, Kyoto, Japan). The decoction was filtered through a 0.45-μm membrane filter (Toyokagaku Sangyo, Tokyo, Japan) and subjected to HPLC. Gardeniae Fructus (3 g) was also treated according to the procedure mentioned above and the sample obtained was subjected to HPLC. Further, a mixture of Gardenia Fructus (4 g), Artemisiae Capillaris Herba (4 g) and Rhei Rhizoma (1 g) in 120 ml of water was stood at 25°C or 60°C for 2 h. In the same manner, Gardeniae Fructus (3 g) in 120 ml of water was also stood at 25°C or 60°C. These extracts were subjected to HPLC analysis following filtration through a membrane filter. The volumes injected into the HPLC columns were 5 μl for GEN analysis and 20 μl for GAR, DAM, SSM and MTM analysis.

Calibration graph

Standard samples supplemented with various concentrations of GAR (0.05–0.2 mg/ml), DAM (0.05–0.2 mg/ml), SSM (0.05–0.2 mg/ml) or GEN (0.3–1.2 mg/ml) were prepared for HPLC analysis. The peak heights were calculated in order to construct a calibration graph. Every sample was analysed in triplicate and the results were averaged.

RESULTS

Determination of GAR and its acid reaction products

A chromatogram obtained using acetonitrile–water (5:95, v/v) as the eluent is compared with that obtained using acetonitrile–tetrahydrofuran–water (4:1:95, v/v) in Fig. 1. Both chromatograms showed the successful separation of GAR, DAM, SSM and MTM. The addition of tetrahydrofuran accelerated the elution without giving overlapping peaks. We therefore used the latter eluent for the effective determination of GAR, DAM, SSM and MTM. By linear regression analysis of the calibration graphs for GAR, DAM and SSM in amounts from 1 to 4 μg, a good linear relationship was obtained between peak height and amount (Fig. 2). The detection limit for these compounds was 4 ng, and this sensitivity is high enough for their determination.

GAR, DAM, SSM and MTM in Inchinkoto and the extract of Gardeniae Fructus (3 g) were determined using the above eluent system under various conditions. The chromatogram of Inchinkoto prepared at 100°C is shown in Fig. 3. The three peaks of GAR, DAM and SSM were separated completely even in Inchinkoto. The peak of MTM was not detected in this chromatogram.

Table I shows the changes in the contents of GAR, DAM and SSM at different extraction temperatures (25°C, 60°C and 100°C). The DAM and SSM contents in both extracts increased with increase in the extraction temperatures. The GAR content of both extracts, however, decreased slightly between 60°C and 100°C. Further, greater increases in DAM and SSM and decreases in GAR were observed in the
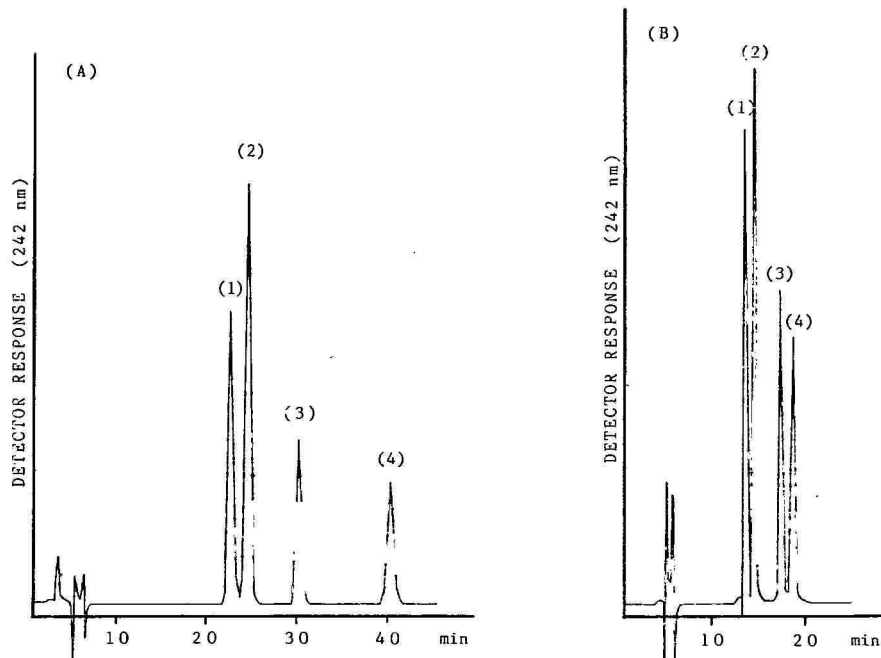
Fig. 1. Effect of tetrahydrofuran on the separation of (1) MTM, (2) DAM, (3) GAR and (4) SSM. Eluent: (A) acetonitrile–water (5:95); (B) acetonitrile–tetrahydrofuran–water (4:1:95).
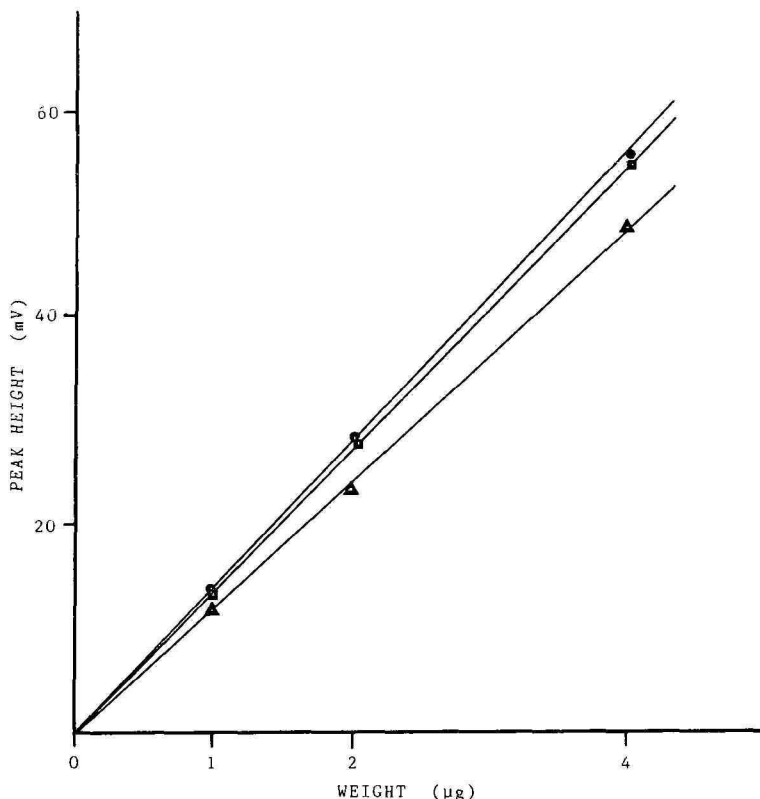
Fig. 2. Calibration graphs for (O) GAR, (■) DAM and (▲) SSM.

extract of Gardeniae Fructus than in that of Inchinkoto. As it is known that iridoid compounds are unstable towards acids, and GAR is easily converted into DAM, SSM and MTM by treatment with hydrochloric acid, the relationship between the pH of these decoctions and the extraction temperature was investigated. The results are shown in Fig. 4. The pH of the extracts of Gardeniae Fructus was lower than that of Inchinkoto at all temperatures. Although the pH of the extract of Gardeniae Fructus is hardly effected by heat, the pH of Inchinkoto increased with increase in the extraction temperatures. This result explains the easier transformation of GAR into DAM and SSM in Gardeniae Fructus than that in Inchinkoto (Table I).

Determination of GEN

As shown in Fig. 5, the separation of GEN from Inchinkoto was achieved by using acetonitrile–tetrahydrofuran–water (8:2:90, v/v) as the eluent. The calibration graph for GEN was linear over the range 1.5–6 μg (Fig. 6), and the limit of detection is 2 ng. GEN showed a single peak under the conditions used. The determination of GEN in Inchinkoto and the extract of Gardeniae Fructus at extraction temperatures of 25°C, 60°C and 100°C is illustrated in Table II. The amount of GEN in each extract increased with increase in the extraction temperature.
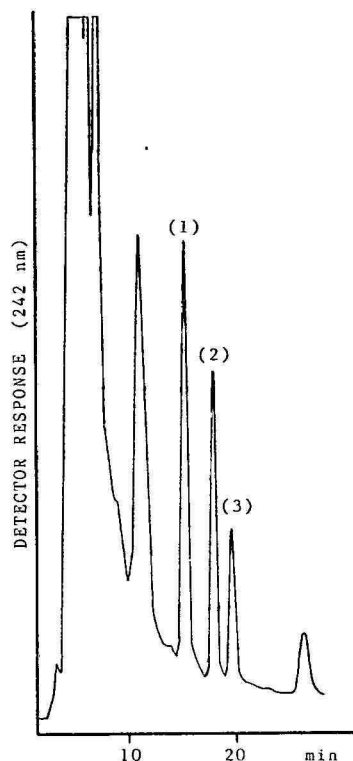
Fig. 3. Chromatogram of (1) DAM, (2) GAR and (3) SSM in Inchinkoto.

DISCUSSION

Recently, traditional Chinese medicines (Kampohozai), decoctions of a mixture of crude drugs, have been used for the treatment of chronic inflammatory diseases in Japan. The Inchinkoto used in this experiment is a decoction of a mixture of Gardeniae Fructus, Artemisiae Capillaris Herba and Rhei Rhizoma, and is applied to the treatment of hepatitis, especially choleresis. The evaluation of traditional Chinese medicines and their component crude drugs is of growing importance owing

TABLE I

DETERMINATION OF GAR, DAM AND SSM IN PREPARED SAMPLES AT VARIOUS TEMPERATURES

Each value represents the mean ± S.D. (mg/decoction) of three determinations.

Compound	25°C		60°C		100°C	
	Inchinkoto	Gardeniae Fructus	Inchinkoto	Gardeniae Fructus	Inchinkoto	Gardeniae Fructus
GAR	9.14 ± 0.09	9.40 ± 0.04	9.79 ± 0.08	11.33 ± 0.23	9.22 ± 0.32	8.33 ± 0.15
DAM	5.31 ± 0.06	4.48 ± 0.07	6.67 ± 0.27	7.07 ± 0.33	12.62 ± 0.36	16.33 ± 0.28
SSM	4.39 ± 0.03	4.41 ± 0.02	4.69 ± 0.07	5.62 ± 0.17	6.63 ± 0.38	8.36 ± 0.20
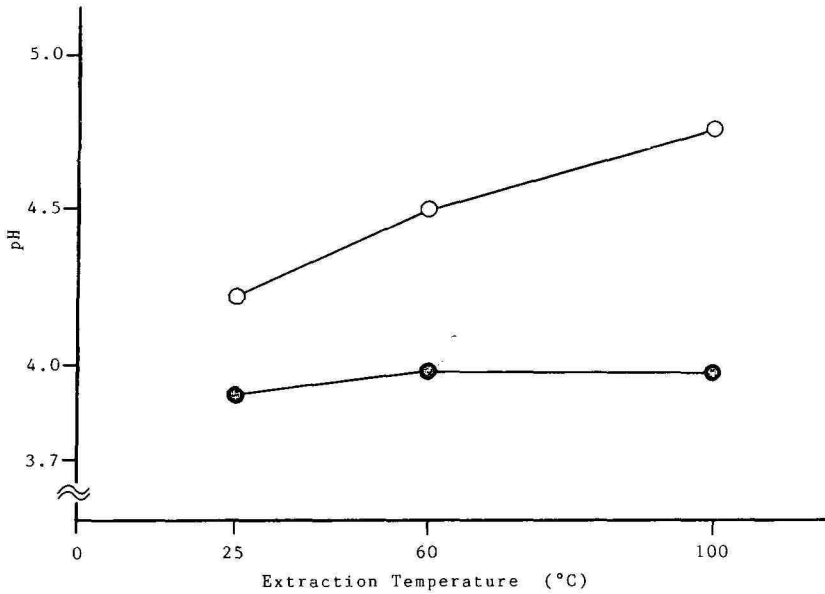
Fig. 4. Relationship between pH of extract and extraction temperature in (○) Inchinkoto and (●) extract of Gardeniae Fructus.

to the variety of cultivation conditions. Recently, gas–liquid chromatography (GLC) and HPLC have been used extensively to determine the main components present in crude drugs. Nevertheless, there have been no reports on the GLC or HPLC analysis of GAR and GEN in Gardeniae Fructus or traditional Chinese medicine because
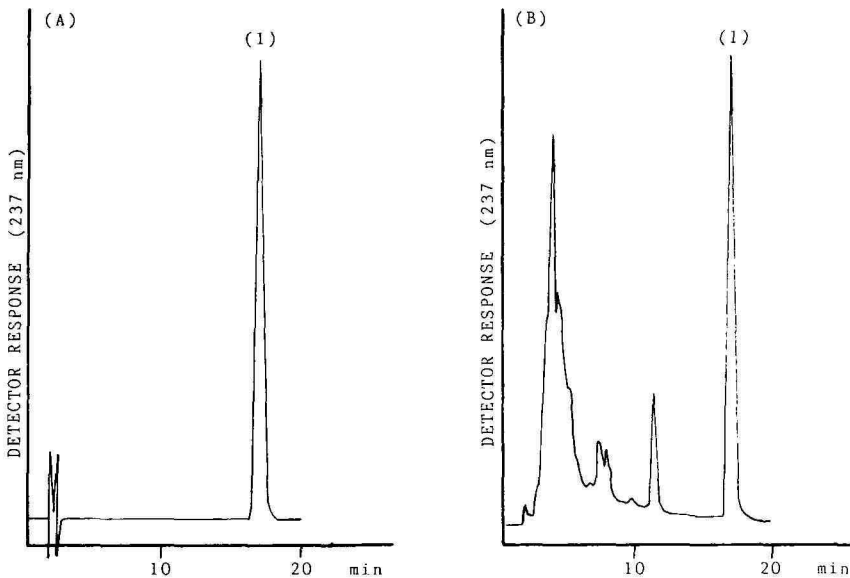
Fig. 5. Chromatograms of GEN (peak 1): (A) standard sample; (B) Inchinkoto.
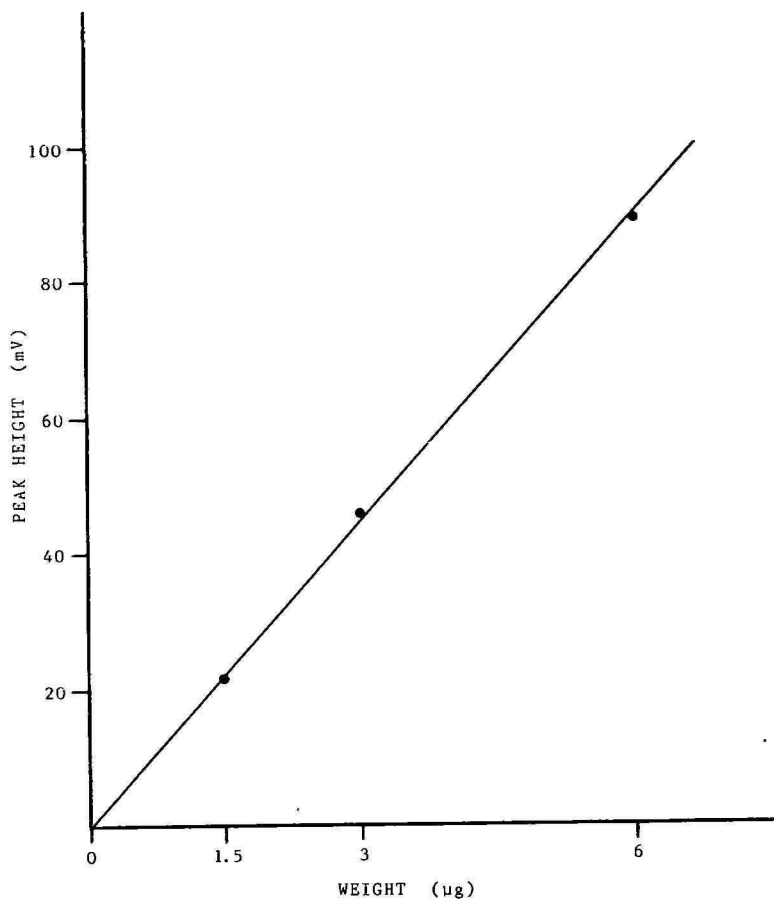
Fig. 6. Calibration graph for GEN.

