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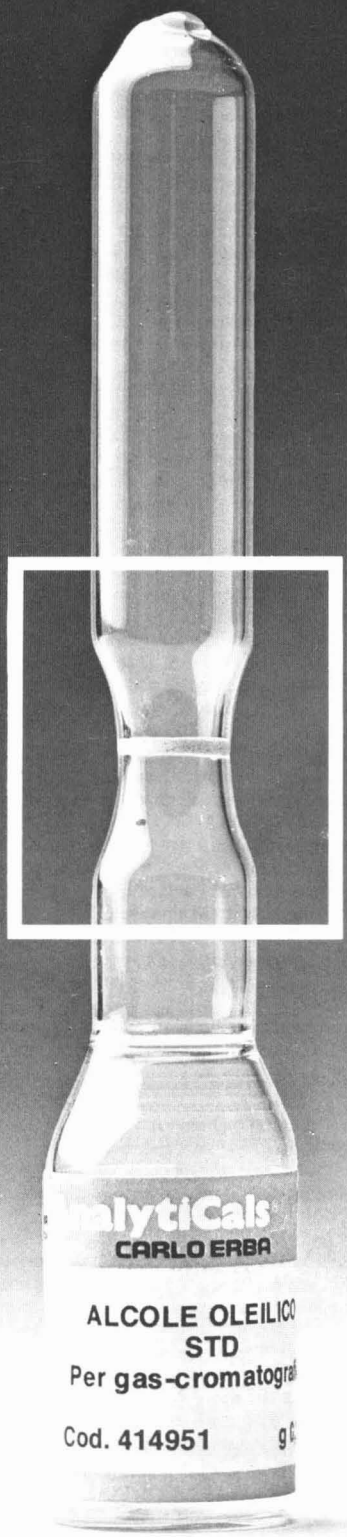

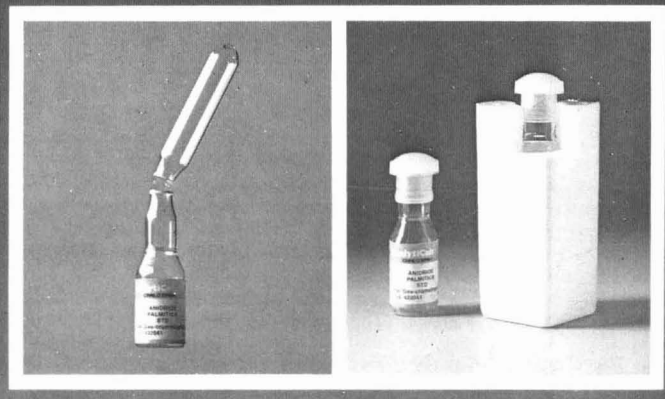

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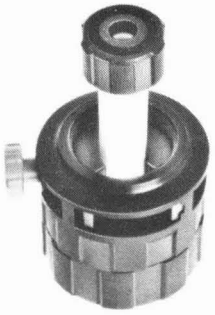

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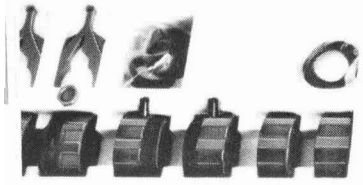

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COMPUTER SIMULATION AND EXPERIMENTAL VALIDATION OF ISOELECTRIC FOCUSING IN AMPHOLINE-FREE SYSTEMS

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SUMMARY

An abbreviated version of a mathematical model of the steady state in isoelectric focusing is presented. Details of the mathematical transformations leading to a model suitable for computer implementation and numerical solution are given in the Appendix. The model describes the structure of concentration, pH, conductivity and potential gradients arising from the focusing of electrochemically defined ampholytes. Some of the results of computer simulations of two and three component systems are of particular interest to the experimentalist and are presented together with experimental validation of this model.

INTRODUCTION

Isoelectric focusing (IEF) is a powerful separation method first proposed by Kolin[1] and reduced to practice by Svensson[2]. The method has since gained widespread popularity, mainly because of the development by Vesterberg[3] of Ampholine (Tradename of LKB, Bromma, Sweden), a polyaminopolycarboxylic acid buffer mixture produced by random polymerization. The constituents of this mixture migrate electrophoretically to stationary zones in the separation field, thereby establishing a relatively stable "natural" pH gradient.

Ampholine and other similar commercial carrier ampholytes are well suited for analytical purposes, but for preparative applications there are several shortcomings: they result in product contamination with a chemically and biologically ill-defined material; they are rather expensive; and they do not allow for custom-design of pH profiles. Our recent development of a large scale preparative IEF apparatus based on a novel recycling principle[4–6] has prompted us to reexamine the possible development of Ampholine-free buffering systems using chemically defined mixtures.

In the last few years there have been several attempts to develop such buffering systems, using a variety of approaches[7–14]. In particular, Chrambach and his coworkers[7–9] have made many attempts to construct useful pH gradients using various ampholyte mixtures, while others[10–13] have also considered non-amphoteric buffer mixtures. In contrast, Caspers *et al.*[14] have used amino acids and buffers to modulate the pH gradients formed by Ampholine. It was generally appreciated that the quan-

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tities of individual components utilized and their specific characteristics must uniquely determine the shape of the pH gradient established, the sample capacity, and the resolution. It should also be appreciated that most of these approaches were purely empirical and have suffered from the absence of an explicit model demonstrating the contribution of individual components to gradient formation.

It appeared, therefore, that further progress required a better understanding of IEF, *i.e.*, a theory describing the structure of concentration, pH, conductivity and potential gradients generated by focusing a limited number of electrochemically defined ampholytes to the steady state. Unfortunately, the very usefulness of Ampholine has also alleviated the need for the development of such a theory. Most prior theoretical treatments of IEF assumed the preexistence of linear pH and/or conductivity gradients[15,16], a situation fairly approximated if Ampholine is employed. The best model of IEF hitherto available is that of Almgren[17], but it has only partially fulfilled the need, as its simplifying assumptions were too limiting for practical applications.

A suitable detailed theory of IEF has recently been developed by Palusinski *et al.*[18] in a form suitable for computer modeling. In the present paper we wish to report an abbreviated version of the model, limited to two ampholytes, as well as some of the results of computer simulations of IEF which we believe are of particular interest to the experimentalist. In addition, we are presenting some of our laboratory data which validate, at least qualitatively, these computer simulations.

MATERIALS AND METHODS

A DEC-10 processor is used for the maintenance and editing of our programs. All computations are performed on the CYBER 175 processor by transferring the files from DEC-10 on simple commands. The differential equations are solved using DARE P software[19]. The program can readily be implemented on any computer provided with a FORTRAN compiler and will be supplied on request.

The amino acids and peptides used in this work were purchased from Vega Biochemicals (Tucson, AZ, U.S.A.). Other chemicals were purchased from Eastman-Kodak (Rochester, NY, U.S.A.).

Experimental IEF was carried out in three systems: thin-layer polyacrylamide gels, the LKB Model 8101 focusing column, and the recycling IEF apparatus developed in this laboratory[4–6]. Gel pH and concentration profiles were determined by sectioning the polyacrylamide gels in 0.5-cm long segments and elution in 0.01 *M* KCl. Amino acid concentrations were determined using the Durrum automatic amino acid analyzer.

MODEL FORMULATION

A detailed account of our model of the steady state in IEF has been recently presented[18]. Briefly, the treatment considers only simple ampholytes, *i.e.*, it is assumed that only the two apparent dissociation constants (p*K*s) nearest the isoelectric point (p*I*) of an ampholyte influence its behavior in the steady state. The analysis is one-dimensional in a stationary fluid with no electroosmosis and at constant temperature.

At present, we wish to present a simplified version of our model, restricting it to binary mixtures only. The construction of the model rests upon the following basic concepts:

(a) The concentrations of component subspecies are described by equations of chemical equilibria.

(b) In the steady state, a balance exists between the mass transports resulting from electromigration and from diffusion.

(c) Electroneutrality is preserved within the relevant physical dimensions.

In such a system, the conductivity σ is given by

$$\sigma = e \sum_{i=1}^{i=8} z_i^2 w_i n_i \tag{1}$$

where e is the Faraday constant (96,500 Coulombs/mole), z_i valences (dimensionless 0, +1, −1), w_i mobility coefficients ($cm^2/V \cdot sec$), n_i concentrations ($mole/cm^3$). Subscripts 1 and 2 refer to hydrogen and hydroxyl ions, 3 and 6 to the neutral species of ampholytes 1 and 2, respectively, 4 and 7 to the cationic species of the two ampholytes, and 5 and 8 to their anionic species. The mobility coefficients w_i are related to the conventional electrophoretic mobilities μ_i by $\mu_i = w_i z_i$, and to the diffusion constants D_i by the Einstein relation $D_i = RTw_i/e$.

The ampholyte conservation equation is

$$e(w_4 n_4 - w_5 n_5)\frac{d\varphi}{dx} + RT\left(w_3 \cdot \frac{dn_3}{dx} + w_4 \cdot \frac{dn_4}{dx} + w_5 \cdot \frac{dn_5}{dx}\right) = 0 \tag{2a}$$

$$e(w_7 n_7 - w_8 n_8)\frac{d\varphi}{dx} + RT\left(w_6 \cdot \frac{dn_6}{dx} + w_7 \cdot \frac{dn_7}{dx} + w_8 \cdot \frac{dn_8}{dx}\right) = 0 \tag{2b}$$

where φ is the local potential (V), x is the distance (cm), from the anodic end of the column, R is the universal gas constant ($g \cdot cm^2/sec^2$) and T is the absolute temperature (°K).

The conservation of charge equation has the form

$$\sigma \frac{d\varphi}{dx} + RT \sum_{i=1}^{i=8} z_i w_i \frac{dn_i}{dx} = -J \tag{3}$$

where J is the current density (A/cm^2).

In addition to the above differential equations the algebraic relation

$$n_1 - n_2 + n_4 - n_5 + n_7 - n_8 = 0 \tag{4}$$

describes the electroneutrality of the solution.

The chemical equilibria for each ampholyte are described by the usual proton dissociation equations defining the constants K_{11} and K_{12} for the first ampholyte and the constants K_{21} and K_{22} for the second ampholyte.

The above equations are non-linear and cannot be solved analytically. Thus, a numerical approach was necessary, requiring the transformations of equations described in the Appendix. The algebraic equations are used to eliminate all concentration terms except those of the two neutral subspecies, n_3 of the first ampholyte and n_6 of the second ampholyte. These transformations produce

$$\frac{dn_3}{dx} = \frac{J}{RT} \cdot a\,(n_3, n_6)\left(\frac{w_4}{K_{11}} \cdot n_1 - w_5 K_{12} \cdot \frac{1}{n_1}\right) n_3 \tag{5}$$

$$\frac{dn_6}{dx} = \frac{J}{RT} \cdot b\,(n_3, n_6)\left(\frac{w_7}{K_{21}} \cdot n_1 - w_8 K_{22} \cdot \frac{1}{n_1}\right) n_6 \tag{6}$$

which define the profiles of the concentrations of the above two species along the IEF column axis. The functions a and b are defined in the Appendix.

Eqns. 5 and 6 lead to the following important conclusions:

(a) The two factors, the applied current density and the distance over which the gradients are developed, have a reciprocal relationship. The same relative profiles will be obtained as long as the product of column length (x) and applied current density (J) is kept constant. Thus, the voltage needed to obtain full development of the gradients across any column is independent of its length.

(b) The sign of the concentration gradients dn_3/dx and dn_6/dx is determined by the sign of the terms in parenthesis in eqns. 5 and 6, since all other factors are positive. This yields the following inequalities

$$n_1^2 > w_5 K_{12} K_{11} / w_4 \tag{7}$$

$$n_1^2 < w_8 K_{21} K_{22} / w_7 \tag{8}$$

for the growth of n_3 and decrease of n_6. These inequalities explain the concentration maxima reported in Fig. 3.

RESULTS OF SIMULATION STUDIES

The foregoing equations have been suitably transformed for computer implementation, as partially shown in the Appendix, and have been exercised to simulate the IEF process in binary and tertiary mixtures of ampholytes[18]. At present we wish to present some of the simulation studies which are of particular importance for experimental IEF. Our studies comprised modeling with hypothetical ampholytes, to which arbitrary electrochemical parameters were assigned, as well as with real compounds for which literature cited parameters were used. Most simulations have been conducted with binary mixtures, as the rational selection of more complex mixtures requires a prior understanding of the interaction between any two ampholytes focused adjacently in the separation field.

Our model requires the input of the following parameters:

(a) Dimensions of the column: all data to be presented assume a length of 1 cm and a cross-section of 1 cm^2.

(b) The mobility coefficient w of all components of the mixture. Different mobility coefficients can be assigned to the positive, neutral and negative subspecies of each ampholyte, as they may vary by as much as 5% due to the greater hydration of the carboxylate group[20]. Most of the mobility coefficients for real ampholytes were calculated from the diffusion constants given by Longsworth[21] or arrived at by interpolation as a function of molecular volumes[21,22].

(c) The dissociation constants of all components. For ampholytes with more than two dissociating groups, only the constants nearest the pI were used. This results in a negligible error if the remaining dissociation constants differ by more than an order of magnitude from those used.

(d) The initiating (boundary) concentrations of the two ampholytes at any arbitrary point along the column axis.

(e) The applied current density.

The output of a computer simulation is:

(a) The concentration profiles of all subspecies of both ampholytes along the column axis, as well as the total amounts in the column.

(b) The pH, conductivity, and electrical potential profiles along the column axis, as well as other complementary data.

Simulations with hypothetical ampholytes

The assignment of arbitrary electrochemical parameters to hypothetical ampholytes has permitted a systematic evaluation of the effects of the input parameters on simulated IEF.

The data presented in the composite Fig. 1 illustrate the effects of applied current density on the concentration, pH and conductivity profiles obtained on simulated focusing of a binary mixture of ampholytes in the neutral pH region. The input parameters are listed in Table I. The plots labelled A were obtained at the lowest current density, plots labelled B and C at three and nine times higher current densities, respectively. The concentration plots show that the more acidic ampholyte (1) accumulates at the anode, the more basic one (2) accumulating at the cathode, their concentrations decreasing monotonically towards the opposite electrodes. As shown previously[18], the ampholytes are essentially isoelectric at the electrodes, with the proportion of charged species increasing as the total concentration decreases. As a result, the pH curves span the range between the isoelectric points of the two ampholytes (pI 6 and 7, respectively) and the conductivity is maximal in the center of the column, decreasing towards both electrodes. An increase in current density (plots B and C) results in the contraction of the effective distance over which full deployment of gradients is obtained. This is dictated by eqns. 5 and 6 which show that an X-fold increase in current density will result in an equivalent reduction in column length over which full deployment of profiles is obtained. The computer calculated total voltages for the above three systems were 0.43 (A), 1.53 (B), and 4.83 (C). These voltages are substantially lower than voltages customarily applied in IEF using commercial carrier ampholytes. Experimental work, which will be discussed, has confirmed this computer prediction. Thus at high applied potentials, no smooth pH or concentration profiles can be expected, the step-gradients obtained being more reminiscent of isotachophoresis than of IEF.

It should also be pointed out that in the above simulations the total amount of

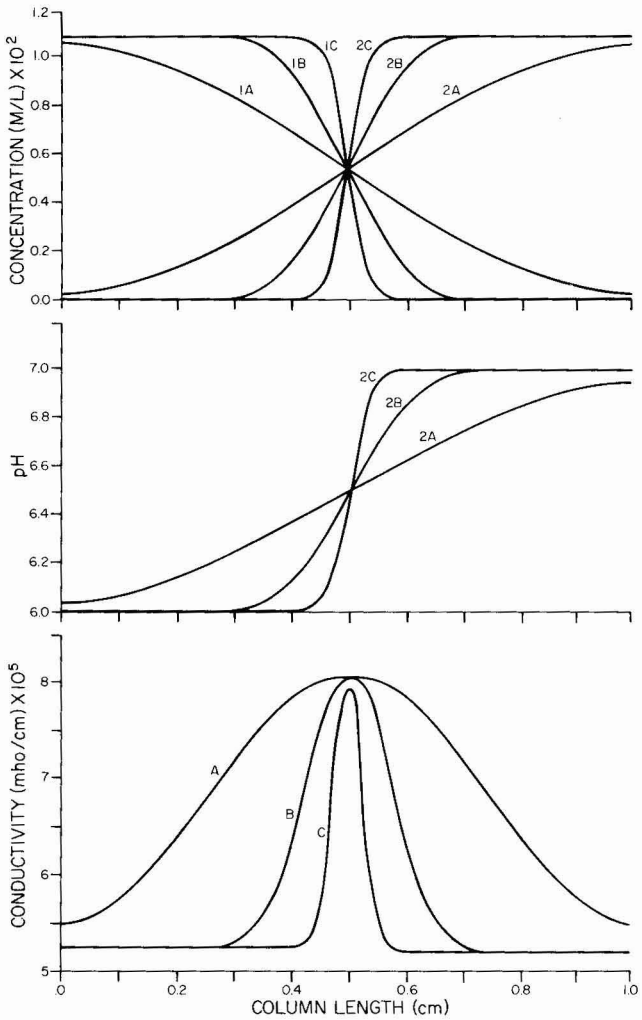


Fig. 1. Effects of current density on computer generated profiles of concentration, pH, and conductivity at the steady state in IEF. Current densities: A, 0.0288 mA/cm^2; B, 0.0864 mA/cm^2; C, 0.2592 mA/cm^2. All other input parameters are listed in Table 1.

each ampholyte in the column remains unchanged by the alteration of applied current, since the profiles remain symmetrical. The general shape of the plots shown in Fig. 1 will remain virtually unchanged if concentrations and applied current are varied by the same factor. Total ampholyte content and conductivity will be, of course, proportional to concentration. Similarly, the plots are not changed noticeably if mobility coefficients for both ampholytes and the applied current are altered by the same factor.

In the above plots, all profiles are essentially symmetrical. This is due to the symmetry of the assigned electrochemical parameters and the pH range being close to neutrality. In particular, the mobility coefficients for all subspecies of both ampho-

TABLE I

INPUT ELECTROCHEMICAL PARAMETERS FOR SIMULATIONS REPORTED IN FIGS. 1–4

Figure No.	*Component No.*	pK_1	*pI*	pK_2	*Mobility coefficient* $\times 10^4$		
					Cation	*Neutral*	*Anion*
1	1A–C	5.0	6.0	7.0	3.0	3.0	3.0
	2A–C	6.0	7.0	8.0	3.0	3.0	3.0
2	1D	5.0	6.0	7.0	3.0	3.0	3.0
	2D	6.0	7.0	8.0	3.0	3.0	3.0
	1E	5.0	6.0	7.0	3.15	3.0	2.85
	2E	6.0	7.0	8.0	3.15	3.0	2.85
3–4	1F	1.5	2.5	3.5	3.0	3.0	3.0
	2F	2.5	3.5	4.5	3.0	3.0	3.0
	1G	1.5	2.5	3.5	3.15	3.0	2.85
	2G	2.5	3.5	4.5	3.15	3.0	2.85
	1H	9.5	10.5	11.5	3.0	3.0	3.0
	2H	10.5	11.5	12.5	3.0	3.0	3.0

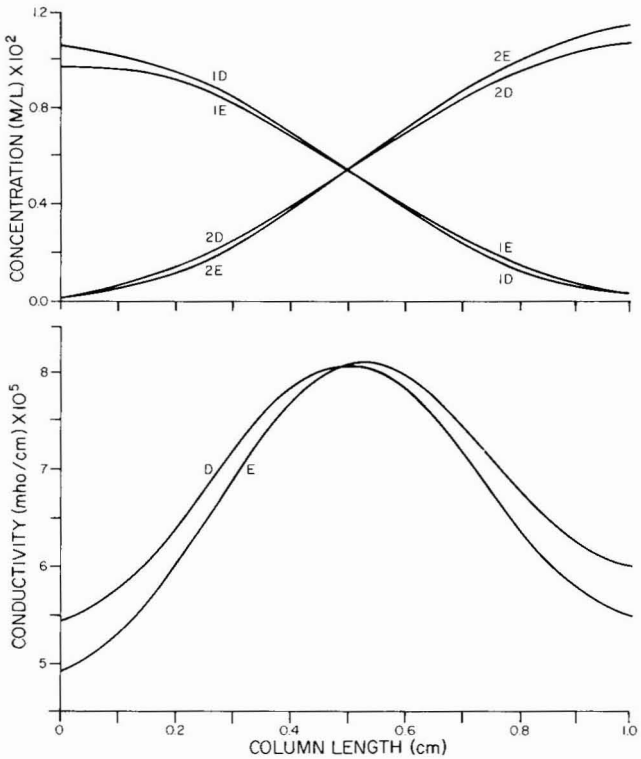


Fig. 2. Effects of differences in mobility coefficients of cationic, neutral and anionic ampholyte species on computer generated profiles of concentration and conductivity in IEF. Current densities were 0.0288 mA/cm² for both systems, all other input parameters being listed in Table I.

lytes were assumed to be identical, which is not the case in actuality. If the cationic subspecies of both ampholytes are assumed to have a higher mobility coefficient than the more hydrated anionic subspecies[20], the profiles are no longer symmetrical, as shown in Fig. 2. The input parameters are presented in Table I. The pH curves were omitted, as they were essentially superimposable.

Symmetry is also lost if the simulation is performed outside of the neutral pH region, due to the contributions of hydrogen or hydroxyl ions to the conductivity. This is illustrated in Fig. 3, which shows the concentration and conductivity plots for binary mixtures similar to those presented in the previous figures, except that they were centered around pH 3 (plot F for components with identical mobility coefficients for all subspecies and plot G where higher cationic mobility coefficients were assigned) and around pH 11 (plot H, with identical mobility coefficients). The simulation input parameters are also listed in Table I. Because of the higher conductivities of these systems significantly higher current densities had to be applied than in the previous simulations.

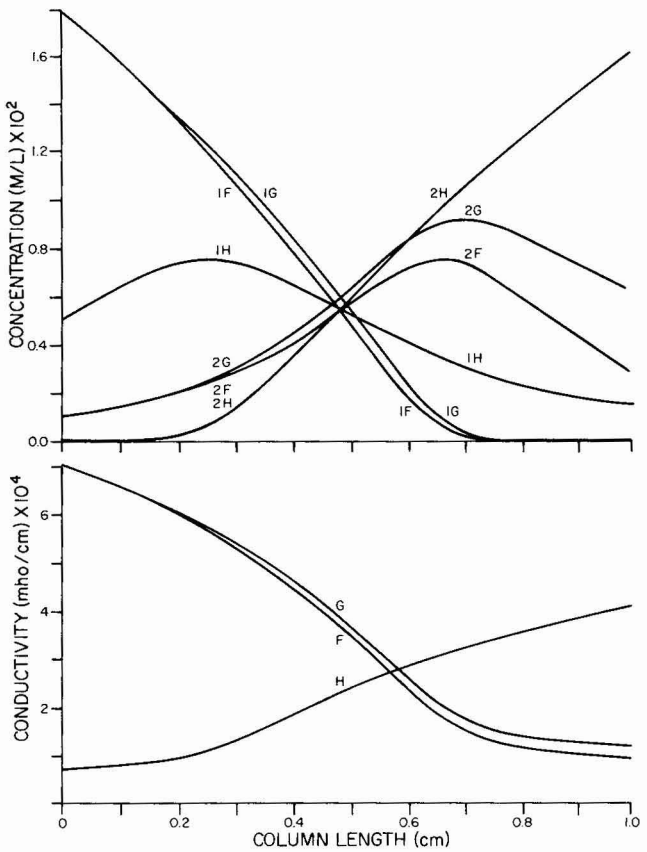


Fig. 3. Effects of low or high pH values on computer generated profiles of concentration and conductivity at the steady state in IEF. Current densities: F and G, 0.20 mA/cm^2; H, 0.10 mA/cm^2. All other parameters are listed in Table I.

The most surprising result of these simulations was that the concentrations of some ampholytes no longer rise monotonically towards the electrodes: at low pH values, the concentration of the more basic ampholytes, 2F and 2G, go through a distinct maximum, while at high pH values the concentration of the more acidic ampholyte, 1H, goes through a maximum. This behavior was quite unexpected, but is explained by the inequalities 7 and 8.

The corresponding pH profiles are given in Fig. 4. It is significant that the pH profiles no longer span the range between the p*I* values of the two constituent ampholytes, but are shifted towards more neutral values. This is in line with the observation of Rilbe[22], who has shown that the pH values of dilute solutions of isoelectric ampholytes do not correspond to their p*I* values (except in the neutral pH region), because of the "buffering action" of water.

In our previous paper[18] we examined the effects of varying the spread between the p*K* values for each ampholyte in binary mixtures, and the effects of varying the

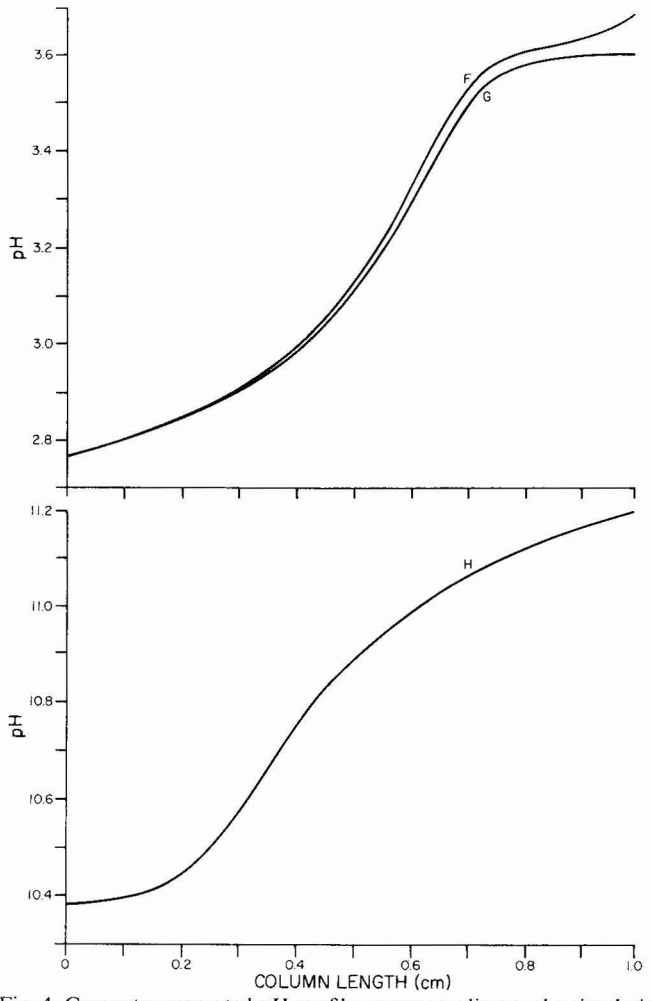


Fig. 4. Computer generated pH profiles corresponding to the simulation data presented in Fig. 3.

spread between the p*I* values of the two components. These simulations were performed in the neutral region, as in acid or alkaline regions the superimposition of the pH effects demonstrated above obscures the results. These pH effects must also be considered if concentrations or mobilities are altered.

The above simulations as well as those previously presented[18] illustrate the effects of the most important experimental variables: the electrochemical properties of the ampholytes (mobility, p*K*, p*I*) and the physical parameters (column dimensions, applied current, and concentrations).

Simulations have also been performed with hypothetical three component systems and will be presented in a future paper. A simulation of a three component system with actual compounds is presented in a following section.

Computer simulations of IEF of real compounds

Following the simulations of IEF of imaginary compounds, the same procedure was applied to real ampholytes. Unfortunately, while hypothetical compounds provide a limitless choice of parameters, there are far fewer actual ampholytes to choose from, *i.e.*, ampholytes for which the required electrochemical parameters have been reported in the literature. The binary systems investigated are listed in Table II. The last column refers to results of the simulation: good refers to systems

TABLE II

INPUT PARAMETERS AND RESULTS OF IEF SIMULATIONS OF REAL AMPHOLYTES

System components	pK_1	pK_2	ΔpK	ΔpI	*Mobility coefficient* $\times 10^4$	*Current* (mA/cm^2)	*Results*
m-ABA	3.12	4.74	1.62	2.96	3.01	0.16	Aborted
β-Ala	3.6	10.19	6.59		3.63		
m-ABA	3.12	4.74	1.62	0.99	3.01	0.18	Poor
α-Asp-His	3.02	6.82	3.80		2.11		
p-ABA	2.41	4.85	2.44	3.36	3.28	0.24	Aborted
β-Ala	3.60	10.19	6.59		3.63		
p-ABA	2.41	4.85	2.44	2.07	3.28	0.18	Aborted
Tyr-Tyr	3.52	7.68	4.16		1.56		
β-Ala	3.60	10.19	6.59	0.70	3.63	0.36	Poor
His	6.0	9.17	3.17		2.85		
β-Ala-His	6.83	9.51	2.68	2.59	2.30	0.098	Good
Arg	9.04	12.48	3.44		2.71		
β-Ala-His	6.83	9.51	2.68	1.53	2.30	0.108	Good
Orn	8.65	10.76	2.11		3.11		
α-OH-Asn	2.31	7.17	4.86	2.68	2.95	0.08	Good
His-Gly	6.27	8.57	2.30		2.40		
Asp	1.88	3.65	1.90	0.98	2.97	0.40	Good
m-ABA	3.12	4.74	1.62		3.01		

TABLE II *(continued)*

System components	pK_1	pK_2	ΔpK	ΔpI	*Mobility coefficient* $\times 10^4$	*Current* (mA/cm^2)	*Results*
Asp	1.88	3.65	1.90	0.58	2.97	0.80	Conc. max. *p*-ABA
p-ABA	2.41	4.85	2.44		3.28		
Asp	1.88	3.65	1.9	0.28	2.97	0.144	Conc. max. Glu
Glu	2.19	4.25	2.13		3.23		
Asp	1.88	3.65	1.90	2.75	2.97	0.40	Aborted
Gly-Gly	3.15	8.25	5.10		3.08		
α-Asp-His	3.02	6.82	3.80	2.67	2.11	0.079	Good
His	6.0	9.17	3.17		2.85		
α-Asp-His	3.02	6.82	3.80	0.93	2.11	0.25	Fair
Isogln	3.81	7.88	4.07		2.96		
β-Ala-His	6.83	9.51	2.68	1.53	2.30	0.096	Good
Orn	8.65	10.76	2.11		3.11		
Glu	2.19	4.25	1.90	2.47	3.23	0.24	Aborted
Gly-Gly	3.15	8.25	5.10		3.08		
Gly	2.35	9.78	7.43	3.63	4.11	0.10	Good
Orn	8.65	10.76	2.11		3.11		
Gly-Gly	3.15	8.25	5.10	2.47	3.08	0.08	Good
β-Ala-His	6.83	9.51	2.68		2.30		
His	6.0	9.17	3.17	0.58	2.85	0.18	Good
β-Ala-His	6.83	9.51	2.68		2.3		
His	6.0	9.17	3.17	0.30	2.85	0.384	Poor
His-His	6.8	7.8	1.0		1.49		
His	6.0	9.17	3.17	1.09	2.85	0.096	Good
Tyr-Arg	7.55	9.80	2.25		1.58		
His-Gly	5.8	7.82	2.02	1.38	2.4	0.20	Good
β-Ala-His	6.83	9.51	2.68		2.3		
His-His	6.8	7.8	1.0	0.89	2.40	0.30	Fair
β-Ala-His	6.83	9.51	2.68		2.30		
Isogln	3.02	6.82	4.07	1.74	2.96	0.09	Good
His	6.0	9.17	3.17		2.85		
Tri-Gly	3.23	8.09	4.86	1.94	2.59	0.076	Good
His	6.0	9.17	3.17		2.85		
Tyr-Arg	7.55	9.80	2.25	2.08	1.58	0.079	Good
Arg	9.04	12.48	3.44		2.71		
Tyr-Arg	7.55	9.80	2.25	1.02	1.58	0.108	Good
Orn	8.65	10.76	2.11		3.11		
Tyr-Tyr	3.52	7.68	4.16	1.99	1.56	0.06	Good
His	6.0	9.17	3.17		2.85		

where reasonably symmetrical concentration profiles were obtained and reasonably useful pH gradients were projected. Poor refers to concentration profiles which were quite unsymmetrical and where the pH gradients did not appear to be promising. The computer aborted a number of runs, indicating a very unsatisfactory pair, where the concentration of one of the ampholytes reached vanishingly low values. Two systems, aspartic acid–*p*-aminobenzoic acid (Asp/*p*-ABA) and aspartic acid–glutamic acid (Asp/Glu), exhibited concentration maxima of the more basic components (*p*-ABA and Glu, respectively), as were shown for hypothetical compounds in Fig. 3.

