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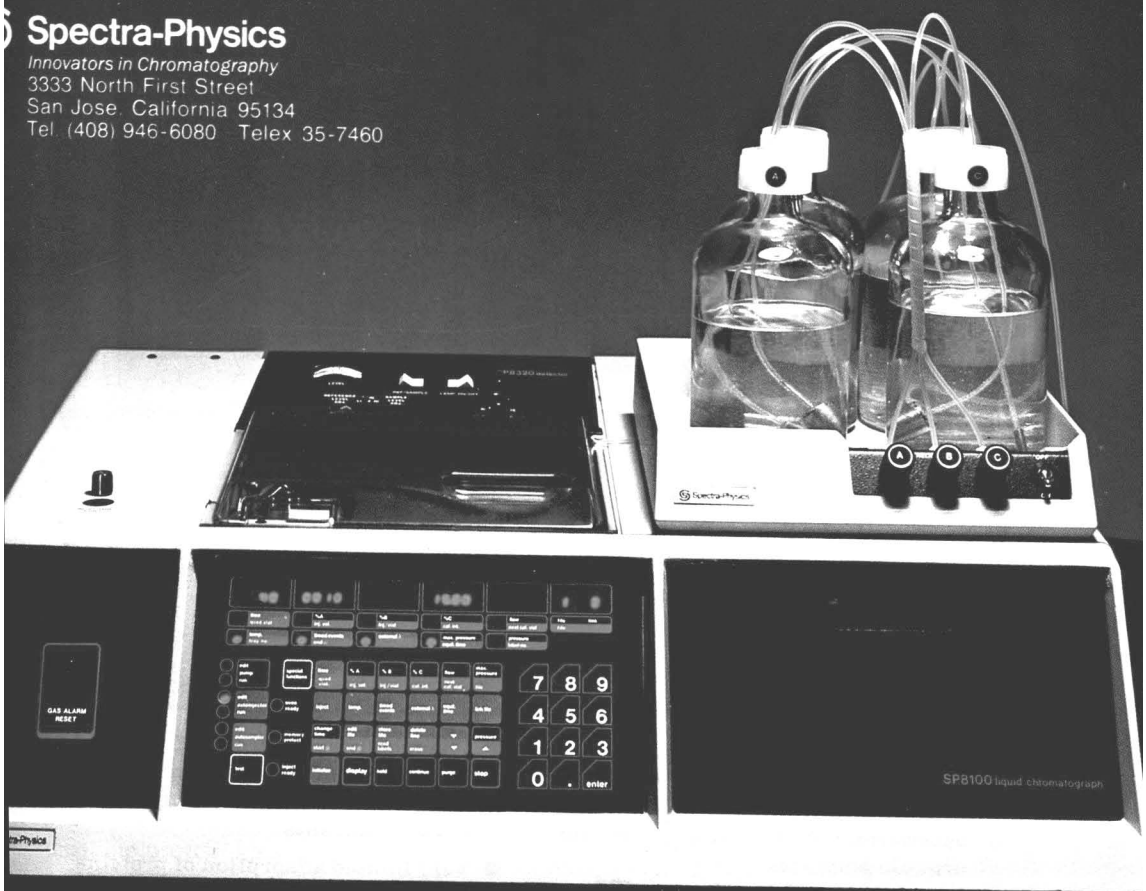

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INVESTIGATION OF THE ACTIVATION OF CROSS-LINKED AGAROSE WITH CARBONYLATING REAGENTS AND THE PREPARATION OF MATRICES FOR AFFINITY CHROMATOGRAPHY PURIFICATIONS

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(Received July 13th, 1981)

SUMMARY

The activation reaction of cross-linked agarose with 1,1′-carbonyldiimidazole (CDI) has been extended to other carbonylating reagents, and has confirmed that CDI allows the facile preparation of activated matrices suitable for affinity chromatographic supports. These studies showed that 1,1′-carbonyldi-1,2,4-triazole (CDT) gave a more reactive activated matrix, while 1,1′-carbonyldi-1,2,3-benzotriazole reacted only slowly and inefficiently. Phosgene, in addition to the disadvantage of toxicity, does not give a high level of activation. The introduction of imidazolyl carbamate groups onto cross-linked agarose by generating CDI *in situ* from phosgene and imidazole gave one-third of the level of activation of that obtained with pure CDI.

All of the activated matrices had sufficient stability to aqueous conditions to allow unhurried isolation of the washed, activated product. All carbonylated matrices when subsequently coupled with monoalkylamines were found to be devoid of any additional charged groups due to the activation process.

The studies have demonstrated that CDI is the most effective and convenient of the carbonylating reagents studied for the preparation of activated matrices to be used in affinity chromatographic experiments. However, the CDT-activated matrix is much more reactive than the CDI matrix and may be useful for the coupling of unstable protein ligands where short coupling times are essential.

INTRODUCTION

In a previous communication[1] we demonstrated that 1,1′-carbonyldiimidazole

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SCHEME A

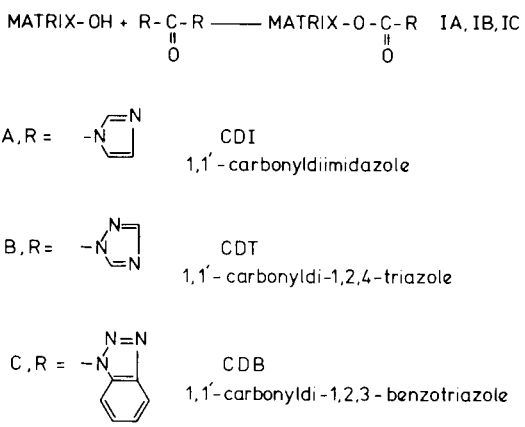

Fig. 1. The chemical reactions involved in the carbonylation of a polysaccharide (Scheme A).

(CDI) could be used to activate cross-linked agarose beads. The imidazolyl carbamate groups (see structure IA, Fig. 1) formed by using this reagent were found to be susceptible to nucleophilic attack by amines in aqueous or organic solutions. Furthermore, the carbamate derivative was found to be relatively stable to oxygen nucleophiles, which thereby facilitated the handling of the activated matrix under aqueous conditions. The attachment of ligands was extended from simple amines to proteins, and the use of the coupled agarose as an affinity chromatography support was subsequently demonstrated[2].

This report investigates the use of other carbonylating reagents *e.g.* 1,1′-carbonyldi-1,2,4-triazole (CDT) and 1,1′-carbonyldi-1,2,3-benzotriazole (CDB) for the activation process shown in Fig. 1. The report describes the activation yield, the stability of the activated matrix, the coupling of amine ligands, and the charge properties of the coupled matrix.

EXPERIMENTAL

Chemicals

CDI and Reactigel™ (6X, 20–25 μmoles of active groups per ml of gel) were purchased from Pierce (Rockford, IL, U.S.A.). CDT and CDB were prepared by standard methods[4]. Phosgene was used as a 17.8% solution in toluene. Acetone was analytical-reagent grade and all other chemicals were of reagent grade.

Solvent exchange of matrices from water

The aqueous suspension of a matrix was washed sequentially with water, water–organic solvent (70:30, v/v), water–organic solvent (30:70, v/v) and five portions of the organic solvent. The matrix was used immediately.

Typical activation procedure

The insoluble polysaccharide (0.2 g dry weight or its moist cake equivalent,

which is *ca.* 3 g in the case of Sepharose CL-6B) was presoaked overnight in, or solvent exchanged into, the appropriate organic solvent (6 ml). It is important that the solvent is anhydrous as the activation reagents used are readily hydrolysed. The activation reagent was added (1 mmol) and the suspension shaken gently for 0.5 h or left longer if desired. The product was washed with the same solvent and either stored under fresh solvent or used immediately.

Typical coupling procedure for simple amines

The activated matrix (from 0.2 g dry weight as above) was sucked dry to a moist cake and added to a solution of amine (5.4 mmol) in aqueous buffer (8 ml), generally either 1 *M* sodium carbonate (pH 10) or 1 *M* N,N,N′,N′-tetramethylethylenediamine (pH 9). For matrices of low degree of activation, a less concentrated buffer may be used. The suspension was shaken overnight at pH 10 or 72 h at pH 9 at room temperature or below. Washing was then carried out with water, 1 *M* salt solution and water again.

Analysis of all carbonylated matrices

The activated matrix was added to a solution of carbonate-free sodium hydroxide (0.15 *M*, 50 ml) in a volumetric flask. The flask was shaken periodically for 0.5 h and the contents allowed to settle. A portion (25 ml) of the supernatant liquid was titrated under nitrogen between the limits of pH 9 and 4 with 1 *M* HCl. This gives the total amount of carbon dioxide and imidazole formed from the active groups. The carbon dioxide was expelled in a stream of nitrogen at pH 3 and the sample was retitrated between the same limits to obtain the amount of imidazole present. The number of active groups present was calculated from the difference between the two figures, that is based on the amount of carbon dioxide expelled. All titrations were carried out using a Radiometer TTT2 pH titrator.

Analysis of coupled matrices

Coupled matrices could generally be analysed by titrations of their amino or carboxyl end-groups. For example, the coupled matrices were washed with HCl (pH 2, 250 ml) if they contained carboxyl end-groups, or NaOH (pH 11.7, 250 ml) if they contained amino end-groups. They were then titrated using the method described above in the presence of 1 *M* salt (10 ml) to pH 7.5 with 0.2 *M* HCl or NaOH as appropriate.

RESULTS AND DISCUSSION

Comparison of activation reagents

CDI was considered on the basis of our preliminary study[1] to be the carbonylating reagent of choice for the activation of cross-linked agarose. In the present study a range of other carbonylating reagents were examined and the results obtained compared with those derived from both the CDI and cyanogen bromide methods. It was apparent from these experiments that other carbonylating reagents could be successfully employed. Data for these experiments are listed in Table I, together with activation yields and efficiencies. None of the listed reagents offers any advantages over CDI in terms of degree of substitution on the matrix. CDT is of comparable efficiency

TABLE I

A COMPARISON OF ACTIVATION YIELDS OF CARBONYLATING REAGENTS ON CROSS-LINKED AGAROSE

Reagent	*Solvent*	*Amount of reagent used (mmol)*	*Yield of active groups (mmol/3 g)*	*Yield (%)*
CDI*	Dioxane	0.93	0.40	43
CDI*	Dioxane–Et_3N(1:1)	0.93	0.26	28
CDT*	Dioxane	0.93	0.32	35
CDB*	Dioxane	0.63	0.08	13
Phosgene*	Dioxane	0.93	0.08	9
Phosgene	Dioxane	4.45	0.31	7
CNBr**	Water	6	0.10	2
CNBr/HCl***	Water	6	0.10	2

* The activation procedure was carried out as described in the Experimental section on 3 g of moist cake.

** This was carried out by the method of March *et al.*[5], on 3 g of moist cake, with 1 *M* Na_2CO_3 as the buffer.

*** The activated agarose (3 g of moist cake) from the CNBr activation was shaken in 1 *M* HCl (10 ml) for 0.5 h. It was washed with deionised water and used immediately.

but is a much less stable reagent and is difficult to handle. CDB reacts only very slowly and inefficiently, possibly because of the steric effect of the bulky benzotriazole groups. In addition, di(*p*-nitroaniline)urea, di-(2-aminopyridine)urea and di-(2-hydroxypyridine)carbonate were tried as activating reagents, but were found to be quite unreactive with cross-linked agarose.

Phosgene, in addition to the disadvantage of toxicity, does not give a high level of activation. The active groups derived from the phosgene reaction were shown to be cyclic carbonates by the absence of chlorine in the product. This cyclisation did not occur with CDI, CDT or CDB in inert organic solvents, and in each case the carbamate derivative (structure I, Fig. 1) was the only identified product. However, if the activation with CDI was carried out in the presence of 50% triethylamine, some cyclisation did occur, and the yield of active matrix was reduced, *i.e.* the active groups present were shown to be 50% cyclic carbonates and 50% imidazolyl carbamates by titrimetric analysis. No cyclisation occurred during the activation of cross-linked agarose with CDB in the presence of triethylamine.

An attempt was made to introduce imidazolyl carbamate groups onto cross-linked agarose by generating the CDI *in situ* from phosgene and imidazole. This experiment was partially successful, but the yield of the imidazolyl carbamate was only *ca.* one-third of that obtained with pure CDI. Furthermore, it was necessary in this case to use an aqueous washing step to remove the amine salts, a procedure which resulted in the hydrolysis of some of the activated matrix. This approach, however, produced little of the cyclic carbonate product. In addition, the procedure could be used successfully to couple 6-aminohexanoic acid, with yields similar to that obtained for the normal CDI-activated matrix.

Cross-linked agaroses, activated by the CDI, CDT or CDB methods, were subjected to hydrolysis under various conditions of pH. The results obtained are

TABLE II

SUSCEPTIBILITY OF ACTIVE CROSS-LINKED AGAROSES TO HYDROLYSIS AT ROOM TEMPERATURE

The matrices, activated as described in the Experimental section (from 3 g of moist cake), were kept at pH 5 by the automatic addition of 2 *M* HCl or at pH 8.5, 10 or 11 by the addition of 2 *M* NaOH. The rate of addition of the acid or alkali was recorded on a Radiometer TTT2 automatic titrator.

Activation	*Reaction time for complete hydrolysis*			
	pH 5	*pH 8.5*	*pH 10*	*pH 11*
CDI	20	30	10	1.5
CDT	–	4	1.5	–
CDB	–	1	1	–

shown in Table II. Clearly all of the activated matrices have sufficient stability to aqueous conditions to allow unhurried isolation of the washed, activated product.

When an active matrix such as a CDI-activated polysaccharide is used to couple proteins it is clearly desirable to have no active groups remaining at the end of the reaction. This is because further nucleophiles inevitably come into contact with the matrix during an affinity chromatography separation. The results shown in Table II therefore give a measure of the time for which a coupling reaction must be left unless the excess active groups are quenched, *e.g.* by the addition of ethanolamine.

The CDT-activated matrix is much more reactive than the CDI matrix and may be used where short coupling times are essential. In particular it could be useful for a more facile coupling of biological molecules sensitive to high pH values. Typically coupling reactions to the CDI matrix should preferably be left 10–18 h at pH 10, or for 36–48 h at pH 8.5 to 9 (both at 4°C). These reactions must be adequately buffered, particularly if high substitution matrices are used.

An investigation of the coupling reaction on activated cross-linked agaroses

A series of couplings was carried out using three simple nucleophilic ligands of differing pK_a values to evaluate the susceptibility of the active groups towards aminolysis compared with hydrolysis. The couplings were carried out on cross-linked agarose activated by the six methods listed in Table III. The ligands selected for this study were 6-aminohexanoic acid, glycine and glycylglycine. In addition, 1,6-diaminohexane was tried in one case to compare couplings under aqueous and non-aqueous conditions. The ligands, all of which have titratable end-groups, were added to the reaction mixture in large (more than ten-fold) molar excess over the active groups on the matrix. The reactions were carried out under well buffered conditions, and their pH values were rechecked just prior to washing. The pH of the glycylglycine reactions, which were carried out at an initial pH of 10, increased to pH 10.5 at the end of the reaction, but in all other cases no pH shift was observed.

The data in Table III show that with the matrices activated by carbonylation, each amine couples best at a pH value within one unit of its pK_a value. For example, under the conditions studied 6-aminohexanoic acid ($pK_a = 11$) couples best at pH 11, glycine at pH 10 ($pK_a = 9.8$) and glycylglycine at pH 9 ($pK_a = 8.1$). The coupling of ligands to the CNBr-activated matrix appears to be less pH dependent, and ap-

ABLE III

OUPLING YIELDS FOR AMINOLYSIS OF ACTIVE CROSS-LINKED AGAROSES

ross-linked agarose (3 g of moist cake) was activated as described above with either CDI or CNBr[5]. For the)upling reactions a constant amount of amine (5.4 mmol) was used in each case. Aqueous coupling reactions were ırried out at pH 9 and 10 as previously described, except 6-aminohexanoic acid which was left for 5 h at pH 11, ıd without additional buffer. The coupling of CDI activated cross-linked agarose to 1,6-diaminohexane in dioxane as carried out overnight. The matrix was solvent-exchanged back into water and washed in the usual way.

ctivation	*Amount of active groups on matrix (mmol)*	*Coupling yield (%)*				
		6-Aminohexanoic acid	*1,6-Diaminohexane*	*Glycine*	*Glycylglycine*	
		pH 9	*pH 10*	*pH 11*	*pH 9*	*pH 10*
DI	0.280	20	40	50	70	25
DT	0.315	27	49	–	49	22
DB	0.070	–	61	–	–	–
hosgene	0.080	–	35	–	–	25
NBr	0.105	61	62	–	32	29
NBr/HCl	0.105	–	–	–	29	13

TABLE IV

THE TRYPSIN CAPACITIES OF AFFINITY COLUMNS PREPARED BY THE IMMOBILISATION OF SOYABEAN TRYPSIN INHIBITOR TO SEPHAROSE CL-6B WITH DIFFERENT CARBONYLATING REAGENTS

*Method**	*Active groups***	*STI coupled at pH 9*		*STI coupled at pH 10*	
		*Trypsin bound*** (mg)*	*Total column volume (ml) in distilled water*	*Trypsin bound*** (mg)*	*Total column volume (ml) in distilled water*
CDI	66	0.68	0.64	1.18	0.46
	192	0.97	0.4	2.1	0.38
CDT	52	0.51	0.6	0.51	0.48
CDB	27	0.18	0.64	0.2	0.58
Phosgene	52	0.37	0.52	1.04	0.38

* The activation procedures described in the Experimental section were used. In this case 1 g of moist cake was activated with the following amounts of carbonylating reagent: CDI, 0.47 and 1.21 mmol; CDT, 0.31 mmol; CDB, 0.63 mmol; phosgene, 4.45 mmol. The activation reaction was left for 0.5 h except for the CDB reaction which was left for 5 days.

** Measured by titration analysis and expressed in μmoles for the whole sample.

*** Soyabean trypsin inhibitor (15 g) was coupled to the activated matrix at pH 9 or at pH 10 using conditions described previously[1]. Crude bovine trypsin (20 mg) was loaded into the column equilibrated in 500 m*M* NaCl–50 m*M* Tris, pH 8.0. The buffer was changed to 500 m*M* NaCl–3 m*M* HCl to elute the bound trypsin. The concentration of bound trypsin was determined spectrophotometrically at 280 nm and values expressed as milligrams per total sample.

parently a higher percentage of activated groups are coupled with this activated matrix than with the CDI-activated matrix, *e.g.* 61% compared with 20% for 6-aminohexanoic acid at pH 9. However, the concentration of ligand groups attached to the matrix is the same for both products (*ca.* 60 μmol) owing to the higher starting concentration of active groups with the CDI-activated matrix.

The coupling of trypsin inhibitor to the activated matrices

Proteins can be attached to the matrix under mildly acidic conditions via a diimide-mediated coupling to a preformed leash with either a free amino or carboxyl group. This procedure has been used in the preparation of high capacity trypsin affinity columns[1]. In addition we described the immobilisation of trypsin inhibitor via CDI-activated cross-linked agarose at pH 9, and the subsequent use of the generated affinity support for the purification of bovine trypsin. These studies have been extended to the other carbonylating reagents, and the results are summarised in Table IV. The results shown in this table confirm that CDI is a very satisfactory reagent for the preparation of an affinity column, although CDT also gives a suitable product. Two levels of CDI were used in this study and it can be seen that the matrix with the higher level of activation (192 μmole per gram of moist cake) showed a decreased swollen volume presumably caused by cross-linking reactions. The matrix still was suitable for the purification of trypsin, as can be seen by the high capacity of the column for this protein. Although soybean trypsin inhibitor is stable to the coupling conditions used in this study, many proteins would be degraded under these conditions. As was shown in the companion paper[6] the coupling reaction can, however, be carried out at pH 8.5, or alternatively a diimide-mediated coupling of the protein to a leash can be carried out at mildly acidic pH values[1].

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INVESTIGATION OF THE ACTIVATION OF VARIOUS INSOLUBLE POLYSACCHARIDES WITH 1,1′-CARBONYLDIIMIDAZOLE AND OF THE PROPERTIES OF THE ACTIVATED MATRICES

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(Received July 13th, 1981)

SUMMARY

This report further characterises a new procedure for the preparation of affinity chromatographic supports, namely the activation of hydroxylic solid-phase supports with 1,1′-carbonyldiimidazole (CDI). Matrices with a controlled degree of substitution can be synthesised by the use of CDI, and if required, a high level of activation can readily be achieved *e.g.* up to 5 mmol/dry gram with cross-linked agarose. The CDI-activated agarose was found to have a half-life of greater than fourteen weeks when stored in dioxane. The conditions for coupling simple amines of differing pK_a values to these active matrices were evaluated and the coupling yields analysed. Based on these results, conditions suitable for the coupling of proteins were established. The linkage of the amino group of the ligand to the support (an N-alkylcarbamate) was shown to possess good stability over a wide pH range. This stability was much greater than that of the isourea linkage obtained with the cyanogen bromide activation method. It is expected that this new activation procedure should prove to be particularly useful for a variety of affinity chromatographic experiments, including those which cannot tolerate the hydrolytic release of small amounts of the insolubilised ligand.

The CDI method has been extended to other polysaccharide matrices *e.g.* cellulose and dextran derivatives, and to glycophase-coated glass beads. The activated glass bead derivative provides a suitable support for attachment of ligands for high-performance affinity chromatography.

INTRODUCTION

Recently we introduced a new procedure for the preparation of activated ma-

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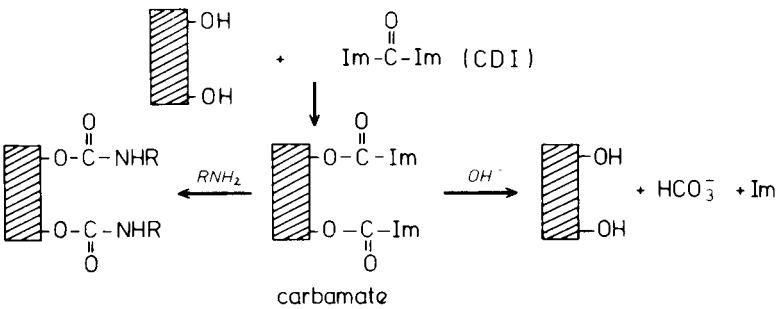
trices suitable for the attachment of affinity chromatography ligands or leashes[1]. This procedure involved the reaction of cross-linked agarose with 1,1′-carbonyldiimidazole (CDI) to form an N-alkylcarbamate (Scheme A, Fig. 1), which could then be smoothly coupled with N-nucleophiles[1,2]. A number of other carbonylating reagents, such as 1,1′-carbonyl-1,2,4-trizaole, were also shown to allow the preparation of activated matrices[3], although CDI remains the reagent of choice, particularly in terms of convenience and activation yields. An important advantage of this new activation method compared with the standard cyanogen bromide method[4,5] is the absence of charged groups in the pH range normally used in affinity chromatography. Other advantages are the ease of handling of the reagent, the ability to achieve a range of substitutions under a variety of readily controlled activation conditions, and the stability of the activated product.