GAR is unstable towards acids and heat, and it is very difficult to separaste GEN, GAR and related compounds produced from GAR during extraction.

In this study, we were successful in the complete separation of GEN, GAR and the related compounds DAM, SSM and MTM by reversed-phase HPLC. A

TABLE II

DETERMINATION OF GEN IN PREPARED SAMPLES AT VARIOUS TEMPERATURES

Each value represents the mean ± S.D. (mg/decoction) of three determinations.

Temperature (°C)	Inchinkoto	Gardeniae Fructus
25	111.7 ± 0.36	106.6 ± 0.32
60	114.9 ± 0.38	132.7 ± 0.09
100	128.8 ± 1.15	148.9 ± 0.17

mixture of acetonitrile and water with a small amount of tetrahydrofuran was a suitable eluent, giving complete separastion. GEN was stable at the extraction temperatures (25–100°C), and no structural changes occurred during the extraction. On the other hand, the content of GAR decreased and the content of DAM and SSM, acid reaction products of GAR, increased with increase in the extraction temperatures. During the extraction, the pH of Inchinkoto increased with increase in temperature, but that of Gardeniae Fructus did not change. These results indicate that there is a possibility of alkaline compounds such as alkaloids in Inchinkoto being extracted with increase in the extraction temperature and pH. Consequently, we conclude that the conversion of GAR into DAM and SSM in both extracts is promoted by heating, and that the difference in the contents of those compounds between two extrascts is due to the effect of acidity. MTM, which was derived from GAR by hydrochloric acid treatment, could not be detected under these mild conditions.

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CHROM. 18 462

RAPID SEPARATION OF HISTONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON C$_4$ REVERSED-PHASE COLUMNS

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(Received December 30th, 1985)

SUMMARY

A very rapid separation of H1 and core histones by reversed-phase high-performance liquid chromatography using a Bio-Rad Hi-Pore butyl (C$_4$) silica-based column in a single run is reported. The histones were dissolved in water containing 0.1% trifluoroacetic acid and fractionated within 20 min by means of a linear gradient system consisting of water–acetonitrile containing 0.1% trifluoroacetic acid. For the detection of histones the eluate was monitored at 210 nm. The identity and purity of eluted proteins were confirmed (1) by acid–urea and Triton–acid–urea polyacrylamide gel electrophoresis, and (2) by comparison with the retention times of pure histone markers. Despite the short elution time, a high resolution of the different histone fractions could be obtained. The eluted histones were recovered in the following order: H1 (LHP), H1 (MHP), H2B, H2A (LHP), H4 plus H2A (MHP), H3 (LHP), and H3 (MHP) (where LHP and MHP refer to less hydrophobic and more hydrophobic histone variants). The reported system is preferable to time-consuming electrophoretic systems for the separation of histones.

INTRODUCTION

Histones are the most intensively studied group of basic nuclear proteins and are of great importance with regard to the organization of chromatin structure and control of gene activity[1]. In the nuclei of all eucaryotic cells, DNA is packed in basically the same way: two each of the histones H2A, H2B, H3 and H4 form the fundamental chromatin subunit, the nucleosome; an additional part of DNA acts as linker between adjacent nucleosomes, which may be associated with histone H1[2,3].

It is apparent that four of the five major histone classes contain different variants[4–8]. Furthermore, the histones and their variants undergo several postsynthetic modifications such as acetylation, phosphorylation, methylation and ADP-ribosylation, which are thought to be important for regulatory functions[9,10]. To study these histone functions it is essential to isolate and fractionate the histones, and different kinds of gel electrophoresis are generally used. Sodium dodecyl sulphate gel electrophoresis[11,12] separates the five major histone fractions, but the modified forms of the

histones are not resolved. Acetic acid–urea gel electrophoresis, as described by Pan-yim and Chalkley[13], can also resolve the five major fractions and in addition may also discriminate some of the modified forms. According to Franklin and Zweid-ler[14,15], the use of non-ionic detergents in acetic acid–urea gels enables the separation of histones and some non-allelic variants as well as modified forms.

However, these methods are laborious and time-consuming. It seemed desirable, therefore, to replace it by a more rapid and simplified technique.

The application of high-performance liquid chromatography (HPLC) to histones has been described by several authors[16–22]. Certa and von Ehrenstein[16] tried, as did Gurley et al., to separate whole histone preparations by reversed-phase HPLC. Employing a relatively long time of analysis (80 min) they were able to fractionate the histones and some histone variants. However, irreversible adsorption of histones on the column packing resulted in a low recovery and, moreover, H3 variants were not separated.

Using a new gradient system (water–acetonitrile–trifluoroacetic acid) Gurley et al.[17] fractionated the histones within 2 h, except H2A from H4. They found two H3 variants but no H1 variant. Their conditions allowed a 90% recovery of all proteins applied to the column. By varying the type of column and the TFA concentration in the gradient, Gurley et al.[18,19] succeeded in separating H4 from H2A during 2 h. To differentiate between H1 and H1^0, Gurley et al.[20] and D'Anna et al.[21] requires 5 h and a tandem arrey consisting of μBondapak cyanopropyl silane and C_{18} columns.

Kurokawa and McLeod[22] recently reported a reversed-phase HPLC system that permits the fractionation in one step (40 min) of five H1 variants, and in another step (60 min) the core histones and different variants.

Despite all these efforts, however the separation of histone modifications by HPLC has not yet been successful. Even in cases where HPLC would be a good alternative to an electrophoretic separation of histones the long time of analysis still remains unsatisfactory.

Studies in our laboratory have been concerned with attempts to get more information on the biological consequences of changes in the extent of histone modification[23,24]. In the electrophoretic systems, as described above, all histone variants and most histone modifications can be separated, but owing to the complexity of gel electrophoresis it is almost impossible to identify and quantify each band exactly. Thus it would be advantageous to be able to fractionate the histones readily before electrophoresis.

In this report we describe a simple and rapid HPLC system. Not only two H1 variants but also the core histones, except H2A (MHP) from H4, can be separated in a single run within 20 min.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and water were obtained from Merck (Darmstadt, F.R.G.), trifluoroacetic acid (TFA) and standard histone H2A (calf thymus, type VI-S) from Sigma (Munich, F.R.G.). The other histone reference substances H1 and H2B (calf thymus) were purchased from Boehringer-Mannheim (Mannheim, F.R.G.).

Calf thymus chromatin was prepared as described by Goodwin et al.[25]. The chromatin was treated with 0.35 M sodium chloride, the residual pellet extracted with 0.4 N sulphuric acid and the histones obtained according to Gurley et al.[17].

High-performance liquid chromatography

All HPLC experiments were performed on a Bio-Rad HPLC-gradient system equipped with two Model 1330 pumps, a microprocessor-controlled solvent programmer (Apple), and a Model 1305 A variable-wavelength UV–VIS monitor. The detection signal was documented on a Bio-Rad Model 1322 dual channel recorder.

Reversed-phase HPLC was carried out on histone preparations using a Bio-Rad Hi-Pore RP-304 C_4 column (250 × 4.6 mm I.D.) packed with 5-μm spherical particles (pore size 330 Å).