To illustrate these results, Fig. 5 shows the quite symmetrical concentration profiles obtained with the aspartic acid–*m*-aminobenzoic acid (Asp/*m*-ABA) system, resulting in a nearly linear pH profile. This can be contrasted with the histidylhistidine–histidine (His-His/His) pair (Fig. 6), which we expected to be an excellent combination, both being "good" ampholytes, *i.e.*, having quite small ΔpK values. The simulation data present grossly unsymmetrical concentration profiles and the pH profile appears to be virtually useless.

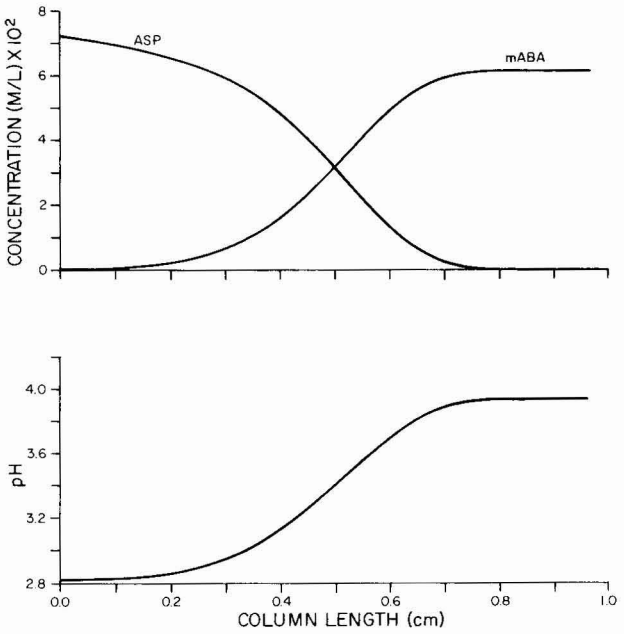


Fig. 5. Computer generated concentration and pH profiles for the focusing of aspartic acid (pI = 2.95, ΔpK = 1.91) and *m*-aminobenzoic acid (pI = 3.93, ΔpK = 1.62) at a current density of 0.40 mA/cm^2. The computer calculated voltage across the column was 0.59 V.

Experimental validation of computer simulations

The experimental validation studies were aimed at confirming the two most striking results of the simulations: (i) the low voltages needed to obtain full deployment of the concentration and pH gradients and (ii) the steep gradients obtained at higher voltages.

The existence of the steep gradients was confirmed experimentally in the LKB focusing column, using the system glutamic acid–histidine and the indicator dye

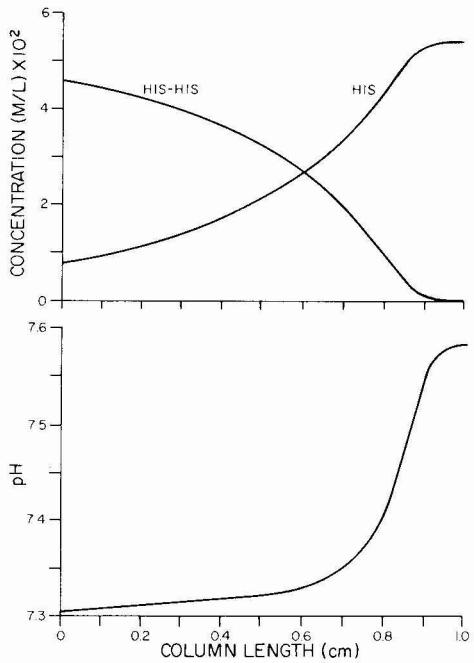
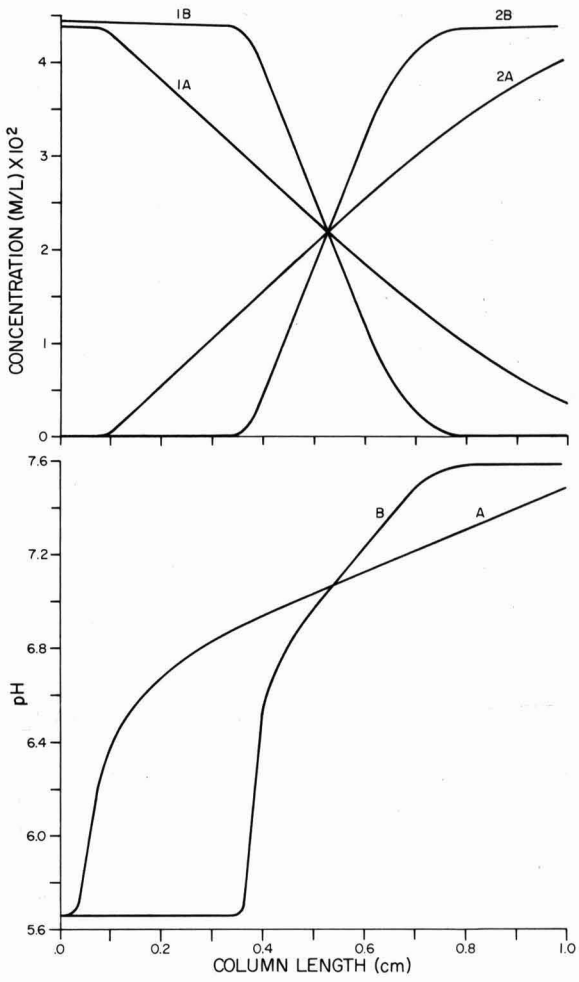


Fig. 6. Computer generated concentration and pH profiles for the focusing of histidylhistidine (pI = 7.3, ΔpK = 1) and histidine (pI = 7.59, ΔpK = 3.17) at a current density of 0.38 mA/cm^2. The computer calculated voltage across the column was 2.4 V.

methyl red as sample. The focusing was carried out at 1000 V, for 43 h. At the end of the focusing, the dye was localized at the center of the column in a narrow band of only 6 mm width, the upper half of the band being yellow and the lower red. Thus, this confirmed the existence of a step pH gradient between pH 3.2 and pH 7.6, which was projected by the simulation.

The validation of focusing obtainable at very low voltages was undertaken using the glycylglycylglycine–histidine (Tri-Gly/His) system. Fig. 7 shows the computer predicted concentration and pH gradients for this system, chosen because it encompasses the pH range where hemoglobin should focus. The model was exercised at the current densities of 0.07 mA/cm^2 and 0.2 mA/cm^2, the computer predicted voltages being 1.3 V and 10 V, respectively.

It was deemed essential to validate the prediction that focusing could be obtained at such low voltages. A sample of porcine hemoglobin was focused in a thin-layer polyacrylamide gel, 5% acrylamide, 0.17% bisacrylamide, 20% glycerol, made 25 mM with respect to Tri-Gly and His. A constant voltage of only 5 V was applied across a distance of 10 cm of the gel, and the focusing was continued for 6 days. The final current density was 0.042 mA/cm^2. The pH gradient was similar to the predicted one, except that it was skewed towards lower pH values, presumably due to the persulfate used as the polymerization catalyst. A photograph of the gel is shown in Fig. 8 and it clearly shows that the bulk of the hemoglobin has focused, leaving a trailing edge, there being two additional rather sharply focused protein bands at lower pH values. This experiment has been repeated a number of times, with quite



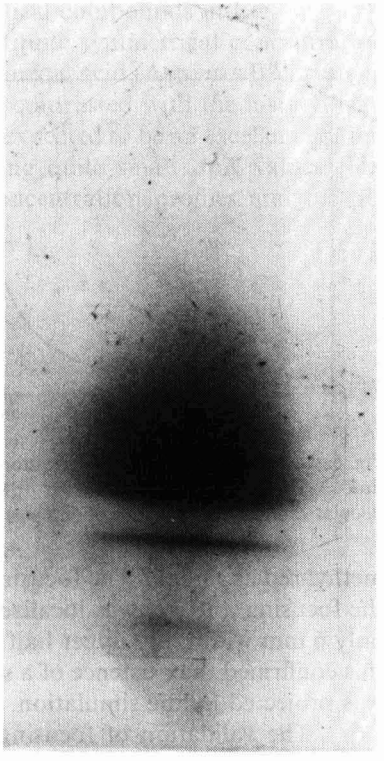


Fig. 7. Computer generated concentration and pH profiles for the focusing of glycylglycylglycine (1A and 1B) (pI = 5.65, ΔpK = 4.86) and histidine (2A and 2B) (pI = 7.59, ΔpK = 3.17). Current densities: A, 0.07 mA/cm^2 at 1.2 V; B, 0.20 mA/cm^2 at 10.1 V.

Fig. 8. Photograph of the focused pattern of a sample of porcine hemoglobin in a polyacrylamide gel. The pH gradient was established using 25 mM each of glycylglycylglycine and histidine. Focusing was carried out for 6 days at a potential of 5 V across 10 cm of the gel. The anode is at the bottom.

similar results. Analysis of the distribution of the two components, Tri-Gly and His, has shown that their concentrations overlap at such low voltages, as predicted in plot A of Fig. 7, but are nearly completely separated at voltages higher than roughly 20 V.

The simulation of a three-component system, glutamic acid–cycloserine–histidine (Glu/C-Ser/His), is shown in Fig. 9. The concentration profiles show that the two end components (Glu and His) are widely separated by a non-Gaussian zone of C-Ser. It should be pointed out that the concentration of C-Ser in this zone is not constant, but decreases slightly from glutamic acid to histidine. The pH profile shows two steep pH gradients due to the interactions of Glu/C-Ser and C-Ser/His, respec-

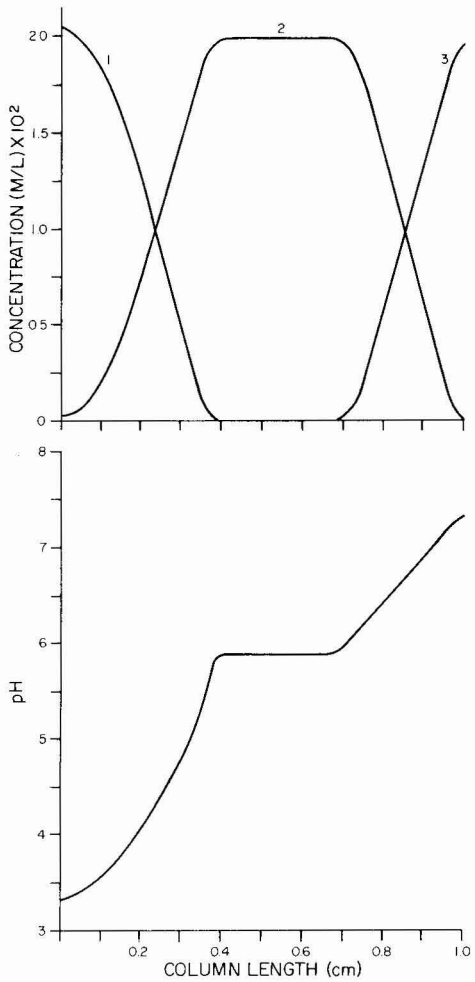


Fig. 9. Computer generated concentration and pH profiles for the three-component mixture of glutamic acid (1) (pI = 3.23, ΔpK = 2.13), cycloserine (2) (pI = 5.90, ΔpK = 3.0), and histidine (3) (pI = 7.59, ΔpK = 3.17) at a current density of 0.13 mA/cm^2 and a potential of 1.8 V.

tively. The conductivity profile, not displayed, exhibits two peaks, corresponding to the two steep pH gradients, separated by a "conductivity gap" due to C-Ser. Such a system was expected to resolve any two proteins with pI values on either side of the C-Ser generated pH plateau. This has been verified in polyacrylamide gels, the LKB focusing column and the recycling IEF apparatus[4–6] developed in our laboratory. Fig. 10 shows the separation of bromophenol blue-stained albumin and red hemoglobin in the recycling apparatus. With all three methods excellent focusing of the two proteins was obtained, the blue albumin focusing in the Glu/C-Ser pH gradient, and the red hemoglobin in the C-Ser/His pH gradient. In the recycling IEF apparatus, the same final protein distribution was obtained whether the albumin and hemoglobin were added as a mixed sample or whether their relative initial applications were

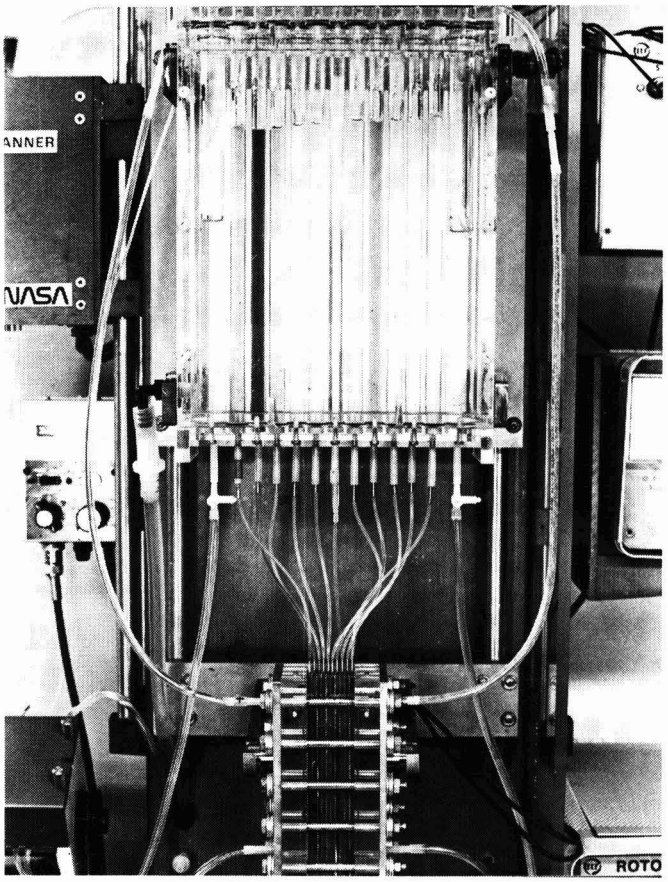


Fig. 10. Photograph of the preparative separation of 500 mg each of porcine hemoglobin (pI = 7.4) and human serum albumin (pI = 4.8) in the recycling isoelectric focusing apparatus (for its description see refs. 4–6). The pH gradient was established using the system modeled in Fig. 9, containing 10 mM each of glutamic acid, cycloserine and histidine. The photograph shows the ten-channel heat exchange reservoir at the top and the focusing cell at the bottom. The mixed sample was added to channel 5 of the heat exchanger at the time of current application. The separation was complete in 1 h, with a final focusing voltage of 300 V. Hemoglobin focused mainly in channel 2 (from the cathode), with trace amounts in adjacent channels, and albumin in channel 7 of the heat exchanger. The albumin was visualized by the addition of a small amount of bromophenol blue dye.

reversed with regard to their final position, *i.e.*, the albumin was applied in the channel where hemoglobin was finally focused and *vice versa*. It is significant, however, that this was obtained only if the proteins were added initially, before the ampholytes were focused. Once the pH gradient was formed, no migration of added samples was obtained, due to the conductivity gap. This separation is controlled by the pI of C-Ser (pI = 5.9), which can be replaced by any other ampholyte with similar characteristics. In fact, comparable separation of the above two proteins was obtained with Gly-Gly (pI = 5.7) or with Gly-Gly-Gly (pI = 5.65) replacing C-Ser.

DISCUSSION

The mathematical model of IEF presented in this and the previous paper[18] was developed in the hope that it would be of value in the design of experimental buffer systems using chemically defined components, thus eliminating the need for the ill-defined commercial carrier ampholytes (Ampholine, etc.) in preparative applications. The experimental data presented here have validated, at least qualitatively, the predictions of the computer simulations. These simulations have been carried out only for two- and three-component systems, as more complex mixtures may be constructed only when the interaction between adjacent pairs of ampholytes is understood.

Some general conclusions can be drawn about the relationships between simulation input parameters and output data. These are summarized in Table III, where a value of 1 is assigned to all "standard" input and output data, and the independently varied parameters are in italics. Each horizontal row of the table summarizes the effects, if any, of the change of the underlined input parameter on the other parameters.

TABLE III

COMPUTER PREDICTED EFFECTS OF INPUT PARAMETERS IN FOCUSING

Computer input parameters				*Computer output data*			
Current	*Column length*	*Mobility coefficient*	*Initiating concn.**	*Voltage gradient*	*Total volts*	*Conductivity gradient*	*Total amount*
2	1	1	1	1	1	1	1
1	*2*	1	1	1/2	1	1	2
1	1	*1/2*	1	1	1	1/2	1
1	1	1	*1/2*	1	1	1/2	1/2

* Initiating concentrations are assigned at the midpoint of the column, where the concentration profiles of the two ampholytes are made to intersect.

For example, lines 1 and 2 indicate that reducing the applied current by half requires doubling the column length to obtain identical profiles, with the exception of the voltage gradient profile, which is halved. The longer column will contain twice the total amount of ampholytes, however, the total voltage required is the same. This holds true for all pH regions.

Similarly, line 3 indicates that reducing the mobility factor by half will reduce the conductivity by nearly the same amount. Thus, half the current will be needed for equivalent gradient deployment. Line 4 indicates that halving the initial concentration of both ampholytes will produce the same effect on the profiles as halving the mobility factor, except that it reduces the total ampholyte content of the column. These two relationships hold true only in the neutral pH region. The further from neutrality, the greater the differences in profiles obtained, due to the contributions of hydrogen and hydroxyl ions to the conductivity, as shown in Fig. 3.

In addition, the following conclusions may help in the selection of ampholytes. The most important factors to be considered are the spread between the p*I* values

(ΔpI) of the two ampholytes, and the spread between the pK values (ΔpK) for each ampholyte:

(1) In the neutral pH region, the pH gradient will cover the span between the pI values of the two components[18]. In acidic or alkaline pH ranges, there will be a shift towards neutrality, as shown in Fig. 3.

(2) Quite similar concentration and pH profiles will be obtained at constant ΔpI, irrespective of the ΔpK values of the individual components[18]. However, the greater the ΔpK, the lower the buffering of the system.

(3) Any ionizable group will buffer only within one pH unit of its dissociation constant. Thus, in a two component mixture only the basic group (pK_{12}) of the more acidic component and the acid group (pK_{21}) of the more basic component may exert a buffering action within the established pH gradient.

All of the above three factors should be considered in the selection of components, the optimum being the smallest possible ΔpI and ΔpK values which provide buffering in the desired pH region.

The most surprising outcome of the simulations is the low current density and, therefore, low voltage needed to obtain full gradient deployment, irrespective of column length. These low voltages are sufficient to cause the focusing of proteins, but only with impractically long focusing times. The application of reasonable potentials appears to require on the order of five components per centimeter of column length, as first suggested by Almgren[17]. With fewer components, and at the high potentials usually used in IEF with commercial carrier ampholytes, step-gradients will be obtained, which are more reminiscent of isotachophoresis[23,24] than of IEF. The similarity with isotachophoresis is also shown in the concentration plots in the three component system shown in Fig. 9. The concentration profile of the intermediate component, C-Ser is not Gaussian, but presents a "square" profile, typical of isotachophoresis.

In conclusion, it would appear that for analytical purposes, where nearly linear gradients spanning a relatively broad pH range are desired, custom designed mixtures of the type discussed do not seem to be promising. A great number of components would be required, some of which would certainly be prohibitively expensive. Thus, Ampholine and other similar commercial carrier ampholytes, remain the buffering agent of choice.

For preparative purposes high voltages are important to minimize the focusing time and maximize the resolution. Any separations will be dependent on finding appropriate "spacer" ampholytes with the desired electrochemical properties, primarily the right pI value. The similarity with isotachophoresis is again evident, except that selecting spacers is easier in isotachophoresis, where the net mobilities can be modified by pH adjustment, whereas the pI values of ampholytes are fixed. Nevertheless, the usefulness of simple ampholyte mixtures for preparative purposes was demonstrated by our separation of albumin from hemoglobin, using the three component system Glu/C-Ser/His. While this is an admittedly simple separation, in a subsequent paper we will report the separation of human hemoglobins A and C, using leucylhistidine as the spacer and C-Ser and Lys as terminal components.

APPENDIX

Basic transformation relations

The equations describing the chemical equilibria

$$K_{11} = n_1 n_3 / n_4 \qquad K_{12} = n_1 n_5 / n_3$$

and

$$K_{21} = n_1 n_6 / n_7 \qquad K_{22} = n_1 n_8 / n_6$$

for the first and second ampholyte respectively, together with the well-known water dissociation constant $K_w = n_1 n_2$ and the electroneutrality eqn. 4 (see text) form a system of algebraic equations which can be used to express the variables n_1, n_2, n_4, n_5, n_7 and n_8 in terms of n_3 and n_6, which represent the concentration of the uncharged species. Solving these equations by simple elimination we obtain

$$n_1 = \left(\frac{K_w + K_{12} n_3 + K_{22} n_6}{1 + (n_3/K_{11}) + (n_6/K_{21})} \right)^{\frac{1}{2}} \tag{A1}$$

and

$$n_2 = K_w / n_1 \tag{A2}$$
$$n_4 = n_1 n_3 / K_{11} \tag{A3}$$
$$n_5 = K_{12} n_3 / n_1 \tag{A4}$$
$$n_7 = n_1 n_6 / K_{21} \tag{A5}$$
$$n_8 = K_{22} n_6 / n_1 \tag{A6}$$

The variable n_1 was not eliminated to simplify the writing. The relations A1–A6 are used to eliminate all variables except n_3 and n_6 in differential eqns. 2 and 3 (see text). These equations contain the derivatives of all variables, therefore the same elimination must be performed with respect to all of these derivatives, with the exception of dn_3/dx and dn_6/dx.

Using eqn. A1 it is derived

$$\frac{dn_1}{dx} = A_1 \cdot \frac{dn_3}{dx} + A_2 \cdot \frac{dn_6}{dx} \tag{A7}$$

where $A_1 = \bar{A_1} A$ and $A_2 = \bar{A_2} A$.

The symbols A, $\bar{A_1}$ and $\bar{A_2}$ represent the following quantities:

$$A = \tfrac{1}{2} \left[K_{11} K_{21} / (K_{12} n_3 + K_{22} n_6 + K_w)\,(K_{21} n_3 + K_{11} n_6 + K_{11} K_{21})^3 \right]^{\frac{1}{2}} \tag{A8}$$

$$\bar{A_1} = K_{12} K_{11}\,(n_6 + K_{21}) - K_{21}\,(K_{22} n_6 + K_w) \tag{A9}$$

$$\bar{A_2} = K_{22} K_{21}\,(n_3 + K_{11}) - K_{11}\,(K_{12} n_3 + K_w) \tag{A10}$$

Analogously from eqns. A2–A6 it is derived:

$$\frac{dn_2}{dx} = -\frac{K_w}{n_1^2} \cdot A_1 \cdot \frac{dn_3}{dx} - A_2 \cdot \frac{K_w}{n_1^2} \cdot \frac{dn_6}{dx} \tag{A11}$$

$$\frac{dn_4}{dx} = \frac{1}{K_{11}} (n_3 A_1 + n_1) \frac{dn_3}{dx} + \frac{1}{K_{11}} \cdot n_3 A_2 \cdot \frac{dn_6}{dx} \tag{A12}$$

$$\frac{dn_5}{dx} = \frac{K_{12}}{n_1} \left(1 - \frac{n_3}{n_1} \cdot A_1\right) \frac{dn_3}{dx} - \frac{K_{12} n_3}{n_1^2} \cdot A_2 \cdot \frac{dn_6}{dx} \tag{A13}$$

$$\frac{dn_7}{dx} = \frac{n_6}{K_{21}} \cdot A_1 \cdot \frac{dn_3}{dx} + \frac{1}{K_{21}} (n_6 A_2 + n_1) \frac{dn_6}{dx} \tag{A14}$$

$$\frac{dn_8}{dx} = -\frac{K_{22} n_6}{n_1^2} \cdot A_1 \cdot \frac{dn_3}{dx} + \frac{K_{22}}{n_1} \left(1 - \frac{n_6}{n_1} \cdot A_2\right) \frac{dn_6}{dx} \tag{A15}$$

Transformation of model equations

Conservation of charge. We shall use the notation:

$$\kappa = \sum_{i=1}^{8} z_i^2 w_i n_i \tag{A16}$$

Note that the conductivity, s, can be expressed by $s = e\kappa$. Using eqns. A1–A15 in eqn. 3 results (after some manipulations) in

$$\frac{e}{RT} \cdot \frac{d\varphi}{dx} = -\frac{J}{RT} \cdot \frac{1}{\kappa} - C_1 \cdot \frac{dn_3}{dx} - C_2 \cdot \frac{dn_6}{dx} \tag{A17}$$

where:

$$C_1 = A_1 \cdot \frac{1}{n_1} + \frac{1}{\kappa} \left(\frac{w_4}{K_{11}} \cdot n_1 - \frac{w_5 K_{12}}{n_1}\right)$$

$$C_2 = A_2 \cdot \frac{1}{n_1} + \frac{1}{\kappa} \left(\frac{w_7}{K_{21}} \cdot n_1 - \frac{w_8 K_{22}}{n_1}\right)$$

Conservation of ampholytes. The eqns. 2a and 2b are divided by RT, then A17 is used to eliminate (e/RT) $(d\varphi/dx)$. Using eqns. A2–A6 and A11–A15 to eliminate n_4, n_5, n_7, n_8 and their derivatives we obtain (after some manipulations)

$$a_{11} \cdot \frac{dn_3}{dx} - a_{12} \cdot \frac{dn_6}{dx} = \frac{J}{RT} \cdot \frac{B_{11}}{\kappa} \tag{A18}$$

$$-a_{21} \cdot \frac{dn_3}{dx} + a_{22} \cdot \frac{dn_6}{dx} = \frac{J}{RT} \cdot B_{21} \tag{A19}$$

where:

$$a_{11} = w_3 + \frac{w_4}{K_{11}} \cdot n_1 + \frac{w_5 K_{12}}{n_1} - \frac{B_{11}^2}{\kappa} \cdot \frac{1}{n_3}$$

$$a_{12} = \frac{B_{11} B_{21}}{\kappa} \cdot \frac{1}{n_6}$$

$$a_{21} = \frac{B_{11} B_{21}}{\kappa} \cdot \frac{1}{n_3}$$

$$a_{22} = w_6 + \frac{w_7}{K_{21}} \cdot n_1 + \frac{w_8 K_{22}}{n_1} - \frac{B_{21}^2}{\kappa} \cdot \frac{1}{n_6}$$

$$B_{11} = \left(\frac{w_4}{K_{11}} - \frac{w_5 K_{12}}{n_1^2} \right) n_1 n_3$$

$$B_{21} = \left(\frac{w_7}{K_{21}} - w_8 K_{22} \cdot \frac{1}{n_1^2} \right) n_1 n_6$$

In order to obtain eqns. 5 and 6, it is necessary to invert the matrix A with non-linear entries a_{ij}. This is possible only when the matrix A is non-singular, *i.e.*, when its determinant is non-zero.

Matrix inversion and determinant computation. It is shown below that the determinant of matrix A (det A) is non-zero, therefore eqns. A18 and A19 can be presented in the form:

$$\frac{\mathrm{d}n_3}{\mathrm{d}x} = \frac{J}{RT} \cdot \frac{B_{11} a_{22} + B_{21} a_{12}}{\kappa \det A} \tag{A20}$$

$$\frac{\mathrm{d}n_6}{\mathrm{d}x} = \frac{J}{RT} \cdot \frac{a_{11} B_{21} + a_{21} B_{11}}{\kappa \det A} \tag{A21}$$

Taking into account the definitions of symbols and the expression for (κ det A) computed below, the eqns. A20 and A21 are transformed easily to forms 5 and 6, where:

$$a(n_3, n_6) = \frac{w_6 + (w_7 n_1 / K_{21}) + (w_8 K_{22} / n_1)}{\kappa \det A}$$

$$b(n_3, n_6) = \frac{w_3 + (w_4 n_1 / K_{11}) + (w_5 K_{12} / n_1)}{\kappa \det A}$$

Determinant computation. In order to simplify the manipulations we introduce the notation:

$$\begin{aligned} w_4 n_1/K_{11} &= p \qquad w_8 K_{22}/n_1 = s \\ w_5 K_{12}/n_1 &= q \qquad w_1 n_1 + w_2 K_w/n_1 = t \\ w_7 n_1/K_{21} &= r \end{aligned} \tag{A22}$$

Using eqns. A1–A6 in A16 and taking into account that $z_i = 1$ ($i = 1, 4, 7$), -1 ($i = 2, 5, 8$), 0 ($i = 3, 6$) we obtain

$$\kappa = w_1 n_1 + w_2 K_w \cdot \frac{1}{n_1} + \frac{w_4}{K_{11}} \cdot n_1 n_3 + w_5 K_{12} \cdot \frac{n_3}{n_1} + \frac{w_7}{K_{21}} \cdot n_1 n_6 + w_8 K_{22} \cdot \frac{n_6}{n_1} \tag{A23}$$

and using A22 we can write:

$$\kappa = t + (p + q) n_3 + (r + s) n_6$$

Using the definitions of a_{ij} (A18, A19) the determinant of matrix A (det $A = a_{11}a_{22} - a_{12}a_{21}$) can be computed and taking into account A22 expressed in the form:

$$\det A = (w_3 + p + q)(w_6 + r + s) - (w_3 + p + q)(r - s)^2 n_6 \cdot \frac{1}{\kappa} - (w_6 + r + s)(p - q)^2 n_3 \cdot \frac{1}{\kappa}$$

Using eqn. A23 and the above, the following expression results

$$\kappa \det A = (w_3 + p + q)[t(w_6 + r + s) + (r + s) n_6 w_6 + 4rsn_6] + (w_6 + r + s)[(p + q) n_3 w_3 + 4pqn_3]$$

which is needed in the eqns. A20 and A21. It also demonstrates that the determinant is not zero, since κ is positive.

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LIQUID CHROMATOGRAPHIC SEPARATION OF PURINES, PYRIMIDINES AND THEIR NUCLEOSIDES ON SILICA GEL COLUMNS

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SUMMARY

The high-performance liquid chromatography of pyrimidine and purine derivatives on plain microparticulate silica gel was studied. Mixtures of dichloromethane, methanol and aqueous ammonium formate–formic acid solutions were used as the mobile phase. Retention data are reported for more than 50 compounds, including the biochemically important nucleobases and nucleosides. By varying the composition of the eluent mixture the chromatographic system provides elution sequences and selectivities that differ markedly from those characteristic of reversed-phase liquid chromatography.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) on microparticulate octadecylsilica has become the method of choice for the separation of nucleosides and nucleic acid bases in recent years[1,2]. Nevertheless, in some instances plain silica gel was used successfully for the liquid chromatographic separation of these and related substances. Thus, some oxypurines were chromatographed[3] on a Bio-Sil A column with a solvent system consisting of diethyl ether, *n*-propanol and dilute acetic acid. Theobromine, theophylline and caffeine were separated on LiChrosorb SI 60 using dichloromethane–ethanol–water mixtures[4] and also on Partisil 10 with dichloromethane–methanol–aqueous ammonia eluents[5]. Further examples include the separation of five uracil derivatives on LiChrosorb SI 100 using a mixture of water-saturated dichloromethane and isopropanol as the eluent[6] and the chromatography of adenine, adenosine and several cytokinins on a Hypersil column with an ammoniacal chloroform–methanol–water system[7]. The most interesting example in this series is the paper of Evans *et al.*[8] on the separation of urinary pyrimidine bases and nucleosides on LiChrosorb SI 100 with mobile phases composed of dichloromethane, methanol and ammonium formate buffer.