Scheme A



Scheme B

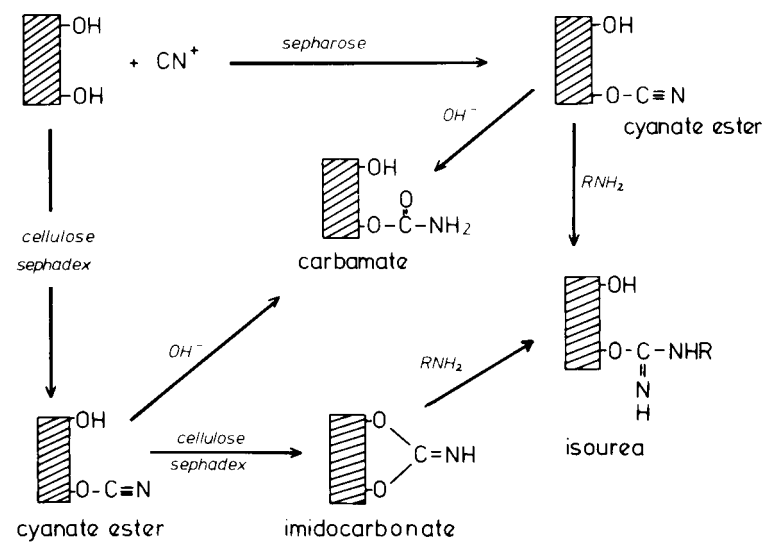


Fig. 1. The chemical reactions involved in the carbonylation of a polysaccharide (Scheme A) and the activation of a polysaccharide with CNBr (Scheme B).

In this report the CDI approach has been extended to other insoluble polysaccharide matrices, such as cellulose derivatives and glass beads. Additional data on the derivatisation of cross-linked agarose are discussed. Experiments demonstrating the stability of both the activated and coupled products are also described.

EXPERIMENTAL

Reagents

CDI, Reactigel™, (6X, 20–25 μmoles active groups/ml gel) and carbohydrate-coated glass beads (glycophase-G™/CPG40) were purchased from Pierce (Rockford, IL, U.S.A.); cross-linked allyl dextran (Sephacryl S-200, superfine), cross-linked agarose (Sepharose CL-6B), and cross-linked dextran (Sephadex G-25) from Pharmacia (Uppsala, Sweden); regenerated cellulose (75–150 μm) from Viscose Group Ltd. (Great Britain); cellulose powder (Whatman cellulose CC-31) from Whatman Ltd. (Great Britain); agarose–polyacrylamide copolymer (Ultrogel AcA-44) from Industrie Biologique Francaise (France); and cellulose beads from the Institute of Macromolecular Chemistry (Czechoslovakia). Trypsin (type III) and soybean trypsin inhibitor (type IIs) were purchased from Sigma (St. Louis, MO, U.S.A.). Acetone was analytical-reagent grade and all other chemicals were of reagent grade. Radioactivity was measured on a Packard Model 2002 Tricarb scintillation counter.

Methods

The procedures used for solvent exchange of the matrices from water, a typical activation procedure, a typical coupling procedure for simple amines and analysis of all carbonylated matrices except glass beads are described in the companion publication[3].

Analysis of carbonylated carbohydrate-coated glass beads

Since the active beads are not stable to the previous analytical method, the beads were hydrolysed at pH 3 for 4 h. The pH was maintained by automatic addition of acid during this period. The suspension was then titrated between the limits of pH 9 and 4 to obtain the amount of imidazole present. This figure was used to calculate the number of active groups present. The method does not allow for the presence of cyclic carbonates; however, previous studies have indicated that negligible amounts of cyclic carbonate are formed under these conditions[1].

RESULTS AND DISCUSSION

Comparison of the activation of cross-linked agarose with the CDI and CNBr methods

Table I shows the efficiency of carbonylation of cross-linked agarose with CDI and compares this procedure with the CNBr method[4,5]. In the CDI method the activated matrix contains imidazolyl carbamates which couple smoothly with N-nucleophiles to yield N-alkylcarbamates (Scheme A, Fig. 1). The CNBr method produces a cyanate ester on reaction with agarose[6], and probably an imidocarbonate with Sephadex or cellulose[7]. Both "active species" couple with N-nucleophiles to give affinity chromatography ligands attached via N-substituted isoureas (Scheme B, Fig. 1). However these groups can, prior to coupling, undergo hydrolysis to unreactive

TABLE I

COMPARISON OF ACTIVATION YIELDS FOR THE CDI AND CNBr METHOD ON CROSS-LINKED AGAROSE

Reagent	*Solvent*	*Amount (mmol)*	*Yield of active groups*	
			(mmol/3 g)	*Percent*
CDI*	Acetone	0.93	0.45	49
CDI*	Dioxane	0.93	0.4	43
CDI*	Dioxane	2.7	1.73	65
CNBr**	Water	6	0.1	2
CNBr**	Water	24	0.11	0.46

* The activation procedure was carried out as described in the *Methods* section on 3 g of moist cake.

** This was carried out by the method of March *et al.*[5], on 3 g of moist cake, with 1 *M* Na_2CO_3 as the buffer.

carbamates (see Scheme B, Fig. 1). The cyanate esters present were estimated by Kjeldahl nitrogen analysis before and after hydrolysis with 1 *M* HCl (see Scheme B, Fig. 1). The ratio of inactive to active groups was shown to be *ca.* 2.5 to 1. The level of active groups obtained with CNBr activation, as shown in Table I, has been corrected for the presence of unreactive carbamates. The concentration of active groups obtained with CDI activation was determined by titration analysis of the carbonate and imidazole liberated by basic hydrolysis (see Scheme A, Fig. 1)[3]. The comparison shown in Table I clearly establishes that even small amounts of CDI can be used to produce much higher levels of activation than with CNBr.

We found that under the reaction conditions described by March *et al.*[5] for the CNBr method, the activation yield can be improved only 10% even with a four-fold excess of CNBr (Table I). By contrast, the following study showed that the CDI method can be used to prepare highly substituted matrices by a proportional increase in the amount of reagent. Cross-linked agarose was activated in dioxane with varying amounts of CDI and the yields obtained are shown in Fig. 2. The maximum activ-

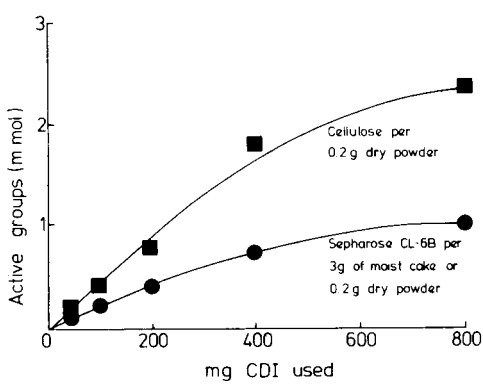


Fig. 2. The yield of formation of active matrix from the reaction of cross-linked agarose or cellulose with varying amounts of CDI. The dry weight of 3 g of moist cake of Sepharose CL-6B is *ca.* 0.2 g. The cellulose derivative used was HP-Regenerated Cellulose.

ation potential is *ca.* 5 mmol per dry gram of matrix (or 1 mmol per 3 g of moist cake). This is about ten times the value obtained for CNBr activation. Such a highly activated matrix is not always desirable in the case of cross-linked agarose where shrinkages can occur, and the original gel volume is not regained even after an aqueous coupling has taken place. For most affinity chromatography work only the lowest point on Fig. 2 (100 μmol per 3 g of moist cake) is relevant as greater activation levels can lead to multi-attachment of peptide and protein ligands, with consequent reduction in biological activities. However, with the attachment of a simple ligand for use as a leash or spacer, a high substitution may be useful.

In addition, the CNBr method requires both the reactions and washing of the active matrix to be carried out quickly. Activation time is not critical with the CDI method and the washing of the activated matrix is carried out under conditions whereby negligible hydrolysis occurs.

In all cases, the coupled products derived from the carbonylated agaroses and 6-aminohexanoic acid were devoid of charged groups at alkaline pH values (as determined by titration analysis). By contrast the corresponding product from the CNBr-activated matrix exhibited on titration analysis a second charged group (pK_a 9.5), which was attributed to the N-substituted isourea linkage arising from the coupling of the ligand to the activated matrix. The chemical complexity of the CNBr reaction has been also documented by other groups[8–10], and leads to a certain amount of non-specific binding of contaminants to the matrix during an affinity purification.

Stability of CDI-activated cross-linked agarose to storage and to aqueous coupling conditions

The activated cross-linked agarose can be readily stored for extended periods (*e.g.* 6 months) in the refrigerator at 4°C under dry acetone. Dioxane is unsuitable for this purpose because of its high freezing point. The stability of the activated matrix was also investigated by storing a sample under fresh dioxane at room temperature in the dark. Samples were withdrawn at different time intervals and coupled with 6-aminohexanoic acid. Despite the fact that the matrix was stored at 20°C rather than 4°C, the product still had some 60% of its initial activity after fourteen weeks of storage. It is therefore a practical proposition to activate a large amount of reswollen agarose and store the product until required. This is an advantage which the cyanogen bromide method does not afford.

Comparison of the coupling efficiency of the CDI-activated matrix with that of the CNBr-activated matrix

Fig. 3 shows that at the same excess of ligand (in the case glycine), the CDI-activated matrix is at least as reactive as the CNBr-activated matrix. The coupling of 6-aminohexanoic acid is a good model for attachment of proteins to the CDI-activated matrix, since the pK_a value of the ε-amino group of the amino acid lysine is similar to that of 6-aminohexanoic acid. Many proteins are sensitive to extremes of pH. This lability precludes couplings being carried out at pH 9–10, which would give the most efficient coupling conditions for CDI-activated gels[3]. However, coupling can be carried out in the range pH 7.5–9. Under these conditions a high coupling yield can still be obtained provided an activated matrix of moderately high substitution is used[2]. This approach is particularly valuable for the coupling of proteins which are

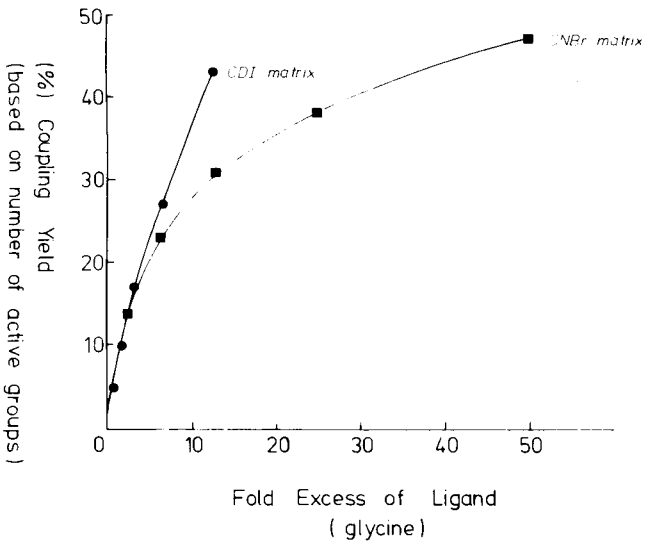


Fig. 3. The coupling yield of different amounts of glycine with either the CNBr- or CDI-activated matrix (0.28 mmol of active groups in each case). In each study the activated matrix (moist cake) was treated in 5.3 ml of water with 1 *M* N,N,N′,N′-tetramethylethylenediamine at pH 9. The calculated excess of glycine was added as a solid to the reaction immediately after the addition of the aqueous buffer to the activated matrix. The amount of coupled ligand was determined by titration analysis.

available only in small amounts. Table II illustrates the excellent linkage yields which can be achieved for a variety of proteins, when the coupling reaction is carried out at pH 8.5 with a matrix at a moderate level of activation. At activation levels much higher than 0.5 mmol per gram of moist cake, the possibility of multipoint attachment of a protein becomes more significant. The use of highly activated matrices could lead in some cases to a lower biological activity of the immobilised protein. However, with more robust proteins, particularly protein antigens, multipoint attachment may prove advantageous as weaker affinity interactions can result in a more facile desorption of retained components. The immobilised proteins listed in Table II demonstrate that a variety of other materials can be bound to cross-linked agarose with good retention of biological activity[2].

The CDI method is clearly very attractive for the coupling of leashes. This can be achieved most readily and efficiently with aqueous buffers at pH 11 for bis-amines of the form $NH_2(CH_2)_nNH_2$ (refs. 1 and 2). Alternatively dioxane can be used as a solvent for 1,6-diaminohexane and other diamines soluble in this solvent. For alkali-sensitive matrices such as glass beads, glycylglycine, which can be coupled at pH 8, has proved an excellent leash. The protein can be attached to the matrix under mildly acidic conditions via a diimide-mediated coupling to a preformed leash with either a free amino or carboxyl group. This procedure has been used in the preparation of high-capacity trypsin affinity columns[1]. In this earlier communication, we described the immobilisation of trypsin inhibitor via CDI-activated cross-linked agarose at pH 9, and the subsequent use of the generated affinity support for the purification of bovine trypsin.

TABLE II

SOME EXAMPLES OF THE COUPLING YIELDS OF LIGANDS TO A CDI-ACTIVATED MATRIX USING MILD COUPLING CONDITIONS

Sepharose CL-6B (3 g of moist cake) was activated with 1 mmol of CDI. The ligands were added to the activated matrix which was buffered with 0.1 *M* borate buffer, pH 8.5. The coupling yield was estimated by decrease in optical density (at λ_{max} of the sample) of the washings relative to the coupling reaction. The protein concentration on the matrix was determined by amino acid analysis of a 1 ml sample which had been hydrolysed with 6 *N* HCl, 110 C, 24 h.

Ligand	*Coupling yield (%)*	*Protein concentration on the matrix (μmol/ml)*
Sulphanilic acid azo-bovalbumin	90	1.0
Bovine thyroglobulin	94	0.4
Bovine thyroid stimulating hormone	88	2.3
Porcine insulin	100	2.9
Human immunoglobulin	87	1.0
3,3',5'-Thyronine	78	33

Stability of the coupled matrix

It is well known that ligands coupled to agarose through amino groups by the CNBr method are slowly released into solution, particularly at alkaline pH values[11]. It has been demonstrated that this release occurs mainly as a result of hydrolysis at

TABLE III

RELEASE OF [^{14}C]GLYCINE FROM COUPLED CROSS-LINKED AGAROSE, VIA CDI METHOD

Cross-linked agarose (6 g of moist cake) was activated in dioxane with CDI (0.9 g) in the usual way. It was coupled to glycine (0.04 g) containing [^{14}C(U)]glycine (28.2 mCi/mmol) at pH 9 in the usual way at 4 C. The coupled agarose was washed, divided into six equal portions, which were suspended in 10 ml of the following 0.1 *M* buffer solutions: 1, potassium hydrogen phthalate, pH 3.5; 2, potassium phosphate, pH 6; 3, potassium phosphate, pH 7; 4, TRIS, pH 8; 5, potassium carbonate, pH 10; 6, sodium phosphate, pH 11.5. The samples were stored at 4 C and aliquots of each (0.5 ml) were withdrawn after 1, 3, 7, 14 and 42 days.

Reaction time (days)	*Loss of [^{14}C]glycine (% of total)*,***					
	pH 3.5	*pH 6*	*pH 7*	*pH 8*	*pH 10*	*pH 11*
1	0.3	0.6	0.5	0.2	0.4	0.8
3	0.3	0.4	0.3	0.3	0.6	1.0
7	0.4	0.3	0.3	0.7	0.8	1.4
14	0.3	0.2	0.2	0.3	0.9	4.0
42	0.4	0.3	0.3	1.1	2.0	6.7

* The amount of glycine remaining attached to the matrix was determined by digesting the matrix with 10 *M* HCl at 60 C. After cooling, a 0.5-ml aliquot was neutralised and the cpm of ^{14}C was measured. This value was used to calculate the total ^{14}C cpm in the sample.

** Expressed as a percentage of total counts per gram of moist cake of coupled agarose.

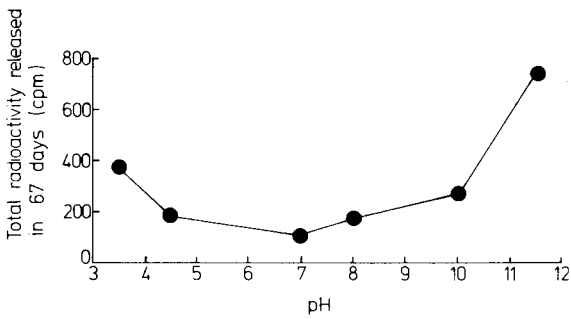


Fig. 4. The relative stability of agarose OCONHCH$^{*}_{2}$COOH when left for up to 42 days at 4 °C at different pH values. The experimental procedure was described in the footnotes to Table III and the values shown in this graph are the total of all values obtained at a given pH.

the isourea groupings[11] particularly in the presence of buffers which contain amines[7]. It was therefore of interest to compare the CNBr and CDI activation methods in terms of their abilities to produce products resistant to ligand leakage. The stability of the CDI-activated matrix was determined by measuring the release of [^{14}C(U)]glycine from the coupled matrix at various pH values over a period of six weeks. The results, expressed as percentages of the total [^{14}C(U)]glycine present, are shown in Table III. The linkage shows good stability at the pH values normally used in affinity chromatography. Fig. 4 shows the relative pH stability for the glycine-matrix, which again illustrates that the carbamate linkage is stable except at extremes in pH.

Tesser *et al.*[11] have studied the solvolytic detachment of [^{14}C(U)]alanine and cAMP from CNBr-activated matrices. Fig. 5 compares the data from their study with

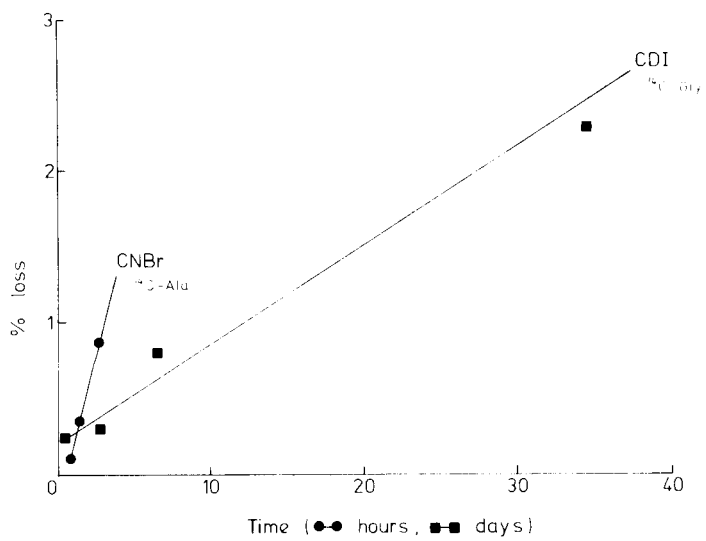


Fig. 5. A comparison of the rates of solvolytic detachment at pH 8 of [^{14}C(U)]-glycine which was insolubilised by the CDI method and [^{14}C(U)]-alanine which was insolubilised by the CNBr method. The CNBr data were derived from Fig. 2 in a study by Tesser *et al.*[11].

our data on the corresponding CDI-activated matrix. This comparison shows that the urethane linkage (CDI method) is *ca.* 25 times more stable than the isourea linkage (CNBr method). This figure is probably a minimum as the slight amount of radioactivity present in the supernatant of glycine matrix prepared by the CDI method and stored at pH 8 did not increase significantly with time of incubation. The low level of radioactivity in the supernatant (up to 1 % of total) is probably due to the presence of fines in the sample rather than glycine released by hydrolysis of the carbamate linkage. The greatly improved stability of attachment of a ligand to the matrix activated by CDI should prove to be invaluable in experiments which are adversely affected by the presence of free ligands, *e.g.* the localisation of hormone receptors on a cell surface. No ligand leakage was observed when sulphanilic acid azo-bovalbumin linked to Sepharose by the CDI method (see Table II) was stored at pH 7 at 4°C for 2 months. Tesser *et al.*[11] have already commented on the unsuitability of the CNBr-activated matrix with such ligands. Similar leakage has been noted for deoxycortico-

TABLE IV

ACTIVATION YIELDS OF POLYSACCHARIDE MATRICES WITH CDI

*Dry matrix presoaked in DMF**		*Matrix presoaked in water and solvent-exchanged***			
Matrix	*Yield*** (0.93 mmol of CDI)*	*Solvent*	*Yield****		
			0.47 mmol CDI	*0.93 mmol CDI*	*1.86 mmol CDI*
Cellulose (Whatman cellulose powder CC-31)	0.1	A	0.08	0.1	0.14
		B	0.06	0.08	0.14
CL-Dextran (Sephadex G-25)	0.4	A	0	0	0
		B	0.16	0.38	0.56
Regenerated powder cellulose	0	A	0.12	0.20	0.28
		B	0.08	0.12	0.34
HP-Regenerated cellulose (Regenerated cellulose after hydroxypropylation)	0.44	A	0.20	0.32	0.56
		B	0.16	0.24	0.50
Glass beads (carbohydrate-coated glass beads, Glycophase-G-CPG40)	0.06	A	0.06	0.07	–
Matrix as aqueous slurry[§]					
CL-Agarose (cross-linked Sepharose 6B)		A	0.16	0.30	0.48
Agarose–polyacrylamide (Ultrogel AcA-44)		A	0.12	0.18	0.24
CL-Allyl dextran (Sephacryl S-200)		A	0.10	0.22	0.38
Cellulose beads (Cellulose regenerated in bead form)		A	0.16	0.26	0.38

* In all cases 0.2 g of the dry matrix was presoaked in DMF for 16 h and then treated with CDI for 0.5 h.

** The dry matrix (0.2 g) was preswollen in water for 16 h and then solvent-exchanged into either dioxane (A) or DMF (B).

*** Yield of active groups determined by titration analysis and expressed as mmol/0.2 g dry wt. of matrix.

[§] The wet equivalent of 0.2 g dry weight was used for those matrices supplied as aqueous slurries.

steroids[12], estradiol[13], catecholamines[14], and ε-DNP lysine[15] from agarose conjugates prepared via the CNBr method.

Activation of other matrices by CDI

Although agarose in bead form is one of the most commonly used matrices for affinity chromatography, it was necessary to use cross-linked agarose beads for this activation procedure with CDI because of the requirement of an organic non-protic solvent for the activation step. Agarose beads without cross-linking lost their mechanical stability and flow-rates deteriorated after activation with CDI in dioxane. However, there are many other hydroxyl-containg matrices available which are amenable to organic solvents. All of those examined and reported in Table II were found to be reactive under optimal conditions. In all cases the active groups on the matrices were shown to be imidazolyl carbamates as found for cross-linked agarose.