The lyophilized histones were dissolved in water (2 mg/ml) containing 0.1% TFA. To remove insoluble material, the solution was filtered through a 0.2-μm Sartorius Minisart filter. The pure histone solutions were prepared in the same manner (0.5 mg/ml). After equilibration of the column with the initial chromatography sol-
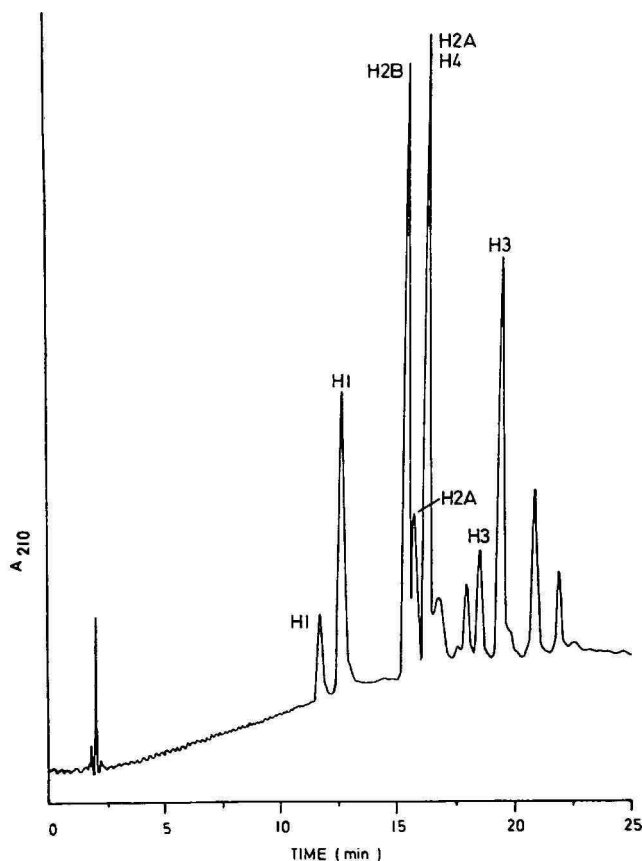
Fig. 1. Separation of calf thymus histones in an analytical run. 40 μg of protein were added to a Bio-Rad Hi-Pore RP-304 C_4 column (250 × 4.6 mm I.D.). Flow-rate, 1.5 ml/min; monitoring wavelength, 210 nm.
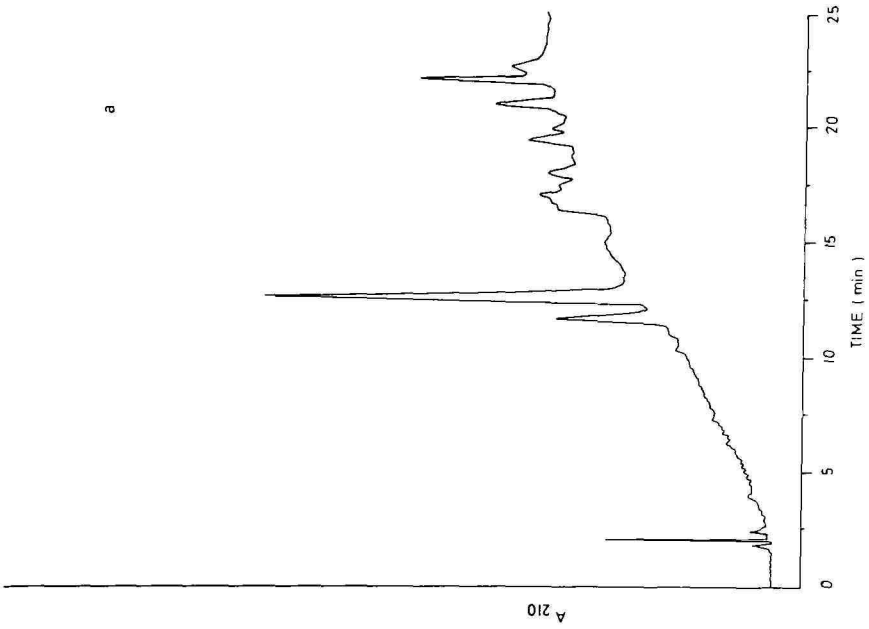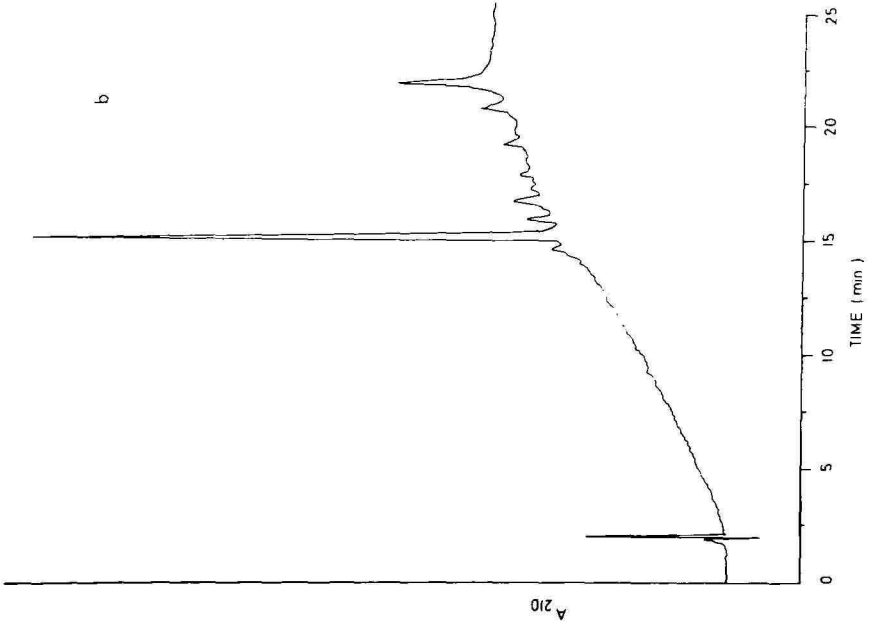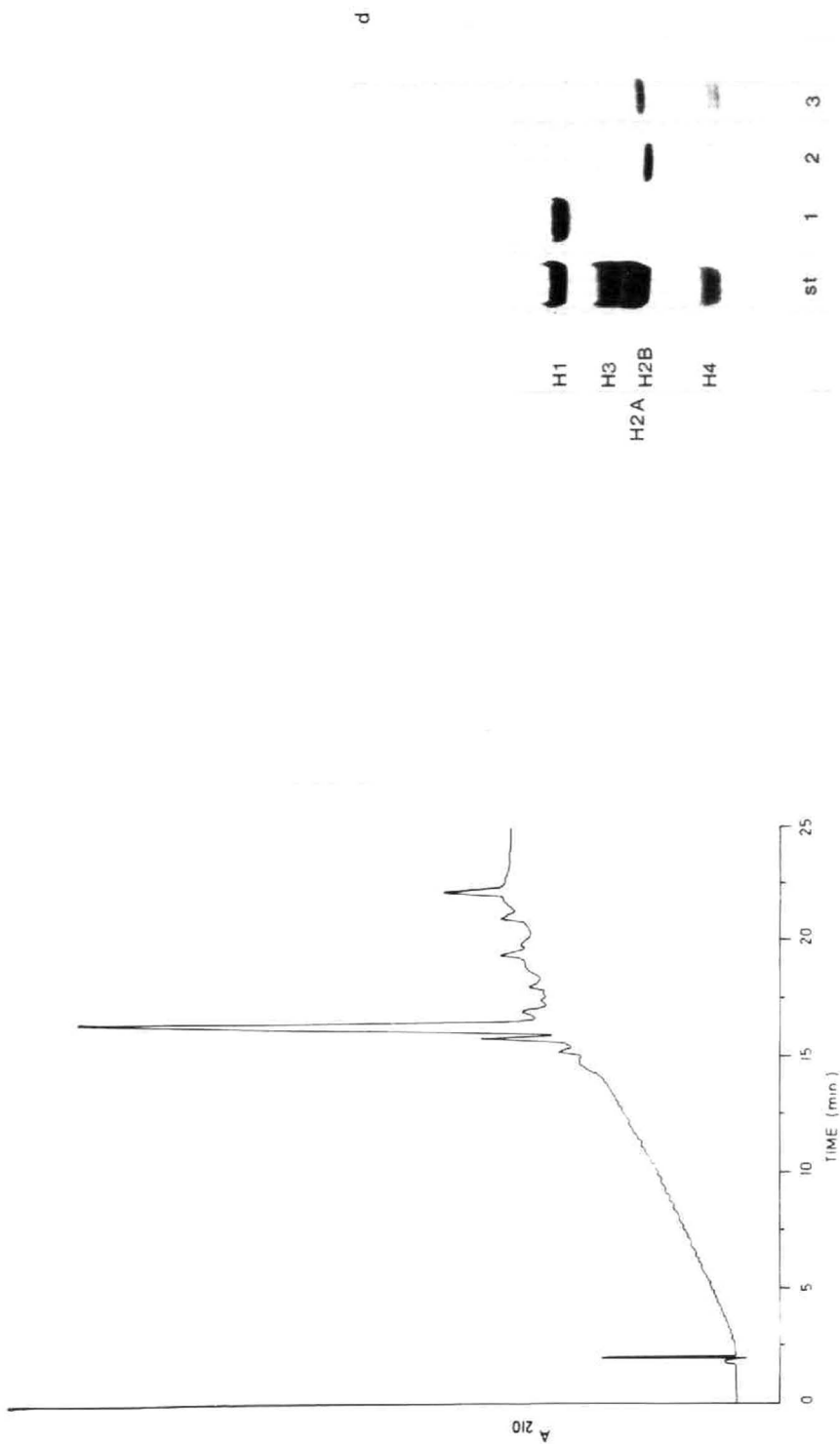
Fig. 2. Chromatograms and electrophoresis of histone markers. The retention times of (a) H1 (b) H2B and (c) H2A are shown. Each histone marker (10 μg) was subjected to HPLC, under the same conditions as for Fig. 1, including UV detection. As another control (d), the samples were submitted to electrophoresis on an acid–urea slab gel in the following order: H1 (lane 1), H2B (lane 2) and H2A (lane 3). The histone fractions were identified by comparison with a calf thymus histone standard (st) (total histones). Lane 3 (H2A) shows contamination with H4 (two minor bands).
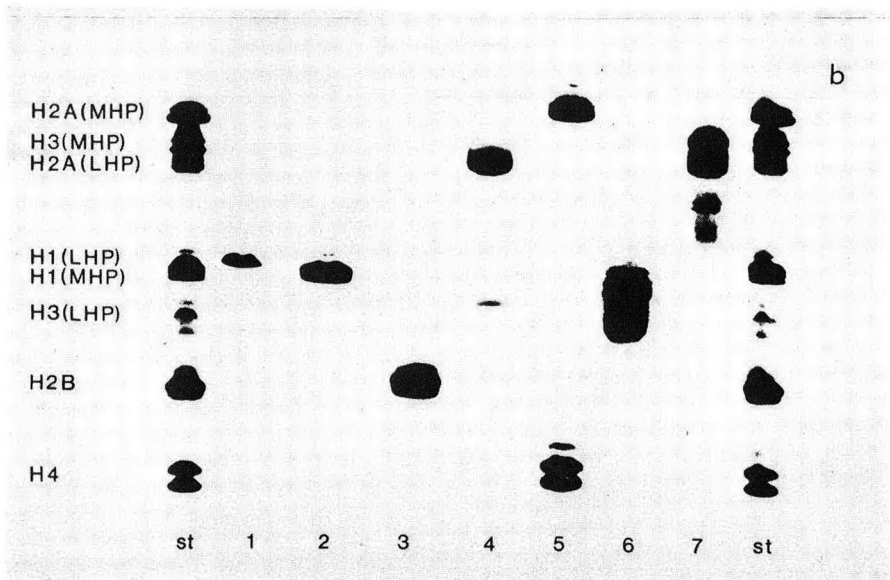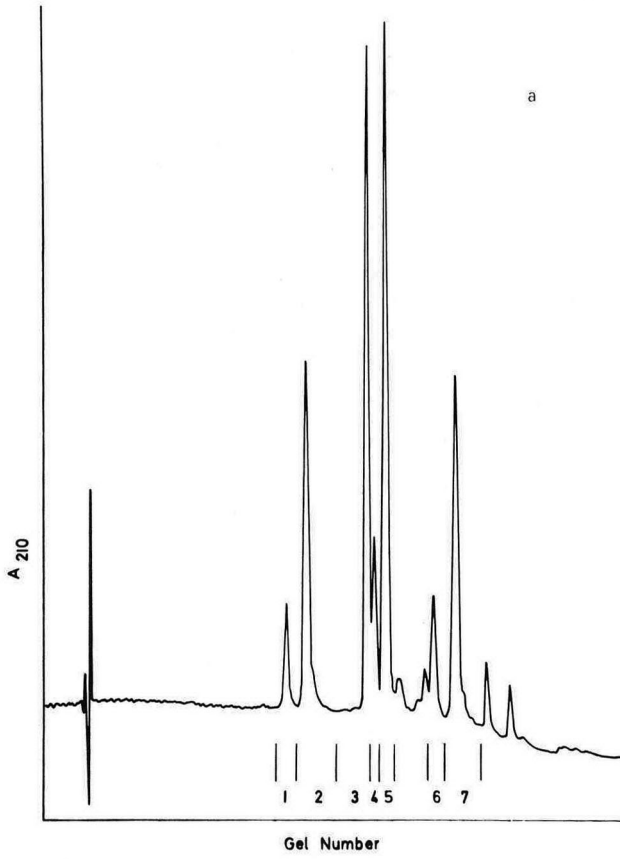
Fig. 3.
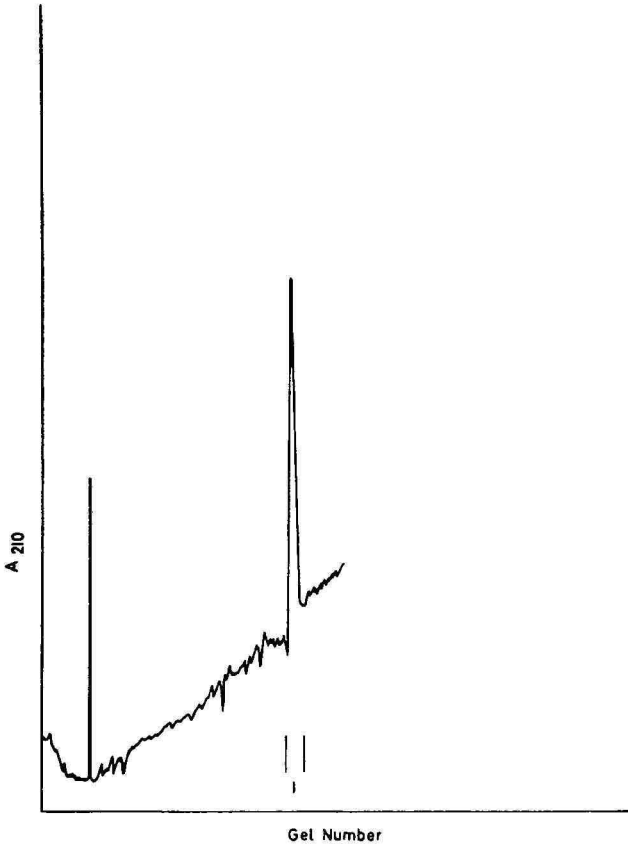
Fig. 3. Separation of calf thymus histones by HPLC and identification of the isolated fractions by Triton–acid–urea polyacrylamide slab gel electrophoresis. (a) The analysis of 1 mg of calf thymus histones (whole histones) in the HPLC system described in Fig. 1. Fractions 1–7 were lyophilized and subjected to electrophoresis. (b) Triton–acid–urea polyacrylamide slab gel electrophoresis of the HPLC fractions 1–7 as indicated on the UV absorbance profile in (a). The histone fractions were identified by comparison with a calf thymus histone standard (st). In (c), 0.5 μg of protein from fraction 1 was rechromatographed in the same HPLC system.

vent, the histones were injected on to the sample loop (20 μl). For preparative runs a more concentrated protein solution (10 mg/ml) was prepared and 100 μl were injected (1 mg of protein). The histones were eluted at room temperature by means of a linear gradient from 80:20 A–B to 20:80 A–B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 70% acetonitrile) for 20 min. The flow-rate was 1.5 ml/min and the eluent was monitored at 210 nm. The preparative runs were performed under the same conditions as the analytical separations.

Electrophoretic analysis of histone fractions

On the basis of the UV absorption, each HPLC peak was collected separately in a tube as indicated. Because of the short analysis time the use of a fraction collector was not required. The fractions were lyophilized to dryness and stored at −20°C

until used for electrophoretic analysis. Electrophoresis of each HPLC fraction as well as of pure histone reference substances was performed on polyacrylamide slab gels (160 × 0.75 mm) of two different types: (1) the acid–urea gel system of Panyim and Chalkley[13], and (2) the Triton X-100 gel system according to Zweidler[15]. The special conditions for the acid–urea gel were 15% polyacrylamide–0.9 M acetic acid–6.25 M urea, and for the Triton X-100 gel were 12% polyacrylamide–0.9 M acetic acid–8 M urea–0.37% Triton X-100, respectively. The gels were stained for 1 h with 0.1% Serva Blue R in 40% ethanol–5% acetic acid and destained in 20% ethanol–5% acetic acid.

RESULTS AND DISCUSSION

Several column types were tested for the rapid fractionation of the histones: Bio-Sil TSK™ columns, DEAE-cellulose, ion-chromatography and RP columns (C_3 and C_4 types). In addition, various solvent combinations and gradients were checked. Poor resolution, long processing times, and low recoveries were the main problems encountered.

The best results were obtained with a Bio-Rad RP-304 C_4 column and a linear gradient system from 80:20 A–B to 20:80 A–B. Fig. 1 shows the separation of histone proteins by HPLC and the retention times achieved under these conditions. We found eleven peaks on separating the calf thymus histones prepared as described under Experimental, seven of which were identified as individual histones. The unlabelled peaks in Fig. 1 were contaminants and non-histone proteins (data not shown). The individual histone peaks were identified (1) from retention times in comparison with purchased individual histone standards (Fig. 2a–c), and (2) by preparative runs and subsequent electrophoretic analysis of the various fractions (Fig. 3a–c). Fig. 2a shows the chromatogram from the H1 standard. Two peaks were detectable, and were in accordance with the ratios of the areas and the retention times of the first two peaks of Fig. 1. Electrophoresis of the H1 standard showed (Fig. 2d, sample 1) the uniformity of the reference. Therefore, we conclude that these two peaks contain at least two H1 variants. The minor peak with the higher mobility in the chromatogram was characterized as H1 (LHP) the second one as H1 (MHP), where LHP and MHP refer to the less hydrophobic and more hydrophobic variant, according to the nomenclature of Gurley et al.[17].

The separations of the H2B and H2A standards are shown in Fig. 2b and c. H2B was identified in the same manner as described for H1. The purity of the H2B standard was also determined by electrophoresis (Fig. 2d, sample 2). However, by electrophoretic control of the H2A reference, some contamination with H4 was detected and, therefore, it was impossible to determine exactly the location of the H2A histone among the HPLC fractions. For this reason, and the non-availability of individual H3 and H4 standard substances, preparative runs characterizing these proteins were performed.

For the separation of histone proteins by HPLC, 1-mg protein samples were loaded on the Bio-Rad C_4 column. The corresponding fractions of the eluate indicated on the abscissa of Fig. 3a were collected, lyophilized and subjected to Triton–acid–urea slab gel electrophoresis (Fig. 3b). Compared with the mobilities of calf thymus standard histones, the HPLC fractions were identified as follows: No. 1, H1; No. 2, H1; No. 3, H2B; No. 4, H2A; No. 5, H2A plus H4; No. 6, H3; No. 7, H3

(*cf.* Fig. 1). Fractions 1 and 2 in the electrophoresis of Fig. 3b show two H1 proteins with different electrophoretic mobilities. Sample 1, the protein with the lower mobility in the electrophoresis and the higher mobility in the HPLC system, is characterized as H1 (LHP), and the protein of fraction 2 as H1 (MHP). Fraction 3 contains only H2B. However, two proteins were detectable in fraction 4; the main component was an H2A variant H2A (LHP) and there were traces of a non-classified protein. In fraction 5 two histone species, H2A (MHP) and H4, were found.

In contrast to the application of the H2A reference alone (contaminated with H4 histone), an unambiguous specification of H2A and H4 was possible in this case. Moreover, Fig. 3b shows the identity and purity of the protein in fraction 6 with an H3 variant H3 (LHP). In the last fraction of the HPLC separation (number 7), H3 (MHP) was detectable as the main product.

The advantage of our HPLC separation method (using a Bio-Rad C_4 column) is demonstrated in Fig. 3c: Fraction 1 was rechromatographed without additional treatment (enrichment), and within 12 min the separation of H1 (LHP) from the residual proteins could be checked. H1 (LHP) shows the same retention time as in the first run and no contamination with the other H1 variant H1 (MHP).

This approach offers some advantages over currently used methods for separating and analysing the histone proteins: our method is very fast (20 min); not only two H1 variants, but also the core histones, except H2A (MHP) from H4, and some variants, H2A (LHP), H3 (LHP) and H3 (MHP) could be separated in a single run. Furthermore, the recoveries of the total histones from the column were more than 85%.

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Note

A rotating unit for preparing circular chromatographic plates at elevated temperatures

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(First received June 24th, 1985; revised manuscript received January 30th, 1986)

The Harrison Chromatotron (Harrison Research, Palo Alto, CA, U.S.A.) is used to perform centrifugally accelerated preparative thin-layer chromatography. The instruction manual suggests that if the circular chromatography plates are rotated as they dry, a more uniform adsorbent layer will be produced which in turn will provide better separations[1]. A phonograph record player may be used to rotate these circular plates continuously as they dry at room temperature; the bands of separating compounds formed with such plates are regular and concentric to within ± 1 mm. We have found, however, that if layers of silica gel G are dried at room temperature while rotating and then developed on the Chromatotron with highly polar solvents, such as ethyl acetate–methanol–water (104:72:26, v/v/v), the plates may be used for only one separation. If a second run is attempted, we have repeatedly observed that the entire silica gel G layer separates from the plate, frequently as a single sheet. When the plates were, however, dried at 70°C with rotations through an arc of 120° at 5-min intervals, the layers did not separate from the glass plate, even after many uses with highly polar solvents [*e.g.*, methanol–water (3:1, v/v)]. Unfortunately, the layers produced in this way were manifestly less uniform than those obtained when the circular plates were dried with constant rotation at room temperature. Since commercial phonograph record players cannot be operated in 70°C ovens, we fabricated the unit described in this report to rotate the glass plates in an oven while they are drying. We have observed that plates dried at 70°C with constant rotation provide tighter bands of separating compounds than do plates dried at the same temperature with intermittent rotation. The unit can be fabricated and assembled for less than US $ 200.