All of the mobile phase systems mentioned above consist of a relatively non-polar, water-immiscible organic solvent, an alcohol and a small amount of either pure water or an aqueous solution of an ionizable substance. No matter what the exact

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retention mechanism on silica gel with such hydro-organic eluents may be (true liquid–solid adsorption or partition mechanism into a more polar, water-rich liquid stationary phase, developed from the eluent *in situ* in the column), it should differ essentially from RPLC with alkylsilica packings so that different and specific separations could be possible. Nevertheless, a very limited number of purines and pyrimidines have been chromatographed with systems of this type so far. In order to explore better the potentialities of such systems, the present work was undertaken and the dichloromethane–methanol–ammonium formate eluent of Evans *et al.*[8] was modified.

In our studies of nucleic acid chemistry during more than 20 years[9–11] we have often encountered difficulties in the separation and isolation of reaction products and particularly in the identification of nucleic acid components and their analogues in biological materials. These difficulties led us to the utilization of high-performance liquid chromatography (HPLC). The suitability of this method for the separation of closely related compounds was demonstrated in the separation of (*E*)- and (*Z*)-isomers of methyl glyoxylate semicarbazones and in the determination of the *E*/*Z* ratio in their mixtures[12], which are otherwise very difficult to separate[13]. Similarly, HPLC was used successfully in the separation and determination of single epimers of 2′,3′-cyclic sulphites of nucleosides at the rare isomerism located on the sulphur atom[14]. Further, HPLC was used for the quantitative determination of nucleosides and their metabolites in biological materials and the time course of the blood level of arabinosylcytosine and the course of its deamination in rats were determined[15]. The rate of deamination of arabinosylcytosine and its derivatives by deaminase was followed quantitatively by HPLC[16], and this method was similarly used for the quantitative determination of 5-fluorouracil derivatives in connection with their inhibition of *Escherichia coli*[17]. In order to obtain chromatographic retention data for our future work in nucleic acid chemistry, a systematic study of the separation and identification of a series of pyrimidine and purine derivatives was undertaken.

EXPERIMENTAL

The liquid chromatograph was assembled in this laboratory and consisted of a Milton Roy Model 396-57 minipump, a Model 709 pulse damper, a Model 1203 UV III monitor (all from Laboratory Data Control, Riviera Beach, FL, U.S.A.), a home-made septum injector and a Model EZ 13 electronic recorder (Laboratorní Přístroje, Prague, Czechoslovakia). The absorbance of the effluent was monitored at 254 nm.

The columns were made from 6.3 mm O.D., 4.2 mm I.D. LiChroma stainless-steel tubing, 25 cm long, purchased from Applied Science Labs. (State College, PA, U.S.A.). The solvent reservoir was maintained at 20°C and the columns were thermally insulated. Silica gel was packed into the columns with a home-made slurry-packing apparatus; chloroform served as the dispersing medium, the maximum pressure applied during packing being 50 MPa. Three types of silica gel were tried in the preliminary experiments: LiChrospher SI 100, particle size $d_p = 10$ μm; LiChrosorb SI 60, $d_p = 10$ μm; and LiChrosorb SI 100, $d_p = 5$ μm (all from E. Merck, Darmstadt, G.F.R.). Only minor differences were observed in their chromatographic performances. Under comparable conditions, LiChrosorb SI 60 (the material with the largest surface area) displayed the most pronounced tendency to give tailing peaks.

The quantitative retention data given further were obtained with the most efficient 5-μm LiChrosorb SI 100 column.

Mobile phase mixtures were made up by volume from dichloromethane, methanol (both of spectroscopic grade) and ammonium formate/formic acid solutions in deionized water. The relative proportions of the principal constituents of the eluent systems investigated are given in Table I.

TABLE I

COMPOSITIONS OF ELUENT SYSTEMS

System	*Content (parts by volume)*		
	Dichloromethane	*Methanol*	*Aqueous solution*
A1	75	22	3
A2	70	27	3
B1	80	18	2
B2	73	25	2

In addition to the influence of the overall composition (dichloromethane : methanol : water ratio), the influence of the concentrations of ammonium formate and formic acid was also studied. Initial tests revealed that varying the salt concentration from 0.5 to 2.0 *M* had no significant effect on retention; hence, a standard concentration of 0.5 *M* ammonium formate was chosen for the aqueous part of the eluent. On the other hand, the concentration of formic acid, determining the pH of the aqueous solution, proved to be important. Detailed experiments were conducted for three pH levels of the aqueous part, *viz.* pH 4.2 (resulting from the addition of 0.20 mole/l formic acid to the 0.5 *M* ammonium formate solution), pH 3.0 (1.22 *M* formic acid, 0.5 *M* ammonium formate) and pH 2.5 (2.65 *M* formic acid, 0.5 *M* ammonium formate).

Commercially available nucleobases and nucleosides were purchased from Lachema (Brno, Czechoslovakia). The other pyrimidine and purine derivatives were synthesized in this Institute. Standard solutions were prepared in methanol–water mixtures (*ca.* 1:1) so as to contain the solutes at concentrations of roughly 50–200 μg/ml and were injected into the chromatograph in 0.5–2.0-μl volumes, using SGE Type B syringes (Scientific Glass Engineering, Melbourne, Australia).

The emergence time of a peak (or baseline disturbance) obtained by injecting pure methanol into the column was taken as the elution time of a non-retained solute, t_M (ref. 18), and the retention (capacity) factors were calculated in the usual way from $k = (t_R - t_M)/t_M$, t_R being the retention time of the considered compound. Each k value was the mean of at least three measurements.

RESULTS AND DISCUSSION

Tables II and III give the capacity factors (k) of pyrimidine and purine derivatives on silica gel for the different mobile phase compositions used.

As could be expected, mobile phase systems with a higher proportion of the

TABLE II

RETENTION FACTORS (k) FOR PYRIMIDINE DERIVATIVES ON SILICA GEL

Column: packing, LiChrosorb SI 100, 5 μm; length, 25 cm. Mobile phase systems: see Table I.

	Mobile phase							
	A1			*B1*			*A2*	*B2*
pH of the aqueous solution	2.5	3.0	4.2	2.5	3.0	4.2	3.0	3.0
Formic acid concentration (M)	2.65	1.22	0.20	2.65	1.22	0.20	1.22	1.22
2-Hydroxypyrimidine	1.18	1.38	1.58	1.59	1.79	1.91	—	0.87
2-Hydroxy-5-methylpyrimidine	0.84	0.91	1.03	1.06	1.17	1.29	0.47	0.66
Uracil (Ura)	0.75	0.93	1.15	1.04	1.16	1.27	0.37	0.43
Uridine (Urd)	1.66	2.15	2.90	2.92	3.13	3.85	0.60	0.80
2′-Deoxyuridine (dUrd)	1.07	1.33	1.48	1.64	1.87	1.99	0.44	0.57
1-β-D-Arabinofuranosyluracil	1.53	1.92	2.61	2.59	2.97	3.29	0.55	0.70
Thymine (Thy)	0.53	0.66	0.79	0.72	0.75	0.85	0.28	0.31
5-Methyluridine	1.30	1.64	2.17	2.18	2.39	2.82	0.51	0.66
Thymidine (dThd)	0.82	1.01	1.27	1.28	1.36	1.51	0.38	0.47
6-Methyluracil	0.55	0.69	0.82	0.76	0.83	0.94	0.31	0.34
6-Methyluridine	1.12	1.40	1.82	1.80	1.99	2.47	0.47	0.63
6-Methyl-2′-deoxyuridine	0.87	1.00	1.19	1.18	1.28	1.37	0.40	0.52

1,5-Dimethyluracil	0.25	0.26	0.28	0.25	0.26	0.31	0.15	0.20
5,6-Dimethyluridine	0.87	1.03	1.30	1.25	1.38	1.56	0.38	0.49
5-Hydroxymethyluracil	1.47	1.86	2.41	2.34	2.69	2.96	0.57	0.75
Orotic acid	4.85	6.15	8.33	10.34	11.70	—	1.46	2.74
Orotidine	10.73	—	—	—	—	—	2.44	5.11
4,6-Dihydroxypyrimidine	1.67	3.19	7.91	3.00	4.22	—	0.75	1.36
4,6-Dihydroxy-5-methylpyrimidine	1.06	1.29	2.81	1.63	1.94	3.43	—	0.70
4,6-Dihydroxy-5-methyl-1-β-D-Rbf-pyrimidine*	1.56	2.01	4.35	2.77	3.32	5.39	—	0.87
Barbituric acid (B.A.)	0.88	1.12	1.70	1.35	1.61	2.02	0.33	0.59
Cytosine (Cyt)	3.20	3.64	4.00	5.44	5.56	5.46	1.43	2.22
Cytidine (Cyd)	4.57	5.75	7.12	9.20	9.52	11.10	1.58	2.63
2′-Deoxycytidine (dCyd)	3.49	4.20	4.82	6.52	6.84	7.22	1.28	2.23
1-β-D-Arabinofuranosylcytosine	4.20	5.31	6.75	8.66	9.04	10.25	1.44	2.48
1-Methylcytosine (m^1Cyt)	1.74	1.76	1.71	2.36	2.35	2.13	—	1.31
6-Methylcytosine (m^6Cyt)	2.44	2.66	2.66	3.90	3.86	3.56	0.97	1.78
5-Methylcytidine (m^5Cyd)	3.75	4.50	5.38	7.05	7.32	8.19	1.48	2.32
Isocytosine	1.37	1.64	1.90	2.09	2.26	2.34	0.69	0.99
Isocytidine	4.17	5.38	8.34	8.60	9.51	10.56	1.67	3.08
4-Amino-6-hydroxypyrimidine	1.41	1.65	1.99	1.99	2.21	1.96	0.74	0.99
4-Amino-6-hydroxy-1-β-D-Rbf-pyrimidine*	2.18	2.67	3.52	3.61	3.91	—	0.89	1.19

* Rbf = Ribofuranosyl.

TABLE III

RETENTION FACTORS (K) FOR PURINE DERIVATIVES ON SILICA GEL

Column: packing, LiChrosorb SI 100, 5 μm; length, 25 cm. Mobile phase systems: see Table I.

	Mobile phase							
	A1			*B1*			*A2*	*B2*
pH of the aqueous solution	2.5	3.0	4.2	2.5	3.0	4.2	3.0	3.0
Formic acid concentration (M)	2.65	1.22	0.20	2.65	1.22	0.20	1.22	1.22
2-Hydroxypurine	2.15	2.66	3.20	3.57	3.83	4.30	1.59	1.54
Hypoxanthine (Hyp)	1.39	1.68	2.06	2.21	2.38	2.62	0.68	0.92
Inosine (Ino)	2.50	3.21	4.35	4.78	5.17	6.13	1.20	1.40
1-Methylinosine (m^1Ino)	1.40	1.63	1.99	2.23	2.30	2.55	–	0.92
Xanthine (Xan)	1.11	1.40	1.96	1.74	1.88	2.24	0.55	0.61
Xanthosine (Xao)	2.61	3.58	8.09	5.06	6.23	–	0.97	1.47
Theophylline	0.15	0.16	0.14	0.17	0.17	0.20	0.11	0.16
Caffeine	0.06	0.08	0.07	0.08	0.08	0.10	0.08	0.10
Uric acid (U.A.)	2.33	3.36		4.52	5.84	7.10	0.84	1.28
Adenine (Ade)	0.92	1.05	1.15	1.27	1.29	1.33	0.62	0.72
Adenosine (Ado)	1.21	1.46	1.77	1.88	2.06	2.27	0.65	0.89
2′-Deoxyadenosine (dAdo)	0.90	1.00	1.07	1.30	1.38	1.45	0.55	0.72
1-Methyladenosine (m^1Ado)	12.50						5.15	
N^6-Methyladenine	0.56	0.61	0.64	0.69	0.73	0.75	0.41	0.49
N^6-Methyladenosine	0.74	0.86	0.98	1.06	1.11	1.13	0.45	0.56
N^6,N^6-Dimethyladenine	0.25	0.28	0.27	0.27	0.32	0.33	0.22	0.25
N^6,N^6-Dimethyladenosine	0.41	0.44	0.48	0.55	0.59	0.64	0.30	0.32
Guanine (Gua)	2.41	3.05	3.80	4.43	4.82	4.87	1.50	1.66
Guanosine (Guo)	3.50	4.56	5.94	7.05	7.97	9.05	1.24	1.93
2′-Deoxyguanosine (dGuo)	2.48	3.18	3.86	4.68	4.90	5.58	0.97	1.50
1-Methylguanosine (m^1Guo)	2.37	2.90	3.65	4.25	4.59		0.93	1.46
Isoguanosine	4.39	5.68	7.56	9.25	10.40		1.65	2.86

less polar component (dichloromethane) act as weaker eluents, and an increase in the methanol and/or water content leads to shorter retention times. With systems A1 and B1 (Table I) the k values lie in the optimal chromatographic range (*ca.* $1 < k < 10$) for most of the substances investigated. Systems A2 and B2 would be useful for the separation of the more retained compounds, such as cytosine derivatives and carboxylated pyrimidines, but for most of the compounds they are rather fast. Also, the influence of pH was less pronounced with these two systems than with either A1 or B1, so that data are given only for a single pH value (pH 3).

With systems A1 and B1, however, changes in the pH (formic acid concentration) of the aqueous part caused considerable effects on both the retention and selectivity of separation. This is demonstrated graphically in Fig. 1, where experimental k values are plotted against formic acid concentration for several selected substances displaying typical behaviour in this respect (data for system B1 were used in Fig. 1; for A1 the dependences, shifted to the lower k region, are similar.) It can be seen that several compound pairs co-eluting under certain conditions can be well resolved when the concentration of the acid has been changed, and in some instances even reversals

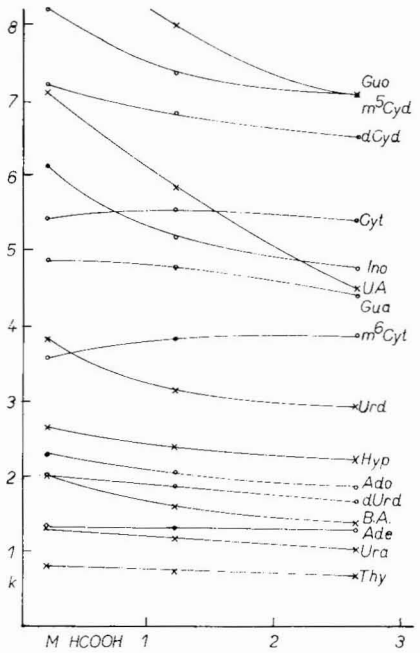

Fig. 1. Dependence of k values on the molarity of formic acid in the aqueous part of the mobile phase system B1.

of the elution order are effected. Obviously, the overall composition of the mobile phase cannot be manipulated at will, as there is a miscibility gap in the underlying ternary solvent system dichloromethane–methanol–water, and additions of formate and formic acid influence the mutual solubilities. Nevertheless, it is evident that the variations attainable in this way provide enough flexibility to affect the retention and selectivity parameters in chromatographic separations of the pyrimidines and purines concerned.

Remarks on structure–retention relationships

Because of the complexity of the interactions necessarily involved, it would hardly be possible to explain the retention behaviour of the main simple pyrimidines and purines in terms of their structures and physico-chemical properties. Nevertheless, some generalizations can be made for similarly substituted compounds, as follows.

(1) In logical contrast to RPLC, methyl groups generally decrease the retention parameters, in proportion to their number. This statement, however, can apply only when the alkyl substituent does not alter the primary structure. Among the compounds investigated, such a notable exception is m^1Ado, which elutes with an extremely high k value after Ado, whereas m^1Ino and m^1Guo emerge before Ino and Guo, respectively, and behave in the normal way. The anomaly must obviously be related to the fact that m^1Ado exists predominantly as the imino tautomer[19], whereas the other adenine derivatives are in the usual amino form. It may be assumed that the two tautomeric structures undergo different retention interactions.

(2) Ribosides possess systematically higher retention factors than their parent bases, which seems to be a logical consequence of the presence of a hydroxylated sugar moiety and their more polar nature. Curiously, in RPLC the order of elution of a certain ribonucleoside–base pair is just the same and by no means 'reversed'; it has been proposed recently[20] that this striking phenomenon may be explained by stacking interactions. Another general observation that can be derived from the data collected here is that the selectivity of separation for ribosides and corresponding bases (*i.e.*, the ratio $k_{\text{nucleoside}}/k_{\text{base}}$) increases significantly when the formic acid concentration is lowered under otherwise comparable conditions.

(3) The 2′-deoxyribonucleosides elute systematically before the corresponding ribonucleosides, in this instance contrary to their behaviour in RPLC[21,22]. The situation is more complicated, however, when the retention of deoxynucleosides is compared with that of parent bases, and cannot be explained in simple terms of 'polarity'; whereas dUrd and dThd always have distinctly higher k values than Ura and Thy, respectively, the differences are small for Ade, Gua and Cyt derivatives and in some instances the deoxyriboside elutes before the base.

Influence of formic acid

As stated above and illustrated in Fig. 1, variations in the concentration of the formic acid additive (all other conditions remaining unchanged) generally have a marked influence on retention parameters. Some characteristic features can be pointed out in this respect.

For most of the substances investigated, the k values increase when the acid content is decreased. The dependence is strong with structures bearing true hydroxyl groups, such as 5-hydroxymethyluracil, uric acid, barbituric acid and, of course, all the nucleosides and deoxynucleosides. For oxygenated compounds that exist predominantly in the lactam tautomeric form (Ura, Hyp, Xan, etc.), this dependence is weaker but still pronounced. On the other hand, Cyt, Ade, Gua and other purines and pyrimidines containing an amino group (apart from their nucleosides, where the effect of the hydroxyls apparently prevails) are less affected and with the B1 mobile phase system their retention is almost insensitive to changes in the acid concentration; in some instances, their k values even fall in the less acidic region. Obviously, these findings reflect the complex character of the chromatographic retention mechanism. The acidity of the aqueous solution was varied in the pH range 2.5–4.2, but these pH values cannot be taken as a quantitative measure of the entire aqueous–organic eluent mixture. Nevertheless, it is clear that just those compounds which exhibit the greatest retention–acidity dependence must exist as neutral, non-ionized molecules over the whole range of mobile phase compositions used. Thus, the apparent pH influence cannot be attributed to changes in the ionic state of such solutes and must be related to variations in the chromatographic mobile phase–stationary phase system. On the other hand, all of the aminopyrimidines and aminopurines possess basic pK_a constants around 3–4 and should become protonated in a more acidic environment. The relative pH insensitivity of their retention factors could then be explained by assuming that two retention-governing factors cancel out: (a) the chromatographic system as a whole becomes less retentive with the increase in acidity, but (b) the amino compounds change gradually from the neutral to the cationic form and the latter is retained more strongly.

Practical chromatographic aspects

The chromatographic systems described here possess some features that compare favourably with the possibilities offered by RPLC for the separation of nucleic acid components. The biochemically important nucleic acid bases elute in the order Thy < Ura < Ade < Xan < Hyp < Gua < Cyt. In contrast, on octadecylsilica, with weakly acidic aqueous eluents, the typical sequence is Cyt < Ura < Hyp < Gua < Xan < Thy < Ade[2,21], with adenine eluting far from from the other bases. Because of the separate position of Ade and its derivatives in RPLC, it is necessary to use gradient elution in order to separate the whole range of compounds in an acceptable time[21,23]. With plain silica gel and the hydro-organic eluents used in this work, separations of this type are easy to achieve under isocratic conditions. An example is given in Fig. 2, which shows the chromatogram of an artificial mixture of thirteen bases and nucleosides. The excellent resolution of Xan, Hyp, Gua and Urd may also be noted, as these substances come very close together in RPLC systems[23,24].

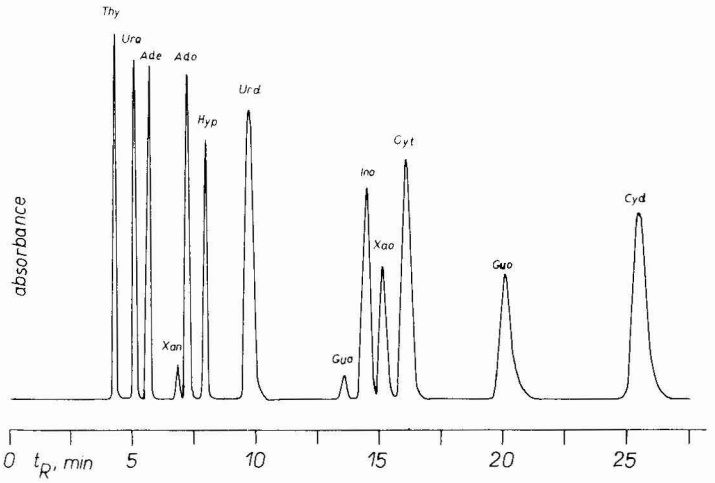

Fig. 2. Separation of bases and nucleosides on silica gel. Column: LiChrosorb SI 100, 5 μm, length 25 cm. Mobile phase system B1 with 2.65 *M* formic acid solution (pH 2.5). Flow-rate, 1.05 ml/min; pressure drop, 7.6 MPa; temperature, 20°C; detection, 254 nm; 0.032 a.u.f.s.; sample size, *ca.* 100 ng of each solute (except for Xan and Gua).

Both column efficiencies and peak shapes were very satisfactory. Plate numbers in the range 10,000–14,000 were typically obtained at flow-rates of 0.7–0.8 ml/min for a 25 cm × 4.2 mm I.D. column packed with 5-μm particles. An interesting finding, however, is that under most conditions the ribonucleosides displayed systematically lower plate numbers (roughly 6000–8000) than the other compound types. Peak profiles were evaluated in terms of the asymmetry factor, $F_t = 100a/b$, where a is the distance between the front peak boundary and the perpendicular drawn from the peak maximum to the baseline, measured at 10% of the peak height, and b is the corresponding distance for the rear boundary. The quantitative values were F_t = 80–90 in the most favourable instances, and never dropped below 60.

A certain disadvantage, for scaling-up applications, may be the relatively low sample loadability of the chromatographic systems. On increasing the sample size above 5–10 μg (for a single solute), a limit is reached where individual substances, especially early eluting ones, tend to emerge as sharply shaped peak doublets or even triplets. This phenomenon must be related to their low solubility in the dichloromethane-based hydro-organic eluent, probably causing an overloading effect on the column entrance. On the other hand, no difficulties were encountered upon decreasing the sample size and detection limits in the range 1–2 ng were obtained. Thus, the systems used are suitable for trace analyses.

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DETERMINATION OF ACETYL AND FORMYL GROUPS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the hydrolysis of proteins, amino acids and carbohydrates for the liberation of N- and O-acetyl and formyl groups. The acetyl and formyl phenacyl esters are prepared by means of crown ether catalysis and determined by gas–liquid chromatography using the flame-ionization detector. Quantitative results are given with samples containing about 20 nmol of acetyl or formyl group. The method is also applicable to the determination of N- and O-propionyl groups.

INTRODUCTION

Narita[1] first isolated and characterized the N-acetylated peptide from the N-terminus of tobacco mosaic virus protein. Acetyl groups have since been shown to occur in proteins obtained from a variety of sources and about 80% of the soluble proteins from Ehrlich Ascites cells were reported to be N^{α}-acetylated[2]. N^{α}-Acetyl-amino acids have been reported in a wide range of proteins, including adenylate kinase, carbonic anhydrase, superoxide dismutase, several dehydrogenases, cytochrome *c*, parvalbumins, α-melanocyte stimulating hormone, histones and keratins[3,4]. The presence of N-formyl groups has been well established in protein chain initiation on the ribosome and N^{α}-formyl-blocked proteins have been reported[5,6].

A variety of methods for the determination of acetate were published. Acetate was determined by enzymic methods [7–18]. Acetate in proteins in the range 0.1–1.0 μmol was determined as the 1-acetyl-2-dinitrophenylhydrazine derivative[19,20]. Using 1–2 mg of protein, formyl and acetyl groups were determined as their 1-acyl-2-Dns-hydrazine derivatives, although the yield of the formyl group was only about 10% that for the acetyl group[21]. Mucopolysaccharides were hydrolyzed with methanolic HCl and the methyl acetate determined as the hydroxamate by colorimetry[22]. Acid hydrolysis was used to liberate acetic acid from protein hexosamines and the acetic acid titrated after steam distillation[23,24]. Proteins were hydrolyzed and the N-acetyl[25,26] and the N-formyl[6] amino acids were separated by high-voltage electrophoresis and the amino acid determined. The determination of N^{α}-blocking groups in proteins is possible by mass spectrometry (MS), but about 50 nmol of a short peptide carrying these groups must be isolated first[27].

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Gas chromatography in the free acid form was used for the determination of acetic acid in a variety of biological solutions such as plasma and urine[28-37], in biological materials[38,39] and attached to proteins[40,41] and lipopolysaccharides[42]. Protein acetyl groups were hydrolyzed by methanolic HCl and the methyl acetate formed was determined by gas–liquid chromatography (GLC)[43,44]. A method using acid fusion reaction gas chromatography for biological materials was reported[45].

We report here on a method for the direct determination of acetyl and formyl groups attached to amino acids, proteins and carbohydrates. The method is equally applicable to propyl groups, although these have not been reported in proteins. After alkaline hydrolysis of the sample the potassium salts of acetic and formic acid are converted to the phenacyl esters prepared by means of crown ether catalysis and determined by GLC. A preliminary report was published[46].

EXPERIMENTAL

Apparatus

A Pye Series 104 Model 24 gas chromatograph, fitted with dual flame-ionization detector (FID) (Pye-Unicam, Cambridge, Great Britain) was used with a Speedomax W 1 mV, 1-sec strip chart recorder (Leeds & Northrup, Birmingham, Great Britain). Integration of the peak areas was carried out with the Kent Chromalog 2 and Vidar Autolab 6300 digital integrators (Anachem, Luton, Great Britain). Nitrogen (99.9% "white spot" from BOC, Wembley, Great Britain) was used as carrier gas.

Materials

Chemicals and stationary phases were obtained as follows: phenacyl bromide, potassium acetate, N-acetylglycine, trimethylchlorosilane (BDH, Poole, Great Britain), dicyclohexyl-18-crown-6 (Aldrich, Milwawkee, WI, U.S.A.), JXR, XF-1105, XE-60, ECNSS-S and EGSS-X (Applied Science Labs., State College, PA, U.S.A.), Dexsil 300 GC and EG isophthalate (Analabs, North Haven, CT, U.S.A.), GMHS, GMS and WOL (Advita, Walton-on-Thames, Great Britain), SE-52, BDS and QF-1 (F & M Scientific, Avondale, PA, U.S.A.), Chromosorb W HP 80–100 mesh (Field Instruments, Richmond, Great Britain), EG distearate and PPSeb (Reoplex 100) (Griffin & George, Alperton, Great Britain), SE-30 (E301) (ICI, Stevenston, Great Britain), MS-200 (Hopkin & Williams, Chadwell Heath, Great Britain), Antarox CO-990 (Perkin-Elmer, Beaconsfield, Great Britain), *n*-octadecane, OV-17, OV-25, OV-61, OV-225, Celite 560, 85–100 mesh (Phase Separations, Queensferry, Great Britain), Porapak Q (Waters Assoc., Milford, MA, U.S.A.), BDA, NPGA, NPGG, NPGP, NPGS and NPGSeb were previously prepared[47], N-acetyl-DL-leucine, N-acetyl-L-tyrosine and N-acetyl-DL-phenylalanine (L. Light & Co., Colnbrook, Great Britain) and N-acetyl-L-aspartic acid, N-acetyl-L-glutamic acid, O-acetylserine, N-formyl-L-leucine, N-formyl-L-methionine, N-formyl-L-tyrosine, N-formyl-DL-valine, chicken egg albumin and egg white lysozyme (Sigma, St. Louis, MO, U.S.A.). Microcapped centrifuge tubes (0.5 ml) were obtained from Hughes & Hughes (Haroldwood, Great Britain).

Acetylated cholesterol and carbohydrates were a gift from Dr. N. R. Williams (Birkbeck College, University of London, London, Great Britain) and carbonic anhydrase from Dr. N. Spencer (King's College, London, Great Britain).

Hydrolysis of samples and preparation of esters

N-Acetyl compounds were hydrolysed with 1 *M* KOH in 0.5 ml polypropylene micro-capped centrifuge tubes in an autoclave at 15 p.s.i. (123°C) for 3 h. After hydrolysis, the tube contents containing 20–100 nmol of acetate or formate were transferred to glass tubes (5 × 0.5 cm I.D.) followed by 5 × 25 μl washings of distilled water. The solution was neutralised to phenolphthalein end-point with HBr and taken to dryness. Phenacyl bromide and dicyclohexyl-18-crown-6 in the proportion 10:1 (mol/mol) in benzene solution (25 μl) were added. The tube was sealed and incubated at 80°C for 30 min with occasional shaking. A five-fold molar excess of phenacyl bromide over the expected acetate was used. Toluene was not a satisfactory substitute for benzene. Aliquots of 1 μl were injected onto the GLC column.

RESULTS AND DISCUSSION

Successful methods were reported for the determination of volatile fatty acids by chromatography in their free acid form[28–42]. Acetic acid was determined after preliminary acid hydrolysis of N-acetylamino acids and N-acetylglucosamine[38] and of proteins[40,41]. Some preliminary experiments were carried out with hydrolysis of N-acetylamino acids in sealed tubes with 2 and 6 *M* HCl at 110°C for periods up to 24 h[38,41]. The acetic acid liberated was determined by GLC. Protein samples with 8–17 μmol acetate were used[40], but here with samples containing 50–100 nmol of acetate inaccurate results were obtained, despite precautions taken to prevent volatility losses. In addition, the analysis of free fatty acids with the injection of aqueous solutions leads to problems arising from the accumulation of non-volatile compounds at the injection head of the column, destruction of stationary phase and ghosting (see review ref. 48) and the known effect of water on the response of the FID[29,49]. The problems with tailing solvent[32,37,44,50] and free acid peaks[32,36,37] were shown. Some of these problems could be avoided by isolation of free acetate by steam distillation[28] or by microdistillation[15,38,39,43] and some workers extracted the acetate into a non-aqueous medium, such as *tert.*-butyl ethyl ether[11,41]. However, these methods involved additional manipulation which did not commend themselves for work at high sensitivity. The determination of small quantities of acetic acid in its acid form with the FID necessitated the use of low attenuation of the signal, *e.g.* $8 \cdot 10^{-12}$ A/mV, with the attendant problems of a noisy baseline when working near the limits of the apparatus[30].

In order to improve the sensitivity of the method for acetate and also to determine formate in addition with the FID it was considered best to work with high-molecular-weight ester derivatives. The use of crown ethers introduced by Pedersen[51] for ester formation by reaction of the potassium salt of the acid with a primary bromide[52–55] offered a method which was applicable to small samples, and phenacyl esters were selected for the determination of both acetyl and formyl groups.

It was previously shown[46] that the best results were obtained with phenacyl bromide and dicyclohexyl-18-crown-6 in 10:1 mol ratio in benzene solution at 80°C for 30 min. With phenacyl bromide:potassium acetate mol ratios 1.07 and 2.14 the yields of phenacyl acetate were 73.5 and 95.5%, respectively. With molar excess ratios ranging from 3.2 to 32 the yields were 98.5% ± 4.36 for $n = 32$ samples. A five-fold molar excess of phenacyl bromide over acetate was adopted. A large excess of phe-

nacyl bromide interfered with the desired derivative peaks. A similar method was reported for valproic acid in serum[56].

Solvent tailing is often a problem with GLC because of its interference with early eluting peaks. Acetonitrile was reported to be the best solvent for rapid ester formation with crown ethers[53]. However, this solvent tailed badly on our column (see ref. 56) and benzene was preferred. Other non-tailing solvents were chloroform and carbon disulphide, but the recoveries were not satisfactory using these solvents for the reaction.

In Table I the retention times for phenacyl formate, acetate and propionate relative to that of phenacyl bromide are given for 23 different stationary phases. Most of the silicone phases gave tailing peaks. The mixed silicone column[57] gave the best separation but the peaks tailed badly. Amongst the ten polyester phases examined only PPSeb and EG distearate showed any effective resolution between the formate

TABLE I

RELATIVE RETENTION DATA FOR PHENACYL ESTERS

The figures are retention times relative to phenacyl bromide taken as 1.00. The retention time in minutes for phenacyl bromide is given in brackets.