Those matrices supplied as aqueous slurries were shown to activate readily by following the same procedure used for cross-linked agarose, *i.e.* solvent exchange into dioxane (see lower half of Table IV). Matrices supplied in the dry form were most conveniently activated by presoaking them directly in dimethylformamide (DMF) for 16 h, followed by activation with CDI for 30 min. The one exception to this was the regenerated cellulose which had been produced by the viscose process, dried and ground to a powder. For activation of this matrix it was necessary to presoak it first in water and then solvent exchange into dioxane or DMF. In all other cases there was no advantage in preswelling the matrix in water first. Presoaking in DMF directly was sufficient to swell these matrices for activation with CDI (see Table IV). However, all matrices failed to react with CDI if the dry forms were put directly into dioxane.

Dioxane and DMF were equally effective for solvent exchanging the matrices from their aqueous slurries. The one exception was Sephadex G-25, which did not

TABLE V

COUPLING YIELDS OF VARIOUS CDI-ACTIVATED MATRICES WITH 6-AMINOHEXANOIC ACID AT pH 10

All matrices (0.2 g dry wt.) were solvent exchanged from water and treated with 0.93 mmol of CDI for 0.5 h. The activated matrix was isolated and treated with 5.4 mmol of 6-aminohexanoic acid at pH 10 for 5 h. No additional buffer was used.

*Matrix**	*Solvent**	*Active groups*** (mmol/0.2 g)*	*Coupled groups*** (mmol/0.2 g)*	*Yield (%)****
Powdered Cellulose	A	0.14	0.042	30
Regenerated Cellulose	A	0.28	0.084	30
Regenerated Cellulose	B	0.34	0.114	34
HP-Reg. Ccell.	B	0.50	0.222	44
Cellulose Beads	A	0.38	0.096	25
Cross-linked Agarose	A	0.48	0.168	35
Agarose-polyacrylamide copolymer	A	0.24	0.090	38
Cross-linked Dextran	B	0.56	0.150	27
Cross-linked Allyl-dextran	A	0.38	0.20	52

* As in Table IV.

** Determined from duplicate activation and titration analysis.

*** Percentage of active groups coupled.

activate in dioxane. In this case there was a pronounced shrinkage of the cross-linked dextran beads upon removal of the water with dioxane. This did not happen with DMF.

The carbohydrate-coated glass beads were similarly activated with CDI and this gives a new route to supports for high-performance affinity chromatography[16].

Regenerated cellulose powder which had been previously treated with propylene oxide to introduce hydroxypropyl groups[17] could be activated to very high levels when treated with large amounts of CDI. This is a result of the well-known lipophilic effects of the hydroxypropyl groups which enable it to swell effectively in organic solvents. Greater than 10 mmol of active groups per gram of dry matrix were introduced in this way (see Fig. 1). As in the case of cross-linked agarose, noticeable shrinkage of the matrix occurred at such high degrees of substitution, but these readily obtainable high levels of activation demonstrate the utility of the CDI method. Specialised supports for hydrophobic chromatography and ion exchange chromatography have been produced by this method.

Table V shows that the same range of matrices, after activation with CDI, coupled readily to 6-aminohexanoic acid at pH 10. The coupling efficiencies were determined by titration of the carboxylic end groups introduced and the yields were generally in the range of 30–50%. The carbohydrate-coated glass beads were coupled to glycylglycine at pH 8 and gave similar yields of carboxylic end-groups.

As a result of these studies it has been shown that CDI is a reagent of general utility for activating hydroxylic supports for affinity chromatography. The active group was always the imidazolyl carbamate, which reacts readily with nucleophilic amines providing stable, unchanged N-alkylcarbamate linkages to ligands and spacer molecules for affinity chromatography.

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TWO-PHASE DERIVATIZATION OF AMITRIPTYLINE AND STRUCTURALLY RELATED TERTIARY AMINES FOR GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A new derivatization technique for tertiary amines has been developed. The amine is extracted at ambient temperature after addition of sodium iodide with methylene chloride containing an aryl chloroformate ester. By use of two new reagants, 2,4-dichlorophenyl and pentafluorophenyl chloroformate, the carbamates formed have high electron-capture response. The derivatization is rapid, often completed within 5 min. Amitriptyline was determined down to 6 ng/ml directly in a spiked plasma sample. A method for selective derivatization of a tertiary amine in the presence of the corresponding secondary amine is given.

INTRODUCTION

Numerous methods for the quantitative analysis of tricyclic antidepressants *e.g.* amitriptyline (AMI) and related compounds have been published[1]. Electron-capture gas chromatography (GC) has been used to a rather limited extent, probably due to the fact that the derivatization step is often very time-consuming[2].

Derivatization of tertiary amines with trichloroethyl chloroformate (TCE) to carbamates with excellent GC performance and high electron-capture response has been reported previously[2,3].

Most of the methods for trace analysis of compounds in complex aqueous matrices, *e.g.* plasma, are laborious and involve several extraction steps before the final assay[1]. Furthermore, precautions against adsorption losses during the extractions are often necessary but not always successful[4,5].

A technique for rapid quantitative determination of tertiary amines in biological material after derivatization with chloroformate esters has recently been reported[6]. The plasma sample is extracted with an organic solvent containing a chloroformate ester. Extraction and derivatization therefore occur in the same procedural step. In such an approach, adsorption losses should be of minor importance. Furthermore, the combined extraction and derivatization procedure can be performed at neutral or slightly acidic pH which is advantageous when dealing with compounds

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unstable at high pH, *e.g.* phenothiazines[5]. The procedure given in ref. 6 could, however, be applied only to compounds containing benzylic or allylic groups at the nitrogen atom.

This study presents a further development of the derivatization technique, which makes the method applicable also to N-methyl substituted tertiary amines. Reaction conditions for determination of amitriptyline in presence of plasma are given.

EXPERIMENTAL

Apparatus

Gas chromatography. A Pye Unicam gas chromatograph Series 106 equipped with a flame ionization detector was used for evaluation of reaction conditions.

Low concentration analysis was performed with a Hewlett-Packard 5710 A gas chromatograph equipped with a ^{63}Ni electron-capture detector. Argon with 5% of methane was used as carrier gas (flow-rate 30 ml/min).

The glass columns (120 × 0.18 cm I.D.) were filled with 3% OV-17 on Gas-Chrom Q, 100–120 mesh, and operated at 310°C for analysis of AMI as the 2,4-dichlorophenyl carbamate.

Mass spectrometry. An LKB 9000 mass spectrometer was used with an ionization energy of 70 eV. The compounds were introduced by GC using a column as above.

Chemicals and reagents

Methylene chloride and trichloroethyl chloroformate (TCE) (Ega-Chemie, G.F.R.) were distilled before use, the latter in vacuo. Toluene Uvasol^{R} (E. Merck, G.F.R.) was used as recieved.

2,4-Dichlorophenyl chloroformate (DCP) and pentafluorophenyl chloroformate (PFP) were synthesized from 2,4-dichlorophenol (Fluka, Switzerland) and pentafluorophenol (Fluka), respectively, by treatment with 20% phosgene in toluene (Fluka) according to the general procedure described in ref. 7. Assay gave a content of 98% for DCP and 96% for PFP. Alcoholic alkali was prepared by dissolving 2.8 g of potassium hydroxide in 75 g of methanol and 25 g of distilled water. All other chemicals were of analytical grade. The identity of all derivatives was confirmed by mass spectral analysis.

Methods

Evaluation of reaction conditions. The amine ($5 \cdot 10^{-5}$ *M*) was dissolved in phosphate buffer pH 6.0 ($\mu = 0.2$, unless otherwise stated) containing sodium iodide. The appropriate chloroformate ester was dissolved in methylene chloride containing the internal standard. Equal volumes (2.00 ml) of aqueous and organic phases were shaken in centrifuge tubes at 20°C. The reaction was quenched by addition of 5 ml of 0.2 *M* sulphuric acid.

The peak area ratio to the internal standard derivative was calculated. The absolute yield was determined by a comparision with peak area ratio obtained with a reference sample containing known amounts of the derivatives.

The internal standards were: desmethyl-2-chloroimipramine for AMI, nortrip-

tyline (NTP) for 2-chloroimipramine (CIM) and 4-phenyl-1,2, 3,6-tetrahydropyridine for pethidine.

Determination of amitriptyline in spiked plasma samples. Plasma (1.0 ml) in a small centrifuge tube (8 ml) is mixed with 1.0 ml of a phosphate buffer solution pH 6.0 (μ = 0.2) containing AMI (6–85 ng/ml), CIM (35 ng/ml) and sodium iodide (1.0 *M*) and 2.0 ml $3 \cdot 10^{-3}$ *M* solution of DCP in methylene chloride. The mixture is shaken for 10 min at 20°C.

The tube is centrifuged for 2 min, and the organic phase is transferred to another extraction tube containing 0.5 ml of toluene. The organic phase is reduced to *ca.* 0.5 ml by evaporation. Alcoholic alkali (0.5 ml) is added and a homogenous solution is obtained. After 5 min, 2 ml of 2 *M* sodium hydroxide is added and the tube vigorously shaken for 30 sec. A few microlitres of the organic phase is taken to analysis and quantitated by peak area measurements.

Determination of amitriptyline in presence of nortriptyline. A 2.0-ml volume of a phosphate buffer solution, pH 6.0, containing AMI (90 ng/ml), CIM (102 ng/ml) and NTP (1100 ng/ml) is mixed with 2.0 ml of a $4 \cdot 10^{-3}$ *M* solution of TCE in methylene chloride. The mixture is shaken for 5 min, then 0.5 ml of 2.5 *M* sodium iodide and 0.5 ml of $1.5 \cdot 10^{-2}$ *M* DCP in methylene chloride are added and the tubes are shaken for 10 min. The organic phase is treated as above.

RESULTS AND DISCUSSION

Reaction principles in two-phase systems

The aqueous phase containing the amine is extracted with an organic solvent, *e.g.* methylene chloride containing the chloroformate ester. In the organic phase, the amine base reacts with the reagent initially to an intermediate chloride ion pair[8]. The intermediate ion is distributed between the two phases and reacts with nucleophiles present, *e.g.* halide ions, to give a carbamate and an organic chloride (Fig. 1, path I). The intermediate ion can also react with water in both phases, giving the original amine (path II)[6,8,9]. A further reaction pathway involves a nucleophilic attack on the alkoxy part of the intermediate ion. In such a reaction, an alkyl halide and the original amine would be formed (path III)[10].

N-Methyl tertiary amines do not react with alkyl chloroformate esters such as TCE at ambient temperature to give carbamates[2] as is the case for benzyl- or allyl-substituted tertiary amines[6]. This might be due to extensive reactions according to both reaction paths II and III. A reaction according to path III should be of less

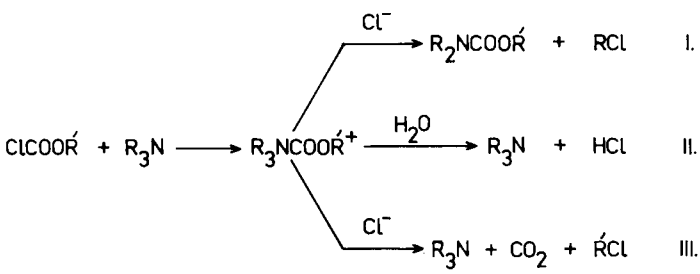

Fig. 1. Reaction pathways for the intermediate ion. R_3N, tertiary amine; ClCOOŔ, chloroformate ester; R_3NCOOR^+, intermediate ion; R_2NCOOR, carbamate.

importance if an aryl chloroformate ester is used[10]. However, the hydrolysis reaction is not eliminated and this leads to consumption of the reagent with a concomitant liberation of hydrogen chloride. This will give a decrease in pH, which in turn will increase the ion-pair distribution of the amine to the organic phase and, as a result, lower the reaction rate for carbamate formation.

If an aryl chloroformate ester, such as DCP or PFP, is used in combination with a strong nucleophilic agent like iodide ion[6], reaction pathways II and III are strongly supressed and rapid carbamate formation occurs even at room temperature.

Reaction conditions

The influence of iodide ion in the aqueous phase on reaction yield is shown in Table I. The reactions were followed up to 30 min. Both AMI and CIM gave a constant yield within 5 min. The low yield obtained at an iodide concentration of 0.05 *M* is ascribed to a higher extent of hydrolysis, which indirectly gives rise to a complete consumption of the reagent (DCP). Essentially the same reaction behavior was found for other tertiary amines, such as promazine and imipramine. When pentafluorophenyl chloroformate (PFP) was used as reagent the effect of hydrolysis was even more pronounced. However, with an increased buffer capacity ($\mu = 0.4$), 0.5 *M* sodium iodide and a tenfold increase of the PFP concentration ($3 \cdot 10^{-2}$ *M*), the yield, *e.g.* for CIM, was quantitative within 5 min.

TABLE I

INFLUENCE OF IODIDE ION CONCENTRATION ON THE YIELD OF 2,4-DICHLOROPHENYL CARBAMATES

Amine	*Yield (%)*	
	Iodide ion concentration (M)	
	0.05	*0.5*
Amitriptyline	29	99
2-Chloroimipramine	52	101
Pethidine	40*	100*

* Reaction times, 25 min; aqueous phase, phosphate buffer ($\mu = 0.2$) pH 6.0; organic phase, $3 \cdot 10^{-3}$ *M* 2,4-dichlorophenyl chloroformate in methylene chloride.

The choice of pH is a key point since the amine only reacts as base. At a low pH, the reaction rate is reduced since the distribution ratio as base is low owing to a high distribution ratio as ion pair with iodide. On increase of pH, the distribution ratio of the amine as base increases but there is also a rapid increase in the hydrolysis rate of the intermediate ion[6].

The influence of the organic solvent on the reaction rate is mainly due to its effect on the distribution of the amine and the intermediate ion pair[6]. Solvents promoting high ion-pair distribution of the intermediate ion to the organic phase are preferred since the hydrolysis rate in the aqueous phase is high. However, hydrolysis can also occur in the organic phase, which is why solvents with low ability to dissolve

TABLE II

ELECTRON-CAPTURE RESPONSE AHD RELATIVE RETENTION FOR 2,4-DICHLOROPHENYL AND PENTAFLUOROPHENYL CARBAMATES

Detector temperature: 325 C. t_R = Retention time.

Amine	*Minimum detectable concentration* ($mol \cdot sec^{-1} \cdot 10^{16}$)		$t_{R(DCP)} \cdot t^{-1}_{R(PFP)}$
	DCP	*PFP*	
Amitriptyline	5	4	5.7
2-Chloroimipramine	4	–	4.8
Pethidine	3	2	6.6

water should be used. Methylene chloride, which fulfills these requirements, is therefore a suitable solvent.

Table II shows the electron-capture response for some of the derivatives determined at optimum detector temperature. In all cases it was found that the response increased slightly with increasing detector temperature, levelling off at about 300°C. The choice of reagent is not very critical with respect to the magnitude of the electron-capture response.

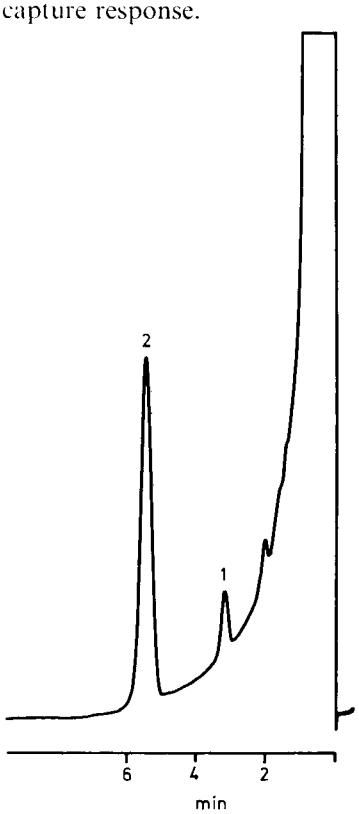

Fig. 2. Gas chromatogram obtained after a two-phase derivatization of amitriptyline (6 ng/ml) in a spiked plasma sample. Peaks: 1 = DCP-amitriptyline; 2 = DCP-2-chloroimipramine.

Analysis of amitriptyline in the presence of plasma

The two-phase reaction can easily be performed with spiked plasma samples. DCP is preferred to PFP as reagent mainly owing to the lower concentration necessary for derivative formation.

Methanolic alkali is added to remove excess of the reagent and other by products that disturb the GC analysis[3,11].

The peak area ratios were the same as those obtained from compounds dissolved in phosphate buffer solutions, indicating a quantitative yield of both AMI and CIM derivatives. The time for quantitative yield of the derivatives was not influenced by plasma.

The standard curves were rectilinear passing the origin in all cases. The lowest concentration determined was 6 ng/ml (Fig. 2).

Selectivity

Chloroformate esters react fast and quantitatively with secondary amines[12]. This means that if a plasma sample containing AMI and its main metabolite NTP (N-desmethylamitriptyline) is treated according to the method for AMI the same derivative is formed from both the tertiary and the secondary amine. However, if the sample is first extracted with a solvent containing an alkyl chloroformate ester, only the secondary amine forms a carbamate. When this reaction is completed, sodium iodide and an aryl chloroformate ester are added and the tertiary amine is derivatized. This principle was tested with AMI and NTP using trichloroethyl chloroformate and 2,4-dichlorophenyl chloroformate as reagents. The peak area ratios obtained did not differ from those obtained when NTP was absent. With an appropriate combination of the two chloroformate esters it should be possible to quantitate both the tertiary and secondary amine within the same chromatographic run.

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ALKYLTHIOLATION FOR THE DETERMINATION OF DOUBLE-BOND POSITIONS IN LINEAR ALKENES

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SUMMARY

The iodine-catalysed addition of dimethyl disulphide to linear alkenes leads smoothly to α,β-bis(methylthio)alkanes. These derivatives are stable to gas chromatographic conditions and provide readily recognizable fragments on electron bombardment. This single-step derivatization procedure is thus eminently suitable for the determination of double-bond positions in linear alkenes.

INTRODUCTION

Spectroscopic methods alone fail in all but a few instances in the determination of the position of a double-bond in a hydrocarbon chain[1,2]. The strategy normally adopted in identifying double-bond positions therefore consists of either oxidative cleavage of or addition across the double bond followed by a combination of chromatographic and spectroscopic techniques to identify the products[3–14]. Owing to difficulties in controlling oxidation, particularly when several functional groups are present, addition is normally the preferred procedure.

Various methods have been used to investigate the derivatives formed[3], but the method of choice is combined gas chromatography–mass spectrometry (GC–MS)[7–14]. The criteria for the use of this combination are that the derivatives formed must be stable during GC and that the MS fragmentation be readily interpreted in terms of the original structure.

Direct addition of two identical groups to the double-bond termini provides a single product and thus makes the interpretation of the resulting spectra easier than when unsymmetrical addition yields two structural isomers. Few such symmetrical additions are known, however, and the two simplest, deuteration[7] and methylene insertion[8], give hydrocarbon products and consequent difficulties in interpretation of the mass spectra. A similar, but more involved, procedure which has been much favoured is the conversion of the alkene to the corresponding diol prior to derivatization[9–11]; the diols themselves present difficulties in both GC and MS interpretation.

We have recently shown that oxyselenation provides derivatives of alkenes that are analytically useful for the GC–MS determination of double-bond positions[15]. This, together with the availability of reactive dialkyl disulphides, prompted us to

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look for methods where the disulphides might be added across alkene double bonds.

Literature reports state that dialkyl disulphides add to vinyl sulphide and to styrene in the presence of iodine[16,17]. Iodine catalysis is also effective in promoting the addition of alkyl sulphides to vinyl acetate[18]. Bis(trifluoromethyl) disulphide adds to alkenes photochemically[19]. Hydrogen fluoride has been used as a catalyst for the addition of disulphides to simple alkenes[20].

A simple procedure based on the iodine-catalysed reactions described above[16–18] was adopted for derivatization. The *trans*-isomer of a linear alkene was added to an excess (6 *M*) of dialkyl disulphide, a catalytic amount of iodine (0.05 *M*) added and the resultant solution allowed to stand for 24 h at room temperature. The reaction mixture could then be analysed by GC–MS without further work-up. If it were necessary to store the products for longer periods prior to analysis, the iodine remaining was removed by washing with alkali, to avoid product decomposition.

EXPERIMENTAL

The GC–MS analysis was carried out on a JEOL JCG-20K gas chromatograph coupled directly to a JEOL JMS D-100 mass spectrometer. The gas chromatograms were obtained by recording the total ion current throughout the chromatographic run with the mass spectrometer operating at an electron bombardment energy of 20 eV. The mass spectra themselves were obtained with the machine operating at 70 eV. The column used for chromatography was made of stainless steel, 6 ft. × $\frac{1}{8}$ in. I.D., packed with 10% SE-30 on Chromosorb W AW. Helium at a flow-rate of 30 ml/min was used as the carrier gas.

The *trans*-alkenes used were prepared by Wittig reaction or dehydration, followed by urea adduction[21], and gave satisfactory physical and spectral data in all instances. Iodine and dimethyl disulphide were obtained from E. Merck, (Darmstadt, G.F.R.).

General derivatization procedure

Dimethyl disulphide (6 mmol) and the *trans*-alkene (1 mmol) were placed in a flat-bottomed flask equipped with a magnetic stirrer and iodine (0.05 mmol) was dissolved in the mixture. The flask was purged with nitrogen and closed. After stirring for 24 h at room temperature, reaction was complete.

The product could be subjected directly to GC–MS analysis, but on standing for several days some decomposition occurred. Such decomposition could be avoided by dissolving the product in diethyl ether and washing with dilute aqueous sodium hydroxide.

RESULTS AND DISCUSSION

Reaction conditions

Initial experiments were carried out using either dimethyl or dipropyl disulphide. No differences in yield or in ease of interpretation of the results were found, and as the bis(methylthio) derivatives were more volatile and hence presented an easier GC problem, dimethyl disulphide was used in the systematic investigation.

The amount of iodine used for catalysis did not seem to be critical, although

TABLE I

RETENTION TIMES OF THE BIS(METHYLTHIO)ALKANES PREPARED FROM THE ALKENES STUDIED

Carrier gas, helium; flow-rate, 30 ml/min. Injector temperature, 250 C. Column: stainless steel, 6 ft. × $\frac{1}{8}$ in. I.D., 10% SE-30 on Chromosorb W AW. Programme: the temperature was increased by 1 C/min from a temperature of 180 C on injection.

Alkene derivatized	*Retention time (min)*	*Alkene derivatized*	*Retention time (min)*
2-Undecene	11.6	1-Tetradecene	29.9
1-Dodecene	18.9	5-Tetradecene	22.1
3-Dodecene	14.6	7-Tetradecene	20.4
6-Dodecene	12.3	1-Pentadecene	36.3
1-Tridecene	23.9	6-Pentadecene	26.3
4-Tridecene	18.0	1-Hexadecene	42.9
		7-Hexadecene	31.5
		8-Hexadecene	31.0

poor results were obtained when much larger or much smaller amounts were used. Ultraviolet irradiation did not appear to improve the reaction yields or rate and was hence not employed.