MATERIALS AND METHODS

The unit is constructed from the following components: a Dayton right angle AC/DC gearmotor, Model number 2Z802A (Dayton Electric Manufacturing Co., Chicago, IL, U.S.A.); a 23 × 15 × 12 cm metal Bud box (Newark Electronics, Chicago, IL, U.S.A.); a 2-meter 18-3 power cord capable of tolerating 100°C for prolonged periods; an appropriately sized strain relief bushing (Heyco Molded Products, Kenilworth, NJ, U.S.A.); a standard 3-pronged plug; Plastilube No. 2 nonmelting grease (Warren Extruded Products, Cleveland, OH, U.S.A.); and a 24-cm diameter disc fabricated from a single piece of steel stock (Fig. 1).
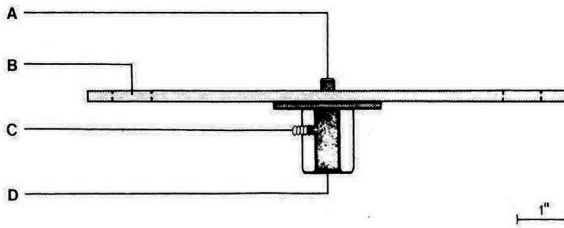
Fig. 1. Diagram of steel disc. (A) centering boss; (B) holes in disc; (C) 0.48-cm set screw; (D) collar.

The motor must be modified for operation at elevated temperatures. To do so, the motor is disassembled and the bearing and gear lubricant is replaced with a high temperature grease, such as Plastilube No. 2. The motor assembly is mounted in the
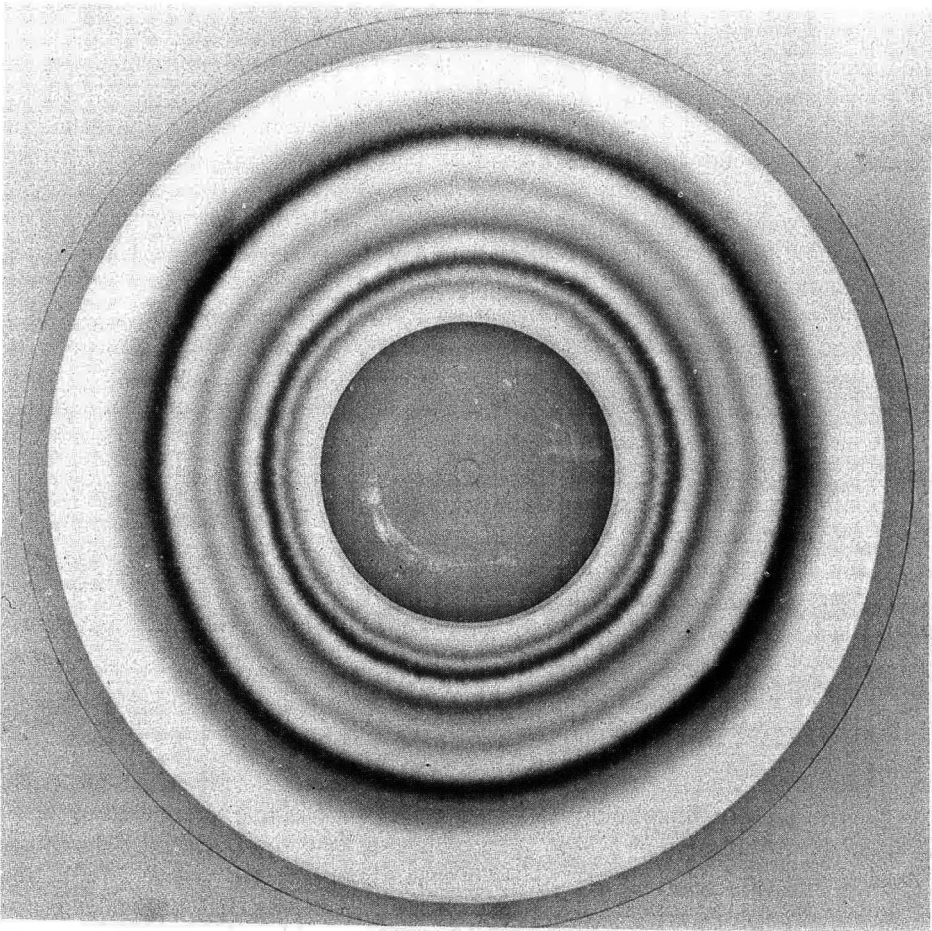

Fig. 2. Illustration of the separation of dyes obtained with the use of a circular disc dried with constant rotation at 70°C. Dye mixture number four (provided by Analtech, Newark, DE, U.S.A.; catalog number 30-04), was applied and the plate was developed with toluene as the solvent.

Bud box with three, 0.635-cm machine screws so that the shaft is positioned vertically. The ground wire is secured to the side of the box via a ring tongue and a 0.318-cm machine screw. The power cord is protected from accidental mechanical removal by securing it to the Bud box via a strain relief bushing. For convenience, a metal handle may be attached to the Bud box.

The steel disc is machined from a single piece of steel stock (Fig. 1) and has a centering boss (A) on the top to accept the hole in the glass Chromatotron plates. The collar (D) is secured to the shaft of the motor with a 0.476-cm set screw so that
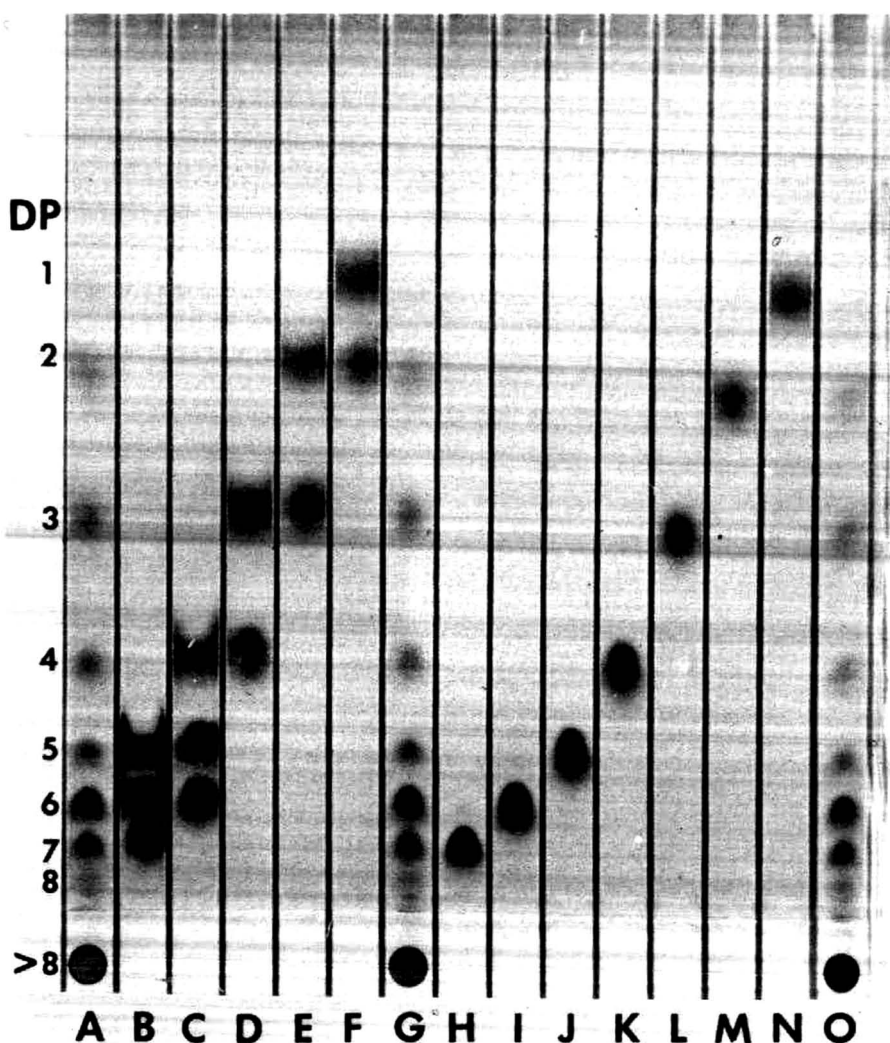

Fig. 3. Thin-layer chromatogram [solvent ethyl acetate–methanol–water (74:80:46, v/v/v)] of glucose oligomeric fractions obtained by centrifugally accelerated preparative thin-layer chromatography [solvent ethyl acetate–methanol–water (104:72:26, v/v/v)]. Lanes A, G and O contain a partial corn starch hydrolysate. Lanes B–F contain fractions isolated on a plate dried with periodic rotation at 70°C. Lanes H–N contain fractions isolated on a plate dried with constant rotation at 70°C. DP refers to degrees of polymerization, i.e., the number of glucose units in the individual glucose oligomer.

the steel disc is positioned horizontally. To facilitate removal of the plate from the disc, two holes (2 2.5 cm in diameter) are drilled opposite each other near the outer edge of the plate (B). To prevent oxidation, the disc should either be chrome-plated or coated with a heat resistant paint. The assembled unit weighs 4.5 kg.

RESULTS AND DISCUSSION

There are several possible variations that may be employed in fabricating the unit. The use of flat cable between the motor and the plug would facilitate closing the oven door. The speed of rotation may be adjusted to the desired rate by the use of a speed control such as a standard laboratory variable transformer. We have found that a speed of 33–45 rpm provides excellent plates when silica gel G is used as the adsorbent. Rather than drilling two holes in the steel plate, one may prefer to machine the steel disc to a diameter slightly less than that of the plates. This approach eliminates temperature variations in the region of the holes, but still permits easy plate removal.

We have found that silica gel G layers prepared with this device can be used repeatedly with ethyl acetate–methanol–water (104:72:26, v/v/v) as the solvent; with such plates, a partial corn starch hydrolysate can be resolved into pure individual oligomers. The oligomeric composition of the various fractions was determined by thin-layer chromatography[2]. If, however, plates prepared by periodic rotation during the drying process are employed, we find that oligomers containing less than four glucose units elute from the circular plate as pairs, whereas those with 5–9 glucose units elute as triplets (Figs. 2 and 3).

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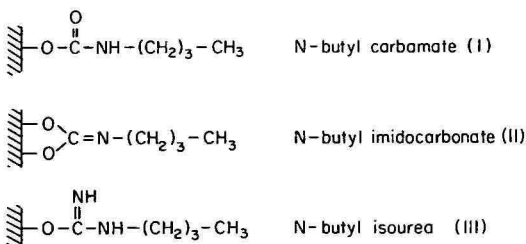
CHROM. 18 477

Note

Origin of the carbamate functional groups in cyanogen bromide-activated, alkylamine-substituted Sepharose

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In a recent study[1], it was shown by using a combination of ion-exchange reactions and NMR spectroscopy that one of the major products obtained on coupling butylamine to cyanogen-bromide-activated Sepharose is an uncharged carbamate derivative together with N-butyl imidocarbonate and N-butylisourea (Scheme 1).
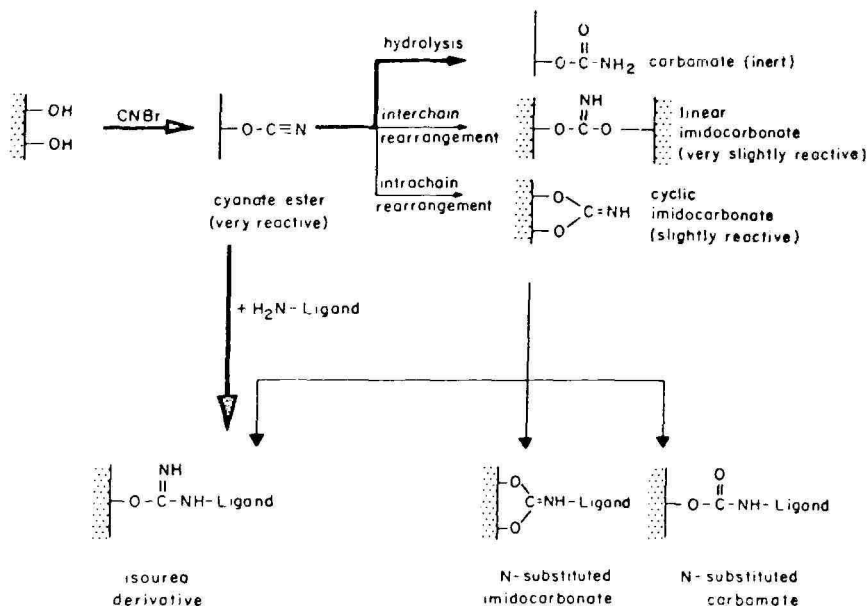

$$\begin{array}{l} \text{—O—C—NH—(CH}_2\text{)}_3\text{—CH}_3 \quad \text{N—butyl carbamate (I)} \\ \\ \text{—O}\diagdown\text{C=N—(CH}_2\text{)}_3\text{—CH}_3 \quad \text{N—butyl imidocarbonate (II)} \\ \\ \text{—O—C—NH—(CH}_2\text{)}_3\text{—CH}_3 \quad \text{N—butyl isourea (III)} \end{array}$$

Scheme 1.

These results appeared to imply that the mechanism for cyanogen bromide activation of polysaccharides, which we have recently described[2–4], is not completely correct (Scheme 2).
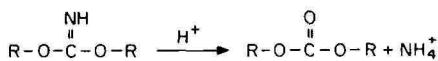
The reason for the discrepancy between the two studies is that in each instance a different type of activated resin was used. In our study, we used a freshly activated Sepharose, which therefore contained only cyanate esters and linear imidocarbonates, both of which give N-substituted isourea as the main product (Scheme 1, III). On the other hand, in the reported study[1] commercially available Sepharose was used, which is known to be treated with acid in order to achieve better stabilization of the activated resin. Such treatment results in a preparation containing cyanate esters and linear carbonates, as acid treatment of imidocarbonate results in the respective carbonate derivative (Scheme 3).

The carbonate can react further with amino-containing ligands to give the corresponding carbamate (Scheme 4).

In order to show that this is the case, we activated Sepharose with cyanogen bromide under basic conditions[4]. The reaction was carried out until no more cyanate ester could be detected[3]. The resulting activated resin (which contained only imido-

Scheme 2.

$$\overset{\overset{\displaystyle NH}{\|}}{R-O-C-O-R} \xrightarrow{H^+} \overset{\overset{\displaystyle O}{\|}}{R-O-C-O-R} + NH_4^+$$

Scheme 3.

$$\overset{\overset{\displaystyle O}{\|}}{R-O-C-O-R} + NH_2R \longrightarrow \overset{\overset{\displaystyle O}{\|}}{R-O-C-NHR} + ROH$$

Scheme 4.

carbonate) was treated with 1 N hydrochloric acid for 1 h in order to form the carbonate. When this Sepharose derivative was treated with butylamine or 1,4-di-aminobutane at pH 9.5 for 24 h, derivatives containing up to 20 μmole of butylamine per millilitre of Sepharose were obtained. The butylamine-substituted Sepharose was devoid of charge, as it contained only carbamates (see Scheme 1, I).

There is therefore no discrepancy between our results and those of Johansson and Drevin[1]; simply two different derivatives of cyanogen bromide-activated Se-pharose were used.