Liquid phase	*Liquid–solid (% w/w)*	*Column temp. (°C)*	*Phenacyl*			
			Bromide	*Formate*	*Acetate*	*Propionate*
Dexsil 300 GC	1	110	(7.38)	1.12	1.60	2.77
SE-30	3	110	(8.86)	1.16	1.71	2.84
SE-52	3	125	(5.91)	1.00	1.47	2.27
JXR	3	125	(6.30)	1.00	1.47	2.41
Mixed silicones*	1	125	(4.72)	1.54	1.79	2.92
OV-17	5	140	(10.43)	1.09	1.53	2.38
OV-25	1	125	(7.29)	1.19	1.65	2.46
OV-61	3	125	(13.40)	1.12	1.60	2.57
OV-225	1	125	(4.33)	1.43	1.67	2.41
XF-1105	2	125	(5.02)	1.24	1.63	2.57
Antarox CO-990	2	150	(10.04)	2.18	2.18	2.63
ECNSS-S	2	150	(6.89)	1.77	1.86	2.09
EGSS-X	2	150	(6.23)	1.89	1.89	2.16
BDA	2	150	(12.99)	1.53	1.53	2.00
BDS	2	150	(4.75)	1.71	1.71	2.23
NPGA	2	150	(12.01)	1.56	1.64	2.20
NPGG	2	150	(8.07)	1.51	1.51	2.07
NPGP	2	120	(7.48)	2.03	2.21	3.00
NPGSeb	2	150	(7.87)	1.25	1.40	2.15
NPGS	5	175	(9.80)	1.52	1.52	1.90
PPSeb	2	150	(15.45)	1.32	1.46	2.01
EG distearate	2	125	(16.90)	1.09	1.44	2.36
EG isophthalate	2	150	(4.92)	1.72	1.72	2.20
GMHS	2	150	(9.55)	1.20	1.58	2.33
GMS	2	125	(14.96)	1.15	1.52	2.52
WOL	2	125	(18.31)	1.17	1.55	2.52
PPSeb–GMS (3:2, w/w)	2	150	(8.37)	1.22	1.43	2.09

* XE-60–QF-1–MS-200 (46:27:27, w/w/w) on Chromosorb W HP, 80–100 mesh[57].

and the acetate. The surfactant phases (GMHS, GMS and WOL) separated phenacyl formate from phenacyl acetate, but phenacyl bromide was very close to the formate.

A liquid phase was developed which consisted of PPSeb and GMS mixed together in the proportions 3:2 (w/w) and coated (2%, w/w) either onto Celite 560 AW DMCS or onto Chromosorb W HP support material. This resolved the phenacyl esters and also avoided interference from the large peak due to the reagent phenacyl bromide which was unavoidably present in large excess, as shown in Fig. 1. During the preparation of the phenacyl esters, small amounts of phenacyl chloride were formed by interchange between phenacyl bromide and contaminating chloride ions[58]. Acetophenone also appeared as a minor component. The identity of the peaks of compounds shown in Fig. 1 were confirmed by GLC–MS. Table II presents the details of the fragmentation patterns obtained for these peaks. In all cases the mass ion was identified and the base peak at m/e 105 was assigned to $(C_6H_5CO)^+$. Similar artifact peaks were obtained with the GLC of pentafluorobenzyl esters prepared by crown ether catalysis[55].

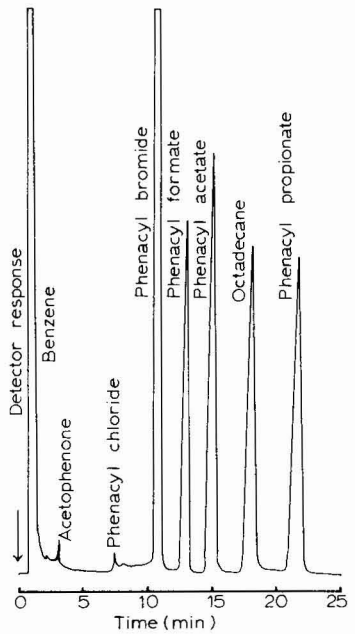

Fig. 1. GLC separation of phenacyl ester derivatives on PPSeb–GMS column. GLC conditions: Pye Series 104 Model 24. FID. Glass column (3 m × 2.5 mm I.D.) packed with 2% mixed stationary phase PPSeb–GMS (60:40, w/w) on Chromosorb W HP 80–100 mesh. Oven temperature, 150°C. Carrier gas, nitrogen, 30 ml/min, hydrogen 30 ml/min, air 450 ml/min. Sample size, 1 μl. Attenuation, $1 \cdot 10^{-9}$ A f.s.d.

The relative molar response values of phenacyl formate, acetate and propionate against octadecane determined with the FID are given in Table III. The response of phenacyl formate was lower than would be expected (by approximately 8–9%) for a linear relationship response/carbon number for the homologous series of esters.

TABLE II

MOLECULAR PEAKS AND MAJOR FRAGMENT PEAKS OF PHENACYL DERIVATIVES AND ACETOPHENONE

The compounds correspond with the peaks shown in Fig. 1.

Compound	$[M]^+$ *m/e (%)*	*Base peak (m/e)*	*Major fragment peaks, m/e (%)*			
Acetophenone	120 (68)	105	77 (71)	51 (55)	43 (48)	91 (23)
Phenacyl bromide*	198 (24) 200 (20)	105	77 (74)	91 (47)		
Phenacyl chloride*	154 (29) 156 (11)	105	77 (80)	91 (37)		
Phenacyl formate	164 (37)	105	85 (99)	29 (77)	91 (68)	119 (63)
Phenacyl acetate	178 (46)	105	77 (99)	43 (99)	91 (77)	119 (56)
Phenacyl propionate	192 (20)	105	57 (60)	77 (56)	119 (30)	91 (24)

* Two molecular ions $[M]^+$ due to natural isotopes.

However, the results were reproducible. The GLC of free acids [C_2 to C_7] using the FID also showed a non-linear response/carbon number relationship[30].

Fig. 2 shows the linear responses obtained when plotting peak areas (integrator counts) against increasing quantities of phenacyl formate, acetate and propionate (1.0–10 nmol) injected onto the GLC column. No breakdown of the phenacyl formate was observed within the experimental range studied.

Conditions were investigated for releasing covalently-bound acetyl groups. Hydrobromic acid was used in order to form potassium bromide for crown ether catalysis and the time course for the hydrolysis of N-acetylglycine with 6 *M* HBr at 110°C in Fig. 3 shows a maximum yield in 3 h. Previous workers used 2 *M* methanolic HCl at 100°C for 4 h to form methyl acetate directly for GLC[22,43]. Aqueous 9 *M* H_2SO_4 (ref. 11) or 3 *M* H_2SO_4 at 105°C for 2 h[41], 6 *M* HCl at 110°C for 24 h[15] and 0.2 *M* HCl at 100°C overnight[42] were used to liberate free acetic acid. This method was appplicable to N-acetyl- and N-formylamino acids. The results for acid hydrolysis are given in Table IV. (The results for alkaline hydrolysis reported for convenience in this table will be discussed later.) Except for N-acetylglycine the recoveries

TABLE III

RELATIVE MOLAR RESPONSE VALUES OF PHENACYL ESTERS DETERMINED AGAINST OCTADECANE = 1

GLC conditions as in Fig. 1. Amount injected, 1 μl.

Phenacyl ester	*Relative molar response ± S.D. (n = 10)*
Formate	0.375 ± 0.0089
Acetate	0.448 ± 0.0067
Propionate	0.506 ± 0.011

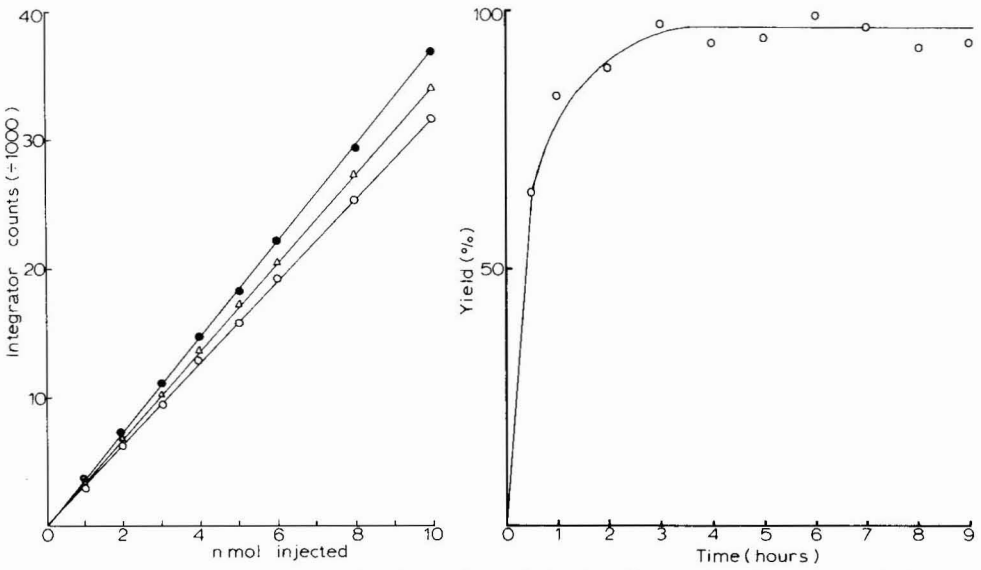

Fig. 2. Response–concentration curves for phenacyl ester derivatives. Preparation of samples: a solution was prepared containing phenacyl formate, phenacyl acetate and phenacyl propionate (10 μmol of each/ml benzene). This solution was further diluted to give the required concentration of phenacyl ester derivatives. Sample size, 1 μl. GLC conditions as in Fig. 1. ●, Propionates; △, acetate; ○, formate.

Fig. 3. Time course for hydrolysis of N-acetylglycine with 6 *M* HBr. Individual samples (683 nmol) were hydrolysed with 50 μl of 6 *M* HBr in sealed tubes at 110°C for increasing periods of time. The tubes were frozen, opened, and the contents allowed to thaw. The contents were neutralized to phenolphthalein end-point with 2 *M* KOH. The tube contents were dried under nitrogen gas at 80°C and the ester prepared as described in the Experimental section. GLC conditions as in Fig. 1.

with acid hydrolysis were below 90%. The low recoveries were attributed to losses of volatile acetic acid despite the use of sealed tubes or Reacti-vials for the hydrolysis and cooling before opening. It was claimed that N-formyl groups in peptides could be removed by refluxing with 0.5 *M* HCl in methanol for 1 h, with yields of 80–95%[59]. We did not investigate acid hydrolysis any further but studied alkaline hydrolysis.

Fig. 4 shows the time courses for the alkaline hydrolysis of N-acetylalanine and N-acetylglycine at 15 p.s.i. (123°C). N-Acetylalanine required 3 h for complete hydrolysis with 1 *M* KOH. About 2 h were required for N-acetylglycine using 2 *M* KOH. The use of higher concentrations of KOH to shorten the hydrolysis time was not studied, because the excess of KBr formed on neutralization subsequently interfered with the formation of the phenacyl esters.

The results for the hydrolysis of N-acetyl- and N-formylamino acids with 1 *M* KOH are given in Table IV. The values were about 10% higher than those for acid hydrolysis and ranged from 93.1% for N-formyl leucine to 97.5% for N-acetylaspartic acid.

Some O-acetylated hexoses and cholesterol acetate were hydrolysed with 0.5 *M* KOH at 123°C for 3 h. The results in Table V show that the expected molar ratios of acetate were obtained for each compound. The N-acetyl group in N-acetylmannosamine required 1.0 *M* KOH for complete hydrolysis. O-Acetyl groups are more easily hydrolyzed[19] and weaker alkali may be used than for N-acetyl groups. It was

TABLE IV

RECOVERY OF ACETATE AND FORMATE AS PHENACYL ESTERS AFTER ACID AND ALKALINE HYDROLYSIS OF N-ACETYL- AND N-FORMYLAMINO ACIDS

Acid hydrolysis for 4 h with 6 *M* HBr at 110°C ($n=3$). Alkaline hydrolysis for 3 h with 1 *M* KOH at 15 p.s.i. (123°C) ($n=5$). GLC conditions as in Fig. 1.

Compound	*Acid hydrolysis*		*Alkaline hydrolysis*	
	Calculated (nmol)	*Recovery (%) ± S.D.*	*Calculated (nmol)*	*Recovery (%) ± S.D.*
N-Acetylalanine	150	87.3 ± 1.76	59	94.9 ± 1.69
N-Acetylaspartic acid	110	83.6 ± 3.96	41	97.5 ± 1.34
N-Acetylglutamic acid	145	85.7 ± 2.11	30	96.1 ± 3.70
N-Acetylglycine	140	93.4 ± 4.67		*
N-Acetylleucine	148	86.9 ± 1.70		*
N-Acetylmethionine	125	89.1 ± 2.55	52	96.3 ± 1.38
N-Acetylphenylalanine		*	72	96.9 ± 1.16
N-Acetyltyrosine	135	85.1 ± 3.73	43	95.1 ± 4.37
N-Formylleucine	150	84.9 ± 4.67	61	93.1 ± 1.37
N-Formylmethionine	131	88.7 ± 4.62	65	96.6 ± 1.28
N-Formylvaline	141	88.6 ± 4.76	80	95.2 ± 1.63

* Not determined.

claimed that O-acetyl groups in lipopolysaccharides could be selectively determined after hydrolysis with 0.05 *M* NaOH for 3–4 h at room temperature[42].

The method was required for application to proteins and peptides and it was necessary to look both for spurious production of formate and acetate resulting from alkali treatment and also any occluded or adsorbed from buffer solutions during the isolation of the protein. Table VI presents the results for the production of formate and acetate from some amino acids and sugars after treating them with 1 *M* KOH at 123°C for 3 h. Sugars gave rise to a lot of acetate and formate. Thus, from 1.0 μmol of galactose, 25 nmol of formate and 19 nmol of acetate were obtained. Amongst the amino acids tested, the highest yields of formate and acetate were given by cysteine. One μmol of cysteine produced about 10 nmol of formate and 20 nmol of acetate, but cystine produced less than twice as much on a mol to mol basis. Serine

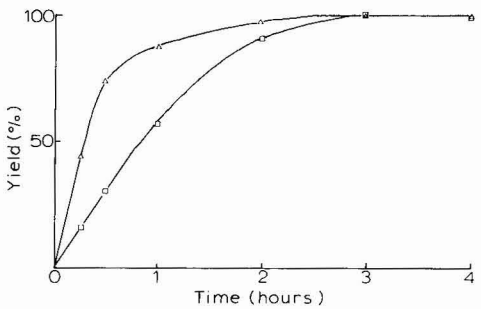

Fig. 4. Alkaline hydrolysis of N-acetylated compounds. △, 2 *M* KOH; N-acetylglycine. □, 1 *M* KOH; N-acetylalanine.

TABLE V

RECOVERY OF ACETATE FOLLOWING ALKALINE HYDROLYSIS OF ACETYLATED SUGARS AND CHOLESTEROL

Hydrolysis with 25 μl of 0.5 *M* KOH at 15 p.s.i. (123°C) for 3 h, except for N-acetylmannosamine 1.0 *M* KOH.

Compound	*Calculated (nmol)*	*Recovery (nmol ± S.D., n = 5)*	*Residues (mol/mol)*
Arabinitol pentaacetate	12.4	61 ± 2.45	5
Epi-2-inositol pentaacetate	8.97	45 ± 2.50	5
Epi-inositol hexaacetate	8.10	47 ± 1.64	6
Epi-inosamine hexaacetate	6.96	40 ± 2.16	6
N-acetylmannosamine	48.0	47 ± 2.12	1
Cholesterol acetate	35.0	32 ± 1.43	1

gave a small amount of acetate (2.5 nmol/μmol serine), whereas threonine gave 0.7 nmol acetate/μmol.

It was reported that acetate in proteins could not be determined after alkaline hydrolysis because of the decompositon of certain amino acids, particularly threonine. Also, sugars yielded both formate and acetate[41]. Our results may be compared with those using acid hydrolysis. Threonine reacted with 3 *M* H_2SO_4 at 105°C for 24 h yielded 9.3 nmol acetate/μmol and with 6 *M* H_2SO_4 at 110°C for 16 h 1.0 μmol of monosaccharide gave 6.5 nmol of acetate[41].

It was claimed that propionyl groups could only be determined in the absence of cystine and cysteine using the hydrazinolysis method[19]. As reported in Table VI only threonine yielded a small amount of propionic acid and this was not sufficient to prevent propionyl group determination.

TABLE VI

PRODUCTION OF FORMATE AND ACETATE FROM SUGARS AND FREE AMINO ACIDS AFTER TREATMENT WITH ALKALI

Sugars (n = 3) and amino acids (n = 4) hydrolyzed with 50 μl of 1 *M* KOH at 15 p.s.i. (123°C) for 3 h.

Compound	*Amount hydrolyzed (μmol)*	*nmol ± S.D.*	
		Formate	*Acetate*
Galactose	1.8	45.0 ± 3.61	34.0 ± 4.72
Glucose	2.1	43.0 ± 3.99	35.0 ± 7.09
Lactose	1.85	54.0 ± 6.11	39.0 ± 6.08
Arginine	2.36	nil	nil
Aspartic acid	3.16	0.36 ± 0.08	0.66 ± 0.06
Glutamic acid	3.04	0.33 ± 0.09	0.26 ± 0.04
Cysteine	2.37	23.2 ± 3.08	45.9 ± 5.57
Cystine	1.64	26.4 ± 5.01	49.2 ± 5.22
Serine	3.64	1.22 ± 0.32	9.02 ± 0.36
Threonine*	3.06	1.89 ± 0.43	2.22 ± 0.40

* Propionic acid 1.31 nmol ± 0.23 S.D. was also found.

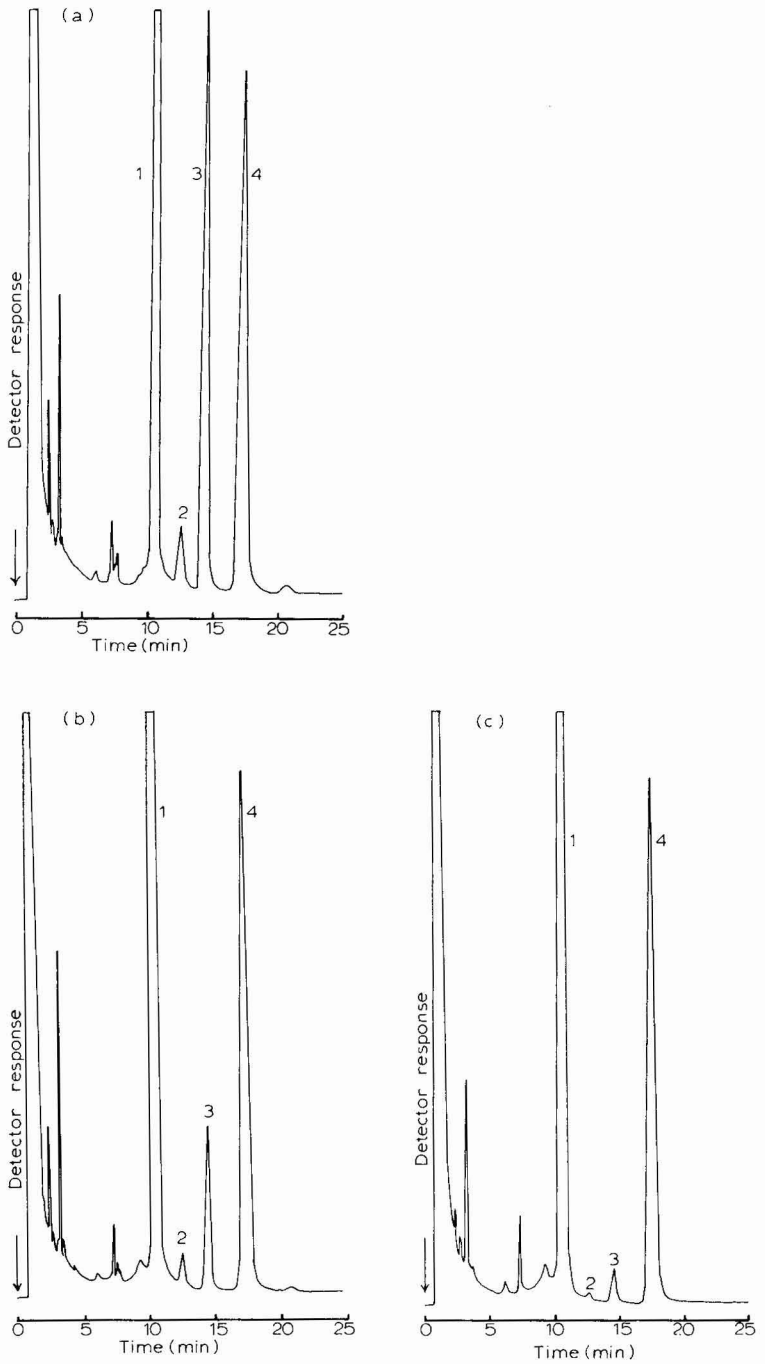

Fig. 5. GLC of phenacyl acetate and formate obtained from proteins after alkaline hydrolysis. (a) Chicken egg albumin (23 nmol), (b) carbonic anhydrase (37 nmol) and (c) egg white lysozyme (100 nmol). Each sample was dissolved finally in 25 μl of benzene and 1 μl injected. GLC conditions as in Fig. 1. Peaks: 1 = phenacyl bromide; 2 = phenacyl formate; 3 = phenacyl acetate; 4 = octadecane (internal standard).

It may be concluded that the spurious production of excess acetate and formate due to side reactions[21] when determining these groups on proteins may be overcome by the appropriate use of control samples. In addition, compensation can be made for acetate and formate contaminants of both the samples and the reagents.

The method was applied to lysozyme, carbonic anhydrase and ovalbumin which were dialysed four times against distilled water and lyophilised. The dialysis residue was then acidified with 1 *M* HBr and lyophilised four more times in order to remove free acetate and formate. However, some free acetate and formate were still detectable. Sephadex gel filtration as a method of removing bound ligands from macromolecules[60] was not satisfactory for removing formate and acetate because of the high blank values obtained from water samples passed through the column. These high values were attributed to traces of carbohydrate material from the Sephadex.

Fig. 5 shows the GLC traces obtained with carbonic anhydrase, chicken egg albumin and egg white lysozyme. All three proteins gave peaks which showed the presence of small amounts of formate. In addition lysozyme showed a peak for acetate. Control values were obtained by preparing the phenacyl esters without previous alkaline hydrolysis. The quantitative results are given in Table VII. The controls yielded values of less than 0.09 mol acetate/mol protein and if these were deducted from the values after alkaline hydrolysis, the results agreed with the expected molar ratios of 4, 1 and 0 for albumin, carbonic anhydrase and lysozyme, respectively.

TABLE VII

DETERMINATION OF ACETATE IN PROTEINS

Hydrolysis conditions: 1 *M* KOH at 123° C for 3 h.

Protein	*Acetate/protein ratio (mol/mol)*		
	Without hydrolysis (n=3)	*With hydrolysis (n=5)*	*Difference*
Albumin	0.0713 ± 0.006	3.971 ± 0.082	3.90
Carbonic anhydrase	0.0868 ± 0.004	1.128 ± 0.033	1.04
Lysozyme	0.0534 ± 0.003	0.092 ± 0.011	0.04

The determination of formate in a protein has not been determined here, although the method gives good results with N-formylamino acids. The determination of phenacyl formate presents no particular problem, but where formic acid was determined as its methyl ester by GLC there were problems with a large tailing solvent peak[50]. GLC offers a more convenient method than that involving hydrazinolysis for the determination of both formyl and acetyl groups in proteins[21]. Enzymic methods required 100–900 nmol[11], 17–250 nmol[10], a minimum of 80 nmol with an average recovery of 90%[15], 5–55 nmol[17] or 3–12 nmol[18] of acetate. It is often difficult to make a direct comparison between GLC methods because certain details are often missing, *e.g.* final volume of sample, the size of the aliquot injected or the attenuation of the recorder[34]. In general, 0.5 ml or more of biological fluid, such as plasma containing 0.05–10 μmol of acetate/ml[34] was used for GLC and no report was found where attempts had been made to scale down the amount used. Protein samples containing 8–17 μmol of acetate were analyzed by GLC[40]. The method described here allows the

determination of 20–100 nmol of covalently bound acetate (also formate and propionate). Further studies are in progress to scale down these quantities.

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METHYLATION OF INORGANIC ANIONS FOR GAS CHROMATOGRAPHIC DETERMINATION

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SUMMARY

Some inorganic anions were converted into their methyl derivatives by reaction with three methylating agents (dimethyl sulphate, methyl *p*-toluenesulphonate and trimethyl phosphate) at 70°C. The derivatives were extracted with dichloromethane or 1,2-dichloroethane, and determined by gas chromatography with a flame ionization detector. By this method, cyanide, thiocyanate, iodide, bromide and sulphide were determined at concentrations of 0.1–1.0 mg/ml.

INTRODUCTION

Some inorganic anions, such as cyanide (CN^-) and sulphide (S^{2-}), are among the important pollutants of the environment. Furthermore, thiocyanate (SCN^-), nitrite (NO_2^-), halides, etc., have biologically important rôles in living organisms. Therefore, the determination of inorganic anions in various environmental and biological matrices is of vital importance. We have investigated the determination of inorganic anions by gas chromatography (GC).

Some compounds cannot be directly determined by GC, and derivatization reactions must be used prior to analysis[1–4]. The application of GC with derivatization to the determination of inorganic anions is a new and as yet relatively unexplored field, because of the non-volatility of simple inorganic anions and the failure to find a universal derivative comparable to the volatile β-diketone chelates of metal cations. In spite of these difficulties, some methods have been developed for the GC analysis of inorganic anions[4–6].

The preparation of trimethylsilyl derivatives of anions has been studied extensively[4]. For example, trimethylsilyl derivatives of the ammonium salts of several common oxyanions (borate, carbonate, oxalate, phosphite, sulphate, arsenite, phosphate, vanadate and arsenate) were prepared and successfully analyzed by Butts[7] and Butts and Rainey[8].

In previous studies[9,10], we converted NO_2^- and CN^- into *p*-bromochlorobenzene and benzonitrile, respectively, according to reactions similar to the Sandmeyer reaction. These products were determined by GC with an electron-capture detector (ECD) and a flame ionization detector (FID), respectively.

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It is well known that some inorganic anions react with methylating reagents, such as dimethyl sulphate, methyl *p*-toluenesulphonate and trimethyl phosphate, to form their methyl derivatives[11]. In the present work, inorganic anions were methylated and the resulting methyl derivatives were determined by GC with a FID. The objective was to investigate the possibility of applying this GC method to the determination of inorganic anions in environmental or biological samples, by using more sensitive detectors than a FID [*e.g.*, ECD, flame thermionic detector (FTD), flame photometric detector (FPD)].

EXPERIMENTAL

Apparatus

A Yanagimoto G-180 gas chromatograph equipped with a dual flame ionization detector was used. The column and column temperature used were dependent on the methyl derivative of the inorganic anion; details are given in Table I. The column packing materials, Porapak Q (80–100 mesh) and T (80–100 mesh), were purchased from Waters Assoc. (Milford, MA, U.S.A.), and 5% PEG-HT on Uniport HP (60–80 mesh) was obtained from Gasukuro Kogyo (Tokyo, Japan). Nitrogen was used as the carrier gas at a constant flow-rate of 45 ml/min. The injection port temperature was maintained at 250°C. The peak areas were measured by a digital integrator (Shimadzu Chromatopac-E1A, Kyoto, Japan).

TABLE I

METHYLATION AND GAS CHROMATOGRAPHIC CONDITIONS

Stainless-steel columns (3 mm I.D.) were used. Column packing: Q = Porapak Q (1 m); T = Porapak T (1 m); PEG = PEG-HT (2 m).

Anion	*Methyl derivative*	*Extraction solvent*	*Column*	*Column temp. (°C)*
CN^-	CH_3CN	CH_2ClCH_2Cl	Q	140
SCN^-	CH_3SCN	CH_2Cl_2	Q	190
I^-	CH_3I	CH_2ClCH_2Cl	T	150
Br^-	CH_3Br	CH_2ClCH_2Cl	Q	140
NO_2^-	CH_3NO_2	CH_2Cl_2	PEG	80
$HCOO^-$	CH_3OCHO	CH_2ClCH_2Cl	Q	140
CH_3COO^-	CH_3OCOCH_3	CH_2ClCH_2Cl	Q	175
S^{2-}	CH_3SCH_3	CH_2ClCH_2Cl	Q	170

Materials

All reagents were of analytical reagent grade and were used without further purification unless otherwise stated. Dimethyl sulphate, methyl *p*-toluenesulphonate and trimethyl phosphate were commercial grade reagents (Tokyo Kasei Kogyo, Tokyo, Japan). Sodium nitrite was dried in an oven at 110°C for 1 h before it was accurately weighed. Deionized water was distilled before use for the analysis. Solutions of the inorganic anions were prepared by dissolving their potassium or sodium salts in water.

Procedure

To 1.0 ml of an aqueous solution of inorganic anion in a reaction vessel was added 0.1 ml of a methylating reagent (dimethyl sulphate, methyl *p*-toluenesulphonate or trimethyl phosphate). In the determination of CN^- and S^{2-}, 0.5 ml of an aqueous solution of KOH (concentration as in Table III) were added before the addition of the methylating reagent. Then the vessel was shaken for a fixed time (see Table III) in an incubator at 70°C. At the end of the reaction period, 1.0 ml of dichloromethane or 1,2-dichloroethane (see Table I) was added to the cooled reaction solution. Then the methyl derivative was extracted by shaking for 10 min at room temperature, and the organic layer was separated from the aqueous layer. An aliquot (1.0 μl) of the organic layer was injected into the gas chromatograph and the methyl derivative was determined with a FID.

RESULTS AND DISCUSSION

Derivatization yield

The derivatization yield for each inorganic anion was estimated for reactions of each methylating reagent with no addition of either alkaline or acid solution. The peak area of the methyl derivative produced from each anion at 0.1 *M* was compared with that of a standard solution. The latter contained the methyl derivative at a concentration of 0.1 *M* which corresponded to a derivatization yield of 100%. The derivatization yields are shown in Table II as the average values from five replicate determination.

TABLE II

DERIVATIZATION YIELDS FOR INORGANIC ANIONS

Number of data: 5. PTS-Me = Methyl *p*-toluenesulphonate.

Anion	*Methylating reagent*		
	$(CH_3)_2SO_4$	*PTS-Me*	$(CH_3)_3PO_4$
CN^-	44.8 ± 1.2	46.7 ± 1.1	53.6 ± 1.1
SCN^-	104.3 ± 2.1	66.4 ± 1.9	16.1 ± 0.7
I^-	73.2 ± 1.8	63.1 ± 1.0	12.9 ± 0.3
Br^-	*ca.* 65	*ca.* 20	*ca.* 2
NO_2^-	20.3 ± 0.8	7.8 ± 0.2	9.2 ± 0.3
$HCOO^-$	11.6 ± 0.8	7.2 ± 0.2	2.6 ± 0.1
CH_3COO^-	11.0 ± 0.5	8.8 ± 0.2	3.1 ± 0.2
S^{2-}	0.4 ± 0.1	9.0 ± 0.6	44.3 ± 1.1

Thiocyanate was converted quantitatively into methyl thiocyanate by dimethyl sulphate. The derivatization yield of CN^- into acetonitrile by every methylating reagent was about 50% as shown in Table II; a large amount of the converted acetonitrile was not extracted with 1,2-dichloroethane from the aqueous reaction mixture. The actual yields are much higher (see Table III). The derivatization yields for bromide (Br^-) were not very accurate because it is very difficult to prepare 0.1 *M*

methyl bromide solution in 1,2-dichloroethane due to the high volatility of methyl bromide (b.p. 4.5°C). Methylation of iodide (I^-) by dimethyl sulphate or methyl *p*-toluenesulphonate, and that of Br^- by dimethyl sulphate, gives fairly high yields. It is interesting that the derivatization yield of S^{2-} by trimethyl phosphate is much higher than that by dimethyl sulphate or methyl *p*-toluenesulphonate.