When higher temperatures (80 C) were used, reaction was complete after 2 h, but the derivative was then contaminated with side-products containing only one methylthio group. A more detailed discussion of this problem will be presented elsewhere[22].

Gas chromatography

GC was carried out on a short packed column (SE-30). Excess of dimethyl disulphide and any remaining alkene were eluted well before the product, which was normally formed cleanly and in good yield (70–90%). The retention times of the derivatives are given in Table I, and it is apparent that they increase with carbon number and as the double bond derivatized nears the chain terminus.

Mass spectrometry

The mass spectra of the derivatives of the three dodecenes studied are given in Fig. 1, and some details of the spectra of the remaining derivatives are given in Table II. The discussion which follows is based on these data.

The mass spectra of the fourteen bis(methylthio)alkanes show considerable uniformity of appearance. In all instances the molecular ion (M) is marked, although the relative intensity decreases from values of about 40% for the derivatives of C_{11} and C_{12} alkenes as the chain length increases. Markedly lower intensities characterize derivatives of terminal alkenes of higher carbon number, *e.g.*, 1-pentadecene and 1-hexadecene. The only other ion observed in the molecular peak region was the considerably less intense M − 47 ion, which is formed by loss of a methylthio radical from the molecular ion.

The low-mass end of the spectra is dominated by hydrocarbon fragments in the series C_nH_{2n-1}. Maximal intensity in this ion series occurs at *m/e* 55 or 69 ($n = 4$ or 5) and the former ion provides the base peak ion in all of the C_{15} and C_{16} alkene

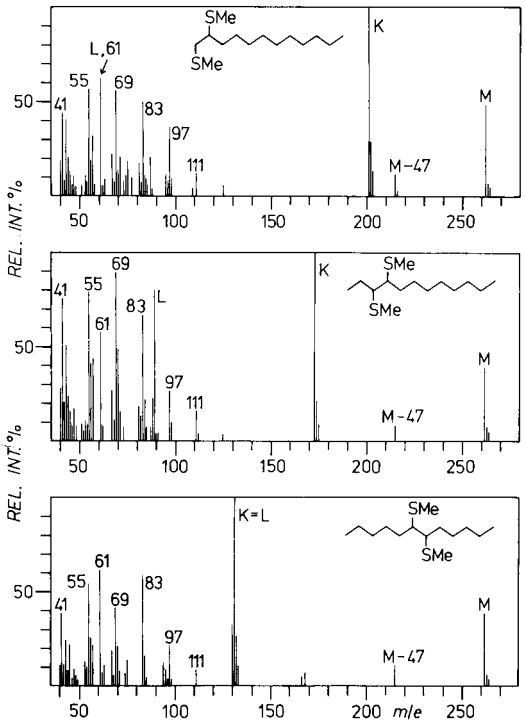

Fig. 1. 70 eV mass spectra of the bis(methylthio)alkanes derived from 1-dodecene, *trans*-3-dodecene and *trans*-6-dodecene. Me = Methyl.

TABLE II

RELATIVE INTENSITIES (%) OBSERVED IN THE MASS SPECTRA OF THE BIS(METHYLTHIO) DERIVATIVES OF ALKENES FOR THE MOLECULAR ION (M), THE CHARACTERISTIC IONS K AND L, AND FOR THE *m/e* 61 ION

Figures in parentheses are *m/e* values. The intensities found for the homologous ion series at *m/e* 41, 55, 69, 83, 97 and 111 are also given.

Alkene derivatized	*M*	*K*	*L*	*m/e*						
				61	*41*	*55*	*69*	*83*	*97*	*111*
2-Undecene	42.3 (248)	100 (173)	57.4 (75)	58.2	50.2	56.2	68.7	52.1	22.3	8.4
1-Dodecene	48.1 (262)	100 (201)	62.2 (61)	62.2	44.3	57.0	56.1	50.4	37.0	12.6
3-Dodecene	39.2 (262)	100 (173)	77.4 (89)	64.2	76.2	79.7	89.2	68.0	27.3	15.5
6-Dodecene	38.6 (262)	100 (131)	L = K	61.9	39.0	54.8	40.5	58.6	21.0	9.1
1-Tridecene	26.9 (276)	100 (215)	70.5 (61)	70.5	69.3	91.0	80.6	78.2	65.4	26.9
4-Tridecene	46.3 (276)	100 (173)	73.3 (103)	75.6	52.2	62.7	68.6	58.2	29.1	13.4
1-Tetradecene	16.2 (290)	83.9 (229)	57.1 (61)	57.1	76.8	100	85.6	78.5	50.1	21.4
5-Tetradecene	45.9 (290)	100 (173)	84.8 (117)	78.4	62.6	76.8	68.2	61.0	40.1	22.2
7-Tetradecene	28.1 (290)	100 (145)	L = K	75.8	67.2	94.3	80.7	67.3	71.8	24.2
1-Pentadecene	10.8 (304)	56.8 (243)	52.6 (61)	52.6	76.3	100	81.6	81.1	57.6	23.7
6-Pentadecene	41.1 (304)	98.4 (173)	92.4 (131)	74.1	83.8	100	96.6	92.8	66.1	40.3
1-Hexadecene	8.7 (318)	41.4 (257)	34.4 (61)	34.4	82.7	100	93.1	92.7	72.4	27.4
7-Hexadecene	31.3 (318)	82.6 (173)	78.2 (145)	66.9	86.9	100	97.3	88.5	79.1	49.5
8-Hexadecene	30.2 (318)	100 (159)	L = K	68.6	52.5	74.4	72.3	56.4	44.9	26.9

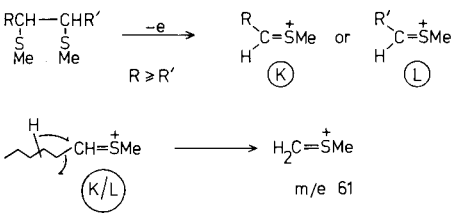

Fig. 2. Origin of the K, L and *m/e* 61 ions in the mass spectra of bis(methylthio)alkanes.

derivatives, except that formed from 8-hexadecene. The marked dominance of the C_nH_{2n-1} ion series over the C_nH_{2n+1} series is probably the result of the ease with which hydrogen transfer to sulphur may take place.

Other ions attracting immediate notice are those due to fragments derived by cleavage through the position of the alkene double bond (see Fig. 2). The ion of higher mass (K) in all instances has greater intensity than the lower mass ion (L), although the difference tends to decrease as the mass difference between K and L decreases. The ion K is sufficiently intense to provide the base peak in the spectra of more than half of the compounds studied.

A further high-intensity ion occurs in the spectra of all of the bis(methylthio)derivatives examined. This ion at *m/e* 61 is presumably due to a rearrangement of the ions K and L such that a hydrogen atom from the alkyl chain is transferred to the carbon atom bearing the charged methylthio moiety, while the hydrocarbon fragment is eliminated as a neutral species, probably as a cycloalkane. It should be noted that this ion arises directly by scission of the chain in terminal alkenes.

In conclusion, the readily identifiable molecular ion, the high intensities of the ions (K and L) due to cleavage of the doubly activated bond formed by derivatization and the general lack of other non-hydrocarbon ions make the mass spectra of the bis(methylthio)alkanes readily usable for the determination of double-bond positions in linear alkenes.

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STUDY OF THE ACIDIC HYDROLYSIS OF CYCLIC TRIMETAPHOSPHATE BY LIQUID CHROMATOGRAPHY

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SUMMARY

The hydrolysis of cyclic trimetaphosphate in acidic aqueous solution ($[H^+]$ = 0.1) was investigated by liquid chromatography. The phosphate in the effluent from an anion-exchange column was detected automatically with the use of Mo(V)–Mo(VI) reagent.

From the rate constants at 10, 20, 30, 40 and 50°C, the Arrhenius activation energy in 0.1 *M* hydrochloric acid solution for scission of a P–O–P linkage was estimated as 21.3 kcal/mole.

INTRODUCTION

The chemical properties of condensed phosphates have been extensively investigated. Hydrolytic reactions of linear-chain phosphates of relatively short chain length have been also reported[1]. However, data on the hydrolysis of cyclic condensed phosphates are scarce. The most common members of cyclic condensed phosphates of the general formula $M_n^I(PO_3)_n$ are tri- and tetrametaphosphates, which have six- and eight-membered rings, respectively.

Several investigators have reported on the hydrolysis reactions of these two cyclic phosphates[2,3]. They used the precipitation method or paper chromatography for the separation of the parent compound and the hydrolysis products. However, it is difficult to obtain reliable data on the hydrolysis kinetics by the use of these techniques.

We have previously reported on the hydrolysis of cyclic octametaphosphate[4], with a 16-membered ring, in acidic media by using combined ion-exchange and gel chromatographic columns. In the study, the effluent from the combined columns was fractionated by a fraction collector and phosphate species in the effluent were analysed spectrophotometrically. The use of column chromatography improved the reliability of the kinetic data compared with the use of paper chromatography.

Nevertheless, the use of a fraction collector is time consuming. We therefore devised a method for the automatic determination of phosphorus concentrations in column effluents. This technique has been already reported by Hirai *et al.*[5], who employed the automatic spectrophotometric determination of phosphate species by

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forming heteropoly blue complexes of the phosphates through a flow cell. We modified their method.

In this study, trimetaphosphate was separated from the hydrolysis products by the use of an anion-exchange column. From the chromatograms obtained automatically, hydrolysis kinetics in 0.1 *M* hydrochloric acid at various temperatures were investigated. In this paper, cyclic phosphates and linear phosphates are abbreviated as P_{nm} and P_n; respectively, where n is the degree of polymerization and m denotes metaphosphate of cyclic structure.

EXPERIMENTAL

Materials

All the chemicals used were commercially available analytical-reagent grade materials and were used without further purification.

Sodium trimetaphosphate was prepared by the usual method[6].

Hitachi Custom Ion-Exchange Resin 2630 anion exchanger was purchased from Nissei Sangyo (Japan).

Apparatus

The chromatographic system is shown schematically in Fig. 1. Pump A and the column were part of a Hitachi Model 635 liquid chromatograph. The spectrophotometer was a Hitachi Model 100-50 with a flow cell unit. The length of the light path and the volume of the flow cell were 8 mm and 8 μl, respectively.

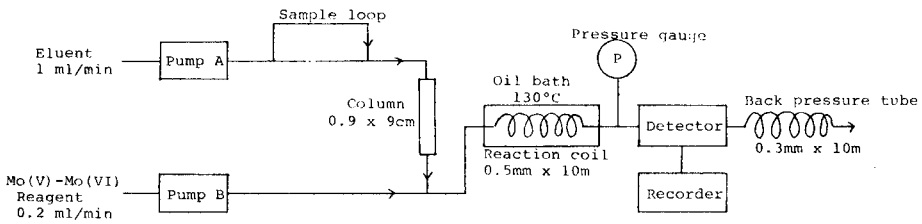


Fig. 1. Flow diagram of chromatograph and detection system.

Separation

The dimensions of the glass column used for packing the ion-exchange resin were 100 × 8 mm I.D. The eluent was 0.5 *M* potassium chloride solution with 0.1 % of EDTA (disodium salt) as a masking reagent, with a flow-rate of 1 ml/min. A 1-ml volume of sample solution was injected into the column through a sample loop.

Detection

The Mo(V)–Mo(VI) reagent was prepared by the method of Hosokawa and Oshima[7]. This reagent was diluted 4-fold with water and passed at a flow-rate of 0.2 ml/min to the stream of column effluent, which had a flow-rate of 1 ml/min. The mixed solution was heated at 130 °C in an oil-bath by passage through 10 m of 1.5 × 0.5 mm I.D. PTFE tubing.

The heteropoly blue complex thus formed was detected at 830 nm in the flow

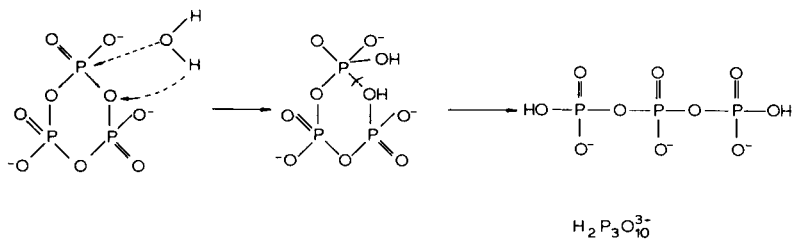
cell. A back-pressure PTFE coil (10 m × 2 mm O.D. × 0.3 mm I.D.) was connected to the end coil of flow cell outlet so as to prevent the formation of air bubbles on heating. The readings on the pressure gauge in the flow system were usually 6–7 kg/cm^2.

Hydrolysis

The initial concentration of the trimetaphosphate solution to be hydrolysed was varied from 0.05 to $6.25 \cdot 10^{-4}$ *M*. Hydrochloric acid was added at a concentration of 0.1 *M* to each solution and the hydrolysis reaction was performed in a water-bath the temperature of which was maintained within ± 0.1°C. At measured time intervals, samples of a few millilitres were withdrawn and neutralized with the same volume of 0.1 *M* sodium hydroxide solution at nearly 0°C. The sample solutions were stored in a refrigerator until taken for analysis.

RESULTS AND DISCUSSION

When trimetaphosphate is hydrolysed as showin in eqn. 1, first linear triphosphate is formed.



(1)

This triphosphate is also degraded to di- and monophosphate. To determine the hydrolysis rate of trimetaphosphate, this parent compound should be separated from the hydrolysis products. This separation was achieved by the use of a Hitachi

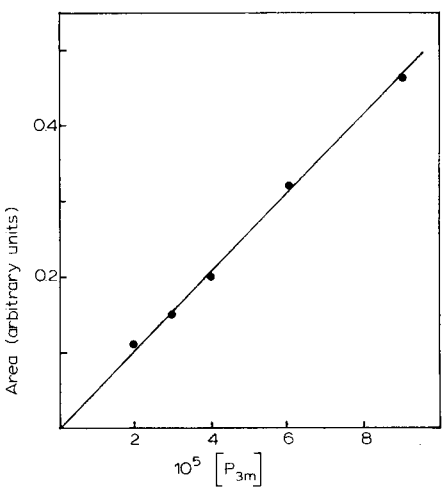


Fig. 2. Calibration graph for trimetaphosphate elution.

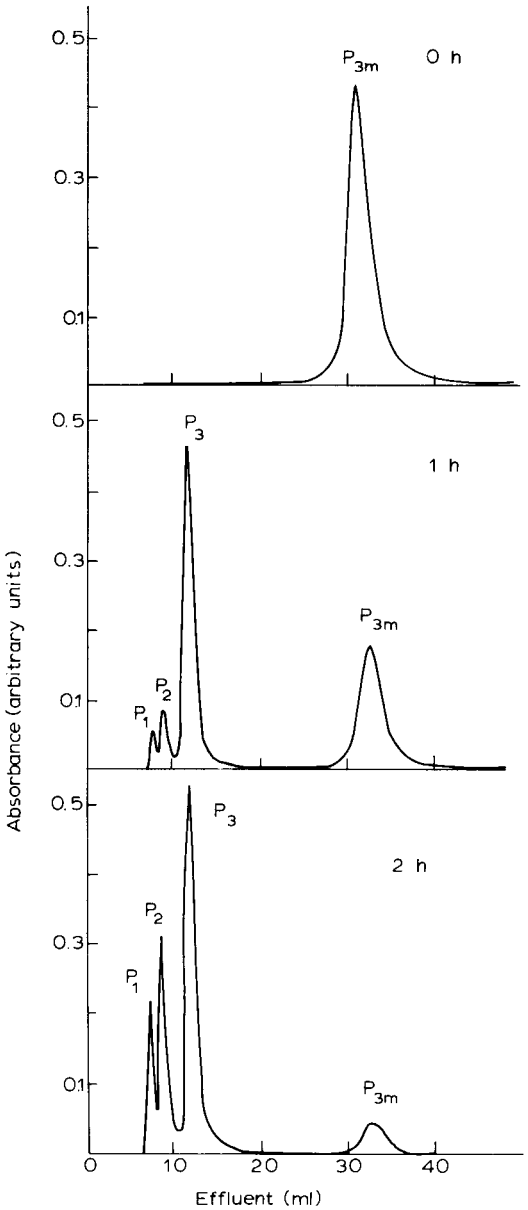


Fig. 3. Chromatograms of hydrolysis samples of trimetaphosphate in 0.1 *M* HCl at 50°C.

Custom Ion-Exchange Resin 2630 column with 0.5 *M* potassium chloride solution containing 0.1 % of EDTA (disodium salt) as the eluent. A good elution pattern of trimetaphosphate was obtained under the conditions described.

On eluting known amounts of trimetaphosphate, a graph of the area of the chromatograms obtained *versus* the phosphate concentration gave a straight line passing through the origin. The result is shown in Fig. 2.

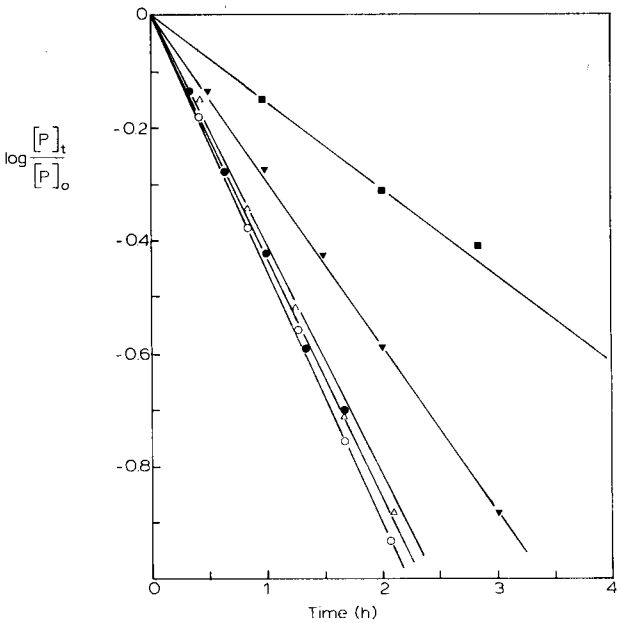


Fig. 4. Hydrolysis rates for various concentrations of trimetaphosphate in 0.1 *M* HCl at 50°C. ■, 0.05 *M*; ▼, 0.01 *M*; △, $2.50 \cdot 10^{-3}$ *M*; ○, $1.25 \cdot 10^{-3}$ *M*; ●, $6.25 \cdot 10^{-4}$ *M*.

As an example, chromatograms of hydrolysis samples in 0.1 *M* hydrochloric acid at 50°C are shown in Fig. 3. From the areas of these chromatograms of trimetaphosphate, the hydrolysis rate could be calculated.

The concentration of trimetaphosphate at times 0 and t were denoted by $[P]_0$ and $[P]_t$, respectively, and log ($[P]_t/[P]_0$) is plotted *versus* time in Fig. 4 for various initial concentrations of trimetaphosphate. As the plots are linear, the hydrolysis reaction of trimetaphosphate is first order and the slope of the straight line is the rate constant of the reaction. As shown in Fig. 4, a constant value was obtained when the

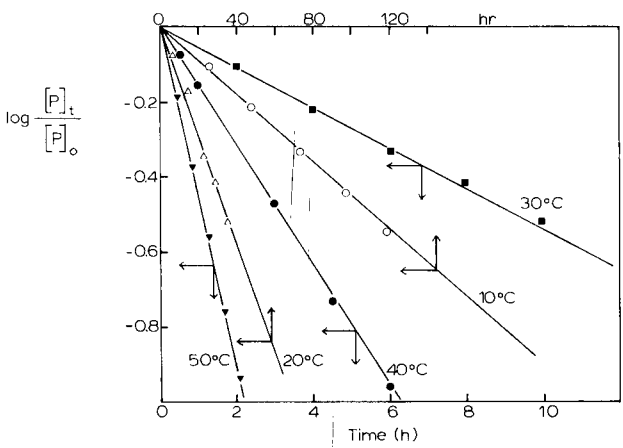


Fig. 5. Hydrolysis rates of trimetaphosphate ($1.25 \cdot 10^{-3}$ *M*) in 0.1 *M* HCl at various temperatures.

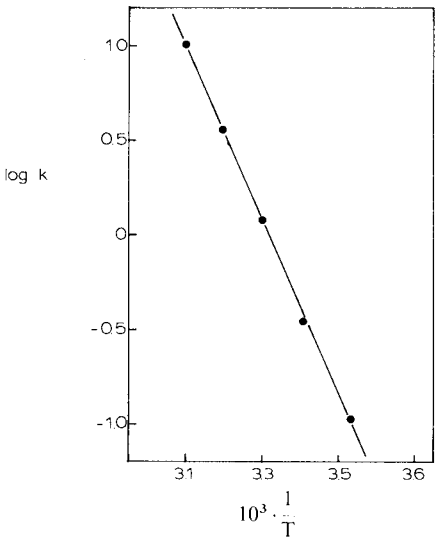


Fig. 6. Arrhenius plot for trimetaphosphate hydrolysis in 0.1 *M* HCl.

initial concentration of trimetaphosphate to be hydrolysed was lower than $2.5 \cdot 10^{-3}$ *M*. When initial concentration was higher than this, an increase in the pH of the test solution was observed. For example, after the hydrolysis reaction was almost completed, the pH of the test solution at an initial concentration of 0.05 *M* changed from 1.0 to 1.6. If the hydrolysis reaction of trimetaphosphate, $P_3O_9^{3-}$, proceeds as in eqn. 1 and only linear triphosphate, $H_2P_3O_{10}^{3-}$, is formed, the pH of the sample solution should be constant. However, as the pK_3 of $H_5P_3O_{10}$ acid is 2.30, $H_3P_3O_{10}^{2-}$ might be formed appreciably in 0.1 *M* hydrochloric acid solution. This effect results in a decrease in hydrogen ion concentration, *i.e.*, an increase in the pH of the test solution. When the initial concentration of trimetaphosphate is very low compared with the hydrogen ion concentration (0.1 *M*), the amount of hydrogen ion consumed by the hydrolysis products can be neglected. A "true" rate constant could then be obtained as the pH of the test solution was kept constant. Log ($[P]_t/[P]_0$) *versus* *t* plots at an initial concentration of $1.25 \cdot 10^{-3}$ *M* and at various temperatures is shown in Fig. 5. Good straight lines were obtained at the temperatures studied. Thus, the trimetaphosphate hydrolysis rate, $-d[P]/dt$, can be represented by

$$-\frac{d[P]}{dt} = k[H^+][P] \tag{2}$$

The first-order rate constants in eqn. 2 and the half-lives of trimetaphosphate at various temperatures are shown in Table I. From these data, linear Arrhenius plots as shown in Fig. 6 were obtained. The activation energy was 21.3 kcal/mole, which energy is nearly equivalent to that obtained for other condensed phosphates in acidic media.