Finally, the bonus from this short study is the introduction of a new activated form of Sepharose containing only carbonate groups. This new type of activated Sepharose can be used to couple amino-containing ligands to the resin, yielding col-umns that consist of stable and uncharged carbamate groups. These activated resins will mainly be useful for coupling low-molecular-weight ligands such as diamino-hexane or aminocaproic acid, as the coupling to the resin has to be performed at high pH (ca. 9.5), owing to the low activity of the carbonate formed. The derivatized Sepharose can be further used to couple proteins under mild conditions and in high

yields. When coupling of protein directly to the carbonate was attempted only low levels of coupling were obtained (0.5 mg of trypsin per gram wet weight of Sepharose or 15 mg per gram dry weight of Sepharose), even when the coupling was carried out at pH 9.

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Note

Determination of carbamates by high-performance liquid chromatography with electrochemical detection using pulsed-potential cleaning

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Carbamate pesticides have come into common use worldwide in recent years. Thus analytical methods for carbamate pesticides are of interest in evaluating contamination of water and agricultural products.

The general interest in analytical methods for carbamate pesticides is evident from the extensive literature. Most authors conclude that gas chromatographic (GC) methods are basically unsuitable since the carbamates are generally unstable at the elevated temperatures needed for GC analysis. Interest has therefore turned to high-performance liquid chromatography (HPLC). The most common detector used with HPLC is the UV detector, and a number of articles discuss analysis for carbamates using HPLC with UV detection. The UV detector, however, is not as sensitive as desired for carbamates. Limits of detection are usually claimed to be in the low nanogram range, although, in a few cases, compounds are claimed to be detectable down to the mid-picogram range[1]. Somewhat lower limits of detection are claimed for post-column derivatization with fluorogenic agents[2,3].

In recent years, electrochemical detectors have been developed and applied to many classes of analytes. For some classes of analytes, electrochemical detectors have proved to be more sensitive that UV detectors. Previous attempts to apply electrochemical detection to carbamates have been successful in a few cases in which the specific carbamate pesticide also contains functional groups such as aromatic amines which can be oxidized readily (*e.g.* Aminocarb[4–7]). Mayer and Greenberg[8] report the detection of eight carbamates using a flow cell with a wax-impregnated graphite electrode. They worked at the positive potential limit for this electrode and obtained limits of detection below 5 ng for only three of the carbamates.

The purpose of this investigation was to apply a newly developed instrument and flow cell to the electrochemical detection of the common carbamate pesticides. The instrument was developed in the School of Chemistry of the Georgia Institute of Technology under a cooperative agreement with the U.S. Environmental Protection Agency[9–11]. The flow cell has a wall-jet configuration and a platinum working electrode. It is a prototype of a new cell under development by Hewlett-Packard.

One of the biggest problems in the use of electrochemical detectors is maintaining a clean electrode surface. Electrochemical detectors are reaction detectors, and reaction products tend to accumulate on the surface of the electrode, where they block the surface and lead to a deterioration of response. The most commonly used

electrodes are made of carbon, and these must be cleaned and polished periodically. Several groups of workers have reported that electrodes can be cleaned *in situ* by periodically pulsing the electrode to extreme potentials[12-17]. In addition, Johnson[18] has reported that platinum electrodes cleaned in this manner are more active than other electrodes and that groups of compounds previously thought to be undetectable can now be detected. This approach was applied in this study to the detection of carbamate pesticides.

In this study a computer-controlled instrument is used. One advantage of such instrumentation is the ability to use the instrument in a wide range of modes by changing the computer software for control and data acquisition. Since the d.c. mode with cleaning pulses was a mode previously unused with this instrument, it was necessary to write a new computer program for this purpose. In addition, it was necessary to include programming to control an instrumental current offset and thus compensate for the high background currents at the extreme positive electrode potentials necessary for the oxidation of the carbamates. This allowed the instrument to be operated at a much higher gain setting and resulted in significant improvements in sensitivity and limits of detection.

EXPERIMENTAL

The instrument has been described in detail[10]. The isocratic HPLC system was the same as before[9], except that a Zorbax ODS column, 150 × 4.6 mm I.D., was used. For most experiments a Spectra-Physics 8770 pump was used. For other experiments a Haskel pneumatic-amplifier pump (No. 26740), as modified by DuPont for their Model 830 chromatograph, was used.

The wall-jet detector cell is a prototype of a cell under development by Hewlett-Packard. The case of the cell is machined from high-density polyethylene. The working electrode is a platinum disk of 0.81 mm diameter, the counter electrode is a length of platinum wire, and the reference eletrode is silver–silver chloride. The effluent enters the cell and is directed perpendicularly to the center of the working electrode by means of a short length of fused silica tubing (57 μm I.D.).

The mobile phase was acetonitrile–acetate buffer, pH 5.50 (1:1). The mobile phase was filtered through a 0.45-μm filter before use. The flow-rate was 1.0 ml/min.

A selection of carbamate pesticides was obtained from the U.S. Environmental Protection Agency, Research Triangle Park, NC, U.S.A., and two others were obtained from the Toxicology Branch of the Centers for Disease Control in Atlanta, GA, U.S.A. The compounds studied, along with their structural formulas, are given in Table I. These compounds were used without further purification.

Preliminary chromatographic experiments were performed using a Chromatronix Model 220 UV detector and injections of approximately 500 ng of each carbamate. Without the availability of a gradient-elution system, it was necessary to compromise on a mobile-phase composition that resulted in acceptable retention and separation of most of the carbamates tested. The primary thrust of this work was the evaluation of the detector, and optimum chromatographic separation was considered to be of secondary importance. Table I summarizes the capacity factors obtained in this study. The multiple retention times indicate the impure condition of these commercial products.

TABLE I

CARRBAMATE PESTICIDES AND CAPACITY FACTORS

Column: Zorbax ODS 150 × 4.6 mm, I.D. Mobile phase: acetonitrile–acetate buffer, pH 5.50 (1:1). Values in parentheses indicate impurities.

Compound	Structure	Capacity factors (k')
Aldicarb	CH$_3$S-C(CH$_3$)(CH$_3$)-CH=N-O-CO-NH-CH$_3$	1.26
Aldicarb sulfoxide	CH$_3$S(O)-C(CH$_3$)(CH$_3$)-CH=N-O-CO-NH-CH$_3$	0.11
Aldicarb sulfone	CH$_3$S(O)$_2$-C(CH$_3$)(CH$_3$)-CH=N-O-CO-NH-CH$_3$	1.44 (0.38)
Aminocarb	(CH$_3$)$_2$N-⟨ring, CH$_3$⟩-O-CO-NH-CH$_3$	1.66
Bendiocarb	O-CO-NH-CH$_3$; ring with O-C(CH$_3$)$_2$-O	1.82
Carbaryl	naphthalene O-CO-NH-CH$_3$	2.05
Chloropropham	Cl-⟨ring⟩-NH-C(O)-O-CH(CH$_3$)$_2$	6.48
Desmedipham	O-CO-NH-⟨ring⟩; ⟨ring⟩-NH-C(O)-O-C$_2$H$_5$	3.44 (0.68, 1.12, 2.36)
Dimethoate	CH$_3$O-P(S)(OCH$_3$)-S-CH$_2$-CO-NH-CH$_3$	2.66 (1.62, 6.11)
Methiocarb	CH$_3$S-⟨ring with CH$_3$, CH$_3$⟩-O-CO-NH-CH$_3$	4.24
Methomyl	CH$_3$S-C(CH$_3$)=N-O-CO-NH-CH$_3$	0.56
Metolachlor	⟨ring CH$_3$, CH$_2$CH$_3$⟩-N(CH$_3$; CHCH$_2$OCH$_3$)(COCH$_2$Cl)	1.65

Preliminary electrochemical experiments were performed in a flow-injection mode without a chromatographic column in the system. Of particular interest were experiments investigating the variation of response with pH. As anticipated, low pH values resulted in decreased response. However, high pH values showed no improvement over neutral values and, in some cases, resulted in poorly defined response peaks on a time basis. Thus the usual range of pH values used with silica-based columns was satisfactory for the electrochemistry. These preliminary experiments also included variations in potential to establish minimum potentials needed for the oxidation of each carbamate. These preliminary experiments showed that all of the carbamates studied were detectable, although there was a considerable range of sensitivities and required potentials.

After these preliminary experiments, the electrochemical detector was connected to the effluent of the chromatographic column and a series of experiments was performed to determine the optimum conditions for the detection of each individual carbamate. The varying factors were the three potentials (reaction potential and first and second cleaning potentials) and the time delay between going to the reaction potential and the start of the current sampling period. Each cleaning pulse and the sampling period were set to 1/60th of a second. The composition and flow-rate of the mobile phase were kept constant as cited above for Table I. All injections were 10 μl containing approximately 180 ng of a carbamate.

The potential of the first pulse was not critical so long as it was greater than 2.0 V. A value of 3.0 V was used for all subsequent experiments. Any adsorbed reaction product is believed to be oxidized at this potential and then removed from the surface of the electrode by the force of the flow stream.

The role of the second cleaning pulse is less certain. If this potential is too negative, hydrogen gas is evolved during the cleaning pulse and large, erratic background currents are observed. If this potential is too positive, the baseline increases during a chromatographic run and sensitivity decreases. A value of 0.5–0.7 V was found to be optimum and was used for all subsequent experiments.

RESULTS AND DISCUSSION

The optimum reaction potential varies with the carbamate being detected. The potential must be postive enough to drive the oxidation reaction. If too positive, performance decreases because the background current increases, and there is also a decrease in current response. Possibly this decrease results from a charge repulsion between the carbamate and the highly positive charge on the electrode. The most striking example is for Aminocarb in acidic solution where the protonated amine group would carry a postive charge. Optimum potentials ranged from a low of 1.3 V for Aminocarb to a high of 2.1 V for Bendiocarb. A value of 1.9 V was adopted for analysis of mixtures of carbamates.

The most surprising results in the optimization experiments came from studies of the time delay between setting the reaction potential and measuring the cell current. Early experiments showed a slowly decreasing background current following the potential jump from the second cleaning pulse to the reaction potential. This background current limited the current-amplifier setting that could be used with the instrument. It was assumed that the desired current response from the carbamate
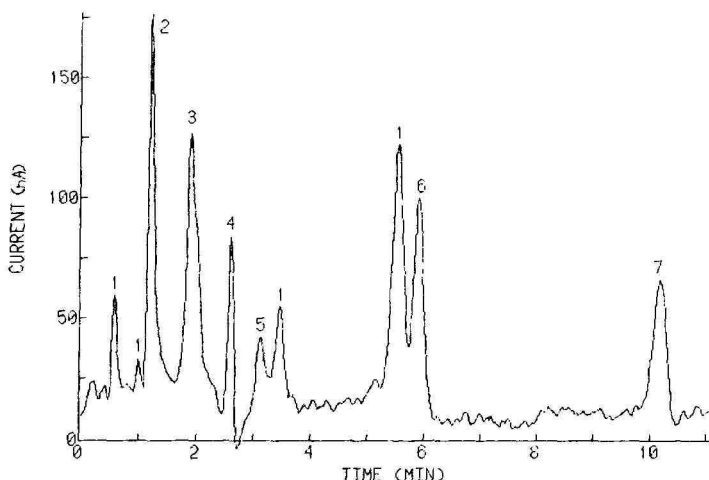
Fig. 1. Chromatogram of eight carbamates at 1.9 V. A 10-μl injection containing 10 ng of each carbamate. Peaks: 1 = Desmedipham; 2 = Aldicarb; 3 = Aminocarb and Bendiocarb; 4 = Carbaryl; 5 = Dimethoate; 6 = Methiocarb; 7 = Chlorpropham.

would also decrease with time after the potential jump, but that perhaps there might be some optimum delay time. The optimization experiments showed the expected decrease in background current and noise level as the delay time increased. However, most carbamates showed a significant increase in current response with increasing delay time. Thus a triple benefit resulted from increased delay time: decreased background which allowed higher current-gain settings, lower noise in the background current, and increased response from the carbamate. The negative aspect of increasing the delay time is that the frequency of sampling is decreased. In order to obtain enough sample points to define adequately the chromatographic peaks under these chromatographic conditions, a minimum sampling rate of one point per second was adopted.

The reason for the increased response with increased delay time is not certain. Johnson[19] suggests that the rate of the oxidation reaction might be proportional to the extent of the oxide layer on the platinum surface and that this layer grows at a relatively slow rate after going to the reaction potential. It is interesting to note that the one carbamate that does not show this increased response with delay time is Aminocarb, which is detected at much lower potentials by the oxidation of its aromatic amine group.

Fig. 1 shows the chromatogram of a mixture of eight carbamate pesticides, each one at 10 ng injected. In this chromatogram, which was run with an electrode potential of 1.9 V, peak number 3 is an unresolved doublet of Aminocarb and Bendiocarb. Fig. 2 is a repeat of Fig. 1 with the electrode potential at 1.3 V where Bendiocarb does not oxidize. An alternative way of resolving the chromatographic overlap would be to use a reduction detector that is sensitive to Bendiocarb but not Aminocarb[20].

Limits of detection for the carbamates vary with the compound, the potential, and the retention time. No attempt was made to estimate optimum limits of detection except for Aminocarb. However, under the conditions of Fig. 1, limits of detection
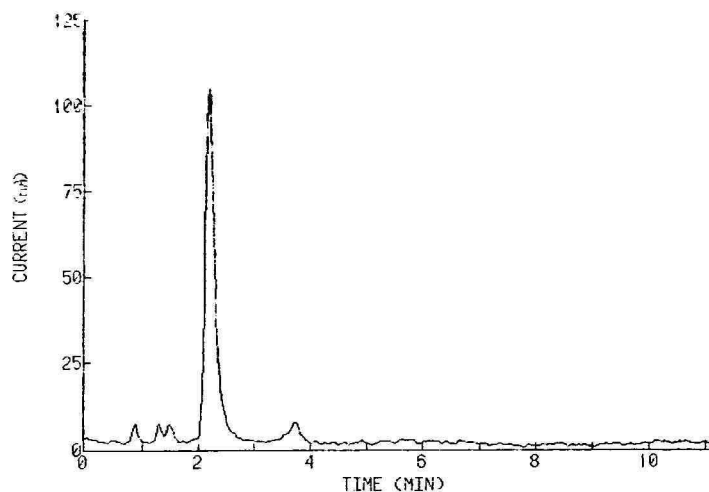
Fig. 2. Chromatogram of eight carbamates at 1.3 V. Repeat of injection of Fig. 1. The only significant peak is that of Aminocarb.

are in the order of 0.1 ng, which is approximately a factor of 10 better than for UV detection and comparable to the post-column derivitization with fluorogenic agents. Since the background current is lower at lower potentials, compounds that react at lower potentials have better limits of detection. Of the carbamates studied, Aminocarb is detected at the lowest potential, and limits of detection were measured for Aminocarb by itself.