TABLE III

OPTIMUM REACTION CONDITIONS AND DERIVATIZATION YIELDS

Anion (1.0 ml)	*Methylating reagent (0.1 ml)*	*Concentration of KOH (0.5 ml)*	*Reaction time (h)*	*Derivatization yield* (%)*
SCN^-	$(CH_3)_2SO_4$	–	0.5	102.9 ± 1.7
SCN^-	PTS-Me	–	4	90.9 ± 0.7
I^-	$(CH_3)_2SO_4$	–	0.5	72.8 ± 1.7
I^-	PTS-Me	–	3	79.0 ± 1.0
Br^-	$(CH_3)_2SO_4$	–	0.5	*ca.* 65
S^{2-}	$(CH_3)_2SO_4$	6 *N*	0.5	42.3 ± 0.8
S^{2-}	PTS-Me	5 *N*	0.5	51.8 ± 0.9
S^{2-}	$(CH_3)_3PO_4$	1.5 *N*	1	62.5 ± 0.8
CN^-	$(CH_3)_2SO_4$	0.5 *N*	0.5	53.5 ± 1.5
				101.1 ± 3.1**
CN^-	PTS-Me	0.5 *N*	1	48.5 ± 1.0
				90.0 ± 1.0**
CN^-	$(CH_3)_3PO_4$	0.5 *N*	2	44.4 ± 2.1
				84.0 ± 1.8**

* The concentration of inorganic anion in the sample is 0.05 *M*. Number of data: 5.
** Value corrected for extraction loss.

Optimum reaction conditions

In order to ascertain the optimum reaction conditions, the effects of pH and reaction time on the derivatization yield were examined.

The pH effect was studied as follows. To 1.0 ml of each aqueous solution of inorganic anion were added 0.5 ml of H_2SO_4 or KOH aqueous solution at different concentrations. Methylation was carried out for 1 h, and the derivatization yield was obtained as above. Fig. 1 shows the effects of the concentrations of H_2SO_4 and KOH on the derivatization yields of SCN^- and Br^- by each methylating reagent. In the cases of SCN^- and Br^-, the yields decreased with increasing KOH concentration, but did not vary with H_2SO_4 concentration. The derivatization yields for I^- do not vary with H_2SO_4 or KOH concentration in the range of 0–1.0 *N*. In subsequent studies of methylation of these three anions, neither H_2SO_4 nor KOH solution was added.

Figs. 2 and 3 show the effects of KOH concentration on the derivatization yields of CN^- and S^{2-}, respectively. The yields decreased to zero on addition of H_2SO_4 in the concentration range 0.1–1.0 *N*. The yields for CN^- became constant at a KOH concentration of *ca.* 0.2 *N*. Thus, in subsequent studies of methylation of CN^-, 0.5 *N* KOH solution was used. All curves in Fig. 3 show maximum derivatization yields; the KOH concentrations which give the maximum yields are shown in

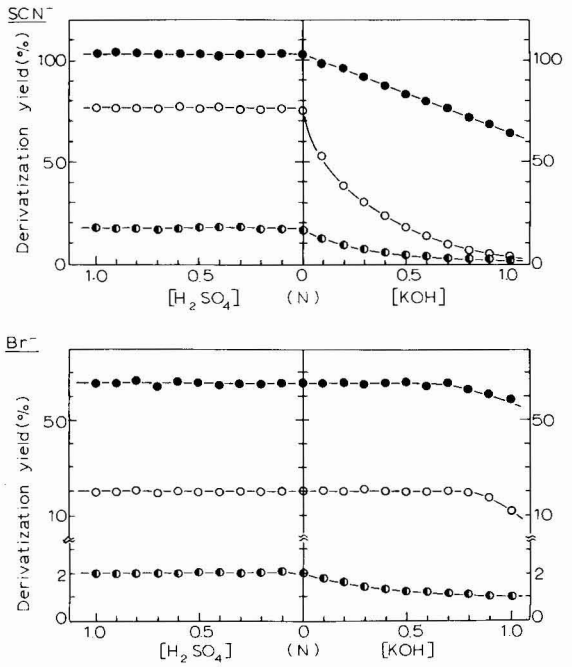

Fig. 1. Effects of H_2SO_4 and KOH concentrations on the derivatization yields of SCN^- and Br^-. Methylating reagent: ●, $(CH_3)_2SO_4$; ○, methyl *p*-toluenesulphonate; ◐, $(CH_3)_3PO_4$.

Table III. KOH solutions with concentrations of 6, 5 or 1.5 *N* were used for subsequent studies of derivatization of S^{2-}.

Methylation of other anions shown in Table II (*i.e.*, NO_2^-, CH_3COO^- and $HCOO^-$) gave relatively low yields ($<50\%$) under a variety of conditions. Each reaction of SCN^-, I^- and Br^- with trimethyl phosphate and that of Br^- with methyl *p*-toluenesulphonate also gave fairly low yields, although the concentration of H_2SO_4 or KOH solution was varied. It is not expected that derivatization reactions with low

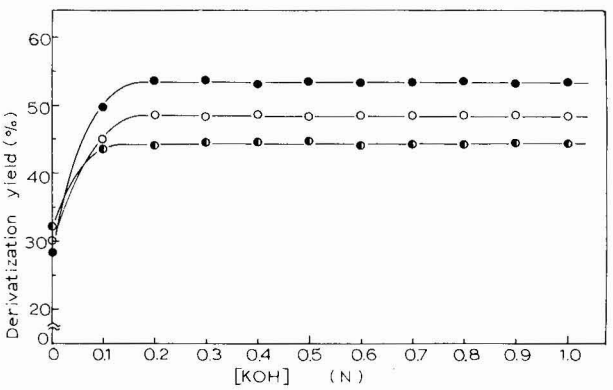

Fig. 2. Effect of KOH concentration on the derivatization yields of CN^-. Methylating reagents as in Fig. 1.

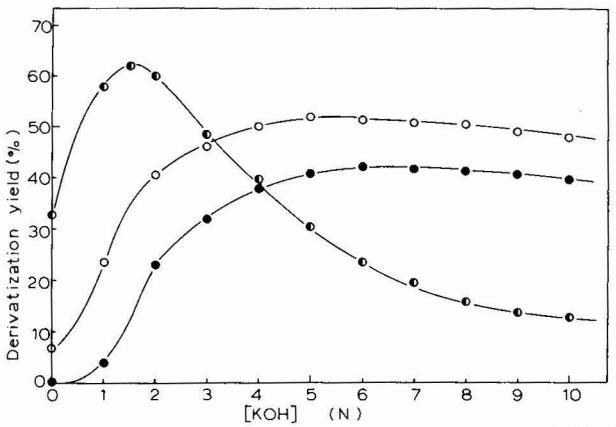

Fig. 3. Effect of KOH concentration on the derivatization yields of S^{2-}. Methylating reagents as in Fig. 1.

yields will be of practical use for the determination of inorganic anions. Therefore, these methylation reactions were not investigated further.

The effect of reaction time on the derivatization yield of each anion (SCN^-, CN^-, I^-, Br^- and S^{2-}) was examined, at the optimum concentration of KOH solution. The results obtained are shown in Figs. 4 and 5. In this study, the 'reaction time' means the period for which the reaction solution is shaken in an incubator at 70°C. It seems that methylation of each anion by dimethyl sulphate proceeds faster than by methyl *p*-toluenesulphonate or trimethyl phosphate. The methylation yield of S^{2-} by dimethyl sulphate reaches its maximum value in a reaction time of 0 min. This shows that this reaction proceeds during the extraction step of shaking at room temperature.

From these results, the optimum reaction conditions were chosen for each methylation reaction (Table III). Table III also shows the derivatization yield of each inorganic anion methylated under the optimum reaction conditions. In the case of CN^-, the resulting product, acetonitrile, was not completely extracted into the organic layer. The derivatization yields corrected for extraction loss by using the distri-

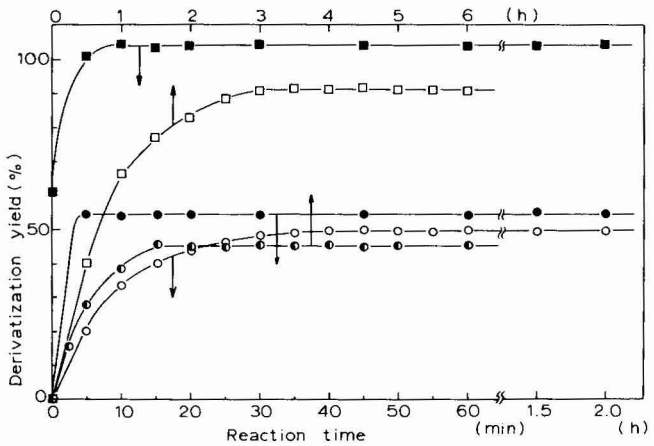

Fig. 4. Effect of reaction time on the derivatization yields of CN^- and SCN^-. Anion (methylating reagent): ●, CN^- ($(CH_3)_2SO_4$); ○, CN^- (methyl *p*-toluenesulphonate); ◐, CN^- ($(CH_3)_3PO_4$); ■, SCN^- ($(CH_3)_2SO_4$); □, SCN^- (methyl *p*-toluenesulphonate).

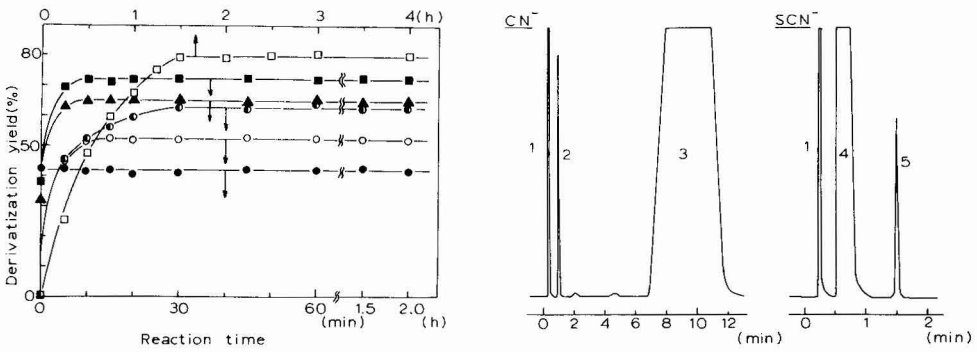

Fig. 5. Effect of reaction time on the derivatization yields of S^{2-}, I^- and Br^-. Anion (methylating reagent): ●, S^{2-} ($(CH_3)_2SO_4$); ○, S^{2-} (methyl *p*-toluenesulphonate); ◐, S^{2-} ($(CH_3)_3PO_4$); ■, I^- ($(CH_3)_2SO_4$); □, I^- (methyl *p*-toluenesulphonate); ▲, Br^- ($(CH_3)_2SO_4$).

Fig. 6. Gas chromatograms of the products of methylation of CN^- and SCN^- by $(CH_3)_2SO_4$ under the optimum reaction conditions. Peaks: 1 = CH_3OH (hydrolysis product of $(CH_3)_2SO_4$); 2 = CH_3CN; 3 = CH_2ClCH_2Cl (solvent); 4 = CH_2Cl_2 (solvent); 5 = CH_3SCN.

bution ratio are also shown in Table III. Methylation of each anion shown in Table III gives a satisfactorily high yield. In particular, CN^- and SCN^- are quantitatively methylated. These high derivatization yields demonstrate the possibility of using these methylation reactions for the determination of inorganic anions by GC with derivatization.

Gas chromatograms and calibration curves

Fig. 6 shows typical gas chromatograms of the products of methylation of CN^- and SCN^- by dimethyl sulphate under the optimum reaction conditions. Fig. 7 shows the calibration curve for CN^-, plotting the peak area of acetonitrile *vs.* the concentration of CN^- aqueous solution, together with the similar calibration curve for SCN^-. The peak area of the methyl derivative produced from 1.0 mg/ml of CN^- or SCN^- solution was arbitrarily assigned a value of 100. Both calibration curves pass through the origin. For the other methylation systems shown in Table III, a good linear relationship between the peak area of the methyl derivative and the concentration of inorganic anion (0.1–1.0 mg/ml) is also obtained.

Comparison with trimethylsilylation

Trimethylsilyl derivatives have been prepared for many anions by reaction with trimethylsilylating reagents. During derivatization, it is necessary to maintain anhydrous conditions since trimethylsilylating reagents and trimethylsilyl derivatives are sensitive to water. The silylation of anions in water, therefore, requires a pretreatment step: extraction of the anions into an organic solvent from an aqueous sample solution, evaporation to dryness, etc. In contrast, the methylating reagents used in this work are much more stable in water and give methyl derivatives which are insensitive to water. Consequently, methylation can be performed directly by adding the methylating reagents to an aqueous sample solution, without pretreatment, although the present inorganic anions methylated were not the same as those trimethylsilylated.

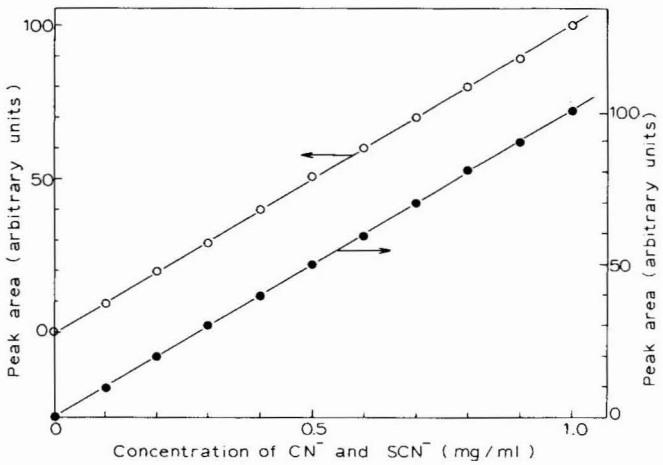

Fig. 7. Calibration curves for CN^- (○) and SCN^- (●). Methylating reagent: $(CH_3)_2SO_4$.

CONCLUSION

It is found that the inorganic anions CN^-, SCN^-, I^-, Br^- and S^{2-} at concentrations of 0.1–1.0 mg/ml can be determined by methylation followed by flame ionization gas chromatography. This concentration range is much higher than the concentrations found in environmental or biological samples. Moreover, the methyl derivatives do not give as high a FID response as compounds containing more carbon atoms. Consequently, in order to determine these anions at low concentrations, a detector must be used which exhibits a higher response to the methyl derivatives. Fortunately, a FTD is highly sensitive to nitrogen-containing compounds (for CN^- and SCN^-), an ECD to halogen-containing compounds (for I^- and Br^-) and a FPD to sulphur-containing compounds (for S^{2-} and SCN^-).

ACKNOWLEDGEMENT

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DETERMINATION OF DISTEARYLCARBAMOYL CHLORIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and specific method for the determination of distearylcarbamoyl chloride in different extracts by high-performance liquid chromatography (HPLC) has been developed. In the method hexane extract containing this compound is evaporated and then treated with sodium thiophenolate to give the stabile thiocarbamate. This is determined by HPLC on a silica gel column with UV detection, using *n*-hexane–dichloromethane (7:3) as eluent. The sensitivity is about 0.1–0.5 μg.

INTRODUCTION

Carbamoyl chlorides are reactive compounds important as intermediates in the synthesis[1] of, *e.g.*, carbamates, thiocarbamates and substituted ureas. However, few methods for the analysis of these compounds have been published. Normally, large amounts of carbamoyl chlorides are analyzed[2] by titration of liberated chloride ions after reaction with an appropriate amine. Recently Rusch *et al.*[3] determined dimethylcarbamoyl chloride in air using a spectroscopic technique after reaction with 4-(*p*-nitrobenzyl)pyridine.

Distearylcarbamoyl chloride (DACC) has been found to be an excellent sizing agent for paper[4]. In the paper making process cellulose fibres are made hydrophobic by the reaction between cellulose hydroxyl groups and the carbamoyl chloride. Here we describe a high-performance liquid chromatographic (HPLC) method of determining distearylcarbamoyl chloride by derivatization with sodium thiophenolate (Fig. 1) to give the stable, UV-absorbing thiocarbamate. The method has been used routinely for small amounts of dialkylcarbamoyl chlorides in extracts from paper treated with a sizing agent (Kenotaf®) containing mainly distearylcarbamoyl chloride.

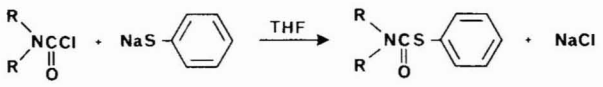

Fig. 1. Structure of distearylcarbamoyl chloride (DACC). On reaction with sodium thiophenolate a stable and UV-absorbing thiocarbamate is formed. R = mainly $C_{18}H_{37}$ containing about 10% $C_{16}H_{33}$ and small amounts of $C_{12}H_{25}$ and $C_{14}H_{29}$.

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MATERIALS AND METHODS

Reagents

n-Hexane, methylene chloride, chloroform and toluene were reagent grade from E. Merck (Darmstadt, G.F.R.). Tetrahydrofuran (THF) (E. Merck, reagent grade) was purified immediately before use by filtration through a column of Al_2O_3 (W 200, basic, activity super 1; ICN Pharm., Eschwege, G.F.R.). Sodium thiophenolate was prepared from thiophenol, pract. grade, (Fluka, Buchs, Switzerland) according to Jenden *et al.*[5].

Silica gel for column chromatography (Si 60, 0.063–0.2 mm and 0.2–0.5 mm) and silica gel thin-layer plates (HF_{254}) were purchased from E. Merck.

Chromatography system

An HPLC system based on a Waters 6000 pump (Waters Assoc., Milford, MA, U.S.A.) was used throughout this work in conjunction with an Altex 153 UV detector (Altex Scientific, Berkeley, CA, U.S.A.), fitted with filters for 254 nm, and a Waters U6K loop-injector. The column was a stainless-steel tube (30 cm × 3.9 mm I.D.) prepacked with 10-μm silica gel (Waters Assoc.). The system was kept at ambient temperature.

Distearylcarbamoyl chloride (DACC)

This compound, prepared from distearylamine and phosgene, is available from KemaNobel (Stockholm, Sweden) as Kenotaf®. Since the amine used as a starting material is synthesized from hydrogenated tallow fatty acids, DACC will comprise *ca.* 80% stearyl, 10% palmityl and 10% lauryl and myristyl carbon chains.

DACC technical grade (85% carbamoyl chloride), containing amine hydrochloride and alkylurea as impurities, was purified by passing through a silica gel column. About 100 g of DACC in 150 ml of carbon tetrachloride–chloroform (8:2) were rapidly passed through silica gel (200 g, 0.063–0.2 mm) in a glass filter (G3, diameter 10 cm). The gel was washed with 400 ml solvent. Evaporation of the combined washings yielded 75 g of DACC, free from amine hydrochlorides.

Preparation of reference sample

Purified DACC (5 g, 7.7 mmoles) and sodium thiophenolate (2.27 g, 17.2 mmoles) were refluxed in THF (30 ml) for 2 h under nitrogen. After evaporation the residue was distributed between 2 *M* sodium hydroxide (100 ml) and *n*-hexane (2 × 60 ml). The hexane layers were collected, dried (anydrous sodium sulphate) and evaporated. The residue was purified by silica gel column chromatography using toluene as eluent. The reaction and purification was followed by thin-layer chromatography (TLC) (silica gel, toluene). Three spots could usually be seen: DACC (R_F = 0.54, ninhydrin positive); unidentified by-product (R_F = 0.5, UV-absorbing); DACC-thiocarbamate (R_F = 0.39, UV-absorbing). The DACC-thiocarbamate was identified by NMR and mass spectrometry (see Discussion).

Extraction

The extraction studies were made on unbleached sulphate paper-board, basc paper weight 300 g/m^2. The paper was coated on both sides with polyethylene, 60

g/m^2. However, both uncoated and coated paper were extracted. Either the paper was cut into pieces and extraced in a flask or the extraction cell test was used (ASTM F 34-68).

The extractions were performed with 10 g paper (or 6 dm^2 in the ASTM cell) using 200 ml *n*-heptane, 50% aqueous ethanol, 3% aqueous acetic acid and water. The aqueous solutions were extracted with *n*-hexane (2 × 50 ml). The combined hexane extracts were dried with anhydrous Na_2SO_4, filtered and the solvent was evaporated in a rotary evaporator at 45°C. The *n*-heptane extract was filtered directly and evaporated as above.

Derivatization

The hexane or heptane extracts were evaporated to near dryness in a rotary evaporator and transferred by means of a few millilitres of dichloromethane to a screw-cap vial (3 ml, No. 13222; Pierce, Rockford, IL, U.S.A.) and the solvent was evaporated using a nitrogen stream. Sodium thiophenolate (20 mg) and THF (1 ml) were added, the air was removed by flushing with nitrogen and the vial was closed (PTFE-lined caps) and heated at 60°C for 2 h.

The reaction mixture was transferred to a test-tube (the vial being thoroughly rinsed with small amounts of hexane and 2 *M* NaOH). A 15-ml volume of 2 *M* NaOH was added and the solution was extracted with *n*-hexane (2 × 5 ml). The combined hexane extracts were collected, dried with anhydrous Na_2SO_4, filtered (through a small glass-wool plug in a Pasteur pipette) and evaporated by means of a stream of nitrogen. The tubes and the Na_2SO_4 were rinsed several times with small amounts of *n*-hexane which were then filtered. The filtered solution was evaporated to dryness (nitrogen) and the residue dissolved in *n*-hexane–dichloromethane (7:3) (0.5 ml). A 5-μl volume was immediately injected into the chromatograph.

Liquid chromatography

An HPLC silica gel column containing 10-μm particles and with *n*-hexane–dichloromethane (7:3) at 1.5 ml/min as eluent was used. Before running the samples a standard solution of DACC-thiocarbamate (*e.g.*, 0.2 mg/ml solution in the eluent) was run as a control of the retention time and column efficiency. After a number of runs with samples from the paper extracts the column deteriorated and no adequate separation was obtained. The column was regenerated by successively washing with dichloromethane (150 ml), *n*-hexane (150 ml) and *n*-hexane–dichloromethane (7:3) (50 ml).

Sample clean-up by column chromatography

Some extracts especially from polyethylene-coated papers gave poor chromatograms due to impurities obscuring the DACC-thiocarbamate peak. In order to achieve better separation and longer HPLC column life, a clean-up was performed by chromatography on a small silica gel column (13 × 1 cm, 5 g, Merck silica gel 60, 0.2–0.5 mm particle size) in *n*-hexane–dichloromethane (8:2). After filtration and evaporation, the derivatized sample was dissolved in 1 ml *n*-hexane–dichloromethane (8:2) and placed on to the column. Most of the impurities comprising low-molecular-weight polyethylene and diphenyl disulphide were eluted with 60 ml *n*-hexane–dichloromethane (8:2) and discarded. The solvent was then changed to dichloromethane

and 70 ml were collected in a round-bottomed flask. After evaporation of the solvent the residue was transferred to a test-tube, again evaporated in a stream of nitrogen, dissolved in 0.5 ml *n*-hexane–dichloromethane (7:3) and submitted to HPLC as above.

Calibration curve

A calibration curve was constructed using solutions containing known amounts of DACC and *n*-hexane extracts from non-DACC-treated papers (= blank extract) corresponding to *ca.* 10 g paper. A 1-ml chloroform solution of a blank extract was transferred to each of five screw-cap vials and evaporated by means of a stream of nitrogen. Sodium thiophenolate (20 mg) was added to each vial and then 1 ml THF containing 0.05, 0.1, 0.25 or 0.5 mg DACC. The derivatization, extractions and HPLC were performed as described above.

RESULTS AND DISCUSSION

Before considering the use of new chemicals in food or in contact with food, authorities in most countries not only demand toxicological studies but they also request specific studies of the amount of the chemicals which might contaminate the food. Since DACC is used for sizing of paper intended for food packaging a series of extractions have been performed. Under constant conditions, food simulating solvents, *e.g.*, *n*-heptane, 50 % aqueous ethanol, 3 % aqueous acetic acid and water, were used. DACC was to be determined in small amounts in these different types of extracts.

The complete extraction of DACC from water and the aqueous solutions described above with *n*-hexane was confirmed by the following experiment. DACC (0.5 mg) in hexane (50 ml) was extracted with 200 ml of the appropriate solvent. The DACC content in the hexane solutions was determined by HPLC and compared with the same amount of DACC in a hexane solution not partitioned with any solvent (see Table I). Thus, since DACC is a very lipophilic molecule and can be fully extracted with hexane the problem was reduced to determining DACC in dilute solutions of hexane (or heptane).

DACC is a reactive compound. It was not possible to use gas chromatography (GC) for quantitative analysis, due to the instability and the high molecular weight. Furthermore, GC separates DACC into four peaks due to the combinations of C_{14},

TABLE I

EXTRACTION OF DACC FROM *n*-HEXANE WITH AQUEOUS SOLUTIONS

DACC content determined by HPLC.

Hexane phase	*DACC content in n-hexane phase (peak height in mm)*
Not extracted	153
Water extracted	157
3 % CH_3COOH extracted	163
50 % C_2H_5OH extracted	159

C_{16} and C_{18} carbon chains. Also underivatized DACC decomposed to the secondary amine when submitted to liquid chromatography on silica gel. However, by treating DACC with sodium thiophenolate (Fig. 1) a stable compound was formed which gave only one sharp symmetrical peak in HPLC. One further advantage of the derivatization was that UV detection could be used.

The structure of the thiophenyl carbamate of DACC was confirmed by nuclear magnetic resonance (NMR) and mass spectrometric studies. In ^{13}C NMR the signal from the carbonyl group is shifted from 149.0 ppm in DACC to 166.1 in the thiocarbamate, an expected low field shift due to the phenyl group. The thiocarbamate also exhibits signals in the region 128–136 ppm from aromatic carbons. It is interesting that CH_2–N in DACC gives two signals at 51.3 and 50.0 ppm, as for example in amides, but only one signal at 48.2 ppm is found for the thiocarbamate.

Mass spectral data for the thiophenyl carbamate of DACC are summarized in Table II. Using electron ionization it was impossible to differentiate between DACC and its thiophenyl carbamate derivative. The spectra of both compounds contained no M ion, but an ion with the same mass corresponding to M – Cl and M – thiophenyl respectively. By using chemical ionization the MH^+ ions were detected and a cleavage of MH^+ – thiophenyl confirmed the structure of the thiophenyl carbamate.

TABLE II

MASS SPECTRAL DATA OF THE THIOPHENYL CARBAMATE OF DACC (FIG. 1)

Collected on a Micromass 7070F instrument (V. G. Micromass, Winsford, Great Britain) using the direct inlet system. EI = Electron ionization; CI = chemical ionization with isobutane.

R groups in R_2N	*Theoretical m/e*		*Found m/e*		
	M	*M – thiophenyl*	*with EI for M – thiophenyl*	*with CI*	
				MH^+	MH^+ – *thiophenyl*
C_{14}, C_{16}	573	464	464	574	464
C_{14}, C_{18}	601	492	492	602	492
C_{16}, C_{16}	601	492	492	602	492
C_{16}, C_{18}	629	520	520	630	520
C_{18}, C_{18}	657	548	548	658	548

The retention time of this synthetic thiophenyl carbamate of DACC was 4.5 min under the HPLC conditions stated above. A peak appeared at exactly the same retention time in chromatograms from derivatized extracts of DACC-treated papers. Typical chromatograms are shown in Figs. 2 and 3.

To show that quantitative determinations were made on the correct compound, several extracts from DACC-treated paper were combined and submitted to preparative HPLC in which peak A (Fig. 2) was collected. The ^{13}C NMR spectrum of the collected substance was identical with that obtained from a reference thiophenyl carbamate of DACC.

The HPLC method was calibrated by adding known amounts of DACC (0.05–

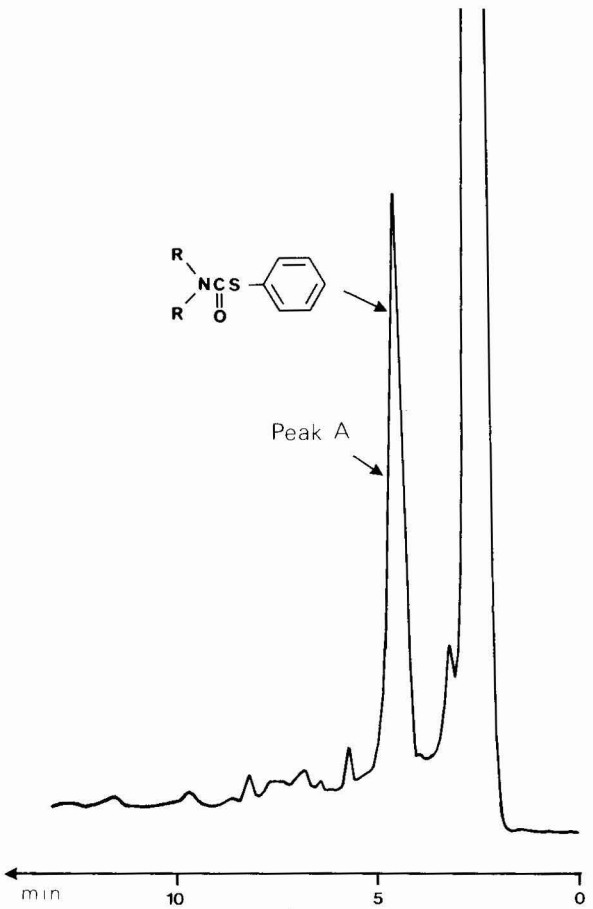

Fig. 2. Chromatogram of 5 μl of derivatized heptane extract from a paper containing 0.35 mg DACC. Conditions: 250 × 3.9 mm I.D. column containing silica gel (10 μm) with *n*-hexane–dichloromethane (7:3) as mobile phase, flow-rate 1.5 ml/min; UV detection at 254 nm with 0.08 a.u.f.s.

0.5 mg) to extracts from paper not treated with DACC and taking these standards through the assay procedure. The amount of DACC in paper extracts was then determined from a calibration curve (Fig. 4) constructed by plotting the height of the thiophenyl carbamate peak *versus* the amount DACC added to the extract.

It was found important to investigate the specificity of the derivatization reaction since both tetraalkylurea and the ethyl carbamate (Fig. 5) could be formed from DACC during extractions of DACC-treated papers in water and alcohol solutions respectively. However, no peak with the same retention time as the thiophenyl carbamate of DACC was detected in HPLC when 0.5 mg of the tetraalkylurea or the ethyl carbamate were submitted to the derivatization procedure.

The reproducibility of the method was investigated by taking four replicate samples of 0.05–0.5 mg through the whole procedure, including extraction from a water solution. The results are shown in Table III. Each solution was injected four times into the chromatograph.

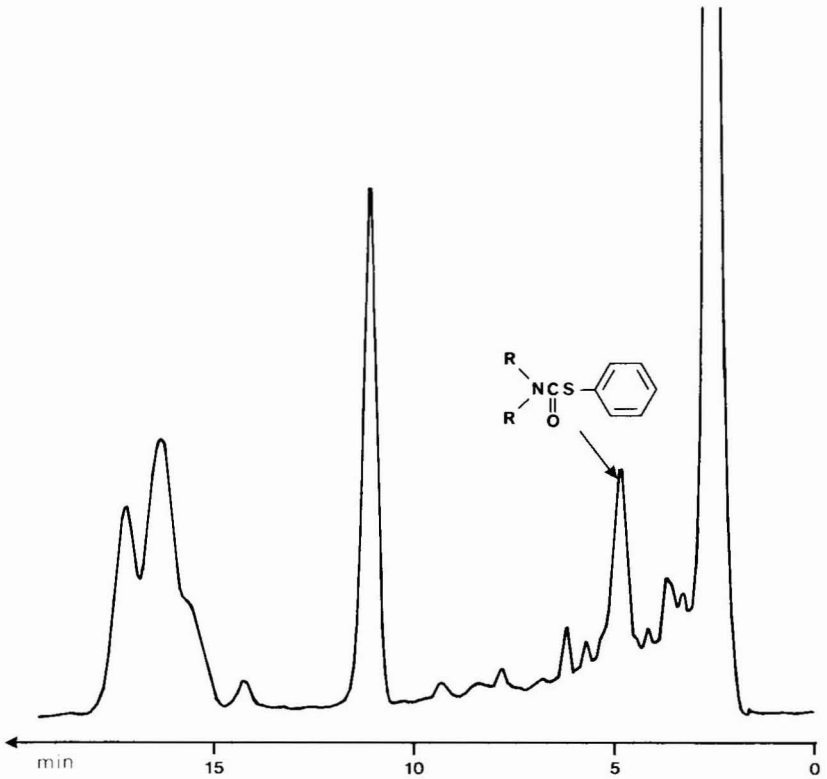

Fig. 3. Chromatogram of 0.05 mg of DACC in *ca.* 9 mg of an extract from bleached untreated paper (140 g/m^2). Conditions as in Fig. 2.