The data on the trimetaphosphate hydrolysis rate under the conditions given in this paper have not previously been published. Nevertheless, the half-life of trimetaphosphate at 50°C and pH 1.0 was assumed to be 0.87 h by extrapolation of the data in

TABLE I

KINETIC DATA FOR TRIMETAPHOSPHATE HYDROLYSIS IN 0.1 *M* HYDROCHLORIC ACID SOLUTION

*Parameter**	*10°C*	*20°C*	*30°C*	*40°C*	*50°C*
$k(h^{-1})$	0.105	0.336	1.22	3.69	10.3
$t_{1/2}(h)$	66.0	20.6	5.67	1.88	0.67

* E_a = 21.3 kcal/mole.

Fig. 10 on p.169 of ref. 1. This value is slightly higher than our reliable value of 0.67 h.

Trimetaphosphate is the smallest six-membered ring in the cyclic phosphate series and is very readily hydrolysed in both acidic and basic media. This instability might be due to the strain in the six-membered ring.

We are now investigating the hydrolysis reactions of other cyclic phosphates in acidic aqueous solution by this automatic liquid chromatographic system.

ACKNOWLEDGEMENTS

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GAS CHROMATOGRAPHIC SEPARATION OF DIASTEREOMERIC ISOPRENOIDS AS MOLECULAR MARKERS OF OIL POLLUTION

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SUMMARY

By means of high-performance glass capillary gas chromatography (GC), diastereomeric isoprenoids were resolved into double peaks. The retention indices on three liquid phases and the mass spectra of the diastereoisomers were almost similar. The leading GC peaks represent the isoprenoids of fossil origin, while the rear peaks correspond to those of recent origin. Computerized gas chromatography–mass spectrometery was used for fingerprinting isoprenoids in different samples. The mass fragmentation patterns were characteristic of the branched alkanes.

Hydrocarbon mixtures from four crude oil spills in the sea and from polluted and oil-free oyster tissues were investigated. The relative ratios of *n*-alkanes/pristane or phytane were shown to be strongly dependent on the chromatographic resolution of the isoprenoid peaks. It is suggested that the double GC peaks in the isoprenoid series are an unmistakable sign of oil pollution.

INTRODUCTION

High-performance glass capillary gas chromatography (GC) is recognized as the most powerful tool for determining individual hydrocarbons (HCs), especially when coupled to mass spectrometry (MS). However, it is of primary importance that glass capillary GC is carried out by use of high-efficiency chromatographic system. The purpose of this paper is to show the need of high efficiency glass capillary GC for differentiating fossil HCs from biogenic HCs in the isoprenoid series.

EXPERIMENTAL

Oil samples

Four oil samples were analysed. Three of them were collected in the marine environment polluted by the grounding of oil tankers on the Brittany coast: Gino in 1979, 40,000 tons of carbon-black; Amoco-Cadiz in March 1978, 230,000 tons of crude oil; Tanio in March 1980, 28,000 tons of No. 2 fuel. The fourth sample was obtained from the Ekofisk oil field in the North Sea by courtesy of Dr. J. E. Portmann[1]. The oil samples were purified and fractionated into aliphatics and aromatics as previously described[2].

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Oyster samples

Flat oysters *(Ostrea Edulis)* were collected at four different commercial sites of the Brittany coast. They were collected on June 1978 from the Aber-Benoit estuary (site A) which was heavily polluted by oil spilled from the Amoco-Cadiz. The three others sites were recognized as oil-free: Binic (site B) near Saint-Brieuc and the Crach river (site C) in the Morbihan department on the southern Brittany coast, and Lézardrieux (site D) on the northern coast.

Approximately 10 g (dry weight) of oyster tissues were analysed. The preliminary clean-up steps consisted of Florisil and silica gel chromatographies as previously described[2]. The aliphatic fraction after concentration was redissolved in 0.1 ml hexane. A 2-μl aliquot of the final solution was injected into the gas chromatograph.

Gas chromatography

GC was carried out on a Carlo Erba (Milan, Italy) Model 2150 AC instrument equipped with a flame ionization detector (FID). Injection was made via a split–splitless injector heated at 275°C while the column was maintained at 40°C. After elution of the solvent the oven temperature was rapidly raised to 60°C and then at 0.8°C/min or 0.5°C/min to 280°C. Hydrogen was used as carrier gas with the flow-rate adjusted according to the Grob test[3].

Glass capillary columns

Glass capillary columns (50 or 25 m × 0.3 mm I.D.) were laboratory made according to the Grob procedures. One column, 50 m × 0.3 mm, was prepared by the barium carbonate procedure followed by Carbowax 20M and Triton X-305 deactivations at 280°C[4]. It was coated by the static procedure with a liquid OV-1 film thickness of 0.15 μm. The other columns were persilylated at high temperature according to the Grob procedure[5] and coated with a SE-52 or OV-73 liquid film thickness of 0.12 μm. The straightened column ends were deactivated by rinsing with the silylating mixture and then expelling the liquid plug with a microflame at moderate temperature[6].

The retention indices (I) of the acyclic isoprenoid alkanes were derived from linear interpolation between the n-alkanes in temperature programmed runs.

Gas chromatography–mass spectrometry

MS analysis were carried out with a Nermag R-10-10 B mass spectrometer (Rueil-Malmaison, France). Samples were introduced via a Carlo Erba Model 2900 gas chromatograph equipped with a 50 m × 0.3 mm glass capillary column coated with a SE-52 liquid film. This column was directly coupled to the mass spectrometer. The apparatus was operated in the continuous mode. Data were acquired and processed on a Nermag-Sidar data system.

RESULTS

Chromatographic separation of diasteromeric isoprenoids

The aliphatic and alicyclic fractions of four oil samples were analysed by glass capillary GC on OV-1 liquid phase. The analytical conditions were such that the prominent discrete peaks between n-alkanes, especially pristane and phytane, were

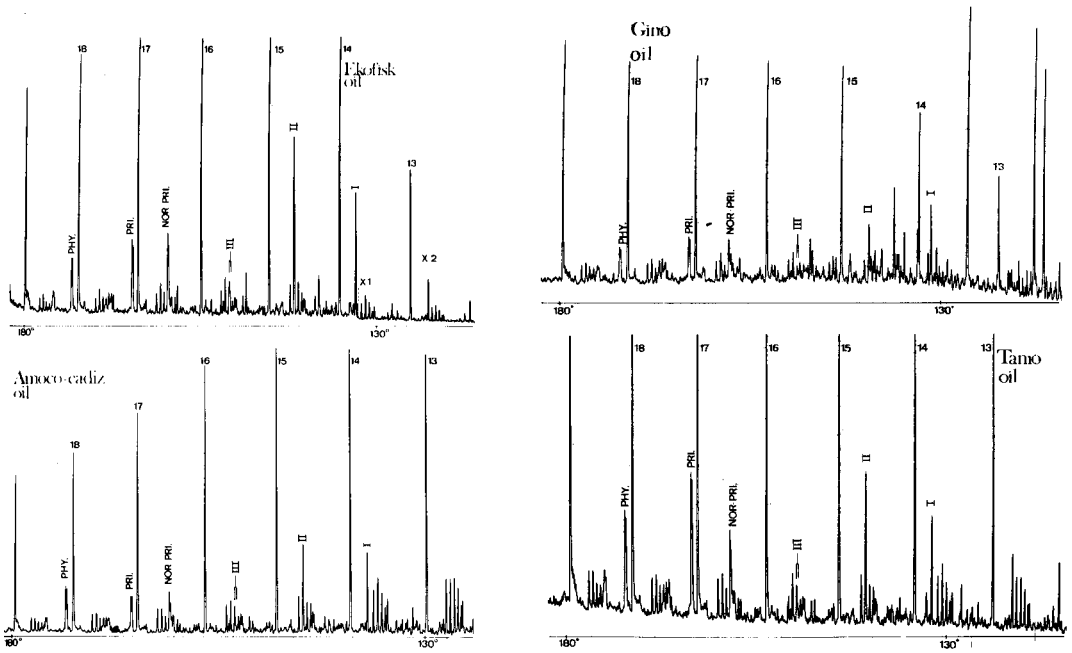


Fig. 1. Sections of gas chromatograms of the aliphatic hydrocarbon fractions of oils spilled in the sea from Gino, Tanio, Amoco-Cadiz and Ekofisk. Arabic numerals indicate the carbon numbers of the *n*-alkanes. Roman numerals refer to the isoprenoids according to Fig. 2. PHY = Phytane; PRI = pristane; NOR-PRI = nor-pristane. Conditions: column 50 m × 0.3 mm I.D., 0.15-μm OV-1; carrier gas hydrogen at 0.8 bar; splitless injection; temperature programme 0.8°C/min.

resolved into doublets. Fig. 1 shows the gas chromatograms of Gino, Tanio, Ekofisk and Amoco-Cadiz oils. They were all characterized by doublets between dodecane and nonadecane. While four double peaks were particularly prominent, two other isoprenoids (I and II) could not be further resolved on OV-1.

These characteristic doublets are due to the presence of diastereoisomers. Fig. 2 summarizes the structures of isoprenoids, the number of asymmetric carbons and the number of possible diastereoisomers. All the compounds of this series have two diastereoisomeric configurations, except for phytane which has four possible diastereoisomers. Of course, liquid phases such as OV-1, OV-73 or SE-52 can resolve

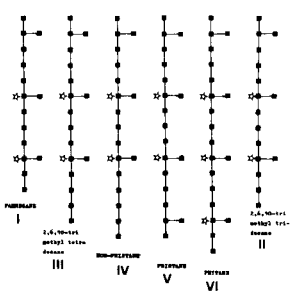


Fig. 2. Structure of isoprenoids. The symbol ☆ denotes an asymmetric carbon atom.

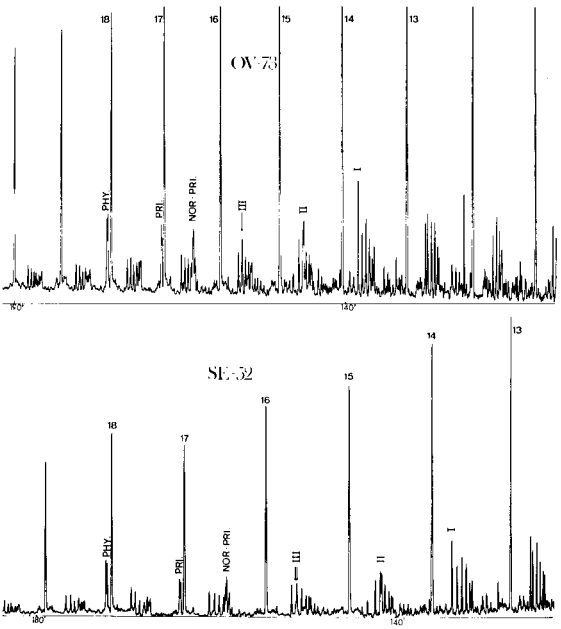


Fig. 3. Gas chromatogram of aliphatic hydrocarbon fraction of Amoco-Cadiz oil on two liquid phases. Conditions: OV-73, 25 m × 0.3 mm I.D., film thickness 0.12 μm, temperature programme 0.5°C/min; SE-52, 50 m × 0.3 mm I.D., film thickness 0.12 μm, temperature programme 0.6°C/min.

only diastereoisomeric molecules and not enantiomers. Accordingly, all isoprenoids of Fig. 2 are capable of resolution in doublets, perhaps in quadruplets for phytane.

Fig. 1 shows that hydrocarbons III, IV, V and VI were effectively resolved into two sub-peaks on OV-1 liquid phase. The second isoprenoid 2,6,10-trimethyltridecane (II) which was not resolved on OV-1 was quite well resolved on OV-73 or SE-52, both methylsilicones with 5.5% and 5% of phenyl groups respectively as shown in Fig. 3. Only the first isoprenoid, farnesane (I), was not resolved while it is capable of resolution into two diasteroisomers.

The resolution of the doublets improved with increasing retention times, *i.e.*, with molecular weight. Table I reports the *I* data of isoprenoid alkanes. The *I* differences were between 1.9 and 1.4. They were insufficient to allow a full resolution of sub-peaks. Table I shows the separation number required in these parts of chromatograms for an adequate separation of doublets. The effective number of theoretical plates, N, required for such separations calculated from[7]

$$TZ = 0.78 \sqrt{N} - 1$$

must be between 390,000 and 600,000, *i.e.*, values attainable with great difficulty in routine analysis.

The *I* values of authentic samples of pristane and phytane (Alltech, Arlington Heights, IL, U.S.A.) were 1710.2 ± 0.1 and 1812.8 ± 0.3 respectively under the same analytical conditions on OV-1 liquid phase. Pristane from biological sources is composed solely of the 6(R),10(S) isomer[8], while in petroleum during the maturation process of crude oil this isoprenoid loses its stereospecificity giving two dia-

TABLE I

RETENTION INDEX (*I*) DATA OF ISOPRENOID ALKANES SEPARATED BY GLASS CAPILLARY GC ON THREE LIQUID PHASES

TZ is the separation number required for separating the diastereoisomers down to the baseline with not more than 1.2% relative overlapping ($R = 1.177$).

Compound	*OV-1*		*OV-73*	*SE-52*
	I ± *S.D.*	*TZ*	*I*	*I*
Phytane	1812.5 ± 0.3	48	1808.3	1808.7
	1810.6 ± 0.3		1806.7	1806.9
Pristane	1710.2 ± 0.1	47	1705.6	1706.6
	1708.4 ± 0.2		1704.3	1705
Nor-pristane	1652.9 ± 0.3	53	1649.3	1650
	1651.5 ± 0.2		1647.8	1648.5
III	1558.2 ± 0.3	61		1564
	1556.8 ± 0.5		1563.6	1562.6
II	1464.2 ± 0.4		1462.8	1462.3
			1461.3	1460.8
I	1377.5 ± 0.2		1374.7	1374.6

stereoisomers. Accordingly, the leading peak of the pristane doublet probably consists of the two enantiomers *RR* and *SS* of fossil origin while the rear peak consists of the *meso*-form (*RS* = *SR*) of biogenic origin.

The situation with phytane is more complex, due to the possibility of four diastereoisomers. Biogenic phytane is assumed to be derived from phytol [*trans*-3,7(*R*),11(*R*),15-tetramethylhexadec-2-en-1-ol]. Accordingly, recently biosynthesized phytane has the configurations 6(*R*), 10(*S*), 14(*R*) or (*S*); it is formed by the racemic hydrogenation and dehydration of phytol in nature. While the rear peak of the doublet contains these two pairs of enantiomers, the leading peak of phytane probably corresponds to the two other diastereoisomers of fossil origin.

The configurations of the other isoprenoid alkanes could not be established experimentally. However, only pristane, phytane and nor-pristane may be useful in the characterization of oil pollution because the other isoprenoids are probably eliminated very rapidly from the marine environment by an evaporation process[1].

Isoprenoids as markers of oil pollution

The characteristic double peaks for the isoprenoids are suggested to be an unmistakable sign of fossil contamination. Fig. 4 shows a gas chromatogram (A) of the aliphatic fraction of tissues from oysters taken from an area polluted by oil spilled from the Amoco-Cadiz compared with those (B–D) of the aliphatic fraction from oysters sampled from three non-polluted areas. These chromatograms illustrate biogenic, petrogenic and anthropogenic HCs. Among these, *n*-C_{16}, *n*-C_{17} and C_{18} may have both biogenic and fossil components. The sample in Fig. 4A was collected 3 months after the spill. Three isoprenoid double peaks are obvious, and it is clear that linear alkanes such as *n*-C_{17} and *n*-C_{18} were preferentially degraded compared to the isoprenoids. Fig. 4B shows that the area from which the samples were taken was not totally oil-free. Indeed, the unresolved complex hump is obvious; such a profile is

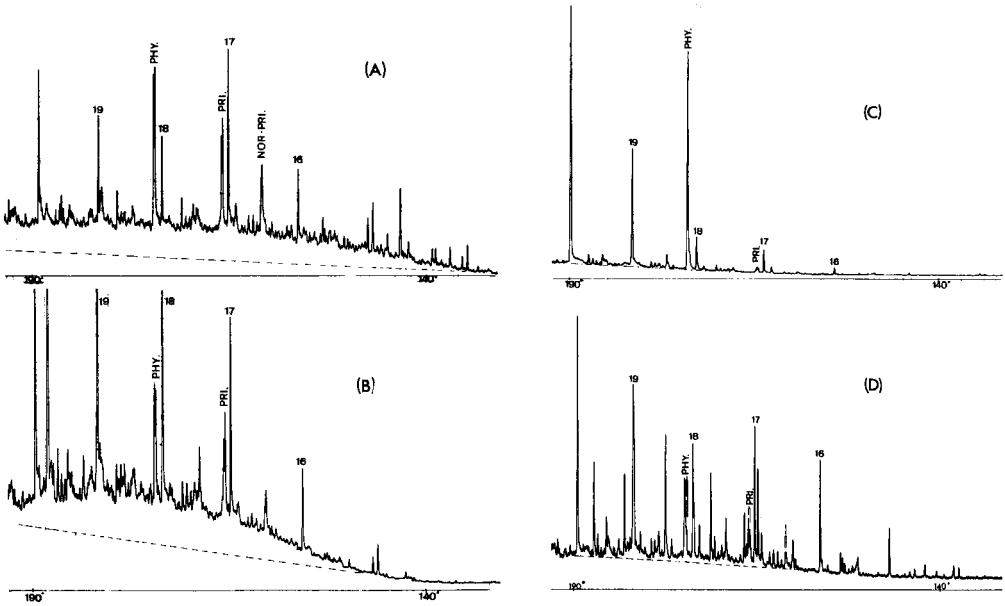


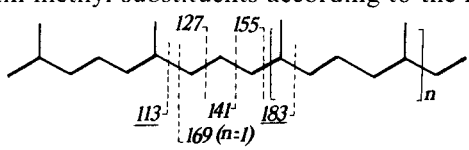
Fig. 4. Sections of gas chromatograms of four aliphatic hydrocarbon fractions extracted from oyster tissues collected from four sites (see Experimental). Conditions as in Fig. 1. The unresolved GC hump is indicated by a dotted line.

characteristic of oil pollution[9,10]. Furthermore, three double peaks of phytane, pristane and nor-pristane occurred.

On the other hand, in Fig. 4C and 4D the unresolved hump is absent. Furthermore, in Fig. 4C the isoprenoid doublets are absent. Only the phytane peak was prominent. This peak (I = 1812.4) was identified due to biogenic phytane. The little shoulder on the phytane peak is probably due to fossil phytane. The situation in Fig. 4D was more complex, due to the interference from other biogenic compounds. The phytane and pristane doublets had a peak height ratio very different from unity, with the biogenic pristane and phytane components predominant. Furthermore, a minor doublet peak of nor-pristane could be detected with I values of 1653.2 and 1651.9. The other peaks, which did not appear to be crude oil alkanes, probably originate from recent biological sources, *e.g.*, alkanes derived from phytol. Therefore, the fossil oil contamination was very low but still discernable. It was ascertained from the doublets of nor-pristane, pristane and phytane.

Fingerprinting by mass fragmentography

Mass fragmentography provides a satisfactory tool for obtaining specific fingerprints for homologous series of compounds resolved by glass capillary GC. Fig. 5 shows an example of a computer reconstructed mass fragmentogram of acyclic isoprenoid hydrocarbons from Amoco-Cadiz oil. Such molecules exhibit prominent ions at m/z = 113 + 70 n, corresponding to the fragmentations induced by the regular side-chain methyl substituents according to the following scheme[11]:



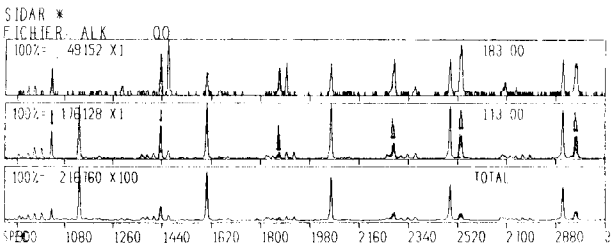


Fig. 5. Computer reconstructed mass-fragmentogram of acylic isoprenoids from an aliphatic fraction of Amoco-Cadiz oil at m/z = 113 and 183. The total ion current is also shown. Isoprenoid peaks are marked by arrows at m/z = 113.

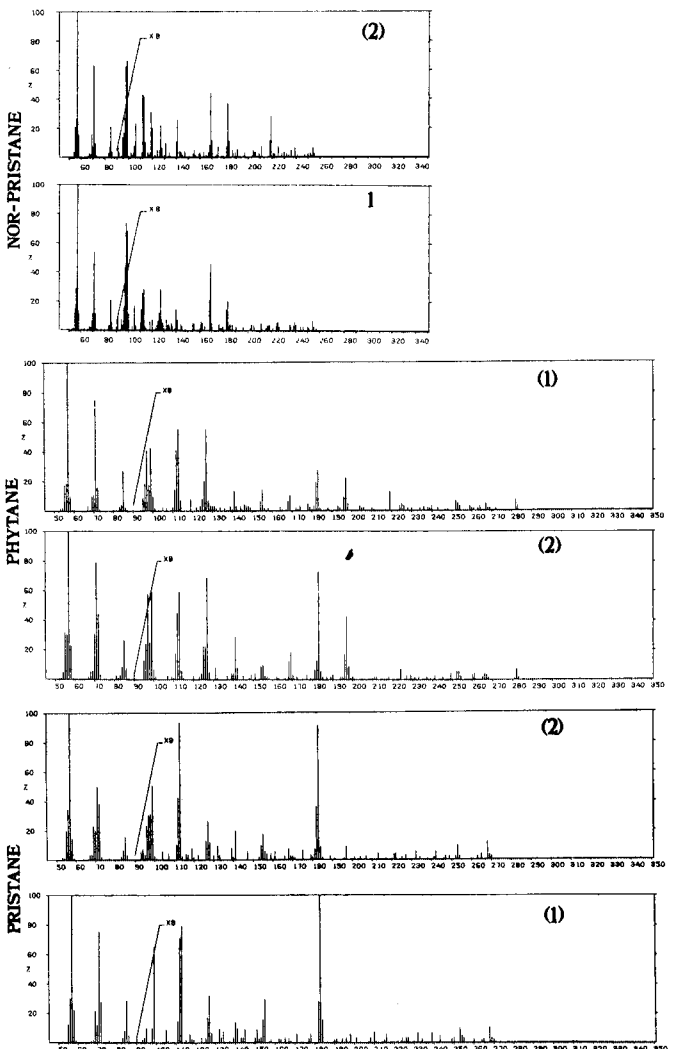


Fig. 6. Mass spectra of the double GC peaks of nor-pristane, pristane and phytane. 1, Leading peak; 2, rear peak of doublet.

The fragmentations at $m/z = 113$ and 183 allowed us to recognize the double peaks of isoprenoids resulting from a thermally induced racemization during maturation and genesis of petroleum. The double peaks of phytane, pristane, nor-pristane and hydrocarbon III are prominent in Fig. 5.