Fig. 3 shows a chromatogram for 500 pg of Aminocarb with the electrode potential at 1.30 V. The limit of detection, calculated as three times the standard deviation of the baseline, is 20 pg. However, it is obvious that the baseline has a
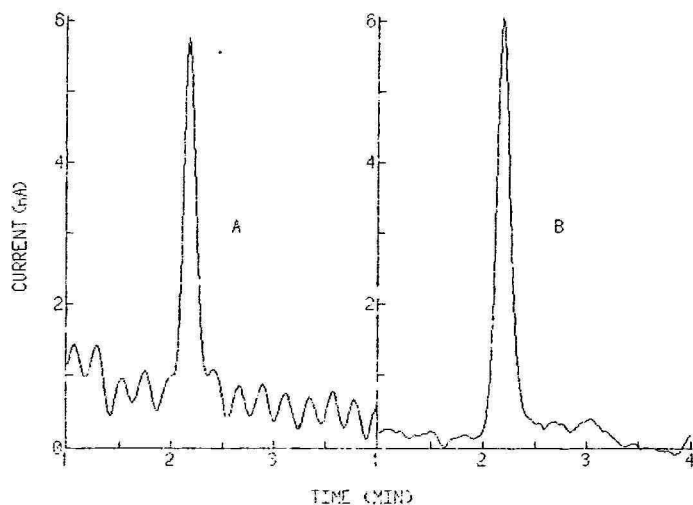
Fig. 3. (A) Chromatogram of aminocarb using Spectra-Physics pump. Potential 1.3 V. Injection is 0.5 ng. (B) Chromatogram of Aminocarb using Haskel pump. Repeat of Fig. 3a, except for pump.

regular noise signal, and further investigation revealed that the frequency of this noise signal is the same as the cycle time of the Spectra-Physics pump. This pump had been selected after comparison trials against other reciprocating piston pumps, and the pump noise had never been noted at higher levels of analyte. To clarify the situation, the chromatogram was repeated after substitution of an air-driven Haskel pump. It is obvious that the regular noise signal is no longer present. For this chromatogram, the limit of detection, as calculated before, is 5 pg, which is approximately an order of magnitude better than reported previously by any method.

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Note

Characterization of modified bovine haemoglobin by molecular filtration and electrophoresis in gradient gels

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(Received December 31st, 1985)

Stroma-free haemoglobin (SFH) and its chemically modified derivatives are of interest as oxygen-carrying perfusion fluids[1-4]. Modification of SFH with pyridoxal-5'-phosphate and glutaraldehyde leads to a very polydisperse system, containing various types of modified and unmodified haemoglobin molecules and molecular aggregates with a relative molecular mass $M_r = 10^4-10^6$. In previous investigations we studied modified, mostly human, haemoglobin by several analytical fractionation techniques[5]. This paper deals with the possibility of using other methods, *viz.*, ultrafiltration, fast protein liquid chromatography (FPLC) on Superose™ 12 and vertical slab electrophoresis on gradient gels, for the characterization of native and modified bovine SFH.

EXPERIMENTAL

SFH was prepared from fresh bovine erythrocytes in the usual manner[6]. The modification of bovine SFH with pyridoxal-5'-phosphate (Fluka, Buchs, Switzerland) and glutaraldehyde (Serva, Heidelberg, F.R.G.) was performed in the presence of lysine (Lachema, Brno, Czechoslovakia) using modified versions of published methods[2,3]. Fractionation of modified bovine SFH by ultrafiltration was performed on XM 50 and XM 100 A membranes (Amicon, Lexington, MA, U.S.A.) as recommended by the producer[7]. FPLC on Superose 12 has been described·elsewhere[8]. The exponential gradient of polyacrylamide gel was 10–22.5% for SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulphate) and 8–20% for PAGE without SDS. The size of the gels was 140 × 180 × 1 mm. The solutions for the preparation of the gel and the buffers for electrophoresis were prepared according to LKB Application Note 320 (ref. 9). A polyacrylamide gel of concentration 3% in buffer of pH 6.8 was used as the stacking gel.

Electrophoresis was carried out at 8°C in a discontinuous buffer system of pH 8.8 according to Laemmli[10], using 30 mA per plate for 6 h. The samples were incubated for 12 h at 37°C in buffer of pH 6.8 in the presence of 8 M urea and 2% SDS prior to SDS-PAGE. PAGE without the addition of denaturing agents was performed under the same conditions.

The densitometric evaluation of the electrophoretograms was carried out on a Clini Scan apparatus (Helena Labs., Beaumont, TX, U.S.A.). A low-molecular-weight calibration kit (Pharmacia, Uppsala, Sweden) was used for calibration in SDS-PAGE.
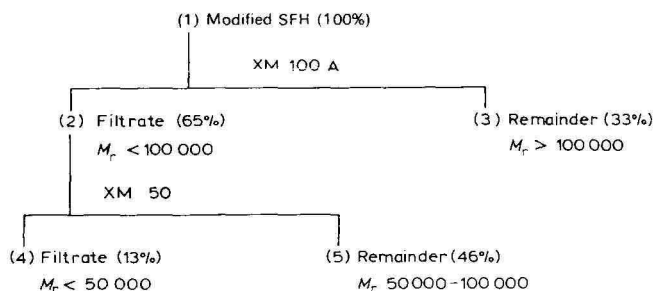
Fig. 1. Scheme of the fractionation of modified bovine SFH by ultrafiltration on Amicon XM 50 and XM 100 A membranes. Numbers in parentheses show the percentages of the individual fractions.

RESULTS AND DISCUSSION

The rough fractionation of haemoglobin samples by ultrafiltration on the Amicon membranes is shown schematically in Fig. 1.

Fig. 2 shows the results of FPLC on Superose 12 achieved with native and modified bovine SFH and also with fractions prepared by ultrafiltration. The native SFH formed a single symmetrical peak (apparent M_r = 48 000 according to the
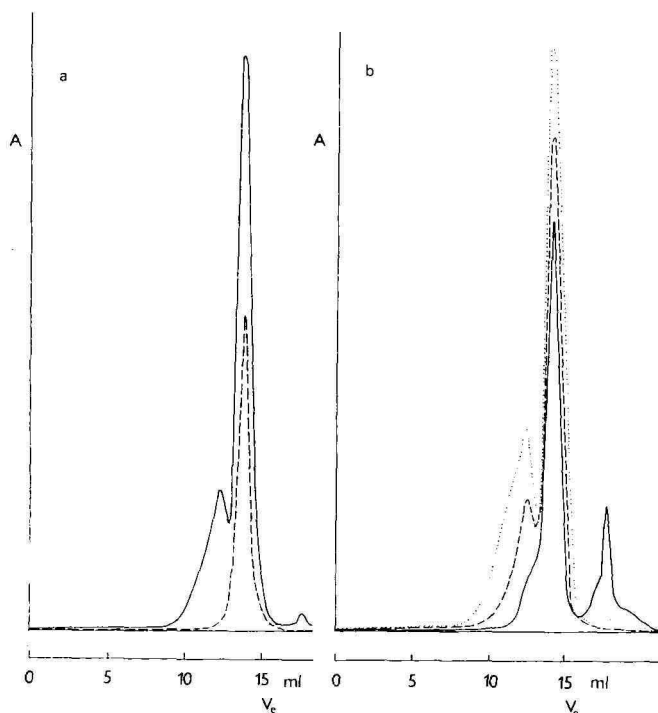
Fig. 2. Gel chromatography of haemoglobin samples on Superose 12. Buffer, 0.05 M sodium phosphate (pH 7.0), 0.15 M NaCl, flow-rate 0.9 ml/min. (a) Full line, modified SFH (see fraction 1 in Fig. 1); dashed line, native SFH. (b) Full line, fraction 4; dashed line, fraction 5; dotted line, fraction 3 (see Fig. 1).
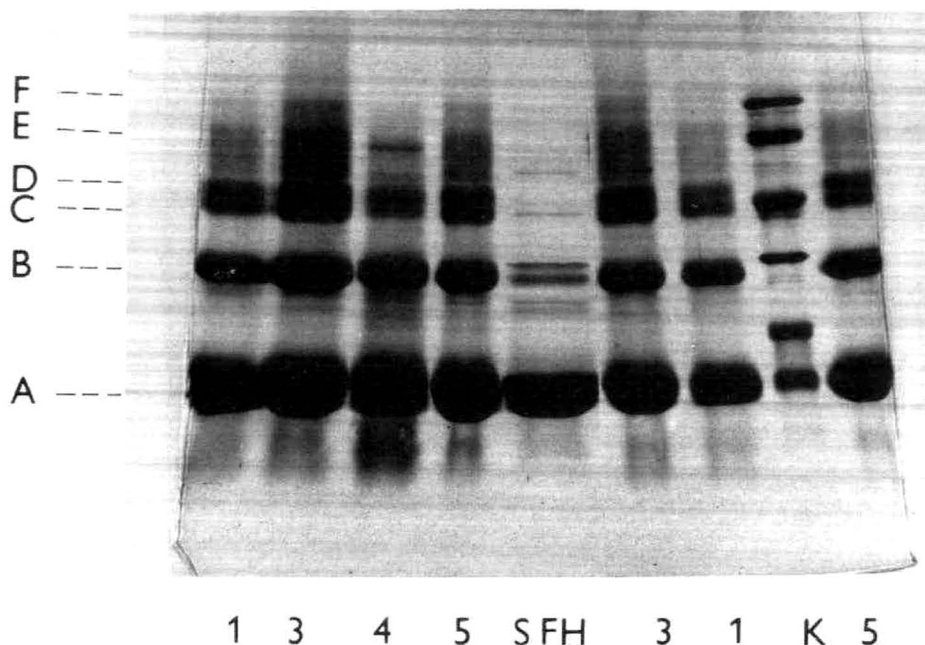
Fig. 3. Electrophoresis of haemoglobin samples on the polyacrylamide gradient gel with sodium dode-cylsulphate (SDS-PAGE). For numbering of samples, see Fig. 1. SFH, unmodified bovine haemoglobin; K, calibration kit. The letters A–F correspond to the zones of the electrophoretic pattern of the modified bovine SFH (see Fig. 4).

calibration graph published in ref. 8). After modification with pyridoxal-5'-phosphate and glutaraldehyde, two peaks appeared. The peak of the molecular aggregates corresponding to a mean M_r of about 150 000 formed 30% and the position of the main peak, forming about 70% of the total mount of proteins, was identical with that of native SFH.

Similar results were obtained with the fractions from ultrafiltration (see Fig. 1). Fractions 5 and especially 3 showed a marked peak, corresponding to aggregates. In fraction 4 the peak of the aggregates was missing, and in addition to the main peak at the position of native SFH a small peak of lower relative molecular mass was found.

FPLC on Superose 12 led to analogous results to chromatography on Sepharose 6B, but better reproducibility and faster separations were obtained comparison with classical chromatography. Similar results were achieved in our previous work[8] on the analysis of human SFH.

PAGE on the gradient gels in the absence of denaturing agents confirmed the high polydispersity of modified SFH. Whereas the native SFH was characterized by a single sharp zone during electrophoresis, the modified SFH, under the same conditions, led to prolonged diffuse strips with the highest intensity at the position of native SFH.

Fig. 3 shows SDS-PAGE on the gradient gel and Table I summarizes the densitometric evaluation of the above electrophoresis. Native bovine SFH is char-

TABLE I

QUANTITATIVE EVALUATION OF FIG. 3

For numbering of the fractions, see Fig. 1.

Sample	Proportion (%)				
	Low M_r	Monomer	Dimer	Trimer	Higher aggregates
Native SFH		93	7		
(1) Modified SFH		60	23	12	5
(3) Fraction with M_r > 100 000		50	24	15	11
(4) Fraction with M_r < 50 000	11	55	24	8	2
(5) Fraction with M_r 50 000–100 000		54	26	14	6

acterized by one intensively stained zone corresponding to the subunits α and β ($M_r = 16\,000$). The modified bovine SFH is characterized by several intense zones with M_r values corresponding approximately to the monomer, dimer, trimer and higher aggregates of haemoglobin subunits. Similar results were achieved with the fractions from ultrafiltration (fractions 3, 4 and 5). The ratio of the individual zones is shown in Table I. The calibration graph for the determination of M_r is shown in Fig. 4 and is related to sample K in Fig. 3.

Electrophoresis in the gradient gels has a significantly higher distinguishing ability than electrophoresis in gels having a uniform pore size. We always prepared the gels in concentration intervals most suitable for the given purpose. In one experiment it was possible to work on two gel plates, which permitted a parallel analysis of twenty samples under the same experimental conditions.
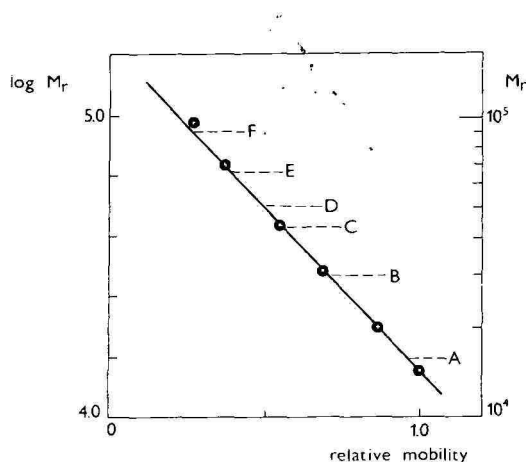

Fig. 4. Calibration graph for the determination of relative molecular mass (M_r) by SDS-PAGE in gradient gel. Relative mobility is related to the fastest zone of the calibration kit (α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, phosphorylase B; full circles). The letters A–F correspond to the zones of the modified SFH (see Fig. 3). Their M_r values read from the calibration graph were as follows: A = 15 900, B = 29 600, C = 43 000, D = 50 100, E = 64 500 and F = 89 000.

The modified bovine SFH prepared in our laboratory generally had a lower degree of aggregation than analogous preparations of this type[2,3]. Our preparation contained about 30% of aggregates, according to FPLC on Superose 12 (Fig. 2) and ultrafiltration (Fig. 1). After treatment with denaturing agents, the presence of about 40% of aggregates was found with SDS-PAGE (Fig. 3). The remainder probably consisted of both unreacted haemoglobin molecules and their subunits and of modified molecules intramolecularly linked by glutaraldehyde to different degrees of aggregation. These molecules were assumed to be partly disintegratable to subunits by denaturing agents[8].

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Note

Analysis of amino acids as *tert.*-butyldimethylsilyl derivatives by gas chromatography

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(First received November 25th, 1985; revised manuscript received February 4th, 1986)

N-Methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) is a relatively new silylation reagent which leads to the *tert.*-butyldimethylsilyl (TBDMS) derivative. Active hydrogen atoms on oxygen, nitrogen and sulfur are quickly replaced[1,2] and yield derivatives that are as much as 10 000 times more stable to hydrolysis than the corresponding trimethylsilyl derivatives[3]. TBDMS derivatives are stable enough so that the derivatives may be concentrated if necessary for improved sensitivity or they may be injected directly with the reaction solvent and reagent[4]. Separation of derivatized amino acids by gas chromatography is much quicker than with conventional amino acid analyzers and involves inexpensive, relatively simple equipment. Therefore, gas chromatographic analysis lends itself to routine analysis of many samples.

In this study MTBSTFA was used to prepare the TBDMS derivatives of the twenty amino acids commonly found in proteins as well as several sulfur-containing amino acids. The derivatized amino acids were separated on a widebore, bonded silicone phase, glass capillary column. The method was tested on two protein hydrolyzated.