The recovery was evaluated by adding 0.25 mg DACC to a water–hexane mixture and then analyzing the hexane phase by the described method. The recovery was determined to be 82 % by comparing the peak height with that obtained by direct injection of the same amount of pure thiophenyl carbamate of DACC.

The minimum amount of DACC detectable depended on a number of factors, *e.g.*, paper qualities, presence of coating and purification of the extract by silica gel chromatography. In extracts from 10 g unbleached paper (300 g/m^2), 0.05 mg added, corresponding to an injected amount of 0.5 μg, gave a peak height of 40 mm at a detector attenuation of 0.08. It was possible under these conditions to detect 0.1 μg, but no efforts have been made to reach a better sensitivity.

Some results obtained are shown in Table IV. The paper-board was produced with 0.2 % DACC in a full scale trial, and assuming 50 % retention the DACC content in the paper should be 3 mg/dm^2. DACC reacts with the fibres during the drying procedure and the storage. The curing was followed by measuring the decreasing amount of absorbed lactic acid. Thus a more hydrophobic paper should show a smaller amount of DACC extractable by heptane. Table IV shows the extractable amounts of DACC and the absorption of lactic acid *versus* storage time.

From toxicological studies a No Toxic Effect Level (NTEL) for a substance can be deduced. In this case it was concluded that 10 mg DACC per kg bodyweight

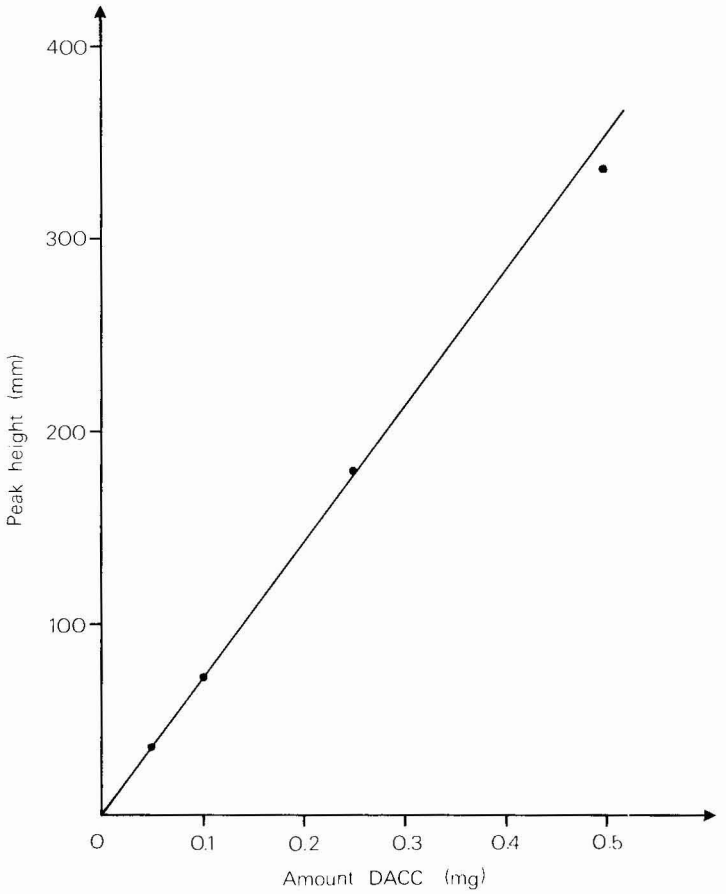

Fig. 4. Calibration curve from paper extracts containing 0.05–0.5 mg DACC. Conditions as in Fig. 2.

per day was a very conservative estimate of the NTEL value, *i.e.*, 600 mg/day (100 mg/dm^2) for a human being. The extracted amounts of DACC from the paper were far below 600 mg, even with a safety factor of 100 (see Table V).

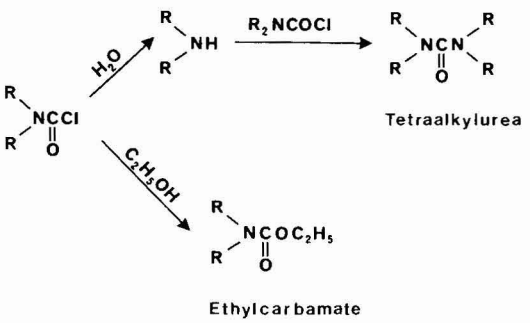

Fig. 5. Reaction products formed when DACC is exposed to water and ethanol.

TABLE III

REPRODUCIBILITY OF THE DETERMINATION OF DACC

DACC was added to a hexane extract from blank paper not treated with DACC. The solution was then extracted with water and taken through the whole derivatization procedure.

Amount of DACC added (mg)	*Mean peak height of 4 samples (mm)*	*S.D. of peak height of 4 samples (mm)*	*Coefficient of variation (%)*
0.5	335.3	15.7	3.14
0.25	179.2	7.3	3.71
0.1	69.2	2.6	4.05
0.05	35.6	1.1	4.67

TABLE IV

INFLUENCE OF CURING TIME ON EXTRACTION OF DACC BY HEPTANE AND PAPER HYDROPHOBICITY MEASURED BY LACTIC ACID ABSORPTION

Age of paper (days)	*Extracted amount of DACC (mg/dm^2)*	*Lactic acid absorption (g/m edge)*
7	0.395	0.91
29	0.28	0.46
80	0.27	0.38
125	0.145	0.38
Fully cured	0.0155	0.36

TABLE V

EXTRACTION OF DACC FROM FULLY CURED PAPER BY DIFFERENT SOLVENTS

Solvent	*Extraction time*	*Temperature (°C)*	*Extracted amount of DACC (mg/dm^2)*
Heptane	48 h	50	0.0155
50% C_2H_5OH	5 days	50	< 0.01
Water	5 days	50	< 0.01
3% CH_3COOH	5 days	50	< 0.01

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Note

Rapid method for packing microparticulate columns packed with a chemically bonded stationary phase for high-performance liquid chromatography

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A number of methods for packing microparticulate high-performance liquid chromatographic (HPLC) columns have been published, but the techniques are still under development. The recent extensive use of microparticulate columns for HPLC has led to the need for simple methods for preparing inexpensive and high-efficiency columns in the laboratory. Several workers[1–4] have discussed the advantages and disadvantages of various packing methods such as the balanced density[5–13], balanced viscosity[7,13–15] and mechanical stirring methods[16–19] and the use of the mobile phase[20], and proposed convenient methods that give good microparticulate columns for routine use. A technique that gives high-efficiency (15.6 μm HETP) silica gel microparticulate columns (*ca.* 3–5 μm) has been described[13]. However, many of the methods are not suitable for chemically bonded stationary phases but only for silica gel columns. So far, few methods that guarantee high-efficiency (HETP less than 20 μm) home-made microparticulate columns packed with a chemically bonded stationary phase have been reported.

In this paper, a rapid packing method that gives high-efficiency chemically bonded normal- and reversed-phase microparticulate columns (*ca.* 5 μm) for routine use is described.

EXPERIMENTAL

Solvents and materials

The solvents used were of special grade from Wako (Osaka, Japan). Methanol was filtered through 0.22-μm Fluoropore filters (Sumitomo Electric, Osaka, Japan) before use. Nonipole 40 (nonylphenyl polyethylene glycol, "4 moles" ether) was purchased from Sanyo Kasei Kogyo (Kyoto, Japan). The composition of the slurry solvent is shown in Table I. Polygosil and Nucleosil materials were obtained from Machery, Nagel & Co. (Düren, G.F.R.) and LiChrosorb RP-18 from E. Merck (Darmstadt, G.F.R.).

Apparatus

The slurry reservoir is shown in Fig. 1. The reservoir is made of 316 grade seamless stainless steel and holds 45 ml of slurry up to the top inlet. The packing

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TABLE I

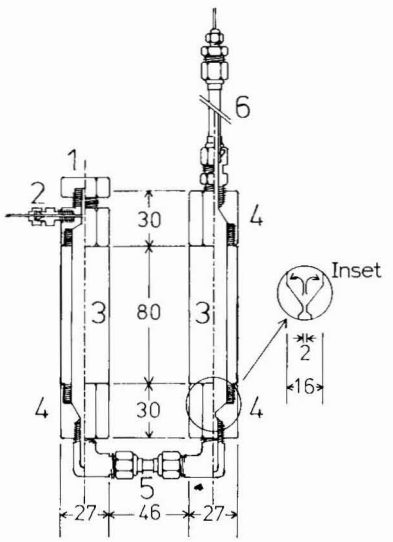
APPROPRIATE AMOUNTS OF PACKING MATERIALS AND COMPOSITION OF SLURRY SOLVENT

Packing material	*Appropriate amount of packing material*		*Solvent composition*	
	Amount (g)	*Column length and I.D. (mm)*	*Component*	*Concentration (%, v/v)*
LiChrosorb RP-18 (5 μm)	1.5	150 × 4.0	Methanol	10.0
Polygosil 60-5C_{18}	3.0	300 × 4.0	Isopropanol	5.0
Polygosil 60-5NO_2	2.0	150 × 4.6	Cyclohexanol	10.0
Nucleosil 5NO_2	2.5	200 × 4.6	Cyclohexane	4.0
Polygosil 60-5NH_2	3.2	250 × 4.6	1,1,1-Trichloroethane	70.0
Nucleosil 5NH_2			Nonipole 40	1.0

apparatus is shown in Fig. 2. A Coulter Electronics (Hialeah, FL, U.S.A.) TA II particle analyser was employed to determine the particle size of the slurried materials by using 4% lithium chloride in methanol[21,22].

Packing procedure

The appropriate amount of packing material for the column dimensions (see



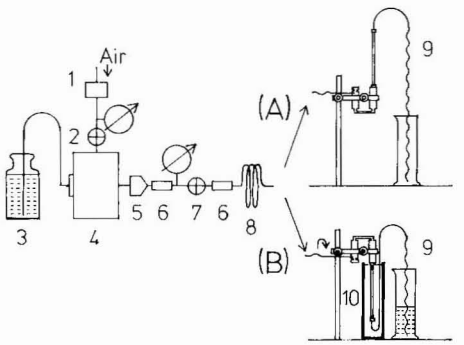


Fig. 1. Slurry reservoir. 1 = Top inlet and removal cap with a PTFE gasket; 2 = eluent inlet; 3 = cell (20 ml); 4 = union; 5 = Tylok (Euclid, OH, U.S.A.) 316 grade stainless-steel 1/4-in. standard connections; 6 = column. Dimensions in millimetres.

Fig. 2. Packing apparatus. 1 = Pressure regulator (0–10 kg/cm^2); 2 = on-off valve; 3 = eluent reservoir (methanol); 4 = Haskel (Burbank, CA, U.S.A.) MPC-110 pneumatic amplifier pump; 5 = damping unit (60 ml); 6 = 10 cm × 4.6 mm I.D. guard column packed with Polygosil 60-2540; 7 = high-pressure valve; 8 = 2 m × 1.7 mm O.D. 316 grade stainless-steel tube; 9 = 1.7 mm O.D. PTFE tube; 10 = heating-bath (60°C).

Table I) was mixed with 40 ml of the slurry solvent and the suspension was placed in an ultrasonic bath for 5 min. The dispersed slurry was poured into the slurry reservoir by using a syringe, and the empty part of the reservoir was filled with the slurry solvent. The packing was started with an upward flow (see Fig. 2A). The packing pressure on to the eluent (methanol) was programmed stepwise from 400 to 700 kg/cm^2 at 50 kg for every 5 cm of the packing. When 15 ml of the eluent had been pumped, the reservoir was inverted (see Fig. 2B), then the column was heated to 60°C. Once the packing was completed, the eluent was allowed to flow at room temperature for 10 min under the final packing pressure. The flow was then stopped and the column was removed from the reservoir and assembled with the frit and the reducing unit.

RESULTS AND DISCUSSION

A relatively non-toxic and very stable slurry solvent was prepared (see Table I). Methanol was used to increase the dispersibility of the solvent and isopropanol and cyclohexanol to control viscosity other than dispersibility. The use of the nonionic surfactant (Nonipole 40) was effective in preparing stable and well dispersed slurries of the packings (*ca.* 5 μm). The use of 0.5–1.5% (v/v) of surfactant minimized the rates of sedimentation and the sedimentation volumes in 24 h for both normal- and reversed-phase materials. The rates of sedimentation, defined as the ratio of height of sedimentation to standing time, were constant for 1 h in the range 0.07–0.11 mm/min for the packing materials used. The use of less or more of the surfactant was less effective in reducing aggregation or flocculation of the dispersed particles. The particle size analysis and direct microscopic observations showed that the slurries were stable and well dispersed.

The reservoir used resulted in minimal use of the packing materials (see Table I), with substantial savings of the packing materials over conventional methods. As slurries usually form aggregates at the bottom, the upward packing technique[1,17,18] may be useful in introducing a dispersed slurry into the column, especially in the initial packing stage. In the initial upward packing with the U-shaped reservoir, the nozzle at the bottom of the second cell (see Fig. 1, inset) was useful for producing mixing effects around the bottom so as to maintain well dispersed particles.

The pressure programming and the column heating were used to maintain constant high flow conditions. The flow-rates in preparing 20 cm × 4.6 mm I.D. columns became stable with 1 min, and were maintained nearly constant until the packing was completed. Typical flow-rates were 7–8 ml/min for LiChrosorb RP-18 and the Polygosil materials and 3–4 ml/min for the Nucleosil materials. Thus, the packing of the columns was completed in a much shorter time than by conventional methods. The high flow-rate may be useful in packing the dispersed slurry into the column before aggregation occurs.

A number of 20 cm × 4.6 mm I.D. microparticle columns were prepared at different times and their performances were examined. Few failures of the packings occurred. Fig. 3 shows typical chromatograms for the evaluation of the column performance. Table II indicates that high-efficiency columns are reproducibly prepared by the proposed method. Occasionally, superior columns with up to 18,000 theoretical plates (11.1 μm HETP) were obtained for LiChrosorb RP-18 and Poly-

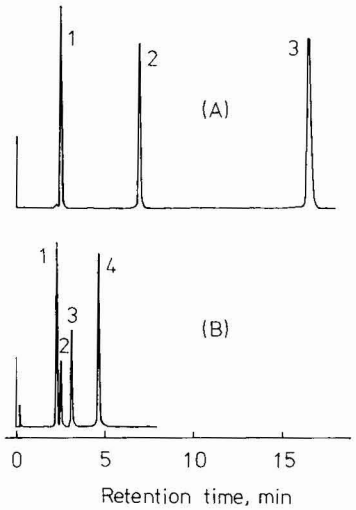


Fig. 3. Typical liquid chromatograms for evaluation of column performance. Columns, 20 cm × 4.6 mm I.D.; mobile phase, acetonitrile–water (60:40); flow-rate, 1.0 ml/min; amount of compound, 0.2–1.5 μg in a 2-μl injection; UV absorbance detector, 254 nm. (A) Polygosil 60-5C_{18}: 1 = uracil; 2 = benzene; 3 = acenaphthene. (B) Nucleosil 5NH_2: 1 = benzene; 2 = *o*-chlorophenol; 3 = uracil; 4 = 2,5-dichlorophenol.

TABLE II

PERFORMANCE OF MICROPARTICULATE COLUMNS PACKED WITH CHEMICALLY BONDED STATIONARY PHASES

Analytical conditions: see Fig. 3. The capacity factors (k') of LiChrosorb RP-18 and Polygosil 60-5C_{18} columns were obtained by using acenaphthene as a reference peak and uracil as an unretained peak, and the k' values of Nucleosil 5NH_2 columns were obtained by using 2,5-dichlorophenol as a reference peak and benzene as an unretained peak.

Packing material	*Column number*	*Column performance*			
		Theoretical plates	*HETP (μm)*	*Reduced HETP*	*k'*
LiChrosorb RP-18 (average size 5.7 μm)	1	10,700	18.7	3.3	8.64
	2	11,700	17.1	3.0	8.61
	3	10,900	18.3	3.2	8.75
	4	11,600	17.2	3.0	8.41
Polygosil 60-5C_{18} (average size 4.9 μm)	1	11,500	17.4	3.6	6.10
	2	12,500	16.0	3.3	6.02
	3	12,800	15.6	3.2	6.09
	4	11,400	17.5	3.6	5.97
Nucleosil 5NH_2 (average size 4.2 μm)	1	11,800	16.9	4.0	1.12
	2	12,400	16.1	3.8	1.09

gosil 60-5C_{18}. The number of theoretical plates obtained here are very similar to or greater than those for columns previously reported or commercially available.

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Note

Packing of Toyopearl columns for gel filtration

IV. Gravitational packing and influence of slurry reservoir size

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We have been investigating packings of Toyopearl (Toyo Soda, Tokyo, Japan), a hydrophilic porous polymer packing material for gel filtration, resistant to pressures up to several atmospheres, and have already reported some results[1–3]. Whereas all columns were packed with a peristaltic pump by a constant-velocity or a semi-constant-pressure method in the previous experiments, this paper reports the results of investigations on the gravitational packing method, which is commonly used with packings of soft gels such as Sephadex[4,5]. In the previous experiments, all columns were also packed using slurry reservoirs consisting of long columns with the same diameters as the chromatographic columns. However, as commercial slurry reservoirs are generally short and have larger diameters than chromatographic columns, the influence of the slurry reservoir size on the performance of packed columns was also investigated.

GRAVITATIONAL PACKING

A schematic diagram of the packing arrangement is shown in Fig. 1.

Toyopearl HW55S (Lot No. 55009-16M) of diameter 20–40 μm, the same material as Fractogel TSK HW55 (0.025–0.037 mm) available from E. Merck (Darmstadt, G.F.R.), was packed gravitationally into various sizes of glass columns as described previously[2]. However, a hydrostatic pressure of 250 cmH_2O was utilized instead of a peristaltic pump to supply the solvent and each packing operation was continued overnight. The packed columns were tested for performance with a mixture of bovine serum albumin and myoglobin as described previously[2].

The results are summarized in Table I and compared with those for constant-velocity packings. The resolution factors for bovine serum albumin and myoglobin, R-(BSA, myoglobin), obtained with gravitational packings were lower than those obtained with constant-velocity packings at optimal velocities. However, the differences were within 10% and were only 5% on average. Furthermore, higher resolutions can be expected with gravitational packings under higher hydrostatic pressures because the resolution increased with increasing final packing pressure up to 0.6 atm with constant-velocity packings. Nevertheless, as a hydrostatic pressure of 250 cmH_2O

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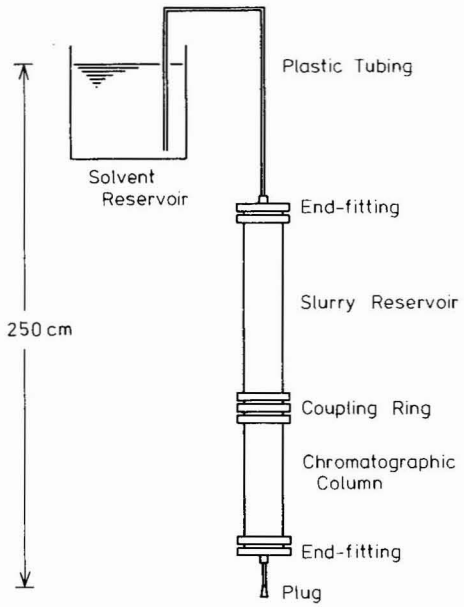

Fig. 1. Schematic diagram of arrangement for gravitational packing.

TABLE I

RESULTS FOR GRAVITATIONAL PACKINGS OF TOYOPEARL HW55S UNDER A HYDROSTATIC PRESSURE OF 250 cmH_2O

Column dimensions (length × I.D., cm)	*R (BSA, myoglobin)*	*R (BSA, myoglobin)**	*Final packing velocity (ml/h · cm²)*
30 × 2.2	1.49 (5%)**	1.57	20.5
	1.43 (9%)		19.8
45 × 2.2	1.78 (6%)	1.90	14.1
	1.89 (1%)		15.8
60 × 2.2	2.03 (9%)	2.22	9.8
	2.10 (5%)		11.9
90 × 2.2	2.63 (1%)	2.65	8.1
	2.53 (5%)		8.7
60 × 1.0	1.94 (2%)	1.98	13.6
	1.96 (1%)		15.5
60 × 1.6	2.12 (4%)	2.21	13.8
	2.06 (7%)		14.1
60 × 3.2	2.08 (8%)	2.25	11.3
	2.19 (3%)		9.4
60 × 4.4	2.11 (5%)	2.23	10.0
	2.07 (7%)		10.1

* Obtained by constant-velocity packings at optimal velocities[2].

** Values in parentheses are differences from *R*(BSA, myoglobin) for constant-velocity packings.

seems to be almost the maximum attainable in most laboratories, it may be concluded that constant-velocity packings with a pump are better than gravitational packings with respect to the resolution of packed columns for semi-soft gels such as Toyopearl. Moreover, the final packing velocities with gravitational packings were half to one third of the optimal packing velocities with constant-velocity packings. This means that columns obtained with gravitational packings must be operated at correspondingly lower velocities. Therefore, it is preferable to use pumps in the packing of semi-soft gels in order to take advantage of hardness of those gels.

INFLUENCE OF SLURRY RESERVOIR SIZE

Toyopearl HW55S (Lot No. 55009-16M) was packed into glass columns of various sizes using commercial slurry reservoirs of the shapes illustrated in Fig. 2. Both the chromatographic columns and the slurry reservoirs were purchased from Amicon (Lexington, MA, U.S.A.) and their sizes are given in Table II. Packings were carried out by constant-velocity or semi-constant-pressure methods.

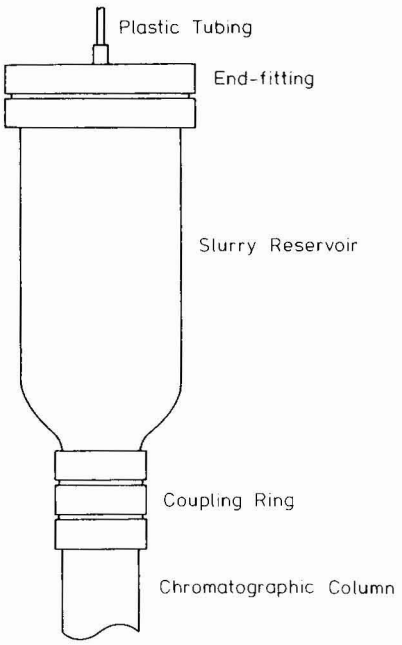

Fig. 2. Commercially available slurry reservoir.

R(BSA, myoglobin) values obtained with constant-velocity packings at various velocities are given in Table II. As R(BSA, myoglobin) was constant at final packing pressures above 0.6 atm with the previously examined constant-velocity packings using slurry reservoirs consisting of long columns of the same diameter as the chromatographic columns, packing was performed under such conditions in the present experiments. However, packings with commercial slurry reservoirs provided slightly lower values of R(BSA, myoglobin) than the previous packings, although the differences were only 2–3 % and the R(BSA, myoglobin) values were independent of

TABLE II

RESULTS FOR CONSTANT-VELOCITY PACKINGS OF TOYOPEARL HW55S USING COMMERCIAL SLURRY RESERVOIRS

Column dimensions (length × I.D., cm)	*Slurry reservoir*		*Inner diameter ratio of slurry reservoir and column*	*Packing velocity (ml/h · cm²)*	*Final packing pressure (atm)*	*R(BSA, myoglobin)*
	I.D. (cm)	*Capacity (ml)*				
60 × 1.0	4.4	280	4.4	78.5	1.80	1.92
				73.0	1.65	1.86
				63.7	1.20	1.77
				54.4	0.95	1.66
60 × 1.6	4.4	290	2.8	55.5	1.55	2.04
				51.9	1.05	2.02
				45.6	1.00	1.97
				39.0	0.60	1.85
60 × 2.2	4.4	570	2.0	52.9	2.00	2.16
				48.5	1.42	2.17
				44.6	1.15	2.15
				39.5	0.98	2.20
				34.8	0.80	2.16
				30.0	0.62	2.15
60 × 3.2	6.1	1110	1.9	38.3	0.98	2.16
				34.1	0.75	2.14
				30.0	0.56	2.07
60 × 4.4	7.1	1470	1.6	31.7	1.05	2.16
				27.9	0.76	2.10
				24.1	0.52	2.15

packing velocity for columns of I.D. 2.2, 3.2 and 4.4 cm. With the I.D. 1.0 and 1.6 cm columns, however, the R(BSA, myoglobin) values increased with increasing packing velocity and even at final packing pressures of *ca.* 1.5 atm they were still 5–10% lower than those obtained previously. On the other hand, semi-constant-pressure packings at higher velocities provided improved column performances even in the columns of I.D. 1.6 cm, as shown in Table III.

TABLE III

RESULTS FOR SEMI-CONSTANT-PRESSURE PACKINGS OF TOYOPEARL HW55S INTO 60 × 1.6 cm I.D. COLUMNS USING COMMERCIAL SLURRY RESERVOIRS

Packing velocity (ml/h · cm²)		*Final packing pressure (atm)*	*R(BSA, myoglobin)*
Initial	*Final*		
85.5	51.9	1.70	2.06
85.5	60.0	1.60	2.15
85.5	46.5	1.48	2.20
85.5	61.5	1.52	2.16
85.5	51.6	1.50	2.08

It can be concluded that slurry reservoirs with the same inner diameter as chromatographic columns are best. With slurry reservoirs with inner diameters less than double the inner diameters of the chromatographic columns, the decrease in resolution of packed columns is only slight. With slurry reservoirs with inner diameters greater than double the inner diameters of the chromatographic columns, the packing velocity must be high in order to obtain reasonably high resolution columns.

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Note

The chemistry of carbazoles

IX. Substituent effect in the gas–liquid chromatography of methylcarbazoles

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Although the presence of methylcarbazoles in natural products has often been reported[1–9], all of them have not fully been identified. In a previous paper[10] dealing with the electronic absorption spectra of a large number of methylcarbazoles, we reported that the additivity of the spectral shifts provides a useful method for determination of unknown methylated carbazoles. In combination with such spectroscopic methods, gas–liquid chromatography (GLC) should serve as the technique for identification of the carbazoles, if some relationship between the retention values and the number or positions of methyl groups can be derived. The chromatographic data on 36 methylcarbazoles presented here provide useful clues for future research on the naturally occurring carbazoles.

EXPERIMENTAL

Methylcarbazoles were prepared by the method described previously[11,12]. Gas–liquid chromatography was performed on a Shimadzu 4PBTF chromatograph with a flame ionization detector (FID). Conditions: stainless-steel column (3 m × 3 mm) packed with 5% silicone SE-30 on Chromosorb W (60–80 mesh); carrier gas (nitrogen) flow-rate, 40 ml/min; column temperature, 200°C; detector temperature, 230°C; injection port temperature, 250°C; sample size, 1 μl (*ca.* 2% acetone solutions).

The nuclear magnetic resonance (NMR) spectra were recorded on a Jeol JNM-PS-100 spectrometer using tetramethylsilane as an internal standard. The infrared (IR) spectra of KBr discs were obtained on a Jasco IRA-2 spectrophotometer. Absorptions in the N–H vibration region were measured by scanning three times for each on an expanded scale.

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TABLE I

RETENTION TIMES (t_R) OF CARBAZOLE AND METHYLCARBAZOLES

No.	*Compound*	t_R *(min)*	*No.*	*Compound*	t_R *(min)*	*No.*	*Compound*	t_R *(min)*
1	Carbazole	4.2	14	2,4-Dimethyl-	7.8	27	2,4,5-Trimethyl-	12.6
2	1-Methyl-	5.2	15	2,5-Dimethyl-	8.1	28	2,4,6-Trimethyl-	10.9
3	2-Methyl-	5.7	16	2,6-Dimethyl-	7.5	29	2,4,7-Trimethyl-	11.0
4	3-Methyl-	5.6	17	2,7-Dimethyl-	7.6	30	3,4,6-Trimethyl-	12.4
5	4-Methyl-	5.8	18	3,4-Dimethyl-	8.7	31	1,2,5,7-Tetramethyl-	15.9
6	1,2-Dimethyl-	7.7	19	3,5-Dimethyl-	7.8	32	1,2,6,7-Tetramethyl-	16.9
7	1,3-Dimethyl-	6.7	20	3,6-Dimethyl-	7.4	33	1,3,4,6-Tetramethyl-	15.1
8	1,4-Dimethyl-	7.4	21	4,5-Dimethyl-	9.9	34	1,4,5,8-Tetramethyl-	14.4
9	1,5-Dimethyl-	7.6	22	1,3,4-Trimethyl-	10.9	35	2,3,5,7-Tetramethyl-	16.3
10	1,6-Dimethyl-	6.9	23	1,4,8-Trimethyl-	9.6	36	2,3,6,7-Tetramethyl-	18.3
11	1,7-Dimethyl-	6.9	24	1,5,7-Trimethyl-	11.0	37	2,4,5,6-Tetramethyl-	18.4
12	1,8-Dimethyl-	6.2	25	2,3,5-Trimethyl-	12.3			
13	2,3-Dimethyl-	8.4	26	2,3,6-Trimethyl-	12.3			

RESULTS AND DISCUSSION

Relation between retention time and the number of methyl groups

Table I lists the retention times determined for carbazole and 36 methylcarbazoles. As shown in Table II, the range of the retention values are separated sufficiently to permit estimation of the number of methyl groups, if the retention time of 4,5-dimethylcarbazole is overlooked. Strictly speaking, however, this relation is effective only up to dimethylcarbazoles, since the boundary between dimethyl- and trimethylcarbazoles remains equivocal on account of the value for 4,5-dimethylcarbazole, and the data are incomplete for the series of trimethyl- and tetramethylcarbazoles.

Relation between retention time and the positions of methyl groups

For the monomethylcarbazoles, the order of retention times is 4-methyl (5) > 2-methyl (3) > 3-methyl (4) > 1-methyl (2), where the numerals in parentheses denote the compounds in Table I. This order is found to hold in certain sets of polymethylcarbazoles.

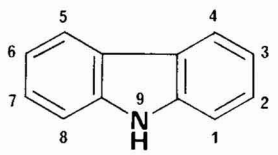
TABLE II

RETENTION TIME RANGES ACCORDING TO THE NUMBER OF METHYL GROUPS

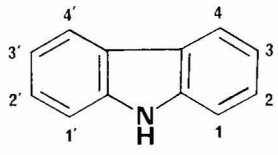
Compounds	t_R *(min)*	*No. of samples*	*No. of isomers*
Carbazole	4.2	1	1
Monomethylcarbazoles	5.2 5.8	4	4
Dimethylcarbazoles	6.2 8.7 (9.9*)	16	16
Trimethylcarbazoles	9.6 12.6	9	28
Tetramethylcarbazoles	14.4 18.4	7	35

* Value for 4,5-dimethylcarbazole.

For the symmetrically substituted dimethylcarbazoles, the retention times increase in the order 4,4′ (21) > 2,2′ (17) > 3,3′ (20) > 1,1′ (12), where the positions of the methyl groups are indicated according to the numbering system B:



A



B

This indicates that the order for monomethylcarbazoles is not altered by the second methyl substitution at the symmetrical positions of the carbazole ring.

The order 4 > 2 > 3 > 1 is applicable to the unsymmetrically methylated carbazoles, with a few exceptions. This trend will be more obvious when the data on the symmetrical dimethylcarbazoles are included, as is shown in Table III. All the carbazoles bearing two adjacent methyl groups exhibit exceptional deviations which are indicated by an asterisk in this table.