The chemical identity of the doublets was confirmed by glass capillary GC–MS. Fig. 6 presents the mass spectra of phytane, pristane and nor-pristane diastereoisomers. The spectra of the two pristane diastereoisomers were practically identical with a base peak at $m/z = 57$, significant fragment ions at $m/z = 253$ ($M-15$), 197 ($M-C_5H_{11}$), 183 ($M-C_6H_{13}$) 155,141, 127,113 ($M-C_{11}H_{23}$) and the molecular ion at $m/z = 268$. The two phytane diastereoisomers also have similar mass spectra. However, the relative intensity of the fragment at $m/z = 183$ was higher for the rear peak of the doublet (see Fig. 5). The two nor-pristane diastereoisomers gave a classical fragmentation for branched alkanes. However, a fragment ion at $m/z = 169$ was characteristic and resulted from the elimination of C_6H_{13}. Any noticeable difference in the relative intensities of ions could be detected. Thus, the doublet peaks probably consist of diastereoisomers.

Finally, the acyclic isoprenoid hydrocarbons could be characterized by mass fragmentography, recording $m/z = 113$, 169, 183 and molecular ions.

TABLE II

PEAK HEIGHT RATIOS OF HEPTADECANE/PRISTANE, OCTADECANE/PHYTANE AND PRISTANE/PHYTANE

Four spilled oils were analysed under different analytical conditions (see Experimental unless given otherwise).

	C_{17}/*Pristane*	C_{18}/*Phytane*	*Pristane/phytane*
Amoco-Cadiz oil			
OV-1	6.13	3.97	0.79
OV-73	5.46	4.04	0.81
SE-52	4.93	3.37	0.64
SE-52*	3.2	4.7	0.87
OV-1**	2.0	2.35	1
Tanio-oil			
OV-1	2.81	4.80	1.44
OV-1***	2.64	3.76	1.44
OV-73	2.77	5.18	1.59
Ekofisk oil			
OV-1	3.87	4.89	1.37
OV-1***	3.27	4.55	1.80
OV-73	4.31	5.06	1.45
Gino oil			
OV-1	5.45	6.73	1.27
OV-73	5.20	4.42	1.43

* Column: 50 m × 0.3 mm. Temperature programme: initial 40 °C, then raised to 110 °C at 4 °C/min

** Column: 25 m × 0.3 mm. Temperature programme: 5 °C/min.

*** Column: 50 m × 0.3 mm. Temperature programme: initial 40 °C then raised to 115 °C at 4 °C/min

Heptadecane/pristane and octadecane/phytane ratios

By using high efficiency GC, the pristane and phytane peaks were shown to comprise two sub-peaks corresponding to the diastereoisomers of these molecules. The *n*-heptadecane/pristane and *n*-octadecane/phytane ratios have long been used as indices of degradation of oil spills during the weathering process[12,13], and it is clear that such ratios may be very dependent on the resolution of the isoprenoid peaks. Thus, a recent intercomparison[1] of HC determinations showed that peak height ratios of straight chain to branched isomers varied greatly between laboratories, *e.g.*, C_{17}/pristane varied from 1.73 to 3.01 and *n*-C_{18}/phytane from 2.07 to 8.14 for Ekofisk oil. Such results are not surprising when the peak heights of isoprenoids are known to be dependent on the separation power of the GC system.

Table II summarizes the peak height ratios for four oils spilled in the marine environment. Different chromatographic conditions were used: three liquid phases, two glass capillary lengths and different temperature programmes. The pristane/phytane ratios showed less variation as a function of analytical conditions than C_{17}/pristane or C_{18}/phytane. Indeed, when the diastereoisomers of pristane were resolved this was true also for the isomers of phytane.

DISCUSSION

In order to estimate oil contamination, the analyst must be able to differentiate recently biosynthesized HCs from petroleum fossil HCs. Indeed, the measurement of environmental hydrocarbons is too often carried out as a whole including the inputs from geochemical, biological and human activities. Many criteria have been suggested for distinguishing between fossil and biogenic HCs[9]. Dastillung and Albrecht[14] proposed the use as molecular markers of pentacyclic triterpanes which occur as diastereomeric isomers and can be separated by GC. The members of this series occurred as 1:1 mixtures of diastereoisomers in geochemical sources. Only one of the two possible diastereoisomers was found in recent marine sediments, while fossil fuels contained a second isomer formed during oil maturation. The same concept is applied in this study for the C_{15}–C_{20} isoprenoids.

Biogenic molecules tend to exhibit a very definite configuration via stereospecific reactions, *e.g.* 6(*R*),(10(*S*) for pristane[8], while this stereospecificity is lost during the maturation process in crude oil. Accordingly, the initial biological substances are racemized giving many diastereoisomers[8,14]. Insofar as such diastereoisomers can be resolved by high-efficiency glass capillary GC it will be possible to differentiate biogenic (one GC peak) from fossil (double peaks) isoprenoids.

This study demonstrates the need of high-performance glass capillary GC which permits the resolution of diastereomeric pairs in the isoprenoid series. Although the double peak shape of pristane and phytane is well known[2,15], the implications of this were never reported, until recently[16,17].

Although the hydrocarbons I–VI are useful for characterizing the degree of geological maturity of oils, only pristane, phytane and to a lesser extent nor-pristane may serve for determining the fossil part of an environmental hydrocarbon mixture. Indeed, hydrocarbons I–IV spilled in the marine environment would be eliminated almost immediately by evaporation.

Thus, nor-pristane, pristane and phytane may serve as molecular markers of oil

pollution in the environment. These molecules are normal constituents of all crude oils and are present in relatively high concentrations. Furthermore, they are persistent in the environment because of their great stability to bacterial degradation. In addition, such recently biosynthesized isoalkanes are assumed to be derived from phytol, retaining its configuration and thus are present in a non-polluted environment as a single configuration. Their stereospecificity is lost during maturation of oil. Thus, the presence of diastereoisomers in the environment can be used as an unmistakable sign of fossil contamination.

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ANALYSIS OF POTATO GLYCOALKALOIDS WITH RADIALLY COMPRESSED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CARTRIDGES AND ETHANOLAMINE IN THE MOBILE PHASE

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SUMMARY

All recorded potato glycoalkaloids of the solanidine series are separated by the use of two reversed-phase (C_8 and C_{18}) and a silica column. The use of radially compressed cartridges and ethanolamine in the mobile phase enables rapid analysis in 3–6 min. The interaction of ethanolamine and the acetonitrile concentration in the mobile phase with the cartridge packing is examined, and, from this information, the optimal mobile phase for each cartridge is determined for maximum separation and minimum retention time. The optimal wavelength for detecting glycoalkaloids is found to be 200 nm.

INTRODUCTION

Screening of the glycoalkaloid content of potato breeding lines is now widely practised[1,2] following the disclosure by Zitnak and Johnston[3] of dangerously high levels of glycoalkaloids in a newly released potato cultivar. Further, a considerable amount of work is in progress to devise techniques for the control of glycoalkaloid synthesis in potatoes, which may occur due to exposure to light, low temperature or injury. Both lines of research are severly hampered by the slow and laborious methods available for glycoalkaloid analysis[4,5].

Current methods of glycoalkaloid analysis virtually all depend on reactions with the stereoid part of the glycoalkaloid molecule and give a result only for total glycoalkaloids[6–9]. Moreover, lengthy extraction and purification processes are necessary in order to obtain accurate analysis, and these processes can introduce considerable errors[4,10]. Recently, gas–liquid chromatographic techniques have been described that are capable of analysis of the individual glycoalkaloids[11–13]. However, these methods require chromatography at high temperatures, which shortens column life, and lengthy hydrolysis and/or derivatization of the glycoalkaloids. Individual

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steroidal alkaloids[14] and glycoalkaloids[15] may also be separated by thin-layer chromatography (TLC). Recent work has considerably shortened the time of analysis, and the application of densitometers enables quantitative analysis[16,17], but a large number of manipulative and preparative steps is still necessary, and the errors involved still make TLC only semiquantitative[5].

The method with the greatest potential for quantitative glycoalkaloid analysis is high-performance liquid chromatography (HPLC); it has the merit of minimal sample preparation, separation into individual glycoalkaloids and rapid, accurate analysis. Several recent papers have explored the HPLC of potato glycoalkaloids; as yet, none has described a method encompassing all the merits potentially achievable. Hunter *et al.* described a preparative[18] and an analytical[19] HPLC method for the individual steroidal alkaloids. Crabbe and Fryer[5] found that, to separate into individual glycoalkaloids, a bonded-phase (Carbohydrate) column was required, with analysis times of 15–20 min. These columns are easily contaminated and have a much shorter life than C_{18} and C_8 reversed-phase columns[5]. Bushway *et al.*[20] reported the separation of glycoalkaloids on bonded-phase (NH_2 and Carbohydrate) columns. The Carbohydrate column was superior, with analysis times of 15–20 min. Neither group of workers[5,20] could separate the major glycoalkaloids on reversed-phase columns.

This paper reports the separation of glycoalkaloids on more robust reversed-phase columns with greatly increased speed and sensitivity of analysis. This was achieved with radially compressed cartridges and the addition of ethanolamine to the mobile phase.

MATERIALS AND METHODS

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) M45 solvent-delivery system, an Altex 210 injector, a Waters RCM-100 compression module and a Varian LC flow cell (Model 02-00173-00), connected to a Varian 635 spectrophotometer. The columns used were Waters Silica, C_8 and C_{18} Radial-Paks, with 10 cm × 8 mm I.D. cartridges of each packing used; a 10 cm × 5 mm I.D. C_{18} cartridge was also used. The volume between injection and the solvent front for the 8 mm cartridges was *ca.* 3 ml; for the 5 mm column, this volume was 1.5 ml. The flow-rate for all analyses was 3 ml/min.

Acetonitrile of HPLC grade was obtained from Waters and ethanolamine (LR grade) and orthophosphoric acid (AR grade) from Ajax Chemicals (Sydney, Australia). Samples of α-solanine and α-chaconine were obtained from Sigma (St. Louis, MO, U.S.A.). Solanidine, solanadiene and β_2-chaconine were prepared from potato sprouts according to Coxon *et al.*[21]. The remaining glycoalkaloids, β- and γ-solanine, β_1- and β_2-chaconine and γ-chaconine were prepared by hydrolysis of α-solanine and α-chaconine, followed by TLC separation according to Lavintman *et al.*[22].

Separations were calculated from the resolution (R_s) equation $R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{k' - 1}\right)$ (see ref. 23), where α is the separation factor, k' is the capacity factor and N is the number of theoretical plates calculated as $16\left(\frac{V}{W}\right)^2$, for V = retention time and W = duration of peak.

RESULTS AND DISCUSSION

Glycoalkaloid detection

The column effluent was initially monitored with a refractive-index detector (Waters, Model 401), but sensitivity was low, the limit of detection (2 × noise) being 5 μg.

The limit of detection was improved greatly when the column effluent was monitored with a UV detector. The UV absorbance of glycoalkaloids is significant only at wavelengths shorter than 220 nm, (previous workers have used 208–215 nm[24] and 205 nm[10]). The UV absorbance of glycoalkaloids is due to the Δ^5-double bond in the alkaloid portion of the molecule[5]; therefore, the absorbance should be identical on a molar basis for glycoalkaloids with a common alkaloid group. The UV spectra of a 1 μg/25 μl injection of α-solanine and of α-chaconine were determined and were virtually identical; this was to be expected, since their molecular weights are extremely close. The maximum UV absorbance would appear to occur in the region 190–195 nm, although high solvent absorbance did not permit operation of the detector in this range. At 195 nm and below, sugars begin to absorb significantly[24], and, since the UV absorbances of individual sugars vary, the equal absorbance of glycoalkaloids on a molar basis no longer applies. The optimal wavelength for detection and quantitation of glycoalkaloids without interference from sugars was therefore 200 nm. The absorbance at 200 nm was only 7% lower than that at 195 nm, and, further, the high background absorbance of the solvent was reduced. Detection of glycoalkaloids at 200 nm with acetonitrile–water was 55% more sensitive than that with methanol–water at 205 nm (ref. 5) and 960% more sensitive than that with tetrahydrofuran–water–acetonitrile at 215 nm (ref. 20). The limit of detection at 200 nm was 0.1 μg, a considerable improvement over that with a refractive index detector.

The present work confirmed the difficulty of separating α-solanine and α-chaconine with a μBondapak C_{18} column[5,20], although addition of ethanolamine to the mobile phase resulted in a slightly forked peak. As separation was still inadequate, C_{18} Radial-Pak cartridges were assessed with encouraging results, but retention times were lengthy and the effect of ethanolamine was unpredictable. In order to understand the effect of ethanolamine more clearly and to reduce retention times, mobile phases of varying acetonitrile and ethanolamine concentrations were examined for the three packings available in Radial-Pak cartridges.

Initially, a negative peak occurred after the solvent front with some mobile phases containing ethanolamine. This was caused by too rapid addition of the orthophosphoric acid when adjusting the pH of the mobile phase from 11 to 3–5, which was the optimal range for peak shape and background absorbance. A reaction in the solvent mixture of ethanolamine with high concentrations of orthophosphoric acid is the most likely cause of this problem, as slow addition of orthophosphoric acid to the mobile phase (with thorough mixing) prevented the negative peak. This solvent mixture is most likely to be unstable at higher ethanolamine and acetonitrile concentrations due to the insolubility of ethanolamine under these conditions.

Addition of ethanolamine to the mobile phase greatly improved the speed of analysis (Fig. 1a–c). The retention time of α-chaconine on the C_8 cartridge was reduced from 18.4 min with a mobile phase of acetonitrile–water (60:40) and 0.01% of ethanolamine to 1.5 min with the same mobile phase containing 0.2% of ethanol-

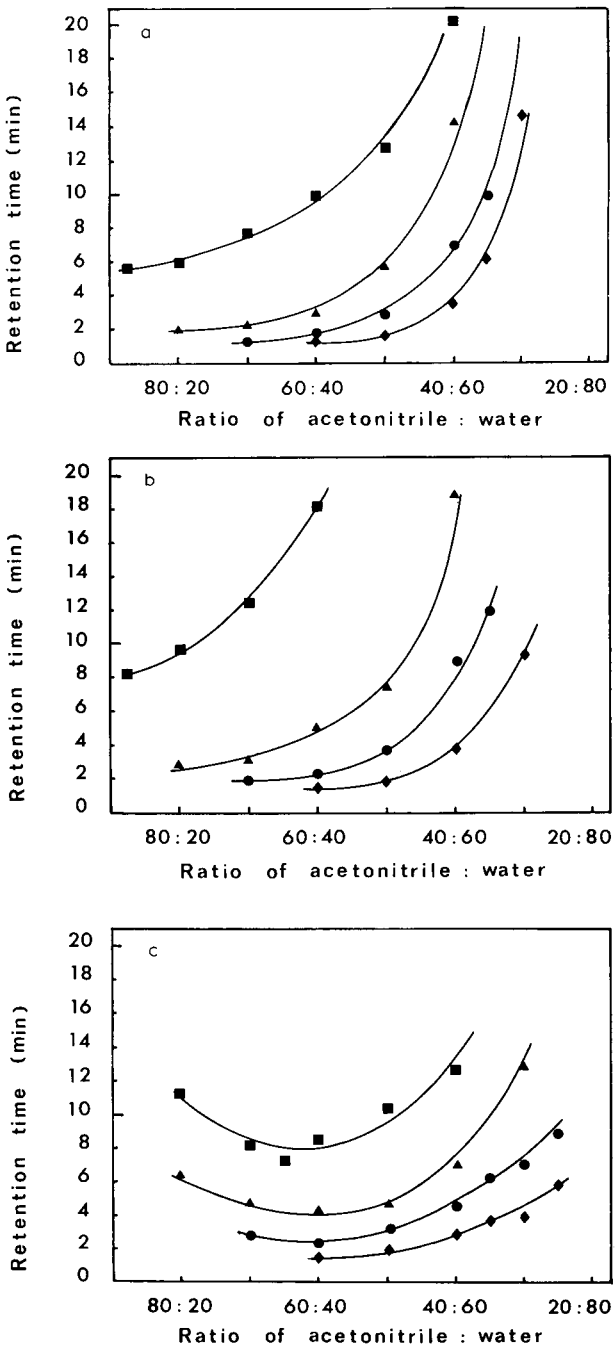

Fig. 1. Effect of concentration of ethanolamine and acetonitrile proportion on retention time. (a) C_{18} 8-mm cartridge; (b) C_8 8-mm cartridge, retention time for α-chaconine (which elutes last from C_{18} and C_8 cartridges); (c) Silica 8-mm cartridge, with retention time for α-solanine, as this elutes last from this cartridge. Ethanolamine concentrations: ■, 0.01%; ▲, 0.05%; ●, 0.1%; ◆, 0.2%.

amine. Moreover, the ratio of resolution (R_s) to retention time (t_R) or R_s/t_R, is greatly improved from 0 without ethanolamine (no separation) to 0.75 with 0.2% of ethanolamine.

The levels of acetonitrile and ethanolamine in the mobile phase also affected retention time and resolution. For example, with the C_8 cartridge and the mobile phase containing 0.05% of ethanolamine, R_s/t_R was 0.3 for acetonitrile–water (80:20) 0.43 for (70:30) and 0.05 for (40:60). With the C_{18} column, the maximum of R_s/t_R was at acetonitrile–water (60:40), whereas R_s/t_R for the Silica column was increased with concentrations of acetonitrile and ethanolamine up to the limits of solubility of ethanolamine in acetonitrile–water. These limits are 90:10 for 0.01% of ethanolamine, 80:20 for 0.05%, 75:25 for 0.1% and 65:35 for 0.2%.

Optimal mobile-phase composition for separation of α-solanine and α-chaconine

An R_s of 1.25 for α-solanine and α-chaconine was considered adequate for analytical work, and the optimal mobile-phase composition that minimised retention time but still achieved this separation was determined for each column. The optimum mobile phase for the C_{18} cartridge was acetonitrile–water–ethanolamine 35:65:0.2; for the C_8 column, the proportions were 50:50:0.2. However, at 200 nm, these mobile phases had high UV absorbance due to ethanolamine. At 0.1% of ethanolamine, this problem was eliminated, the optimal mobile-phase composition then becoming 55:45:0.1 for C_8 and 45:55:0.1 for C_{18}. A 5 mm I.D. C_{18} cartridge gave even more

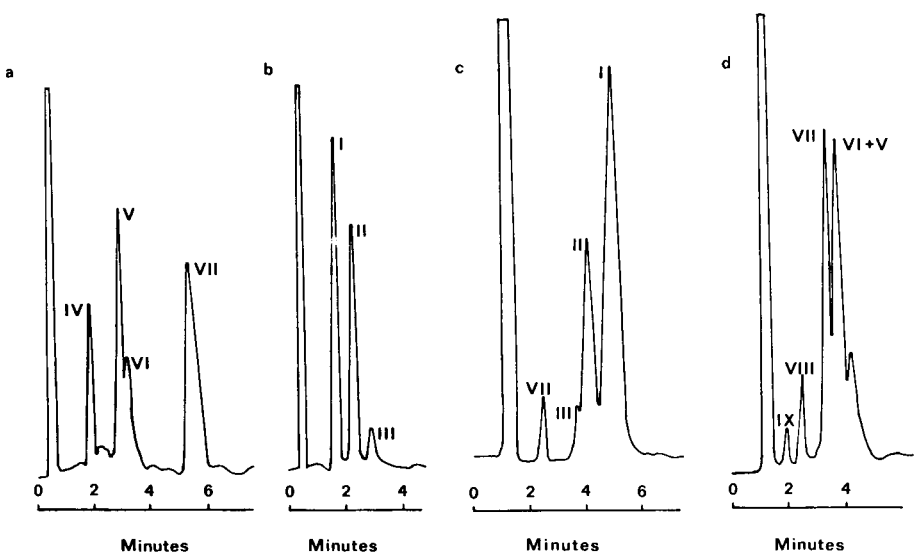

Fig. 2. HPLC separation of glycoalkaloids of the α-solanine and α-chaconine series. Conditions of chromatography: (a) α-Solanine hydrolysate, C_{18} 5-mm cartridge, mobile phase acetonitrile–water–ethanolamine (45:55:0.1), a.u.f.s. 0.025. (b) α-Chaconine hydrolysate, other conditions as in (a). (c) α-Solanine hydrolysate, Silica 8-mm cartridge, mobile phase proportions 77.5:22.5:0.05, a.u.f.s. 0.025. (d) α-Chaconine hydrolysate, other conditions as in (c). Peaks: I = α-solanine; II = β-solanine; III = γ-solanine; IV = α-chaconine; V = β_1-chaconine; VI = β_2-chaconine; VII = γ-chaconine; VIII = solanidine; IX = solanadiene.

rapid analysis with the latter mobile phase, without any significant loss of resolution compared to that from the 8 mm I.D. C_{18} cartridge.

The Silica cartridge showed a behaviour different from that of the C_{18} and C_8 reversed-phase cartridges. At high acetonitrile concentration (>60%), the order of elution from this cartridge was the reverse of that from the C_{18} and C_8 cartridges, α-chaconine being eluted first and being separated from α-solanine. At a lower acetonitrile concentration (<50%), the column operated in a reverse manner, α-solanine and α-chaconine being eluted as one peak, followed by the less polar solanidine. The optimal mobile phase composition for the separation of α-solanine and α-chaconine was established as 77.5:22.5:0.5. For maximum sensitivity, although without separation of α-solanine and α-chaconine, the optimal composition was 45:55:0.1.