EXPERIMENTAL

MTBSTFA, with 1% *tert.*-butyldimethylchlorosilane (TBDMCS) as a catalyst to facilitate derivatization of alcohols and amines, was obtained from Regis (Morton Grove, IL, U.S.A.). About 1–2 mg of dry amino acid(s) were placed in a vial and 100 μl of acetonitrile and 100 μl of MTBSTFA solution were added. Alternatively, aqueous mixtures of amino acids were added to a vial and dried in a vacuum oven at 40°C for 30 min prior to derivatization. Retention times were identified for derivatives of each individual amino acid. Quantitative reaction of most amino acids required heating at 95°C for 1 h. A mixture containing all of the amino acids was differentially derivatized by heating at increasing temperatures for 1 h at each temperature. The vial was first heated at 25°C for 1 h, a sample taken for the gas chromatograph, reheated for 1 h at 60°C, sampled once again, and so on at 70, 86, 102 112 and 125°C. Samples were thoroughly stirred with a vortex device every 15 min to facilitate their solution and, therefore, reaction. By this method, an ideal reaction temperature could be determined to allow quantitative derivatization without deg-

radation of the derivatives. Several reaction solvents were also investigated: aceto-nitrile, tetrahydrofuran, carbon disulfide, and no solvent.

Derivatized amino acids were analyzed with a 60 m × 0.75 mm I.D., SPB-5 bonded silicone phase, 1 μm thick, on a wide-bore capillary glass column (Supelco, Bellefonte, PA, U.S.A.) using hydrogen as the carrier gas at 64 cm/s. On-column injection was used as recommended by some authors when analyzing amino acids on glass capillary columns[5]. Since temperature programming was used and hydrogen was used as the carrier gas, the carrier gas was regulated by flow control instead of pressure regulation to avoid a continuously falling baseline[6]. Temperature programming was used as follows (Tracor Model 560 gas chromatograph, Houston, TX, U.S.A.): 165°C for 5 min; 4°C/min to 300°C; 300°C for 5 min. Nitrogen at 40 ml/min was used as the makeup gas, hydrogen for the flame ionization detector was used at 15 ml/min (in addition to the carrier gas) and air at 300 ml/min.

To determine the precision and accuracy of the method, solutions containing approximately 2, 5, 12.5, 25, 50 or 100 μg of each amino acid and 50 μg of S-meth-ylcysteine (internal standard) were evaporated in triplicate, derivatized at 95°C for 1 h and injected. Since the injection volume was typically 1 μl, 10–500 ng of each amino acid were actually injected into the chromatograph. By applying linear regres-sion with the molar amount of each amino acid as the independent variable against the area of each amino acid divided by the area of S-methylcysteine (the internal standard), the calculated slope represents the molar relative detector response for a particular amino acid.

Two sample proteins, hen ovalbumin (99%, Sigma, St. Louis, MO, U.S.A.) and bovine serum albumin (96–99%, Sigma), were hydrolyzed in 6 N hydrochloric acid for 24 h in tubes first purged with nitrogen and then evacuated, then analyzed for amino acid composition as described above.

RESULTS AND DISCUSSION

Of the solvents tested, acetonitrile was by far superior in terms of amount of derivatization for a given temperature and time, as well as for the least amount of degradation as indicated by extraneous peaks. Derivatization without solvent was not as rapid as using acetonitrile and also led to more extraneous peaks.

The separation of the twenty amino acids is shown in Fig. 1 with their retention times. Norleucine and S-methylcysteine were added as internal standards. All of the twenty amino acids commonly found in proteins, except for glutamate and arginine, were resolved. Arginine was not derivatized until the reaction temperature exceeded 95°C, and when it was derivatized it gave two peaks, the larger of which co-eluted with glutamate. It is possible that they may be separable using a fused-silica column or mass spectrometer to quantitate both of these components.

As shown in Fig. 2, the relative amounts of amino acids present after reaction at each temperature of the stepwise derivatization scheme reveal that there is a trade off between quantitative derivatization and decomposition of some of the derivatized amino acids. The curve for threonine is typical for serine, lysine, and histidine; the curve for glutamine is typical for norleucine and asparagine; and the curve for valine is typical for the remaining of the twenty amino acids. Of the protein amino acids, only glutamine, and to a smaller degree, asparagine, break down under the conditions of derivatization.
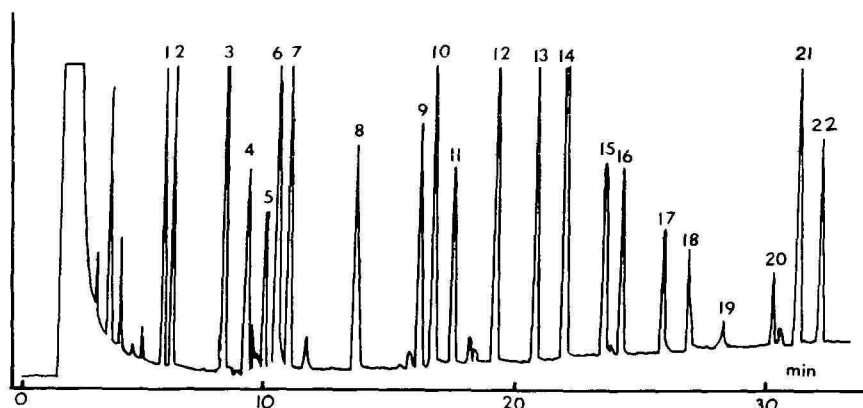
Fig. 1. Separation of the twenty common amino acids, norleucine, and S-methylcysteine. Retention times (min) are given in parentheses; some amino acids not present (NP) are also given. Amounts: 25–100 ng of each amino acid. Peaks: 1 = alanine (5.68); 2 = glycine (6.04); homocysteine thiolactone (7.09 NP); 3 = valine (8.08); 4 = leucine (9.00); partially underivatized serine (9.41 NP); 5 = isoleucine (9.77); 6 = norleucine (10.19); 7 = proline (10.72); hypotaurine (12.74 NP); 8 = S-methylcysteine (13.31); 9 = methionine (15.78); 10 = serine (16.33); 11 = threonine (17.09); ethionine (17.45 NP); 12 = phenylalanine (18.78); 13 = aspartate (20.38); 14 = cysteine (21.56); 15 = glutamate/arginine (23.11); methionine sulfone (23.62 NP); 16 = asparagine (23.83); homocysteine (24.17 NP); cysteine sulfinic acid (24.32 NP); 17 = lysine (25.47); 18 = glutamine (26.46); 19 = arginine-2 (27.75); 20 = histidine (29.92); 21 = tyrosine (31.74); 22 = tryptophan (31.74); methionine DL-sulfoxide (27.00 and 24.23 NP).

The precision and accuracy of this method as a means of quantitating free amino acids is shown in Table I. Linear regression was applied to the triplicate samples at concentrations of 10, 25, 62.5, 125, 250 or 500 ng of amino acid per μl injected after derivatization. Values for glutamate and alanine were corrected for small peaks which were present in a reagent blank. Excellent linear fits of the data were obtained for each amino acid. If any of the three values at a single concentration for a particular amino acid were not within 20% of the mean, those three values were then not included in the regression analysis. This eliminated the 10 ng/μl concentration for cysteine, glutamate, asparagine, lysine, glutamine, histidine and tryptophan, in-
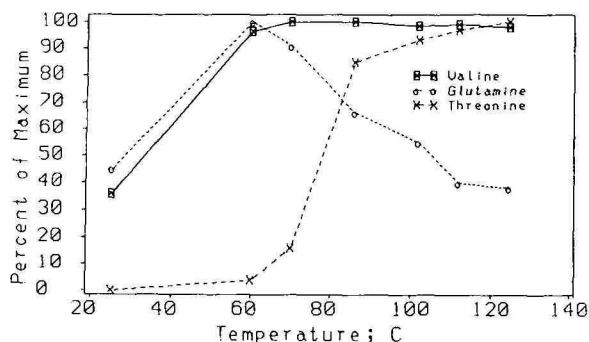

Fig. 2. Stepwise derivatization of some representative amino acids. The amino acids were held at each temperature for 1 h, sampled and heated at the next highest temperature for 1 h.

TABLE I

LINEAR REGRESSION ANALYSIS OF RELATIVE MOLAR RESPONSE AGAINST AMINO ACID CONCENTRATIONS

Relative molar response was calculated as the peak area of the amino acid divided by the peak area of the internal standard, S-methylcysteine.

Amino acid	No. of observations	Slope*	Intercept	Coefficient of determination
Alanine	18	1.007 (±0.8)	0.004	0.999
Glycine	18	0.893 (±1.2)	0.006	0.999
Valine	18	1.106 (±1.4)	0.016	0.999
Leucine	18	1.135 (±1.4)	0.021	0.999
Isoleucine	18	1.171 (±1.4)	0.031	0.999
Proline	18	1.095 (±2.0)	−0.047	0.999
Methionine	18	1.020 (±1.5)	0.015	0.999
Serine	18	1.293 (±0.8)	−0.004	0.999
Threonine	18	0.876 (±5.4)	0.003	0.993
Phenylalanine	18	1.371 (±0.8)	0.015	0.999
Aspartate	18	1.412 (±1.1)	0.010	0.999
Cysteine	12	1.236 (±1.3)	−0.029	0.999
Glutamate	12	1.209 (±4.9)	0.056	0.997
Asparagine	15	1.023 (±2.2)	−0.049	0.999
Lysine	15	1.325 (±2.7)	−0.056	0.999
Glutamine	15	0.885 (±5.3)	−0.096	0.994
Histidine	12	1.118 (±5.2)	−0.187	0.996
Tyrosine	18	1.723 (±4.2)	−0.007	0.996
Tryptophan	15	1.160 (±4.2)	−0.054	0.996

* Numbers in parentheses indicate the 95% confidence interval of the slope as % of slope.

TABLE II

AMINO ACID ANALYSIS OF BOVINE SERUM ALBUMIN AND HEN OVALBUMIN

Amino acid	Composition of bovine serum albumin (%)		Composition of hen ovalbumin (%)	
	Experimental	Literature*	Experimental	Literature**
Alanine	4.44	4.99	4.89	5.36
Glycine	1.46	1.38	2.39	2.32
Valine	4.46	5.01	5.07	5.96
Leucine	8.58	10.59	6.48	7.94
Isoleucine	1.96	2.25	4.10	6.04
Proline	3.90	4.01	2.62	3.04
Methionine	0.68	0.71	3.40	1.57
Serine	3.01	3.50	6.11	6.75
Threonine	3.75	4.95	3.00	3.42
Phenylalanine	4.88	5.87	5.36	6.86
Aspartate	7.44	9.43	7.78	8.04
Glutamate	10.97	14.48	10.93	14.48
Lysine	12.00	11.25	6.67	5.52
Histidine	2.54	3.54	1.61	2.08
Tyrosine	3.84	4.56	3.41	3.33

* From refs. 7 and 8.
** From ref. 9.

dicating that the methods may not be sensitive at this level for these particular amino acids. The 25 ng/μl values for histidine and glutamate were also excluded. Cysteine concentration at 500 ng/μl was not within the linear range; however, all other amino acids tested exhibited a linear response through a maximum concentration of 500 ng/μl.

The excellent resolution and linear response makes this method suitable for analysis of biological samples. The method has been applied to the determination of the amino acid composition of bovine serum albumin and hen ovalbumin (Table II). There is good agreement between the literature values and those obtained by this method. S-Methylcysteine made a good internal standard as it was stable to hydrolysis and its derivatization product is also stable; furthermore, it is usually not found in biological samples in appreciable quantities.

Besides increased stability over trimethylsilyl derivatives, TBDMS derivatives of amino acids offer several advantages over other derivatives used for gas chromatography. It is a simple (one-step) and rapid derivatization process. This derivative has interesting application to gas chromatography–mass spectrometric analysis due to the frequently prominent ion at 57 units less than the molecular ion[10]. Amino acid analysis has not been studied extensively by capillary gas chromatography of the TBDMS derivatives. The method described here should provide for a straightforward approach to be taken in amino analysis of a wide array of biological media.

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Note

Resolution of (+)- and (−)-α-difluoromethylornithine by capillary gas chromatography

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(Received February 6th, 1986)

α-Difluoromethylornithine (DFMO, MDL 71.782, I, Fig. 1) is a selective, enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC). Inhibition of this enzyme, which catalyses the initial, rate-limiting step in the biosynthesis of putrescine and the polyamines spermine and spermidine, leads to *in vivo* depletion of

$$R_1-NH-CH_2-CH_2-CH_2-\underset{\underset{CO_2R_2}{|}}{\overset{\overset{X}{|}}{C}}-NH-R_1$$
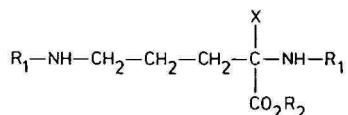
Compound	R_1	R_2	X	Ion m/z
I	−H	−H	−CHF$_2$	−
II	−H	−H	−H	−
III	−COCF$_2$CF$_3$	−C$_3$H$_7$	−H	216
IV	−COCF$_2$CF$_3$	−C$_3$H$_7$	−CHF$_2$	266
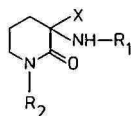
Compound	R_1	R_2	X	Ion m/z
V	−H	−H	−CHF$_2$	−
VI	−COCF$_2$CF$_3$	−H	−CHF$_2$	270
VII	−COCF$_2$CF$_3$	−COCF$_2$CF$_3$	−CHF$_2$	266
VIII	−COCF$_2$CF$_3$	−H	−H	260
IX	−COCF$_2$CF$_3$	−COCF$_2$CF$_3$	−H	388

Fig. 1. Structural formulae of DFMO and derivatives. Numbers refer to compounds as cited in text.

those compounds. The chemotherapeutic implications of the inhibition of polyamine biosynthesis have recently been reviewed[1]. While DFMO is currently available as a racemic mixture, only the (−)-isomer shows significant ODC-inhibiting activity. Hence, in any clinical studies with DFMO, it will be the plasma and tissue levels of the pharmacologically more active enantiomer which are of particular interest. The significance of such stereochemical considerations in pharmacokinetics and clinical pharmacology has recently been emphatically pointed out by Ariens[2]. A previously published assay for DFMO using automated amino acid analysis did not distinguish between the two enantiomers[3].

The enantiomers of ornithine (ORN, II) along with those of many other amino acids have previously been resolved as their di-pentafluoropropionamide propyl esters (*e.g.* di-PFP-ORN-isopropyl ester, III) by capillary gas chromatography on a commercially available chiral stationary phase[4]. The enantiomers of the analogous derivative of DFMO (di-PFP-DFMO-isopropyl ester, IV) failed to resolve on this phase under a wide range of operating conditions. A new approach, involving the lactamisation of DFMO to form 3-amino-3-(difluoromethyl)-2-piperidone (DFMO-lactam, MDL 71.880, V) was adopted.

This compound was further derivatised to the corresponding mono- or di-pentafluoropropionamide, the enantiomers of which were subsequently resolved by capillary gas chromatography. This method may provide a useful basis for establishing a stereospecific assay for DFMO in biological samples.

MATERIALS AND METHODS

(±)-DFMO (I), (+)-DFMO (I), and (±)-DFMO-lactam (V) were gifts from Merrell-Dow Pharmaceuticals. (±)-Ornithine, (+)-ornithine and (−)-ornithine were purchased from Sigma (St. Louis, MO, U.S.A.). Hexamethyldisilazane (HMDS) and pentafluoropropionic anhydride (PFPA) were purchased from Pierce (Rockford, IL, U.S.A.) and were used without further purification. Analytical reagent (AR) grade dichloromethane and acetonitrile were redistilled and dried over molecular sieve (BDH type 4A). AR grade benzene was redistilled and dried over sodium wire.

Preparation of derivatives

(±)-di-PFP-ORN-isopropyl ester (III) was prepared as described previously by Frank *et al.*[4].

(±)-di-PFP-DFMO-isopropyl ester (IV) was also prepared by this method.