TABLE III

ORDERS OF RETENTION TIMES FOR DIMETHYLCARBAZOLES

Carbazoles	*Positions of another methyl group, x, according to the numbering system B*
1,*x*-Dimethylcarbazole	2* (6) > 4′ (9) > 4 (8) > 2′ (11) = 3′ (10) > 3 (7) > 1′ (12)
2,*x*-Dimethylcarbazole	3* (13) > 4′ (15) > 4 (14) > 1* (6) > 2′ (17) > 3′ (16) > 1′ (11)
3,*x*-Dimethylcarbazole	4 (18) > 2 (13) > 4′* (19) > 2′ (16) > 3′ (20) > 1′ (10) > 1 (7)
4,*x*-Dimethylcarbazole	4′ (21) > 3* (18) > 2′ (15) > 2 (14) = 3′ (19) > 1′ (9) > 1 (8)

It is difficult to find a close relation between the chromatographic data and methyl substitution for trimethyl- and tetramethylcarbazoles. Thus, in the case of four 2,4,*x*-trimethylcarbazoles, the elution order is *x* = 4′ (27) > 2′ (29) = 1′ (24) > 3′ (28). However, the order 4 > 2 > 3 > 1 still partially holds in some cases. For several tetramethylcarbazoles, the order 2,4,3′,4 (37) > 2,4,2′,3′ (35) > 2,4,1′,2′ (31) corresponds consistently to the orders 4′ > 2′ and 3′ > 1′, and a tendency 2′,4′ > 1,3′ is obvious from the result 3,4,2′,4′ (37) > 3,4,1,3′ (33).

Relation between retention time and physical properties of methylcarbazoles

The physical properties, which should be related to interactions between monomethylcarbazoles and the stationary phase, are given in Table IV.

Although the boiling point is expected to be a primary factor determining the retention time, the carbazoles are not eluted in the order of their boiling points. Even if the boiling points of 1- and 4-methylcarbazoles are excluded because of the uncertainty in their values, the longer retention time of 2-methylcarbazole compared with that of 3-methylcarbazole is unexpected.

The imino-hydrogen is capable of interacting with the stationary phase through hydrogen-bonding, which influences the elution parameters[15]. But the

TABLE IV

PHYSICAL PROPERTIES OF CARBAZOLES

Compound	*Boiling point (°C)*	*Equilibrium constant of hydrogen-bonding*[13] *(l/mole)*		*NMR in $^2H_6$ acetone: δ (N H) (ppm)*	*IR in KBr: ν (N–H) (cm^{-1})*	*Δν (cm^{-1})*
		In diethyl ether	*In THF*			
Carbazole	354.76*				3420	
1-Methylcarbazole	360**	2.80	4.01	10.15	3410	10
2-Methylcarbazole	363/765 mmHg*	5.00	7.50	10.16	3400	20
3-Methylcarbazole	365/765 mmHg*	3.37	4.30	10.21	3403	17
4-Methylcarbazole	360**	4.00	6.80	10.27	3385	35

* Ref. 14.
** Ref. 1.

proton donating powers of monomethylcarbazoles were reported to be in the order 2 > 4 > 3 > 1 (ref. 13). The NMR chemical shifts of the imino-proton are also independent of the elution order. Only the vibrational frequencies of the N–H bond seem to be correlated to the elution order, but the meaning of this is still obscure. Consequently, there is no information available to justify any conclusion as to the relation of the retention times to the physical properties. However, the interaction between the imino-hydrogen and stationary phase is a dominant factor, because 1-methyl-, 1,8-dimethyl-, 1,4,8-trimethyl- and 1,4,5,8-tetramethylcarbazoles showed the shortest retention times among their respective isomers. Methyl substituents at the 1- and 8-positions of carbazole should sterically hinder the interactions with the stationary phase, which decrease the retention times, as is known for the chromatographic behaviours of 2- and 2,5-alkylated pyrroles[16].

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Note

Capillary column gas–liquid chromatographic analysis of cholesterol derivatives

Application to the autoxidation products of cholesterol

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An accurate and rapid method to detect and assay cholesterol autoxidation products in biological and organic specimens, *e.g.*, in treated human food, and to determine their biological activities, *e.g.*, angiotoxic, carcinogenic, cytotoxic and mutagenic[1], is required. A minimum of 50 sterols has been identified in cholesterol autoxidation products[2–4]. They have been analyzed by liquid chromatography on silica and Sephadex LH-20 columns[5], thin-layer chromatography (TLC)[6], high-performance liquid chromatography[4,7] and gas–liquid chromatography (GLC) on OV-101 or SE-30 columns[8].

The use of capillary columns, impregnated with OV-101, achieves better resolution and enables the detection of quantities of sterols in the range of 10 ng. The method was applied to the analysis of the products of cholesterol autoxidation at 100°C in the presence of air for 30 days. Oxidation of cholesterol occurs both in the rings and in the side chain.

MATERIALS AND METHODS

Reagents

Solvents for extraction and chromatography were double-distilled before use. Pyridine, hexane, hexamethyldisilazane and trichloromethylsilane were Merck reagents. The methoxyamine hydrochloride was supplied by Pierce (Rockford, IL, U.S.A.).

Steroids

Cholestane, dihydrocholesterol, 7α-, 7β-, 25-, (20 *S*)-20-hydroxycholesterol and 3β-acetoxy-5,7-cholestadiene were obtained from Steraloids (Pawling, NJ, U.S.A.). The (22*S*)-22-, (24*R*)-24- and (24*S*)-24-hydroxy-; the (25*R*)-26-hydroxy- and 22ξ,23ξ-dihydroxycholesterol were generously provided by Professor Van Lier (Sherbrooke, Quebec, Canada). 5α,6α-Epoxycholestan-3β-ol, 5α-cholestane-3β,5α,6β-triol and 3,5-cholestadien-7-one[9] were prepared in our laboratory.

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Apparatus

For GLC, a Carlo Erba Fractovap 2300 chromatograph, equipped with a capillary column (25 m × 0.25 mm) impregnated with OV-101, was used and helium was the carrier gas at 285°C and a flow-rate of 1 ml/min. For injection, a 1-μl Pyrex stream splitter, Type ROS (Carlo Erba, Milan, Italy) was used. Other conditions: column temperature, 285°C; injection temperature, 350°C; flame ionization detector; recorder speed, 30 cm/min.

Mass spectra were recorded on an LKB 2091 gas chromatograph–mass spectrometer at 70 eV voltage. The chromatograph was equipped with a column packed with SE-30 (25 m × 0.25 mm). The helium flow-rate was 4 ml/min, and the column temperature was programmed from 250 to 310°C at 10°C/min.

TLC

Silica gel 60 F_{254} plates, 20 × 20 cm, 0.2 mm thick (E. Merck, Darmstadt, G.F.R.) were developed twice with diethyl ether–cyclohexane (9:1). The sterols were revealed by their UV absorption at 254 nm or by spraying with a 50% aqueous solution of H_2SO_4, followed by heating. Preparative TLC was performed in the same manner, but with 0.25-mm thick plates. After scraping of the zones outlined by UV spectroscopy at 254 nm, they were eluted with 50 ml of chloroform–methanol (2:1).

Derivatization of sterols

The residue to be derivatized (generally obtained by preparative TLC) was deposited in a screw-cap culture tube by evaporating the solvents under nitrogen. Then 0.5 ml methoxyamine hydrochloride solution in pyridine (20 mg/ml) were added. The tube was plugged with a PTFE stopper and heated in a water-bath for 4 h at 60 C. After evaporation of the excess of pyridine under nitrogen, 150 μl of pyridine–hexamethyldisilazane–trichloromethylsilane (1:1:1) were added and the tube was allowed to stand at ambient temperature for 12 h. Excess of solvent was evaporated under nitrogen. The residue was taken up in hexane (1 ml per 100 μl) and injected into the gas chromatograph.

Cholesterol autoxidation

A 500-mg amount of Merck cholesterol, recrystallized twice in ethanol, was placed in a tube and heated in an oven at 100°C in the presence of air for 30 days. The crude product was chromatographed on preparative silica plates. After double development with diethyl ether–cyclohexane (9:1), cholesterol and the products having $R_F > 0.8$ were scraped from the plate and collected for subsequent analysis by capillary GLC. The remainder of the products was recovered by elution with chloroform–methanol (2:1) and derivatized by the method indicated.

RESULTS AND DISCUSSION

Twenty-two authentic cholesterol derivatives were chromatographed separately or as mixtures. Their retention times (RRT) relative to cholestane are given in Table I. The accuracy of these values, ±0.02, corresponds to the maximum shift observable during successive injections of the same compound.

The chromatogram of a mixture of these 22 sterols is shown in Fig. 1. Sixteen

TABLE I

RELATIVE RETENTION TIMES OF SOME OXIDIZED CHOLESTEROL DERIVATIVES (ACCURACY ±0.02)

Compound	*RRT*
1 5α-Cholestane	1.00
2 5-Cholestene-3β,7α-diol	1.74
3 5-Cholesten-3β-ol (cholesterol)	1.80
4 5α-Cholestan-3β-ol	1.84
5 3,5-Cholestadien-7-one 6 5,24-Cholestadien-3β-ol (desmosterol) 7 5α-Cholestan-3-one	1.91
8 5,7-Cholestadien-3β-ol 9 5α-Cholest-7-en-3β-ol	2.01
10 4-Cholesten-3-one	2.14
11 5-Cholestene-3β,7β-diol	2.25
12 5α,6α-Epoxycholestan-3β-ol	2.32
13 5-Cholestene-3β,(22*S*)-22-diol	2.41
14 5-Cholestene-3β,(22*S*)-20-diol	2.46
15 3β-Hydroxy-5-cholesten-7-one 16 5α-Cholestane-3β,5α,6β-triol	2.95
17 5-Cholestene-3β,(24*R*)-24-diol 18 5-Cholestene-3β,(24*S*)-24-diol	3.14
19 5-Cholestene-3β,25-diol	3.26
20 5-Cholestene-3β,(23*R*)-23-diol	3.53
21 5-Cholestene-3β,22ξ,23ξ-triol	3.53
22 5-Cholestene-3β,(25*R*)-26-diol	3.67

peaks can be seen in this figure. Dihydrocholesterol (4), having a retention time closely similar to that of cholesterol (3), was chromatographed separately (Fig. 2). The difference in RRT between compounds 3 and 4, 0.04, is the minimum value for effective separation of two sterols.

Peaks 5, 8, 15 and 17 correspond, respectively, to mixtures of compounds 5–7, 8 and 9, 15 and 16, and 17 and 18, which have similar retention times in the proposed system. In the analysis of samples of biological or chemical origin, these overlapping RRT values would be no bar to the identification of compounds eluted together.

Such sterols can easily be separated by preparative TLC prior to GLC. With diethyl ether–cyclohexane (9:1), the following R_F values are obtained: 3,5-cholestadien-7-one (5), 0.53; desmosterol (6), 0.44; 5α-cholestan-3-one (7), 0.66. 3β-Hydroxy-5-cholesten-7-one (15) and 5α-cholestane-3β,5α,6β-triol (16) are also easily separated. Only the pairs 8 and 9 and 17 and 18 are difficult to separate. 5,7-Cholestadien-3β-ol (8) is quite unstable. It changes spontaneously or after heating to an apolar compound, which has the RRT of a triene (probably $\Delta^{3,5,7}$ derived from the dehydration of 8). Consequently, the presence of compound 8 does not preclude the identification of 5α-cholest-7-en-3β-ol, which is stable. The resolution of the pair 17 and 18 by paper chromatography is described by Van Lier and Smith[10].

Oxidation of crystallized cholesterol at 100°C

Determination of cholesterol in the crude autoxidation mixture on an SE-30 column shows that 80% of the cholesterol remains unchanged after 30 days; only 6%

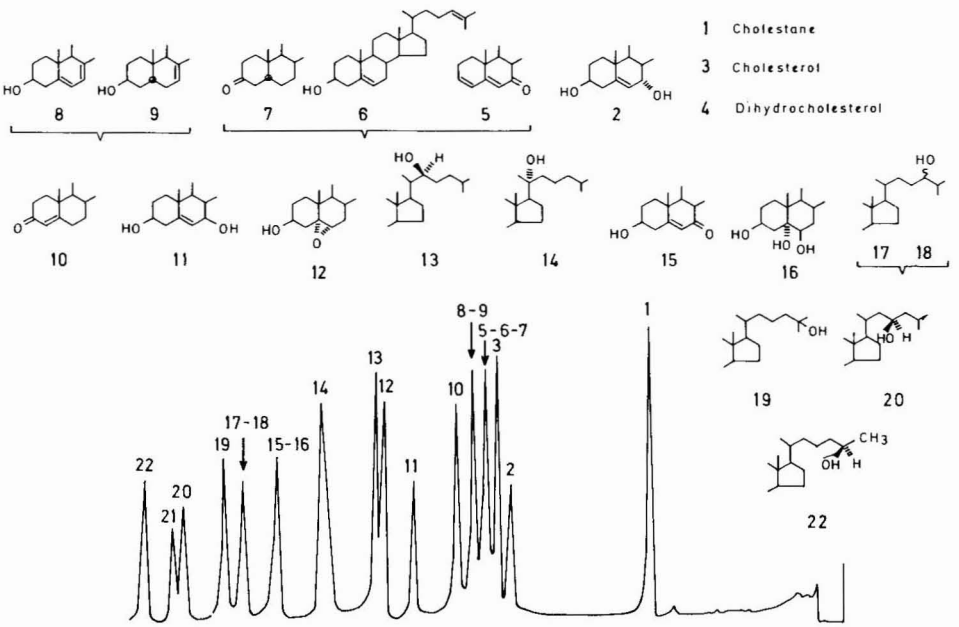

Fig. 1. Chromatogram of oxygenated sterols on a capillary column coated with OV-101.

was detected as oxidation products. This mixture (100 mg) was developed twice with diethyl ether–cyclohexane (9:1) on a preparative plate. The cholesterol was eluted, as previously described, and the remainder was eluted, derivatized and resolved by capillary GLC on OV-101 (Fig. 3). The different constituents are identified by their RRT in comparison with cholestane and by addition of an authentic sample. This identification was confirmed by gas chromatography–mass spectrometry. The proportions of oxidation products were as follows: cholesterol (3), 80%; 25-hydroxycholesterol (19), 1.5%; 7-oxocholesterol (15), 0.75%; 20-hydroxycholesterol (14),

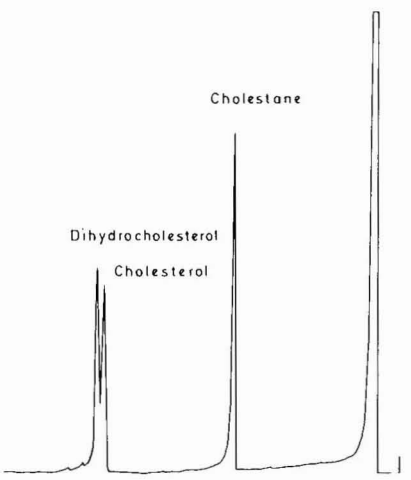

Fig. 2. Separation of cholesterol and dihydrocholesterol on a capillary column coated with OV-101.

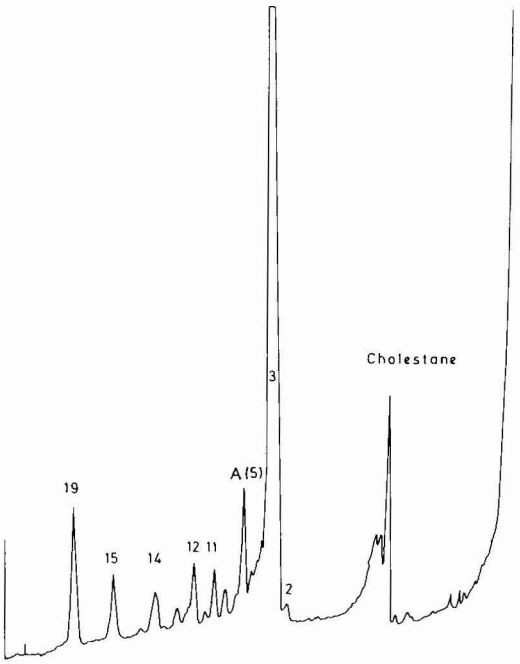

Fig. 3. Chromatogram of crude autoxidation products of cholesterol. A = Unidentified peak eluted together with the dienone (5).

0.75%; 5α,6α-epoxycholesterol (12), 1.1%; 7β-hydroxycholesterol (11), 0.6%; 7α-hydroxycholesterol (2), 0.1%; 3,5-cholestadien-7-one (5) mixed with an unknown compound, 1.1%.

Traces of peroxides were observed at the end of the autoxidation reaction, implying that hydroperoxides were first formed, as in liquid-phase autoxidation[3], although the temperature was 100°C. This view appears to be supported by the fact that mixtures containing a polyethylenic compound [able to provide hydroperoxy radicals, *e.g.*, squalane–cholesterol (1:1)] in addition to cholesterol, enhance the autoxidation reaction and give rise to the formation of large amounts of epoxide (12).

The various products identified are those usually formed in liquid-phase autoxidation[3]. Also observed are the 20- and 25-hydroxycholesterols which are specific to solid-phase autoxidation.

25-Hydroxycholesterol (19) is highly stable at 100°C. It undergoes no change if heated for 6 months in the presence of air. 20-Hydroxycholesterol, on the other hand, is unstable. It decomposes slowly on heating to give compounds of lower molecular weight than the sterols identified here.

The stabilities of the ring B oxidation products are analogous to those described for liquid-phase autoxidation[11]. 7-Hydroperoxycholesterols give rise successively to 7-hydroxy, 7-oxo and 7-oxo-$\Delta^{3,5}$ derivatives. The 7α-hydroperoxy- and hydroxycholesterols epimerize partly to 7β-derivatives as follows:

Mass spectrometry confirms most of the characterizations made for the different sterols. The secondary diols (7-hydroxycholesterols) and secondary/tertiary thiols (20- and 25-hydroxycholesterol) are disilylated. Their mass spectra are characterized by molecular ions at *m/e* 546.5 and fragment ions at *m/e* 531.5 ($M^+ - 15$), 457.5 ($M^+ - 89$) and 368 ($M^+ - 178$). 7-Oxocholesterol is characterized by molecular ions at *m/e* 501 and fragment ions at *m/e* 470 ($M^+ - 31$) and 456 ($M^+ - 45$). The epoxide (12) sometimes appears as a diol, according to its molecular and fragmentation ions.

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Note

Determination of residual epichlorohydrin in middle cut alkylglycidyl ethers by headspace gas chromatography

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Epichlorohydrin (1-chloro-2,3-epoxypropane) has been determined in a variety of matrices by extraction followed by infrared spectrophotometry[1,2], colorimetry[3–5], and by direct injection gas chromatography (GC)[6–8]. When the need developed to determine low levels of residual epichlorohydrin (epi) in some middle cut (C_{12} to C_{16}) alkylglycidyl ethers, also known as alkylglyceryl ethers (AGE), these methods were considered and rejected as too long and involved, too insensitive, and/or unsuited to our matrix. We needed a method which could quantitate epi concentrations below 100 μg/g and be fast as well as simple enough to install in a manufacturing plant laboratory.

Headspace (GC)[9] was the method of choice since it is quite sensitive and reasonably interference-free. The fairly volatile epi (boiling point 118°C), is determined in the vapor above the high-boiling (high-molecular-weight, >200) glycidyl ethers which are the main components of the sample. This simplifies GC conditions by reducing analysis time and lowering column temperatures, and increases the sensitivity of the determination, compared to direct injection of the liquid sample.

EXPERIMENTAL

A Valco (Houston, TX, U.S.A.) zero dead-volume, stainless-steel, 6-port liquid chromatography valve (Model CV-6-UHPa-N60) was attached to the external portion of the injection port of a Perkin-Elmer 3920 gas chromatograph using 1/8-in. Swagelok fittings. The dead volume of the injection port was substantially reduced by placing a glass insert in the injection port and extending a PTFE-lined 1/8 in. O.D. (0.070 in. I.D.) stainless-steel tube from the valve body through the glass insert to the rear of the injection port. The PTFE lining of the tubing prevented decomposition of the analyte due to contact with any hot metal surfaces in the injection port and reduced the dead volume. The valve body and sample loop were also lined with or fabricated of an inert fluorocarbon "Valcon-H". The valve configurations are shown in Fig. 1. For temperature control, we wrapped the valve and loop with heating tape (attached to a Variac) and mounted a thermocouple temperature probe on it. By adjusting the Variac, the temperature could be controlled from approximately 50°C to 150°C. Valco literature indicated the valve could tolerate 175°C.

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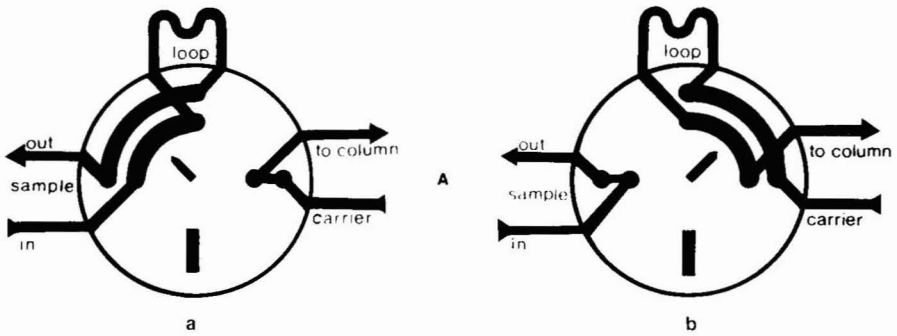

Fig. 1. Internal configuration of the valve in "fill" (a) and "inject" (b) positions. The syringe is attached to "in" during "fill". "Out is connected to a water aspirator, which is used to purge the system between samples. Redrawn from Valco Instrument Company Sales literature, with permission.

Samples (1 ml) of AGE were equilibrated for 15 min at 60° C in 10 ml serum vials (Wheaton Scientific, Millville, NJ, U.S.A.) sealed with PTFE-faced rubber septa. Vapor samples were removed from the vials and introduced to the valve-loop assembly with a heated Hamilton gas-tight syringe. GC conditions are summarized in Table I. Epichlorohydrin was purchased from Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.).

TABLE I

GC CONDITIONS USED FOR THE DETERMINATION OF EPICHLOROHYDRIN

Instrument	P-E 3920 modified for headspace
Detector	Flame ionization
Integrator	P-E Sigma 10
Carrier gas	Nitrogen; 20 ml/min
Valve–sample loop assembly temperature	60° C
Injection port temperature	200° C
Oven temperature	80° C for 8 min, then 32°/min to 140° C
Interface temperature	200° C
Detector temperature	250° C
Column	10 ft. × 1/4 in. O.D. (2 mm I.D.) glass packed with 100–120 mesh Ultra-Bond PEGS (RFR Corp., Hope, RI, U.S.A.)
Transfer syringe	5 ml, Hamilton, gas-tight, *ca.* 60° C
Approximate retention time of epichlorohydrin	4.6 min

Epi-free AGE was prepared by bubbling nitrogen through a stirred portion of the AGE which was heated to approximately 45° C, until the sample analyzed by the method described here did not show the presence of an epi peak. An attempt to verify independently the absence of epi in the blank using a modification of the method of ref. 3 was not successful, presumably due to a matrix effect. The sparged material was

used as the blank for epi determinations and for preparation of standards. Standards were made by adding weighed amounts of epi to the blank and diluting these with additional blank AGE to reach the desired epi concentration.

RESULTS AND DISCUSSION

Quantitation of epi in AGE depends upon equilibrium being attained between the liquid and gas phases. While only a portion of the epi contained in the headspace was measured, the absolute amount of epi was not known or desired. Since we carefully controlled equilibration and sampling conditions, the amount of epi in the vapor was proportional to the original concentration in the liquid phase and therefore could be used to determine the level of epi in the sample. Based on our experiments with epi in AGE and epi dissolved in ethylene glycol we estimated the distribution coefficient for epi–AGE to be 3% vapor–liquid phase.

The equilibration temperature of 60 C was chosen because it was high enough to provide the required sensitivity by vaporizing reasonable amounts of epi in the headspace, but low enough to prevent vaporization of higher boiling components in the sample. Higher and lower equilibration temperatures gave poorer results.

Optimum equilibration time was determined experimentally by analyzing the same sample repeatedly using different equilibration times. An equilibration time of 15 min was chosen for all analyses because, under our conditions, there was little increase in peak area after that length of time (Fig. 2).

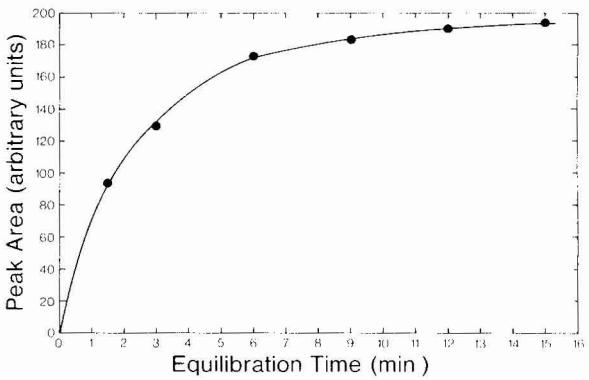

Fig. 2. Equilibration of epichlorohydrin between the liquid and vapor phases. Equilibrium is reached after approximately 12 min, as the epi vapor reaches a maximum and levels off.

A typical chromatogram is given in Fig. 3. The epi peak retention time and calibration curve were checked daily. Adequate separation was attained with an isothermal program, but to prevent possible carry-over from run to run, the temperature was increased to 140 C at 32 C/min after 8 min at 80 C. Average analysis time was approximately 20 min per sample since the start of each equilibration was staggered, to coincide with the GC run.

The calibration curve was linear (correlation coefficient 0.999) for at least three orders of magnitude above the limit of detection. Fig. 4 is the low concentration portion of the calibration curve. No attempt was made to determine whether linearity

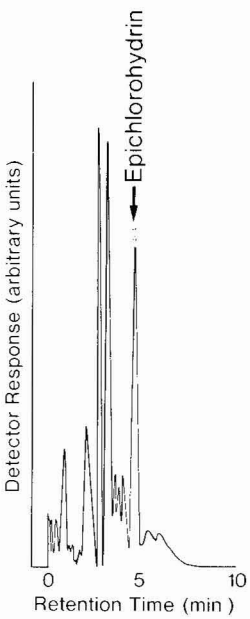

Fig. 3. Typical gas chromatogram of a middle cut alkylglycidyl ether from the headspace GC determination of epichlorohydrin.

extended above approximately 2000 μg/g. The limit of detection was approximately 2 μg/g based on a signal-to-noise ratio of 3; this might be lowered further by using a larger volume sample loop. The reproducibility of the method at several concentration levels is shown in Table II.

Experience with this method for more than a year and hundreds of samples indicates that it is both a simple and reliable method for determining epichlorohydrin

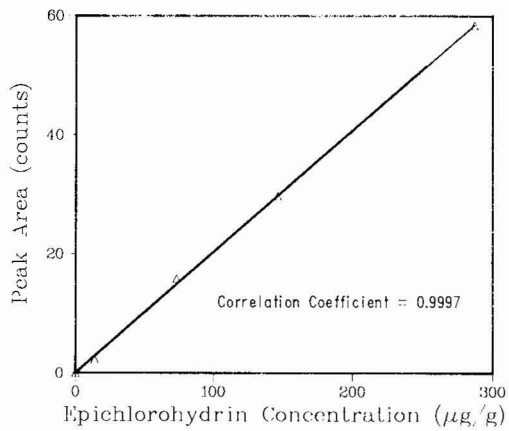

Fig. 4. Calibration curve for epichlorohydrin in a middle cut alkylglycidyl ether as determined by headspace GC. Linearity extended from the detection limit (approximately 2 μg/g) up to at least 2000 μg/g.

TABLE II

PRECISION OF THE HEADSPACE GC EPICHLOROHYDRIN METHOD

Number of replicates	*Mean concentration (μg/g)*	*Standard deviation (S.D.) (μg/g)*	*Relative S.D. (%)*
7	847	16	1.8
8	100	2.6	2.6
10	7	0.93	12.5

in mid-cut alkylglycidyl ethers. It is capable of providing precise results at epi concentrations as low as *ca.* 2 μg/g and can do so on simple, inexpensive equipment in a short period of time.

ACKNOWLEDGEMENT

We thank Karen Nutley and Cathy Ruhlen for their analytical assistance.

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Note

Determination of the carcinogen methylazoxymethyl-β-D-glucosiduronic acid in rat bile and urine

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(Received March 18th, 1981)

Methylazoxymethanol (MAM), the aglycone of cycasin (methylazoxymethyl-β-D-glucopyranoside), administered by any route is carcinogenic to rodents. Carcinomas of the colon are induced in rats upon multiple injections of MAM[1–3]. Injected MAM may be conjugated with glucuronic acid in the liver to form MAM–β-D-glucosiduronic acid (MAM–GlcUA) and excreted with the bile. The MAM–GlcUA moves to the lower intestinal tract and is hydrolyzed by bacterial β-glucuronidase to give the free carcinogen MAM[4].

A sensitive analytical method for the determination of MAM–GlcUA in the bile and urine could be used to establish whether or not MAM injected into an animal is conjugated with glucuronic acid. This paper describes a gas chromatographic (GC) procedure for the quantitative analysis of MAM–GlcUA in bile and urine.

MATERIALS AND METHODS

Chemicals

Methylazoxymethyl-β-D-glucosiduronic acid was prepared by catalytic oxidation of cycasin[5]. MAM was prepared by the method of Kobayashi and Matsumoto[2].

Amberlite XAD-2 resin (non-ionic, polymeric adsorbent; Mallinckrodt, St. Louis, MO, U.S.A.) was purified by extraction with acetone, followed by thorough rinsing with water. Darco G-60 activated charcoal (Matheson, Coleman & Bell, Norwood, OH, U.S.A.) and Celite analytical filter-aid (Johns-Manville, Denver, CO, U.S.A.) were prewashed with several volumes of 95% ethanol and water before use[6]. Tril-Sil concentrate (Pierce, Rockford, IL, U.S.A.) was stored in a desiccator under refrigeration until used. Reagent grade pyridine was distilled over KOH. The silylation reagent was prepared by introducing one volume of Tri-Sil concentrate and four volumes of dry pyridine into a 30-ml serum bottle capped with a PTFE-lined septum[7].

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Gas chromatography

The XAD-2 column was prepared by pouring 5–7 cm of prewashed resin into a glass chromatography tube (15 cm × 15 mm I.D.). The charcoal column was prepared by pouring a water slurry of 7 g of prewashed Darco G-60–Celite (1:1, w/w; thoroughly premixed) into a chromatographic column (25 cm × 17 mm). After rinsing each column with four volumes of water, the XAD-2 column was placed atop the charcoal column. The effluent end of the charcoal column was inserted through a rubber stopper which was placed in a filter-flask. The flask was connected to a vacuum source and a slight vacuum was applied to the eluate receiver during a chromatographic run.

Gas chromatographic analysis was carried out on a Bendix 2500 equipped with a flame ionization detector. Peak areas were determined by an on-line Autolab Vidar 6300 digital integrator. The column was a U-tube (190 cm × 2 mm I.D.) packed with 1.5% OV-17 on Gas-Chrom Q (60–80 mesh). Operating conditions were: column temperature, 230 C; detector temperature, 240 C; injector temperature 240 C; nitrogen flow-rate, 25 ml/min.

A 2-ml volume of silylation reagent was transferred with a syringe into a glass vial, which contained the dried MAM–GlcUA, and capped with a PTFE-lined screw cap. The mixture was stirred with a vortex mixer for 30 sec and allowed to remain at room temperature for at least 1 h prior to GC analysis. Samples of 0.5–2.0 μl were directly injected into the gas chromatograph[8].

Urine and bile collection

Male Wistar rats (250 ± 10 g) were injected intraperitoneally with measured amounts of MAM–GlcUA and placed individually in metabolism cages. Urine was collected in a test-tube which was immersed in ice contained in a small Dewar flask. Control urine was collected from non-injected rats. The common bile duct of male rats was cannulated, the animals were placed in restrainers and bile was collected in small vials.

RESULTS

Gas chromatography of MAM–GlcUA standard

Gas chromatographic analysis of the pertrimethylsilylated derivative of MAM–GlcUA produced a chromatogram with a symmetrical peak against a clean background (Fig. 1). Excellent linearity was obtained for 1-μl injections of standards with concentrations in the 0.09–4.7 mg/ml range. The reproducibility of the first several determinations was excellent, but the sensitivity changed with successive analyses. Thus, it was necessary to construct a standard curve each day and check it at intervals throughout the day. Another problem, that of great fluctuation in detector sensitivity, was alleviated by repacking the inlet end of the column every day. A dark brown deposit derived from the urine and bile samples formed at the inlet after a number of MAM–GlcUA determinations evidently interfered with the analysis. The detector also had to be cleaned about every 2 weeks to remove the build-up of silicon from the silylation reagent and of carbon from pyridine.