Separation of other glycoalkaloids in the solanidine series

Besides α-solanine and α-chaconine, significant levels of β_2-chaconine and solanidine can occur in potato extracts[15,25]. Therefore, the optimal mobile phase–cartridge combinations established for the separation of α-solanine and α-chaconine were tested with other glycoalkaloids; the results (for the C_{18} column only are presented in Figs. 2a and 2b. However, the C_8 column is also capable of separating the hydrolytic products of both α-solanine and α-chaconine. The separation of the combined products of α-solanine and α-chaconine hydrolysis is possible, except for γ-

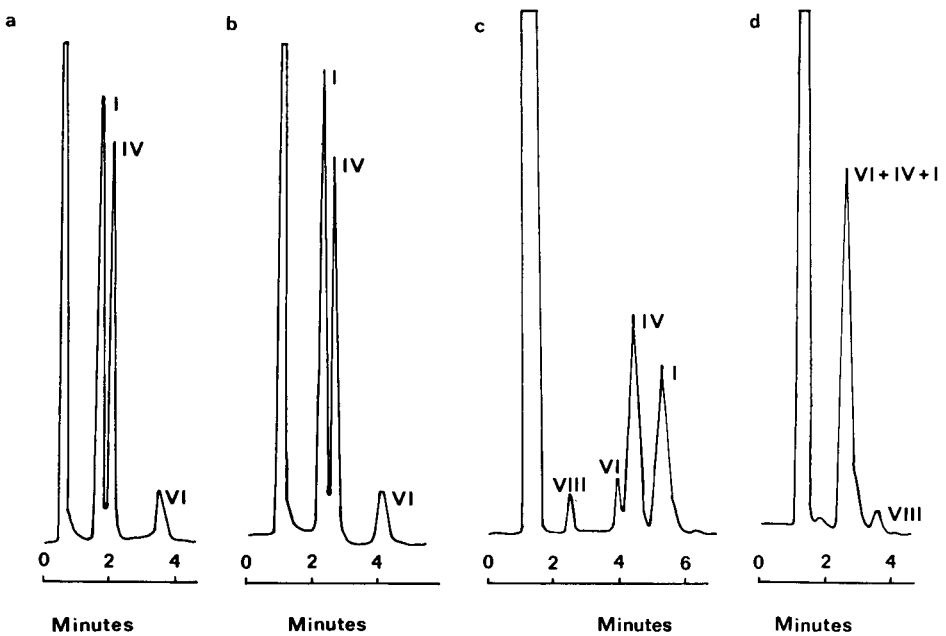

Fig. 3. Separation of glycoalkaloids of potato tubers (cv. Pentland Crown). Glycoalkaloids were extracted with 2% acetic acid, precipitated with ammonia, redissolved in 1 ml of 2% acetic acid and drawn through a filter tip of porosity 2 μm (Supelco, Bellefonte, PA, U.S.A.). Conditions of chromatography: (a) C_{18} 5-mm cartridge, mobile phase acetonitrile–water–ethanolamine (45:55:0.1), a.u.f.s. 0.025; (b) C_8 8-mm cartridge, mobile phase 55:45:0.1, a.u.f.s. 0.025; (c) Silica 8-mm cartridge, mobile phase 77.5:22.5:0.05, a.u.f.s. 0.025; (d) Silica 8-mm cartridge, mobile phase 45:55:0.01, a.u.f.s. 0.05.

solanine and β_1-chaconine, which have similar retention times. Solanidine and solanadiene were retained on the reversed-phase cartridges with all mobile phases tested.

On Silica cartridges, separation and peak shape of the hydrolytic products are not as good as with C_{18} and C_8 cartridges (Fig. 2c and d). However, the hydrolytic products of both α-solanine and α-chaconine were separated, except for β_1- and β_2-chaconine, which were eluted as one peak. The separation of all combined hydrolytic products was not possible, as β- and α-solanine have retention times similar to α-, and to β_1- and β_2-chaconine, respectively. Unlike the reversed-phase cartridges, both solanidine and solanadiene can be eluted and separated with the Silica cartridge at the same time as higher-order glycoalkaloids (see Fig. 2d).

Analysis of tuber extracts

The methods developed were evaluated with acetic acid extracts from a cultivar that contained higher than normal levels of β_2-chaconine and solanidine (levels per g FW in this sample were α-solanine 54.4 μg, β_1-chaconine 79.6 μg, β_2-chaconine 9.7 μg and solanidine 10.8 μg). On the reversed-phase C_{18} and C_8 packings, the peaks were clearly separated from the solvent front with adequate resolution of α-solanine, α-chaconine and β_2-chaconine; however the solanidine peak was not eluted (Figs. 3a and b). On the Silica column, with the 77.5:22.5:0.05 mobile phase, all four compounds were eluted with acceptable resolution (Fig. 3c). With the 45:55:0.1 solvent mixture, α-solanine, α-chaconine and β_2-chaconine were eluted as one peak, solanidine following shortly afterwards (Fig. 3d). Where only total glycoalkaloids are required, this column–mobile phase combination is desirable, both for its speed and increased sensitivity of analysis.

This work reports for the first time a rapid separation of all known glycoalkaloids in the solanidine series, including the separation of β_1- from β_2-chaconine. Similar separations have been made by TLC, but analysis times of up to several hours are required[15,22,26]. For routine analysis of potato glycoalkaloids, the reversed-phase cartridges are recommended, owing to their robust characteristics and long life. These should permit a routine 3-min analysis time, although, when significant levels of β_2-chaconine are present, the time of analysis may be 5 min. When solanidine is expected, the Silica cartridge should be used, either with 45% of acetonitrile, which elutes α-solanine and α-chaconine as one peak followed by solanidine (analysis time 4 min), or with 77.5% of acetonitrile, which elutes solanidine first, followed by α-chaconine and α-solanine peaks (analysis time 6 min).

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A FAST AND SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ARYL HYDROCARBON HYDROXYLASE

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SUMMARY

A simple high-performance liquid chromatographic method using fluorescence detection of the remaining substrate is described for the determination of benzo[*a*]-pyrene hydroxylase activity. This assay is far simpler than the previous ones, as it does not require extraction or centrifugation and the measurement occurs directly after dilution of the total incubation medium. The aryl hydrocarbon hydroxylase (AHH) activities in rat liver microsomes are in agreement with those obtained by radioactive assays. Moreover, this assay allows the routine determination of the AHH activity in animal tissues.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have attracted a great deal of attention because they are widely distributed in the environment and have been shown to cause carcinogenic, mutagenic and cytotoxic effects in various species and tissues[1–3]. For many PAHs, biologically active arene oxides are the initial products formed by the microsomal mixed function oxidase system; epoxide hydrolase (E.C. 3.3.2.3) catalyses the hydration of the arene oxides to biologically inactive dihydrodiols, which may undergo further metabolism, by the microsomal Cyt P_{450}-dependent system, to form highly reactive metabolites.

Although the relationship between tissue inducibility of aryl hydrocarbon hydroxylase (AHH: 1.14.14.2) activity and susceptibility to PAH carcinogenesis is still not clear, several reports have shown that there is a correlation. Thus it is of interest to establish simple and reliable methods for the measurement of AHH activity in the different tissues.

Benzo[*a*]pyrene (B*a*P) is the commonest PAH used for the determination of AHH activity, and in most work the assay procedure used is the fluorimetric method described by Nebert and Gelboin[4], which measures a fraction of the metabolites of B*a*P, as only products that fluoresce in a manner similar to 3-hydroxybenzo[*a*]pyrene are taken into account. More recently, De Pierre *et al.*[5] and Van Cantfort *et al.*[6] developed radiometric assays in which the water-soluble metabolites of B*a*P were measured directly by liquid scintillation counting.

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In this paper, we report an assay consisting of a very simple and reproducible high-performance liquid chromatographic (HPLC) quantification of the unreacted B*a*P, using fluorimetric detection; it allows direct measurement in the incubation medium and is very convenient for the routine determination of AHH activity.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (150–200 g), bred at the laboratory, were allowed an AO3 diet (UAR, Villemoisson, France) and water *ad libitum*. They were starved for 12–15 h before killing.

Chemicals

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were obtained from Sigma (St. Louis, MO, U.S.A.); B*a*P was purchased from Fluka (Buchs, Switzerland). The solvents used for extraction and HPLC analysis were obtained from commercial sources and purified by distillation

Enzyme preparation

Animals were killed by exsanguination to aid removal of blood from organs. The liver was immediately excised, weighed, then chilled and homogenized with a Potter-Elvehjem instrument, fitted with a PTFE pestle, in 4 volumes of ice-cold 0.1 *M* phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 *g* for 20 min. The mitochondria-free supernatant was further centrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in 0.1 *M* phosphate buffer to give final protein concentrations ranging from 2 to 10 mg/ml. The protein concentration of the microsomal suspension was determined by the biuret method of Gornall *et al.*[7] using bovine serum albumin as the standard.

Aryl hydrocarbon hydroxylase assay

The reaction mixture, which contained 250 μmoles of Tris buffer (pH 7.4), 12.5 μmoles of glucose-6-phosphate, 2.5 μmoles of NADP, 2 units of glucose-6-phosphate dehydrogenase and 0.5 ml of microsomes in a final volume of 2.5 ml, was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of B*a*P in 50 μl of acetone, and the concentrations used varied from 2 to 100 μ*M*. The mixture was shaken for 30 min at 37 °C and the reaction was stopped by the addition of 1 ml of cold acetone; the flasks were stored at 4 °C until taken for quantification of B*a*P.

The assays were carried out in duplicate and the AHH activity was obtained by comparing the remaining B*a*P in the incubation flasks with B*a*P measured in identical assays to which acetone had been added prior to incubation.

High-performance liquid chromatography

The HPLC arrangement consisted of a Spectra-Physics Model SP 3500 B pump equipped with a Valco Model 60 sample injection valve (10-μl sample loop) and connected to a Schoeffel FS 970 spectrofluorimetric detector fitted with a 5-μl cell. The excitation monochromator was set at 366 nm and fluorescence emission was measured through a cut-off filter ($\lambda_{em} > 385$ nm). The column consisted of a stainless-

steel tube (250 × 4.6 mm I.D.) packed with 5-μm LiChrosorb RP 18 (Merck, Darmstadt, G.F.R.) using a Chromatem apparatus (Touzart et Matignon, France). The mobile phase consisted of 10% water in acetonitrile, the eluent flow-rate was 1.2 ml/min and all manipulations were performed at ambient temperature.

The incubates were adjusted to bring the volume to 4 ml and, after homogenization on a vortex mixer, a 200-μl aliquot was diluted with acetonitrile in order to obtain concentrations of B*a*P in the 1 μM range. After shaking, 10 μl of the solution were injected for HPLC analysis and the AHH activity was determined by comparing the peak heights obtained in assays in which acetone had been added before or after incubation at 37°C; AHH activity was expressed in nanomoles of hydroxylated B*a*P per minute per milligram of microsomal protein.

RESULTS

BaP assay

As the determination of remaining B*a*P was conducted on an aliquot of the total incubation medium, it was necessary to ascertain that the fluorimetric detection was specific; from this point of view, it was shown that none of the components of the incubation medium gave interfering peaks with the same retention time as B*a*P. It was also observed (Fig. 1) that the same response was obtained when a 200-μl sample was

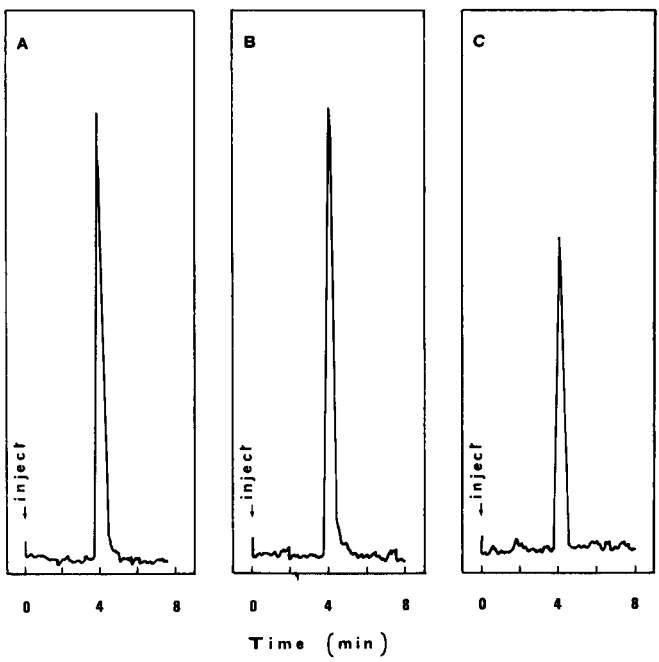

Fig. 1. (A) Liquid chromatogram of a 10-μl sample from a 1 μM solution of B*a*P in acetone–acetonitrile. (B) and (C) Liquid chromatograms of 10-μl samples from diluted incubation mixtures (B = blank incubation; C = assay incubation). After the 30-min incubation period, dilution with acetonitrile was such that the B*a*P concentration in the control flask (B) was 1 μM; the same dilution was applied to the assay flask (C). The difference in the responses gives the hydroxylation rate.

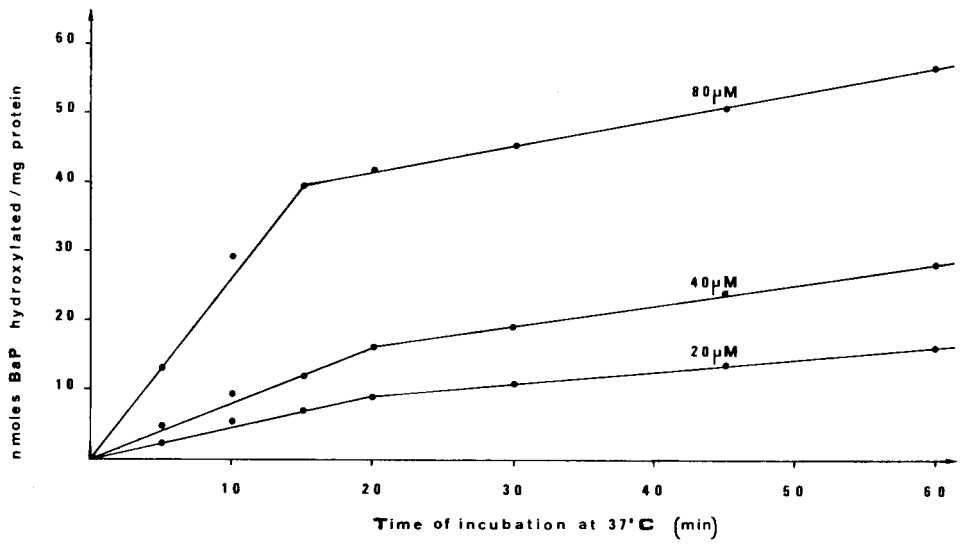

Fig. 2. Relationship between time and B*a*P hydroxylase activity in rat microsomes. Details of the assay procedure are described under Materials and methods, and each point represents the mean of duplicate determinations. The incubation systems contained rat microsomes at a concentration of 6 mg protein per millilitre. The reaction was started by adding sufficient B*a*P (dissolved in acetone) to produce final concentrations of 20, 40 and 80 μM. Incubations were carried out at 37°C for 5, 10, 15, 20, 30, 45 and 60 min.

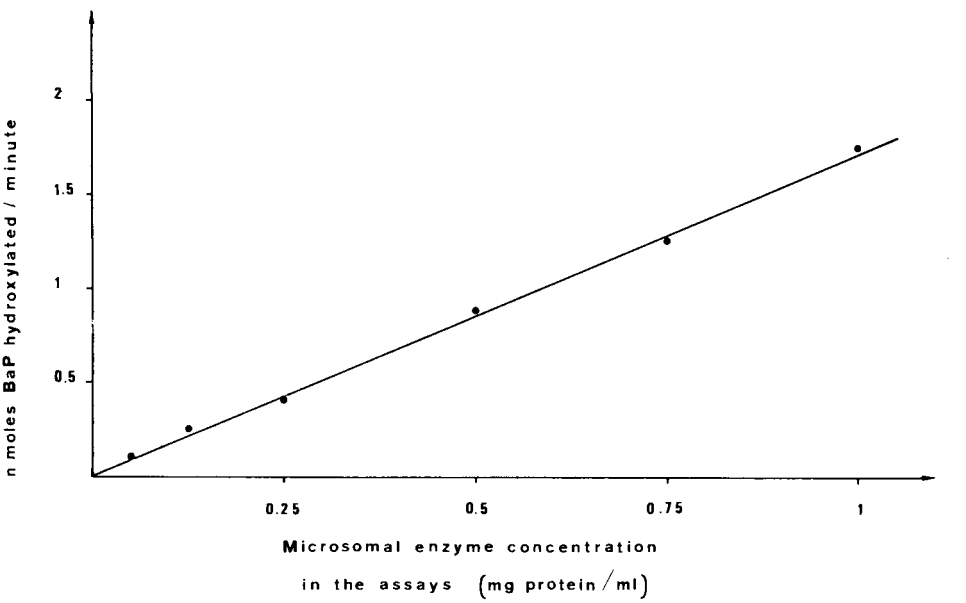

Fig. 3. Dependence of B*a*P hydroxylation on enzyme concentration. The incubation systems used rat microsomes at a concentration of 6 mg protein per millilitre. B*a*P was used at a final concentration of 20 μM and incubations were carried out at 37°C for 30 min.

diluted in acetonitrile, whether it came from a pure solution of B*a*P in acetone at the concentration used in the incubation flasks or from an incubation medium. The possibility of erroneous measurements of the remaining B*a*P after incubation, due to interfering metabolites with the same retention time, was eliminated after analysis by HPLC under the same conditions, after total removal of B*a*P by extraction with *n*-hexane.

All of the assays were carried out in duplicate, and the differences between the duplicate results were less than 2%; under the conditions described, the retention time of B*a*P was about 4 min and the response was linear for the range 0–0.05 nmole injected.

Linearity with time and protein

Fig. 2 shows the relationship between enzyme activity and time; linearity of hydroxylation was observed for at least 15 min; as has been described for the radioactive assay[5], the extent of linearity depends on the rate of formation of the reaction products.

Fig. 3 shows the effect of liver microsomal protein on the AHH activity; hydroxylation of B*a*P is linear when concentrations in the range 0–1 mg of protein per millilitre of incubation mixture are used.

Saturation with substrate

It is of great importance, when comparing in animal tissues the effects of various treatments on enzymatic activity, to use a saturating concentration but not a too high one (which would decrease the precision of the comparison); thus the depen-

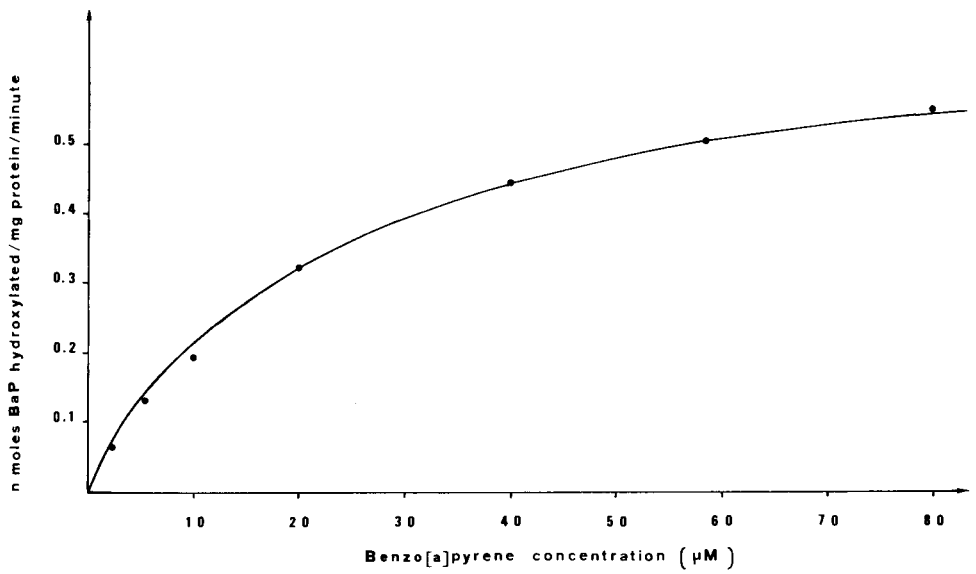

Fig. 4. Dependence of B*a*P hydroxylase on substrate concentration. The assay procedure is described under Materials and methods. The incubation systems used rat microsomes at a concentration of 6 mg protein per millilitre. The reactions were started by adding B*a*P to produce final concentrations of 2, 5, 10, 20, 40, 60 and 80 μM. Incubations were carried out at 37°C for 30 min.

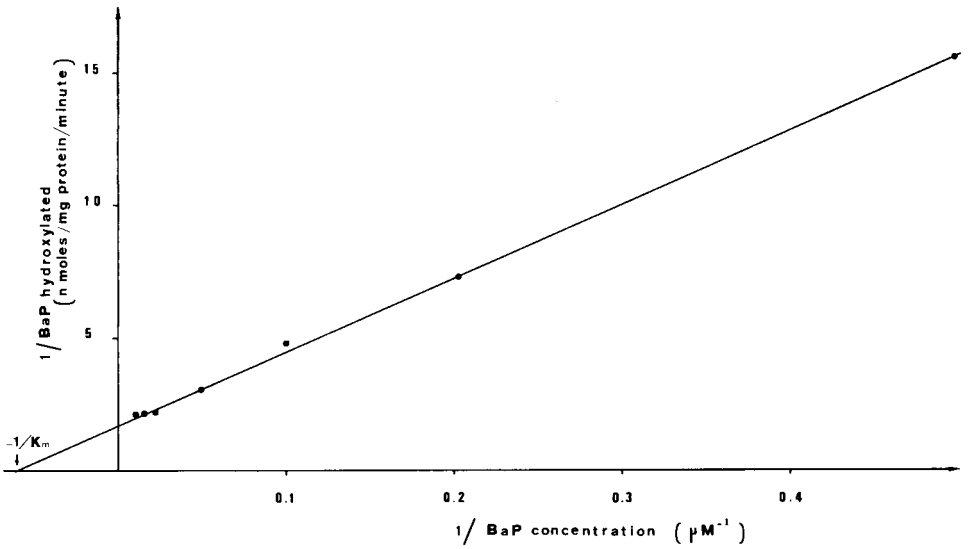

Fig. 5. Lineweaver–Burk plot of B*a*P hydroxylation. Incubation conditions as in Fig. 4. The abscissa indicates the reciprocal of the B*a*P concentrations employed and the ordinate shows the reciprocal of the apparent rate of metabolism.

dence of B*a*P hydroxylase on substrate concentration was determined and is shown in Fig. 4.

It was possible to obtain the apparent K_M for B*a*P hydroxylase by displaying the data on a Lineweaver–Burk plot (Fig. 5). The value of 16 μM is in good agreement with those obtained in other laboratories for similar microsomal protein concentrations.

DISCUSSION

The procedure described allows the determination of unreacted substrate without interference from any of the reaction products. The enzyme activity is linear over reasonable ranges of time and protein concentration, and thus this assay appears to be useful for AHH measurement. Nevertheless, it should be compared with the fluorimetric assay of Nebert and Gelboin[4] and with the radioassay described by De Pierre *et al.*[5] and modified by Van Cantfort *et al.*[6].

Up to now, the fluorescence assay was the most commonly used, owing to its very high sensitivity; however, it is a questionable technique for numerous reasons. It is based on an acetone–*n*-hexane extraction of the products formed but only a portion of the B*a*P metabolites are extracted; on the other hand, spectrofluorimetric measurement of products extractable in alkaline medium (emission peak at 522 nm produced by activation at 396 nm) is limited to the determination of products fluorescing under the same conditions as 3-hydroxybenzo[*a*]pyrene. This method does not assay diols, epoxides, quinones or other poorly fluorescent metabolites, and it has been observed that it measures not more than half of the alkali-soluble metabolites. From this point of view, the radiometric assay is better, as it measures, after *n*-hexane extraction of the

remaining B*a*P, the whole of the radioactivity associated with the water-soluble metabolites formed.