(±)-DFMO-lactam (V) was prepared from (±)-DFMO by a modification of the method described by Pellegata *et al.*[5] for the corresponding lactamisation of ornithine. (±)-DFMO hydrochloride (2.36 mg, 10 μmol) was refluxed for 24 h in a mixture of acetonitrile (0.5 ml) and HMDS (0.2 ml). The reaction mixture was evaporated to dryness under a stream of dry nitrogen.

(±)-PFP-DFMO-lactam (VI) was prepared by dissolving the residue from the previous reaction in dichloromethane (200 μl) and adding PFPA (20 μl). The mixture was then allowed to stand in a sealed vessel at room temperature for 20 min, before evaporation to dryness under a stream of dry nitrogen. The residue was then redissolved in benzene (20 μl).

(±)-di-PFP-DFMO-lactam (VII) was prepared in the same manner except that the acylation reaction was carried out at 80°C for 1 h.

(±)-PFP-ORN-lactam (VIII) and (±)-di-PFP-ORN-lactam (IX) were prepared from (±)-ornithine by the methods described above for the analagous derivatives of DFMO.

Corresponding derivatives of individual enantiomers were prepared by the same methods using the appropriate enantiomer of the starting material.

Gas chromatography–mass spectrometry

Results were acquired using a Pye 204 gas chromatograph equipped with an SGE Unijector capillary injection system and directly coupled, via a heated, glass-lined stainless-steel tube (0.5 mm I.D.) to a VG 70/70F mass spectrometer with 2035 datasystem. Chromatography was carried out on Chirasil-Val columns (Applied Science (Deerfield, IL, U.S.A.), 25 or 50 m long and 0.3 mm I.D. with a film thickness of 0.23 μm. Helium was used as the carrier gas at a flow-rate of 2 ml/min measured at atmospheric pressure and with the column at ambient temperature. Injections were made in the split mode with a split-ratio of 10:1. The oven temperature was programmed from 140 to 200°C at 4°C/min with the injection port and interface temperatures held at 250 and 220°C respectively.

Full mass spectra of reference compounds were recorded initially by direct insertion probe in the electron-impact ionisation mode at 70 eV and 4 kV accelerating voltage. Representative ions for selected ion monitoring were chosen from the resulting spectra.

RESULTS AND DISCUSSION

Under chromatographic conditions which gave baseline resolution of the enantiomers of (±)-di-PFP-ORN-isopropyl ester, no resolution of the corresponding derivative of (±)-DFMO [(±)-di-PFP-DFMO-isopropyl ester] was achieved (Fig. 2). Similarly, the enantiomers of the analogous n-propyl, tert.-butyl and methyl ester derivatives of DFMO could not be resolved. The failure of the present system to resolve the DFMO derivatives must then be attributed to steric and/or electronic influences of the difluoromethyl substituent on the formation of the diastereomeric association complex responsible for the resolution of the analogous derivative of ornithine, as discussed by Frank et al.[6]. It was noted, however, that in some full-scan chromatograms of these DFMO derivatives, two small, partially resolved peaks of equal size were eluted after the derivatives of immediate interest. The mass spectra of these two peaks were identical and corresponded with that of PFP-DFMO-lactam derived from an authentic sample of DFMO-lactam. A second pair of peaks of equal size, baseline-resolved and eluting earlier than any of the other derivatives also occurred in some samples. Full-scan chromatograms showed that these peaks also had identical spectra and corresponded to di-PFP-DFMO-lactam.

Attempts to achieve lactamisation of DFMO via its methyl ester (as described for preparative scale work by Bey et al.[7]) proved unsuccessful at the analytical level. Subsequently, a method described by Pellegata et al.[5] to achieve lactamisation of ornithine was successfully adapted to the analytical scale.

By controlling the conditions of the subsequent halocylation step it was possible to limit the product of this reaction exclusively to PFP-DFMO-lactam. Although di-PFP-DFMO-lactam, formed under more vigorous conditions, gave some-
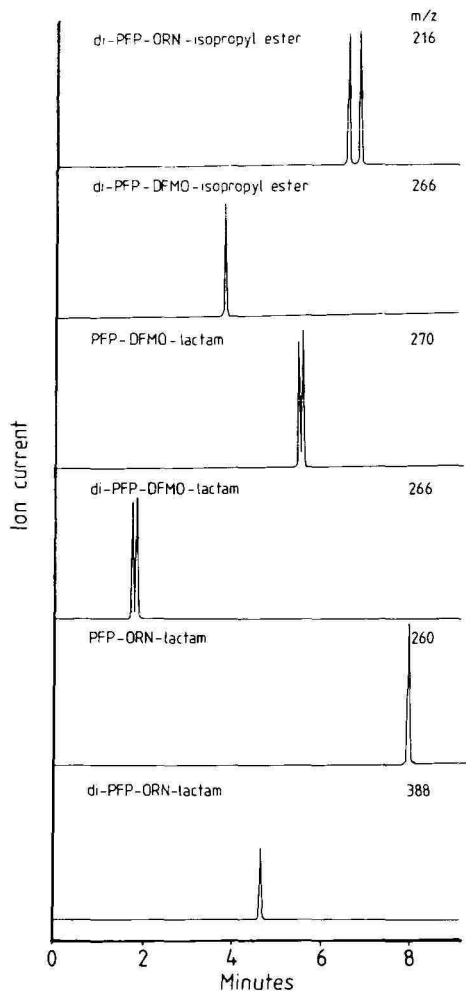
Fig. 2. Selected ion chromatograms of derivatives of ornithine and DFMO chromatographed on a 25-m Chirasil-valine column. Ion current in arbitrary units.
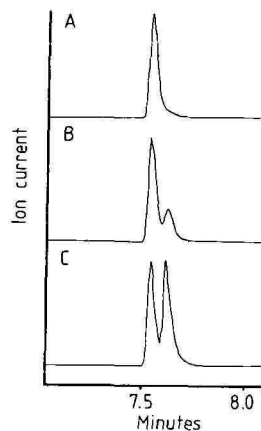
Fig. 3. Selected ion chromatograms (*m/z* 270) of PFP-DFMO-lactam. (A) Derived from (+)-DFMO; (B) derived from (+)-DFMO enriched by 25% with (±)-DFMO; (C) derived from (±)-DFMO. Ion current in arbitrary units.

what better resolution of the enantiomers and had an appreciably shorter retention time than PFP-DFMO-lactam (Fig. 2), it was found that the yield of the former derivative was capricious and hence would not be suitable as a basis for routine analytical work.

Initially, chromatography was carried out using a 25-m column. In an attempt to improve resolution of the more readily produced PFP-DFMO-lactam, subsequent studies were carried out using a 50-m column. Surprisingly, this yielded no measurable improvement in resolution at a flow-rate optimised according to the method of Davies[8], suggesting that factors other than those generally considered to influence

resolution using non-chiral stationary phases may have a predominant effect when using the present chiral system.

Paradoxically, both (±)-PFP-ORN-lactam and (±)-di-PFP-ORN-lactam were not resolved by the present system (Fig. 2) suggesting that the stereospecific interaction of the DFMO derivatives with the chiral stationary phase depends on the presence of both the lactam ring and the difluoromethyl substituent and that the basis of this interaction differs considerably from that responsible for the resolution of ornithine.

Previous studies have shown that the (−)-isomers of amino acids derivatised in the more conventional manner described by Frank et al.[4] consistently elute first when chromatographed on Chirasil-valine columns. However, the study of Pellegata et al.[5] noted that the lactamisation of ornithine proceeded with inversion of optical activity. In the absence of sufficient (+)-DFMO to determine the optical activity of PFP-DFMO-lactam derived from it (and hence whether inversion of optical activity had also occurred in the lactamisation of DFMO) it was necessary to demonstrate which of the two PFP-DFMO-lactam peaks was derived from (+)-DFMO. This was achieved by chromatographing a sample derived from (+)-DFMO and subsequently rechromatographing the same material enriched by 25% (on a molar basis) with material derived from (±)-DFMO. This clearly demonstrated that the first peak of the enantiomeric pair was due to material derived from (+)-DFMO (Fig. 3).

Resolution of the enantiomers of DFMO as the PFP-DFMO-lactam derivatives may thus provide a useful basis for the stereospecific analysis of DFMO in biological samples and further work to this end is being undertaken.

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Note

Preparative scale isolation of 11-*cis*-retinal from isomeric retinal mixture by centrifugal partition chromatography

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(Received January 9th, 1986)

Retinals perform crucial functions in nature because they constitute the chromophore of various rhodopsins. This is exemplified by the visual pigment rhodopsin and the proton-translocating bacteriorohodopsin which contain 11-*cis*-retinal and *trans*-retinal as the chromophore, respectively[1–3]. A common method used in recent years to clarify the properties of these pigments is to study regenerated rhodopsins; these are prepared by detachment of the retinal from the apoprotein and reincubation with various retinal analogues[4] including isotopically labeled compounds. A preparative method for the purification of these light- and heat-sensitive retinals, natural and synthetic, thus becomes highly desirable. Being the most polar and stablest isomer, *trans*-retinal can be obtained rather readily by high-performance liquid chromatography (HPLC)[5,6] or flash chromatography; however, this does not apply to other isomers which are harder to purify. The same is true for synthetic retinal analogues[4] since the elution order generally follows that of natural series. Retinal purification is thus mostly confined to analytical or semi-preparative HPLC which, however, produces only sub-mg quantities per injection. Obtaining the crucial 11-*cis*-isomer in a pure state is particularly difficult because it usually elutes in the middle of the various isomers[6,7]. However, in one case using a μBondapak CN column, 1% ether in hexane[8], the 11-*cis*-isomer could be made to elute first in the four-component mixture, 11-*cis* → 13-*cis* → 9-*cis* and all-*trans* (the normal phase elution sequence is 13-*cis*, 11-*cis*, 9-*cis* and all-*trans*). The μBondapak CN column thus facilitates collection of the 11-*cis* isomer but on an analytical scale.

We recently observed that the elution sequence of the four retinals resulting from the above mentioned support is also obtained in the liquid–liquid distribution pattern with certain solvent systems (see below). This then allowed us to purify relatively large quantities of 11-*cis*-retinal using centrifugal counter-current (or centrifugal partition) chromatography (CPC)[9–12], a technique which has recently been applied to extremely labile natural products[13,14].

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EXPERIMENTAL

Instrumentation and reagents

A centrifugal partition chromatography apparatus (Model CPC-B92-N) manufactured by Sanki Engineering (Nagaokakyo, Kyoto, Japan) was used. It consisted of a continuous flow centrifuge (B92-N) containing twelve cartridges (total volume 180 ml) made of monochlorotrifluoroethylene resin, a constant flow pump (LBP II type triple plungers), a valve connection unit (FCU-II) linked to a 2-ml PTFE sample loop injector, an electric power control unit (PCB II), a recorder, and a fraction collector. Detection of isomeric retinals were carried out by a flow-cell UV spectrophotometer (Jasco UVIDEC-100) and monitored at 420 nm (sensitivity 2.0 a.u.f.s.); this wavelength was used because the intensity at the absorption maxima around 360 nm was too high.

A mixture of cyclohexane–pentane–acetonitrile (5:2:5, v/v) containing 0.1% methanol (MCB solvents, HPLC-grade) served as the biphasic solvent system. The upper phase (mobile phase) was pumped through the lower stationary phase at 2.5 ml/min, thus adopting the "ascending" mode. The rotation speed was adjusted to 1500 rpm which stabilized the pump discharge pressure at 700 p.s.i.

Thin-layer chromatographic (TLC) analysis of the CPC fractions was performed on Whatman HPTLC plates using the upper phase of the solvent system as developing solvent and 3% (w/v) vanillin in absolute ethanol containing 1% (v/v) sulfuric acid as detector. A Perkin-Elmer chromatography station including a Series 4 pump and an ISI 100 auto-sampler both controlled by a PE 7000 laboratory computer was used to monitor the purity of isolated retinals and to identify each isomer by comparison of HPLC retention times with those of authentic samples. The column, 250 × 4.5 mm I.D., was packed with 5 μm/100 Å spherical silica, (YMC, Mt. Freedom, NJ, U.S.A.); the solvent system was 5% ether in *n*-hexane, flow-rate 1 ml/min, or 1,1,2-trichlorotrifluoroethane–methyl *tert.*-butyl ether mixture (97:3)[7], flow-rate 0.7 ml/min. A Kratos spectroflow 773 variable-wavelength detector, monitoring wavelength 360 nm, was employed.

Sample

The retinal mixture (160 mg) was a crude "11-*cis*-" retinal sample stored at −20°C for several years. This was dissolved in 2 ml of the lower solvent phase and injected into the continuous flow centrifuge. The authentic retinals used as reference were available in our laboratory. All experiments were carried out at room temperature and under dim red light.

RESULTS AND DISCUSSION

The centrifugal partition chromatogram obtained under these experimental conditions is shown in Fig. 1. The separation was complete after 2.5 h. The elution sequence of the 11-*cis* and 13-*cis* isomers under these CPC conditions is the reverse of that resulting from silica gel adsorption chromatography with an alkane–diethyl ether solvent system. The shaded portion of the first peak shown in Fig. 1 gave 50 mg of HPLC-pure 11-*cis*-retinal. The slowest eluting peak yielded 40 mg of HPLC-pure *trans*-retinal, whereas the middle fraction gave a total of 40 mg of a mixture of
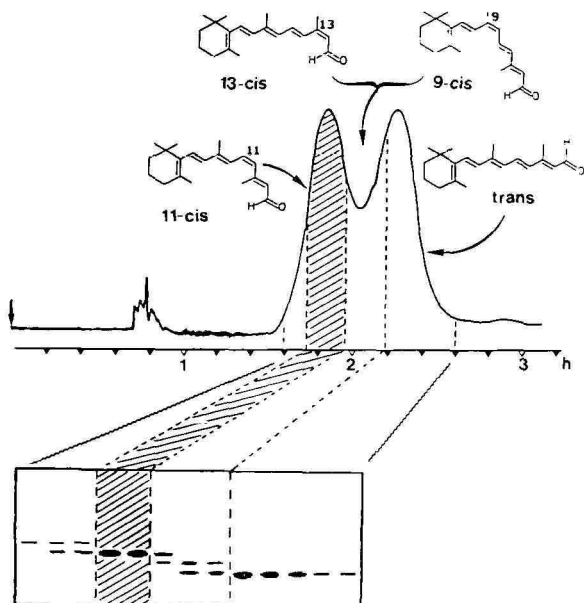
Fig. 1. Upper trace: CPC of 160 mg of crude isomeric retinal mixture consisting of four major retinals. Lower trace: TLC trace of CPC fractions. The solvent system and other experimental conditions are described in the text. Note that the TLC solvent system employed here results in the same retention time sequence as that of CPC.

the four isomers. The volume of mobile phase pumped was *ca*. 240 ml before elution of the solutes and an additional 150 ml for completion of analysis. The rest of the sample which remained in the stationary phase was a polar tar which did not move on TLC. It weighed 30 mg, thus accounting for recovery of all material and showing that pre-purification of crude sample is not necessary.

The centrifugal partition (or counter-current) chromatography technique[9-14] combines the advantages of classical counter-current chromatography and preparative flow-through centrifuge and allows efficient separation of sensitive material on a gram scale. Moreover, in the present application on retinals, the non-aqueous solvent system permitted recovery of solutes under non-isomerizing low temperature conditions.

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MONTH	O 1985	N 1985	D 1985	J	F	M	A	M	J	
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