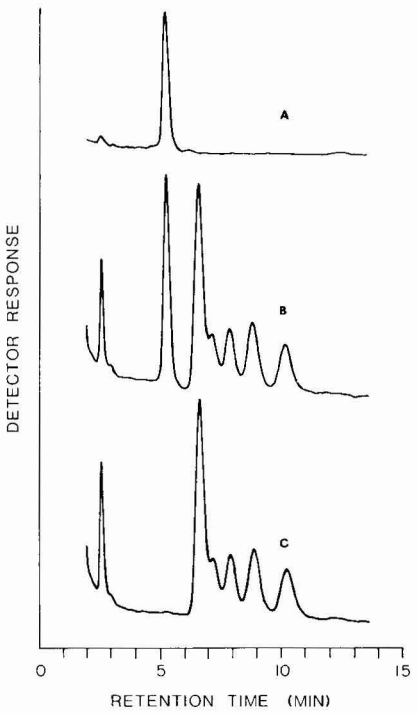

Fig. 1. Gas chromatograms of pertrimethylsilylated MAM–GlcUA (A), MAM–GlcUA added to rat urine (B) and rat urine (C). Operating conditions: column temperature, 230°C; detector temperature, 240°C; inlet temperature, 240°C; nitrogen flow-rate, 25 ml/min.

Gas chromatography of MAM–GlcUA added to urine

Direct determination of MAM–GlcUA in urine after lyophilization of the sample was found to be unsatisfactory, because of the large amount of salt residue which prevented the use of small volumes of silylation reagent. Therefore, a clean-up procedure was devised to remove the salts and lipophilic contaminants in the urine (see Materials and methods). The urine sample was poured through the XAD-2 column and the percolate was allowed to drip directly onto the top of the Darco–Celite bed. The XAD-2 bed was washed with 100 ml water and removed. The Darco–Celite column, through which the 100 ml water had passed, was then eluted with 100 ml of 75% aqueous ethanol.

The alcoholic eluate, which contained the MAM–GlcUA, was evaporated to dryness under vacuum in a Buchi rotary evaporator at 60°C. The solid residue was triturated with 7 ml methanol in three portions and each was filtered through a 5 mm pore size, Mitex membrane filter to remove particulate impurities. The filtrate, collected in a glass vial, was evaporated to dryness under a stream of nitrogen on a warm heating plate. The residue was exhaustively dried in a vacuum desiccator and then silylated.

The chromatogram of control urine subjected to the purification procedure displayed several large peaks (Fig. 1C), but was clear at the retention position of MAM–GlcUA. Urine samples to which MAM–GlcUA was added yielded a chromatogram with a cleanly resolved MAM–GlcUA peak (Fig. 1B).

TABLE I

RECOVERY OF MAM–GLUCOSIDURONIC ACID ADDED TO RAT URINE

The indicated amount of MAM–GlcUA was added to rat urine and the compound was separated by carbon column chromatography and eluted with 75% aqueous ethanol. The dried compound was derivatized with Tri-Sil reagent and the quantity determined by GC.

MAM–GlcUA added (mg)	*Recovery (%) ± S.D. (n = 10)*
	99.0 ± 1.1
2.0	99.7 ± 1.1
5.0	100.5 ± 1.2
10.0	83.3 ± 2.7

Four different quantities, 1, 2, 5 and 10 mg, of MAM–GlcUA were added to 1 ml of control rat urine and the samples were analyzed. The recoveries were good for the three smaller quantities, but recovery dropped for the 10-mg level (Table I). This was probably due to overloading of the column, and thus for experimental work when the quantity was greater than 5 mg a smaller aliquot of the collected urine was analyzed.

Gas chromatography of MAM–GlcUA in bile

Direct pertrimethylsilylation and GC analysis of MAM–GlcUA in bile residue was satisfactory. MAM–GlcUA was added to bile and the mixture was transferred to a small vial and lyophilized. A 2-ml volume of silylation reagent was added to the bile residue in the vial, thoroughly mixed and allowed to remain at room temperature for at least 1 h. A 1-μl volume of the silylated mixture was injected into the gas chromatograph. There were no interfering peaks in the vicinity of the MAM–GlcUA peak.

Four different quantities of MAM–GlcUA were added to 1-ml samples of control bile. Each quantity was determined in triplicate. The recoveries were uniform and averaged at least 93% (Table II).

TABLE II

RECOVERY OF MAM–GLUCOSIDURONIC ACID ADDED TO RAT BILE

The indicated amount of MAM–GlcUA was added to bile and the mixture was lyophilized. The compound was pertrimethylsilylated in the bile residue and the quantity of the derivatized compound determined by GC.

MAM–GlcUA added (mg)	*Recovery (%) (n = 4)*
0.075	95.5*
0.15	93.3
0.50	93.8
1.00	96.3

* Mean from four determinations.

Determination of MAM–GlcUA in bile and urine of rats injected with the compound and MAM

Bile and urine from male Wistar rats injected with MAM–GlcUA were analyzed to determine the detectable amounts. Four dosage levels of MAM–GlcUA were intraperitoneally injected into rats with and without a bile duct cannula and analyzed for quantities of the compound excreted in the bile and urine. Six animals per dosage level were used for urine collection over 24 h, and two animals per dosage level for bile collection over 12 h. In addition, four male rats with bile duct cannula were injected with 20 mg free MAM per kg bodyweight and the bile was collected. The urine was made up to 25 ml and suitable aliquots were analyzed for MAM–GlcUA. A 1-ml volume of the collected bile was used per determination. No MAM–GlcUA was found in the bile. The recoveries of injected MAM–GlcUA in the urine ranged from 95 to 103%. The injected MAM–GlcUA was essentially quantitatively excreted in the urine (Table III).

TABLE III

RECOVERY OF MAM GLUCOSIDURONIC ACID FROM URINE AND BILE OF RATS INJECTED WITH MAM OR MAM–GlcUA

Compound injected	*Amount (mg per kg bodyweight)*	*Recovery (%)*	
		*Urine**	*Bile***
MAM–GlcUA	40	100.3 (95–102)	0.0
MAM–GlcUA	80	102.9 (97–104)	0.0
MAM–GlcUA	160	95.3 (95–96)	0.0
MAM–GlcUA	320	98.5 (98–99)	0.0
MAM	20		0.0***

* Mean from six urine samples. Figures in parentheses indicate range of values.
** Two bile samples for each dosage level of MAM–GlcUA, except where stated otherwise.
*** Four bile samples.

DISCUSSION

Trace quantities of glucuronides, which have small aglycones and are hence highly water-soluble, are difficult to quantitate in biological fluids. Extraction of glucuronides with water-immiscible solvents would be highly desirable since they would remove the bulk of materials which interfere with the quantitation procedure[8]. Steroid glucuronides can be extracted with solvents of high polarity, but very polar steroid glucuronides are not completely extracted[9]. Amberlite XAD-2, a synthetic polystyrene polymer, Sephadex LH-20 and anion-exchange resins have been used for the isolation of high-molecular-weight glucuronides from biological fluids[10]. MAM–GlcUA in urine was not adsorbed by Amberlite XAD-2. In addition, Sephadex LH-20, Sephadex G-10, anion-exchange resin and reversed-phase high-performance liquid chromatography failed, for different reasons, to separate satisfactorily MAM–GlcUA from interfering substances.

The method developed for the determination of MAM–GlcUA offers the advantage of combining high accuracy and sensitivity with brevity of analysis time.

MAM–GlcUA is eluted from a carbon–Celite column with water–ethanol after the bulk of the impurities have been removed with water. The XAD-2 column, while it does not adsorb MAM–GlcUA, removes other substances in the urine which interfere with the GC analysis. The determination of MAM–GlcUA in urine is accurate when the amount of the compound passed through the Darco–Celite column is less than 5 mg, but the recovery drops to 80% when 10 mg of MAM–GlcUA are present. The quantitation of MAM–GlcUA at any concentration can accurately be carried out by utilizing a suitable aliquot of the urine collected. The determination of MAM–GlcUA after direct pertrimethylsilylation of lyophilized bile is sensitive and as low as 0.075 mg/ml of MAM–GlcUA can be determined.

The quantitative recovery of MAM–GlcUA in the urine of rats injected with various amounts of the compound demonstrated that the method is accurate. The slightly less than 100% recovery in some of the urine samples may have been due to incomplete excretion of the compound by rats in 24 h. The observation that no MAM–GlcUA was detected in the bile of animals injected with MAM or MAM–GlcUA is indicative that MAM is not conjugated with glucuronic acid. It is unlikely that minute quantities of MAM–GlcUA excreted with the bile had gone undetected. If a 350-g rat were injected with 160 mg MAM–GlcUA per kg bodyweight and if only 0.5% of the injected compound were excreted in as much as 3.5 ml bile, the compound could be detected with greater than 90% accuracy. A greater percentage of injected compound would be expected to be present if excretion of MAM–GlcUA with the bile was a major route for the elimination of the compound.

ACKNOWLEDGEMENT

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Note

Separation of cAMP from adenine nucleotides and nucleosides by electrophoresis on cellulose acetate membranes

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(First received February 3rd, 1981; revised manuscript received March 10th, 1981)

cAMP is involved in many biological and biochemical events and its metabolism has been intensively studied.

The separation of cAMP from other adenine nucleotides, nucleosides and bases is essential for the determination of cAMP and the activities of its metabolizing enzymes, adenylate cyclase and phosphodiesterase. It has been achieved by specific radioimmunoassay[1], ion-exchange column chromatography[2,3], paper chromatography[4] and chemical methods[5]. The evaluation of the changing levels of cAMP and its metabolites, adenine nucleotides and nucleosides, is required for studies of cAMP metabolism, particularly in crude systems. This paper describes a simple procedure that permits the simultaneous separation of cAMP, ATP, ADP, AMP, adenosine and inosine from each other by electrophoresis on a cellulose acetate membrane at low voltage. The proposed method takes less time than paper electrophoresis and, in contrast to paper electrophoresis, can be performed at 150 V and at room temperature.

EXPERIMENTAL

The cellulose acetate membrane, Cellogel (18 × 5 cm), was a product of Chemetron (Milan, Italy). Nucleotides, nucleosides and bases were purchased from Sigma (St. Louis, MO, U.S.A.). [8-^{14}C]Adenosine, [8-^{3}H]cAMP, [8-^{14}C]AMP and [8-^{3}H]ATP were obtained from the Radiochemical Centre (Amersham, Great Britain). Other chemicals were of analytical-reagent grade.

Electrophoresis

The electrophoresis was carried out in a Type 238 electrophoresis apparatus (Jokyo Sangyo, Tokyo, Japan). The electrophoresis buffer contained 12 m*M* citric acid, 14 m*M* potassium dihydrogen orthophosphate, 14 m*M* boric acid and 2% glycerol, and was adjusted to pH 7.0 with potassium hydroxide solution.

A strip measuring 2.5 by 9 cm was cut from a sheet of Cellogel and soaked in the electrophoresis buffer at least overnight. The strip was placed on an electrophoresis cell after removing excess of buffer with filter-paper. A 3-μl volume of solution containing 4 m*M* each of ATP, ADP, AMP, cAMP, adenosine, inosine and adenine

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was applied on the cathode side of the strip as a 2-cm streak. Electrophoretic runs were carried out at 150 V for 40 min at room temperature.

To ensure the separation of each compound, labelled ATP (8800 cpm) and cAMP (10,000 cpm), or AMP (12,000 cpm) and adenosine (8900 cpm) were applied together with the carrier solution. After electrophoresis, the marker bands and the areas between each band were located under UV light, dried under hot air, cut out and counted in 5 ml of scintillation fluid (OCS; Radiochemical Centre) with a Beckman scintillation counter.

RESULTS AND DISCUSSION

We reported previously the separation of purine derivatives by electrophoresis on a cellulose acetate membrane and its application to enzyme assays[6,7]. Ribonucleotides were readily separated from the corresponding nucleosides and bases in 0.1 *M* borate buffer (pH 9.0)[6], in which the *cis*-hydroxyl groups in the ribose moiety form the chelated derivatives with orthoboric acid. In this system, cAMP was separated from AMP, but it moved together with adenosine and inosine.

In Tris–hydrochloric acid buffer (pH 8.0), cAMP was clearly separated from AMP and nucleosides (Fig. 1A). This system had been used for the determination of phosphodiesterase activity[8]. However, ATP and ADP had the same mobility and the labelled ATP showed trailing and overlapped cAMP. ATP might interact with the Tris cation. This system could not be used for the determination of the formation of cAMP from ATP. In citrate buffer (pH 6.0), ATP, ADP and AMP were separated from each other and cAMP was separated from other adenine nucleotides and nucleosides (Fig. 1B). However, the distances between cAMP and AMP and between cAMP and inosine were too short to measure the radioactivity of cAMP without contamination of the neighbouring compounds.

Complete separation of cAMP from adenine nucleotides and nucleosides was obtained in the specified buffer (Fig. 2). ADP moved close to ATP, and inosine to adenosine and adenine, but the radioactivity of ATP and AMP showed no trailing.

Fig. 1. Electrophoretic patterns of the carrier substances in (A) 0.1 *M* Tris HCl buffer (pH 8.0) and (B) 0.03 *M* citrate buffer (pH 6.0). Electrophoresis was carried out at 150 V for 40 min. From the cathode side: A, adenosine, inosine, cAMP, and AMP + ADP and ATP; B, adenosine, inosine, cAMP, AMP, ADP, and ATP.

Fig. 2. Electrophoretic separation of cAMP from other adenine nucleotides, nucleosides and adenine in the buffer containing 12 m*M* citric acid, 14 m*M* KH_2PO_4, 14 m*M* boric acid and 2% glycerol (pH 7.0). From the cathode side: adenine, adenosine, inosine, cAMP, AMP, ADP and ATP.

TABLE I

RADIOACTIVE MEASUREMENT OF LABELLED NUCLEOTIDES AND NUCLEOSIDES AFTER ELECTROPHORESIS

Substance	*Radioactivity (cpm)*	
	*Experiment 1**	*Experiment 2**
Origin	22	80
Adenine	60	380
Adenosine	321	9618 (109)**
Inosine	85	215
Blank 1	53	20
cAMP	9748 (97)**	62
Blank 2	42	55
AMP	32	11,072 (92)**
Blank 3	85	352
ADP	325	122
ATP	8172 (93)**	74

* In experiment 1, [8-^{3}H]ATP (8800 cpm) and [8-^{3}H]cAMP (10,000 cpm) were applied together with the carrier solution. In experiment 2 [8-^{14}C]AMP (12,000 cpm) and [8-^{14}C]adenosine (8900 cpm) were applied. After electrophoresis, each band and the areas between the marker bands (indicated as Blank) were located under UV light, cut out in order of migration from the cathode side (origin) as shown in Fig. 2, and counted.

** Numbers in parentheses are the recoveries of the ratioactivity of the compounds (%).

The radioactivity of cAMP was measured without interferences from ATP, AMP and adenosine (Table I). This separation method could be easily performed at low voltage and at room temperature in a reasonable time. It might be applicable to the determination of adenylate cyclase and phosphodiesterase activities and for the quantification of ATP and cAMP metabolites, by using the labelled nucleotides, as ATP, ADP, AMP, cAMP and the nucleosides were simultaneously separated from each other.

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Book Review

Proceedings of the 5th symposium on chemical problems connected with the stability of explosives (Bastad, May 28–30, 1979), Parts 1 and 2, edited by J. Hansson, Sektionen för Detonik och Förbränning, Sundyberg, Sweden, 1980, XII + 580 pp., price SwKr. 250.00.

Of the 31 papers, which are reproduced from camera-ready material, 14 deal with chromatography or describe work in which chromatography is used as an auxiliary technique. Some papers are of general interest, *e.g.*, a comparison of high-performance thin-layer chromatography and thin-layer chromatography and a comparison of gas chromatography and high-performance liquid chromatography.

There is also one paper which uses explosives as stationary phases for the study of the interactions of these explosives with various compounds.

In view of the general interest of these papers and the presumable unavailability of these volumes in general libraries, the chromatographic papers are listed below:

Chang Yung-feh, Liu Jung-hwa and Li Chung-chin, Thermal analysis used in the stability study of double-base propellants

T. Lindblom, Determination of stabilizers and their nitro derivatives in double-base powders by high-performance liquid chromatography

R. Ammann, H. Hilty, H. R. Pfeiffer and W. Rauber, Methods for evaluating the chemical stability of propellants using thin-layer and liquid chromatography

A. Sopranetti and H. U. Reich, Possibilities and limitations of high-performance liquid chromatography for the characterization of stabilizers and their daughter products in comparison with gas chromatography

M. Bédard, G. Perrault and R. D. Suart, The use of high-performance liquid chromatography in propellant ageing studies and explosives analysis

C. D. McAuley, Separation of high-explosive components in some common compositions by high-speed liquid chromatography

A. Delpuech, J. Cherville and J. M. Maquin, Relation entre la structure électronique et la sensibilité au choc des explosives. Contribution à l'étude des interactions liant-explosif par chromatographie sur colonne d'explosif

C. P. Ramaswamy and N. S. V. Subba Rao, Problems of stability of spent acid from PETN manufacture

R. Flückiger, E. Daume and E. Rochat, Determination of residual solvents in single- and double-base propellants

A. J. W. Brook, B. Kelso, I. Neil and N. MacLeod, An investigation of some aspects of the stability testing of nitrocellulose-based propellants

A. Sopranetti and H. U. Reich, Comparison of high-temperature stability tests with studies of the conversion of stabilizers in single-base propellants

F. Volk, Alterungsverhalten von Doublebase-Festtreibstoffen und Treibladungspulvern

L. Leneveu, P. Seite and J. Tranchant, Consommation de stabilisant et evaluation de la stabilité des poudres

J. Verhoeff, Comparison of several stability test methods

The two volumes have the general shortcomings of camera-ready reproduction, *e.g.*, the figures are often poor and often at the end of the paper, but on the whole they are readable.

Lausanne (Switzerland) MICHAEL LEDERER

Author Index

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NEW BOOKS

Advances in chromatography, Vol. 19, edited by J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown, Marcel Dekker, New York, Basel, 1981, XVIII + 312 pp., Price SFr. 98.00, ISBN 0-8247-1246-3.

Comparisons of fused silica and other glass columns in gas chromatography, by W.G. Jennings, Hüthig, Heidelberg, New York, 1981, 80 pp., price DM 38.00, US$ 19.00, ISBN 3-7785-0729-X.

Liquid chromatography in clinical analysis, by P.M. Kabra and L.J. Martin, Humana Press (Wiley), 1981, *ca.* 496 pp., price *ca.* US$ 78.00, £ 33.95, ISBN 0-89603-026-1.

Quantitative chemical analysis: a laboratory manual, by R. Belcher, A.J. Nutten, R.A. Chalmers and M.S. Cresser, Ellis Horwood (Wiley), Chichester, 1981, *ca.* 420 pp., price *ca.* US$ 63.25, £ 25.00, ISBN 0-85312-192-3.

The analysis of explosives, by J. Yinon and S. Zitrin, Pergamon, Oxford, New York, 1981, *ca.* 300 pp., price US$ 60.00, £ 25.00, ISBN 0-08-023846-7 (hardbound) or US$ 22.50, £ 9.35, ISBN 0-08-023845-9 (paperback).

Connective tissue research: chemistry, biology and physiology, edited by Z. Deyl and M. Adam, Heyden, London, 1981, 262 pp., price £ 16.40, DM 72.00, ISBN 0-8451-0054-8.

MEETINGS

14th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY
THE CHROMATOGRAPHY DISCUSSION GROUP JUBILEE SYMPOSIUM

The Chromatography Discussion Group, in association with the Royal Chemical Society (Analytical Division), Arbeitskreis Chromatographie der Fachgruppe Analytische Chemie der Gesellschaft Deutscher Chemiker and Groupement pour l'Avancement des Méthodes Spectroscopiques et Physico-Chimiques d'Analyse (G.A.M.S.), is holding the 14th International Symposium on Chromatography at the Hotel Inter•Continental, London, Great Britain, September 13–17, 1982. There will also be an exhibition of chromatographic and ancillary equipment which will be held at the nearby Grosvenor House as part of the Laboratory 82 Exhibition to which symposium delegates will be admitted free of charge.

The programme will be comprised of plenary lectures, discussion papers, and poster sessions dealing with all aspects of chromatography and related techniques. The language of the symposium will be English.

The meeting coincides with the 25th anniversary of the formation of the Chromatography Discussion Group, and a social programme commensurate with the occasion will be available for delegates and their guests.

An abstract of between 250–300 words will be required from prospective authors by November 30th, 1981. These should be sent to Dr. R. Stock, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain.

VI INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY

The VI International Symposium on Column Liquid Chromatography will be held in Philadelphia, PA, U.S.A. from June 7 to 11, 1982. The Symposium will be organized by The Chromatography Forum of Delaware and co-sponsored by The Chromatography Discussion Group, Arbeitskreis Chromatographie der Gesellschaft Deutscher Chemiker, and GAMS. All correspondence regarding the meeting should be sent to R.A. Barford, ERRC–SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A.

The scientific program will consist of oral presentations as well as poster papers dealing with all areas of column liquid chromatography and related techniques. Lectures will be held in English. A poster session will be organized daily, followed by a discussion session on the same topic.

Notification of discussion papers and poster communications submitted for consideration should arrive in Philadelphia, at the address of R.A. Barford, not later than December 4, 1981, together with a one-page, 300 word abstract. A scientific committee will review all papers by January 15, 1982, and make a decision as to which have been accepted for presentation.

The abstracts of the oral and poster communications will be reproduced in a booklet distributed to those attending the meeting. The papers (oral presentations as well as posters) will be published in a special issue of the *Journal of Chromatography*. Authors will be requested to hand their manuscripts to the Editor at the meeting.

In conjunction with the Symposium, an exhibition of liquid chromatography instruments and accessories will be planned. Companies interested in participation in this exhibition should direct their inquiries to R.A. Barford.

NOMINATIONS INVITED

The Delaware Valley Chromatography Forum invites nominations for the Steven Dal Nogare Award. This award is given annually for significant contributions to chromatographic theory, instrumentation, or applications, and consists of an honorarium and an inscribed plaque. The award will be presented at the 1982 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. The recipient will be expected to give an award address. Nominations should be submitted before July 27, 1981, accompanied by a brief bibliography and a list accomplishments related to chromatography. Send nominations to Gerald R. Umbreit, Greenwood Laboratories, 903 E. Baltimore Pike, Kennett Square, PA 19348, U.S.A.

CALENDAR OF FORTHCOMING MEETINGS

Aug. 3–7, 1981 Denver, CO, U.S.A.	**30th Denver Conference on Applications of X-Ray Analysis** Contact: Mrs. Mildred Cain, Denver Research Institute, University of Denver, Denver, CO 80208, U.S.A. Tel. (303) 753-2141.
Aug. 17–21, 1981 Meriden, NH, U.S.A.	**Gordon Research Conference on Ion Exchange** Contact: Dr. A.M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel. (401) 783–4011, or (401) 783–3372. (Further details published in Vol. 209, No. 2)
Aug. 20–21, 1981 Helsinki, Finland	**Symposium on Harmonisation of Collaborative Analytical Studies** Contact: Dr. H. Egan, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London SE1 9NQ, Great Britain.
Aug. 23–28, 1981 Espoo, Finland	**Euroanalysis IV – Triennial Conference of the Federation of European Chemical Societies** Contact: Professor L. Niinistoe, Department of Chemistry, Helsinki University of Technology, SF-02150 Espoo 15, Finland.

Aug. 23–28, 1981 New York, NY, U.S.A.	**182nd American Chemical Society National Meeting** Contact: American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Aug. 30–Sept. 5, 1981 Vienna, Austria	**XI International Congress of Clinical Chemistry – IV European Congress of Clinical Chemistry** Contact: Congress Secretariat: Interconvention, P.O. Box 35, A-1095 Vienna, Austria. Tel. (0222) 421352.
Aug. 31–Sept. 4, 1981 Freiburg i.Br., F.R.G.	**3rd International Symposium on Organic Free Radicals** Contact: Gesellschaft Deutscher Chemiker, P.O. Box 90 04 40, D-6000 Frankfurt/Main 90, F.R.G.
Sept. 1–3, 1981 Houston, TX, U.S.A.	**"EXPOCHEM '81"** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749–2623.
Sept. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).
Sept. 7–10, 1981 Guildford, Great Britain	**4th International Bioanalytical Forum** Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.
Sept. 7–11, 1981 High Tatras, Czechoslovakia	**International Symposium on Clathrate Compounds and Molecular Inclusion Phenomena** Contact: Doc. Ing. Anna Sopková, CSc., Special Group of Inorganic Chemistry in Eastern Slovakia, Slovak Chemical Society at Slovak Academy of Sciences, Moyzesova 11, 041 67 Košice, Czechoslovakia. (Further details published in Vol. 209, No. 2)
Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th National Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: M.A. Kaiser, E.I. du Pont de Nemours & Co., Experimental Station E228/200, CR&D Department, Wilmington, DE 19898, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Complete program published in Vol. 209, No. 3)
Sept. 28–Oct. 4, 1981 Moscow, U.S.S.R.	**International Symposium on Chemical Physics** Contact: Prof. N.M. Emmanuel, Institute of Chemical Physics, Academy of Sciences of U.S.S.R., Vorobyevskoye Chaussee 2-6, SU–117334 Moscow, U.S.S.R.
Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC 81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 20 20 20.
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectrometry** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3).
Oct. 27–29, 1981 London, Great Britain	**Petroanalysis 81** Contact: Miss I.A. McCann, Conference Officer, Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR, Great Britain. (Tel: 01–636 1004, Telex: 264380)

Oct. 28–30, 1981 Gatlinburg, TN, U.S.A.	**Resource Recovery and Environmental Issues of Industrial Solid Wastes** Contact: J.S. Watson, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2).
Nov. 16–17, 1981 Washington, DC, U.S.A.	**International Symposium on HPLC of Proteins and Peptides** Contact: Ms. S.E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. Joan Albaigés, General Secretary, Plaza de Espana, Barcelona-4, Spain. Tel. 223 31 01.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
Jan. 4–9, 1982 Orlando, FL, U.S.A.	**1982 Winter Conference on Plasma Spectrochemistry** Contact: 1982 Winter Conference, c/o ICP Information Newsletter, Chemistry -GRC Towers, University of Massachusetts, Amherst, MA 01003, U.S.A. Tel. (413) 545–2294.
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A.
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 5–8, 1982 Las Vegas, NV, U.S.A.	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 15–17, 1982 Tokyo, Japan	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
April 19–22, 1982 Barcelona, Spain	**International Congress on Automation in Clinical Laboratory** Contact: Dr. R. Galimany, Sección de Automatización, Laboratorio de Analisis Clinicos, C.S. "Principes de Espana", Hospitalet de Llobregat, Barcelona, Spain.
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik 82** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, G.F.R.

May 2–6, 1982 Interlaken, Switzerland	**2nd International Symposium on Instrumental TLC (HPTLC)** Contact: Dr. R.E. Kaiser, Institute for Chromatography, P.O. Box 1141, D-6702 Bad Dürkheim, G.F.R.
May 11–14, 1982 Ghent, Belgium	**4th International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium.
June 6–12, 1982 Frankfurt, G.F.R.	**European Meeting on Chemical Engineering and ACHEMA Exhibition Congress 1982** Contact: DECHEMA P.O. Box 970146, D-6000 Frankfurt/M 97, G.F.R.
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A.
June 28–30, 1982 East Lansing, MI, U.S.A.	**35th American Chemical Society Annual Summer Symposium** Contact: A.I. Popov, Chemistry Department, Michigan State University, East Lansing, MI 48824, U.S.A.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
July 11–16, 1982 Louvain-la-Neuve, Belgium	**6th IUPAC Conference on Physical Organic Chemistry** Contact: Prof. A, Bruylants, Université Catholique de Louvain, Laboratoire de Chimie Generale et Organique, Batiment Lavoisier 1 Place Louis Pasteur, 1348 Louvain-la-Neuve, Belgium.
July 12–16, 1982 Amherst, MA, U.S.A.	**IUPAC Macromolecular Symposium** Contact: James C.W. Chien, Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.
July 19–22, 1982 Prague, Czechoslovakia	**Prague Microsymposium "Selective Sorbents"** Contact: Dr. F. Svec, c/o Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Heyrovského n.2, 162 06 Prague, Czechoslovakia.
Aug. 2–7, 1982 Pretoria, South Africa	**13th International Symposium on the Chemistry of Natural Products** Contact: The Symposium Secretariat – S.219, CSIR, P.O. Box 395, Pretoria 0001, Republic of South Africa.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.
Aug. 23–27, 1982 Budapest, Hungary	**22nd International Conference on Coordination Chemistry** Contact: Prof. M.T. Beck, Institute of Physical Chemistry, Kossuth Lajos University, Debrecen 10, H–4010, Hungary.

Aug. 29–Sept. 4, 1982 Kyoto, Japan	**5th International Congress of Pesticide Chemistry** Contact: Rikagaku Kenysho (The Institute of Physical and Chemical Research), 2–1 Hirosawa Wako-shi Saitama Pref. 351, Japan.
Aug. 30–Sep. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A–1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 12–17, 1982 Kansas City, MO, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington DC 20036, U.S.A.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain.
Sept. 19–24, 1982 Philadelphia, PA, U.S.A.	**9th National Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Division of Analytical Chemistry, American Chemical Society, Department of Chemistry, Notre Dame, IN 46556, U.S.A.
July 17–23, 1983 Edinburgh, Great Britain	**SAC 83: Sixth International Conference on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Royal Chemistry Society, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel. 01–734 9971.
Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.

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PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2					

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications*: Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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Electrodes of Conductive Metal Oxides: Part A

edited by SERGIO TRASATTI, *Laboratory of Electrochemistry, University of Milan, Italy*

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 11

The discovery by Beer in the second half of the sixties that the performances of anodes made of thermally prepared noble metal oxides were better than those of noble metals provoked something of a technological revolution in the large electrolytic industry. Since then an ever increasing number of fundamental studies have been published but the large amount of data has, until now, not been adequately assimilated.

This two-part work provides a general unifying introduction plus a state-of-the-art review of the physicochemical properties and electrochemical behaviour of conductive oxide electrodes (DSA). The text has been divided into two volumes – Part A dealing mainly with structural and thermodynamic properties and Part B, to be published in due course, dealing with kinetic and electrocatalytic aspects. This division came about due to the large amount of material to be treated and also because, in a rapidly developing field, difficulties arise in collecting all relevant material at one given moment.

The editor approaches the subject from a multidisciplinary angle, for example, the electrochemical behaviour of oxide electrodes is presented and discussed in the context of a variety of physicochemical properties – electronic structure, nonstoichiometry, crys structure, surface structure, morphology and adsorption properties. For the first time the different groups of oxide are treated together in orde place emphasis on their simi larities and differences.

This major reference work is mainly directed to electrochemists and those working catalysis. It will also be usefu to those in the fields of mate rials science, physical chemi try, surface and colloid chemistry and in areas wher oxide surfaces may play a major role as in chromatography and photochemistry.

CONTENTS: Chapters. 1. Electr nic Band Structure of Oxides wi Metallic or Semiconducting Characteristics *(J. M. Honig)*. 2. Chemisorption and Catalysis Metal Oxides *(A. Cimino and S. Carrà)*.. 3. Oxide Growth and Oxygen Evolution on Noble Metals *(L. D. Burke)*. 4. Electrochemistry of Lead Dioxide *(J. P. Pohl and H. Rickert)*. 5. Pro perties of Spinel-Type Oxide Electrodes *(M. R. Tarasevich an B. N. Efremov)*. 6. Physicochemi and Electrochemical Properties Perovskite Oxides *(H. Tamura, Y. Yoneyama and Y. Matsumoto)* 7. Properties of Conductive Transition Metal Oxides with Rutile-Type Structure *(S. Trasatt and G. Lodi)*.

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