Van Cantfort *et al.*[6], using HPLC analysis, showed that dihydrodiols and quinones were present in non-negligible proportions in the *n*-hexane extract. After modification of the polarity of the aqueous phase, complete removal of B*a*P from its metabolites (>95%) was obtained. Nevertheless, they specified that a second extraction with *n*-hexane was necessary in order to achieve minimal contamination of the aqueous phase by unchanged substrate, but without any indication on the new yields of the measured metabolites.

Our method is much simpler, as measurement is effected after direct dilution of the incubation medium. Of course, all the cofactors and all the B*a*P metabolites are present but the specificity of the HPLC determination associated with the high dilution used (50- or 100-fold when 80 μM of B*a*P was used) allows a very accurate quantification of the unchanged B*a*P. In addition to specificity, very good reproducibility is achieved as measurements on duplicates did not differ by more than 2%.

In other respects, the classical fluorimetric assay is limited, owing to the decreasing fluorescence of the metabolites when they are stored after alkali extraction from the organic phase; the samples must be extracted one at a time and the fluorescence determined as quickly as possible; with our method, the samples can be stored, before or after dilution of an aliquot with acetonitrile, for several days without affecting the results if protected from sunlight. Thus it is possible to perform a routine determination of AHH activity with an HPLC apparatus fitted with an automatic injector.

If we consider the sensitivities of the methods, the radiometric and fluorimetric assays seem very similar; the lower limit as indicated by Van Cantfort *et al.* is about $2 \cdot 10^{-11}$ moles of metabolites formed per millilitre of incubation medium. It is impossible to compare these sensitivities with that of our assay in which we measure the rate of disappearance of the substrate.

Although we are able to detect $8 \cdot 10^{-14}$ moles of B*a*P, our HPLC measurement of AHH activity is limited by the lowest significant difference measurable between the assays in which acetone is added before or after incubation. Owing to the good reproducibility of the measurements on duplicates, a 5% difference can be considered as significant, corresponding to the hydroxylation of the substrate by the mixed function oxidase (MFO) system. Thus, if we utilize substrate concentrations in the range 40–80 μM, our assay can be used for tissues in which the lower limit of the hydroxylation route is about 100 pmole per minute per milligram of microsomal protein (which is 10-fold lower than the value observed for rat liver).

It has been observed that PAH metabolism differs qualitatively and quantitatively according to the organs or animal species considered[8–11]. Moreover, it has been shown that the relative distribution between the different metabolites of B*a*P (phenols, diols, epoxides, quinones) was not the same when the animal had been treated or not by inducers such as 3-methylcholanthrene or other PAHs[11,12]. As a result, it is impossible to compare the AHH activities using the fluorimetric assay, but the radioisotopic method and our assay are very satisfactory.

Nevertheless, the best method would in fact be the identification and quantification of all of the metabolites of B*a*P produced, in order to determine how patterns of metabolism may vary (between animal species or chemical treatments), and thus

indications could be obtained of the factors influencing carcinogenic risk. If HPLC fulfills the requirements for such an analysis it is impossible to apply it in routine experiments. In addition to the separation of the water-soluble metabolites and of the organic solvent-soluble metabolites, it would be necessary to determine the various metabolites that are covalently bound to protein and which may represent 4–9% of the total metabolites of B*a*P[13,14]. Moreover, taking into account that non-enzymatic conversions may occur, which modify the relative distribution between the metabolites, the systematic quantification of each metabolite is of little interest.

Thus it is necessary to have a simple technique for AHH determination; the fluorimetric assay can be discarded as it measures only part of the mixed function oxidase activity. The radiometric method and our HPLC assay are similar, and give a good determination of the AHH activity. The former method requires two extraction and centrifugation steps, whereas in our method measurement is effected after dilution of the incubation medium. Utilization of labelled B*a*P in the radiometric assay makes it more expensive and requires frequent checking of chemical and radiochemical purities; indeed, the more serious limitation to the sensitivity of a radioactive enzymatic assay is often the background from contaminants in the substrate and from non-enzymatic reactions.

Finally, the HPLC method described here is much simpler and reproducible than the previous assays; the possibility of automation makes it more convenient for large-scale analysis and is of great interest when studying the induction of liver AHH by various xenobiotics in many animal species or when utilizing the AHH measurement as an indicator of petroleum hydrocarbon pollution in the marine environment[15].

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PURIFICATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *ACER PSEUDOPLATANUS* L. USING IMMOBILISED PROCION RED HE-3B*

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SUMMARY

The effect of the support matrix on the capacity of immobilised Procion Red HE-3B for a higher plant $NADP^+$-dependent dehydrogenase is examined. A method is presented for the purification of 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44.) from *Acer pseudoplatanus* L. (sycamore) which includes chromatography on Procion Red–Sepharose. The enzyme was purified 380-fold with 40–50 % recovery and a final specific activity of 80 units per mg protein. Preliminary evidence was obtained for the existence of isoenzymes of 6-phosphogluconate dehydrogenase in *Acer pseudoplatanus* L.

INTRODUCTION

The use of group-specific adsorbents for the purification of $NADP^+$-dependent dehydrogenases is now widespread[1]. Immobilised nucleotides such as $NADP^+$ and 2′,5′-ADP are often suitable as ligands[2–4], but their high cost and low capacities preclude their use in many instances. In addition, the covalent attachment of nucleotides to insoluble support materials requires complex and potentially hazardous synthetic procedures. In contrast, immobilised Cibacron Blue 3G-A (the triazine dye component of Blue Dextran) which binds many nucleotide-dependent enzymes, is easily prepared and relatively inexpensive and consequently has been used as a group-specific ligand for the purification of several kinases and dehydrogenases[5,6]. It has been suggested that the dye may interact with the nucleotide binding site of such proteins[7]. An additional advantage of Cibacron Blue 3G-A is its high operational capacity when compared with group-specific nucleotide adsorbents[8].

A related triazine dye, Procion Red HE-3B, which was first used in the purification of carboxypeptidase G from *Pseudomonas*[9], has more recently been shown to retard several dehydrogenases[8,10]. In particular, there is some evidence that this ligand exhibits a greater selectivity toward $NADP^+$-dependent enzymes than Ciba-

* Dedicated to Professor J. Porath on the occasion of his 60th birthday.

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cron Blue 3G-A[8]. We have investigated the use of immobilised Procion Red HE-3B for the purification of an $NADP^+$-dependent dehydrogenase from *Acer pseudoplatanus* L. While the enzymes of the pentose phosphate pathway from animal and microbial sources have been extensively purified and characterised, relatively little is known of the function and regulation of this process in higher plants. We report here a method for the isolation of 6-phosphogluconate dehydrogenase in high yield including chromatography on immobilised Procion Red HE-3B. This work forms part of a larger study of the regulation of carbohydrate oxidation in *Acer pseudoplatanus* L.[11–13].

MATERIALS AND METHODS

Materials

Cellulose and DEAE-cellulose (DE-52) were obtained from Whatman (Maidstone, Great Britain); Sepharose 6B, CL-6B, Sephadex G-200, Sephacryl S-200 and 2′,5′-ADP–Sepharose were from Pharmacia (G.B.) (London, Great Britain); Ultrogel AcA-54 was from LKB (Croydon, Great Britain); Indubiose Perles A6 was from Réactifs IBF (Clichy, France) and Unisphere P1000 from Hydron Labs. (New Brunswick, NY, U.S.A.). Procion Red HE-3B was a gift from ICI Organics Division (Manchester, Great Britain). 6-Phosphogluconate and $NADP^+$ were purchased from Boehringer (London) (London, Great Britain). Standard marker proteins for sodium dodecyl sulphate (SDS) electrophoresis were from BDH Chemicals Ltd. (Poole, Great Britain) and Minicon-A macrosolute concentrators and Matrex Gel Red A were obtained from Amicon (Woking, Great Britain). All other chemicals were purchased from BDH, and were of the highest purity available.

Culture of Acer pseudoplatanus L.

Non-photosynthetic cell suspension cultures of *Acer pseudoplatanus* L. were used throughout this work. The origin and maintenance in batch culture of this tissue were as described previously[11]. For extraction of 6-phosphogluconate dehydrogenase the cells were harvested at 10 days, since preliminary work established that maximum activity was present at this time.

Immobilisation of Procion Red HE-3B

Procion Red HE-3B derivatives of several support materials were prepared as described[6]. Immobilised ligand concentrations of all materials except cellulose, Unisphere P1000, Sephacryl S-200 and Sephadex G-200 were determined after hydrolysis in 50% (v/v) acetic acid at 100°C for 5 min by measurement of $A_{536\,nm}$ of the resulting solution. The remaining samples were suspended in 10% (v/v) glycerol and the ligand concentration determined directly by measurement at 536 nm against blanks containing an equivalent amount of matrix.

Measurement of enzyme activity

6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44.) was measured at 25°C by following the reduction of $NADP^+$ at 340 nm. The assay comprised 0.05 *M* Tris–HCl (pH 7.5) containing 15 m*M* $MgCl_2$, 2 m*M* 6-phosphogluconate and 0.16 m*M* $NADP^+$. The reaction was initiated by the addition of enzyme and was linear for 1–2 min. A unit of activity was defined as 1 μmole of $NADP^+$ reduced per min at 25°C and specific activity as units per mg protein. Enzyme protein was determined by the

Folin method[14] using bovine serum albumin as standard. Column fractions were monitored for protein by measurement of $A_{280\,nm}$.

Purification protocol

All operations were performed at 4 °C.

(a) Preparation of cell-free extract. Cells (fresh weight 300–400 g) were harvested on a sintered glass funnel, washed successively with distilled water and 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA and suspended in one volume of the same buffer. Cells were disrupted by sonication and the homogenate centrifuged at 50,000 *g* for 30 min to remove particulate debris.

(b) $(NH_4)_2SO_4$ fractionation. The supernatant was stirred on an ice-bath and solid $(NH_4)_2SO_4$ added to give 50% $(NH_4)_2SO_4$ saturation. After 30 min the suspension was centrifuged at 50,000 *g* for 15 min. $(NH_4)_2SO_4$ was added to the supernatant to give 80% saturation and after 30 min the suspension was centrifuged for 15 min at 50,000 *g*. The pellet was dissolved in 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% (v/v) glycerol and dialysed against the same buffer.

(c) DEAE-cellulose chromatography. The dialysed enzyme was applied to a column of DEAE-cellulose 52 (30 × 2.5 cm) equilibrated with 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% (v/v) glycerol. Elution was performed by washing the column with 300 ml of the same buffer followed by a linear gradient of NaCl in buffer (0–0.3 *M* NaCl, total volume 500 ml). The flow-rate was 20–30 ml/h and fractions of *ca.* 7 ml were collected. Fractions containing 6-phosphogluconate dehydrogenase were combined and dialysed under vacuum against 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol.

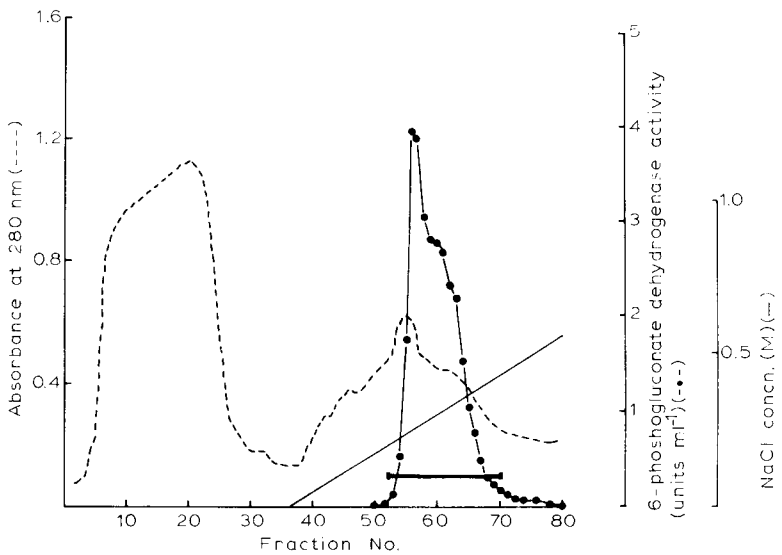

Fig. 1. Elution profile from Procion Red HE-3B–Sepharose 6B. Enzyme from step c (see Materials and methods) was applied at 10 ml/h to a column of Procion Red HE-3B–Sepharose 6B (25 × 1 cm) equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol, washed through with two or three column volumes of buffer, then developed with a salt gradient (0–1.0 *M* NaCl; total volume 200 ml). Fractions (4 ml) were assayed for 6-phosphogluconate dehydrogenase activity (●), absorbance at 280 nm (– – –) and conductivity (——). The horizontal bar represents those fractions which were pooled.

(d) Procion Red HE-3B–Sepharose chromatography. The dialysed and concentrated extract was applied to a 25 × 1 cm column of Procion Red HE-3B–Sepharose 6B equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol. Unretarded protein was removed by washing with two or three column volumes of buffer. Enzyme was eluted in a linear gradient of NaCl in buffer (0–1.0 *M* NaCl, total volume 200 ml). Approximately 4-ml fractions were collected at 10 ml/h. Fig. 1 shows a typical result. Fractions containing enzyme were combined and dialysed under vacuum as described above.

(e) 2′,5′-ADP–Sepharose chromatography. The dialysate was applied to a 15 × 1 cm column of 2′,5′-ADP–Sepharose 4B equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (V/V) glycerol at 10 ml/h. The column was washed with three volumes of buffer and developed with a linear gradient of $NADP^+$ in buffer (0–1.0 m*M* $NADP^+$, total volume 200 ml). Fractions containing 6-phosphogluconate dehydrogenase were pooled and concentrated by vacuum dialysis against column buffer. Further concentration was achieved using a Minicon-A macrosolute concentrator.

Gel electrophoresis

Gel electrophoresis was performed in 7.5% gels and Tris–glycine buffer (pH 8.5)[15] at 2–3 mA per gel and 4°C for 1–2 h. Protein was detected by incubation in 0.25% (w/v) Coomassie brilliant blue in methanol–water–acetic acid (5:5:1, v/v/v) followed by destaining in several washes of methanol–water–acetic acid (3:35:2, v/v/v). Enzyme activity was located by incubating gels in the dark at 37°C in 0.1 *M* Tris–HCl (pH 7.5) containing 0.4 mg/ml phenazine methosulphate, 0.4 mg/ml nitro blue tetrazolium, 0.5 m*M* $NADP^+$, 1.4 m*M* 6-phosphogluconate and 2.5 m*M* $MgCl_2$. Bands corresponding to the position of the enzyme were fixed in water–ethanol–acetic acid–glycerol (2:1:1:1, v/v).

Sodium dodecyl sulphate polyacrylamide electrophoresis was as described[16]. Calibration was achieved by running in parallel a standard mixture of proteins in the mol. wt. range 14,300–71,500. Gels were stained for protein as described above.

RESULTS AND DISCUSSION

Effect of the matrix on ligand capacity

It has been shown that the choice of support material for a ligand may dramatically affect its capacity as an adsorbent in affinity chromatography[17]. We therefore first examined the suitability of several insoluble matrices as supports for Procion Red HE-3B and their influence on the interaction between bound dye and 6-phosphogluconate dehydrogenase (Table I). All gels were covalently coupled to Procion Red HE-3B under similar conditions (see Materials and methods) and most of the prepared adsorbents contained 2–3 μmole dye per ml swollen gel. The capacities of the adsorbents for 6-phosphogluconate dehydrogenase were determined by frontal analysis chromatography[18] as described in the legend of Table I. Attachment of the dye to a support matrix apparently significantly affected its performance as a ligand, since a 200-fold variation was measured between the capacities of the adsorbents tested for enzyme. The results confirm a previous study of interactions between Cibacron Blue 3G-A adsorbents and human serum albumin[17] in that cellulose, Ultrogel

TABLE I

CAPACITIES OF IMMOBILISED PROCION RED HE-3B DERIVATIVES FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE

The capacities of Procion Red HE-3B derivatives were determined by frontal analysis chromatography[18] at 4 C using micro-columns (diameter 6 mm) containing 1 ml adsorbent equilibrated in 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% glycerol. 6-Phosphogluconate dehydrogenase, partially purified from a cell-free extract from *Acer pseudoplatanus* L. by $(NH_4)_2SO_4$ fractionation (see Materials and methods), and dialysed against column buffer was applied at 2 ml/h until the enzyme activity and $A_{280\ nm}$ of the eluate and applied sample were identical. Unretarded protein was eluted with buffer, then bound enzyme was desorbed by a linear gradient of NaCl (0–1.0 *M* NaCl; total volume 20 ml). The frontal elution profile allowed calculation of the amount of enzyme retarded by the column after correction for binding to a column of unsubstituted support under identical conditions.

Support matrix	*Ligand concentration (μmole/ml swollen gel)*	*Capacity for 6-phosphogluconate dehydrogenase (units of enzyme activity bound/μmole immobilised Procion Red HE-3B)*
Cellulose	2.0	0.1
Ultrogel AcA-54	0.6	0.3
Unisphere P1000	2.9	0.4
Sephadex G-200	2.0	23.1
Sephacryl S-200	1.8	8.5
Sepharose 6B	2.0	22.1
Sepharose CL-6B	2.8	5.8
Indubiose Perles A6	0.2	26.0
Matrex Gel Red A	2.8	22.1

and Unisphere apparently restrict access of protein to the ligand. The high capacities of the substituted dextrans and agaroses suggest that these matrices present the ligand in a more accessible manner. Cross-linked dextran (Sephadex G-200) and the un-cross-linked 6% agaroses (Sepharose 6B and Indubiose Perles A6) had the highest molar capacities for 6-phosphogluconate dehydrogenase. Inclusion of polyacrylamide bridges (Sephacryl S-200) or cross-linkage of the agarose (Sepharose CL-6B) decreased ligand effectiveness relative to the corresponding un-cross-linked matrices. In contrast, the molar capacity of Matrex Gel Red A (Amicon; Procion Red HE-3B attached to a cross-linked 4% agarose) was similar to that of our Sepharose 6B adsorbent. Consideration of respective ligand concentrations, capacities for enzyme and flow properties of the adsorbents indicated that agarose (Sepharose 6B) or cross-linked agarose (Matrex Gel Red A) were the preferred supports for Procion Red HE-3B in the purification of 6-phosphogluconate dehydrogenase from *Acer pseudoplatanus* L. The inferior flow properties of Sephadex G-200 make this matrix unsuitable for large scale preparative work. Nevertheless, the ease with which triazine dyes can be coupled to cross-linked dextrans[19] apparently without the damage to the bead structure which is associated with cyanogen bromide activation[20], allows the possibility of their application in molecular sieving combined with affinity chromatography ("affinity gel filtration"[21]) using the dye either directly as a ligand or indirectly as a spacer for other affinity ligands[22].

Chromatography of 6-phosphogluconate dehydrogenase on Procion Red HE-3B–Sepharose 6B.

Cibacron Blue 3G-A is widely used as a general group-specific ligand in the purification of nucleotide-dependent enzymes[5]. Displacement of adsorbed enzyme from immobilised Cibacron Blue is frequently achieved by "biospecific" elution with appropriate coenzyme or substrate at significantly lower ionic strenghts than those at which salt solutions are effective. This has been taken as an indication that the immobilised dye interacts directly with the nucleotide-binding site of proteins[7,22]. While more recent information shows that "biospecific" elution need not necessarily imply any such specificity in the adsorptive mechanism (*cf.*, ref. 6), X-ray crystallographic studies have demonstrated marked similarities between the binding of NAD and Cibacron Blue 3G-A to liver alcohol dehydrogenase[23].

The demonstration that Procion Red HE-3B also retards dehydrogenases[8], acts as an inhibitor in free solution[19] and exhibits some selectivity toward NADP-dependent enzymes[8], indicated that elution of 6-phosphogluconate dehydrogenase by coenzyme might be successful. However, in this instance we were unable to displace this enzyme from Procion Red HE-3B–Sepharose 6B using $NADP^+$ (0–10 m*M*). Furthermore, 6-phosphogluconate, citrate and pyrophosphate (the latter two being competitive inhibitors with respect to both coenzyme and substrate for the mammalian enzyme[24]) could not elute the *Acer pseudoplatanus* L. enzyme at ionic strengths below that at which sodium chloride is effective (*cf.*, ref. 25). The failure of substrate (as opposed to coenzyme) and inhibitors to elute the enzyme confirms recent observations of the chromatographic behaviour of *Bacillus stearthermophilus* 6-phosphogluconate dehydrogenase[19] and bovine liver NADPH-dependent 5′,10′-methylene tetrahydrofolate dehydrogenase[10] on immobilised Procion Red HE-3B. Rather than supporting the idea that the ligand Procion Red HE-3B binds at the dinucleotide fold for the above enzymes, these data seem to us to suggest non-involvement of the substrate binding site. Since presaturation with nucleotide ($NADP^+$) dramatically alters the subsequent chromatographic behaviour of at least one of the above enzymes[19], we might conclude that the dinucleotide fold is occupied by the dye when the enzyme binds to these columns. However, the very effective salt-mediated desorption of these columns (reminiscent of the behaviour of immobilised nucleotides) seems to be inconsistent with this hypothesis because very few kinetically determined binding constants (*e.g.*, k_M for $NADP^+$) are salt-dependent. The view expressed by Edwards and Woody[26], that these dyes do not assume a single unique conformation on different enzymes possessing the dinucleotide fold, seems to be consistent with our data.

The applications of decreasing (*cf.*, ref. 19) temperature, aprotic solvents and dyes as desorption methods have not been widely studied. A solution of Procion Red HE-3B at 4 μM eluted 6-phosphogluconate dehydrogenase, but subsequent difficulty in dissociating the dye–protein complex render this effect of limited preparative use. In the present instance we found desorption was achieved most effectively by an increase in ionic strength. Recoveries of 80–100 % were routinely obtained using a linear 0–1.0 *M* NaCl gradient. At the purely practical level, data which were collected from the behaviour of at least ten different enzymes on 60 different immobilised dyes[27], suggest that elution methods might be selected according to the following order:

$$\text{salt} \approx \text{cofactor} \approx \text{pH} \approx \text{inhibitors} > \text{substrate}$$

TABLE II

PURIFICATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *ACER PSEUDOPLATANUS* L.

Approximately 400 g (wet weight) of cells were used. Data refer to a single experiment. Similar results were obtained in five separate experiments.

Step	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification (n-fold)*	*Yield (%)*
Crude extract	685	1192	253	0.21	1.0	100
$(NH_4)_2SO_4$ fractionation	43	206	211	1.02	4.9	83
DEAE-cellulose	77	66	162	2.45	11.7	64
Procion Red Sepharose	44	16.1	131	8.14	38.8	52
2′,5′-ADP-Sepharose	9.5	1.32	105	79.5	379	41

Purification of 6-phosphogluconate dehydrogenase from Acer pseudoplatanus L.

The result of a large-scale purification of the enzyme, which included chromatography on Procion Red HE-3B-Sepharose 6B, is shown in Table II. Several contaminating proteins co-eluted with 6-phosphogluconate dehydrogenase from the latter adsorbent, but could be removed by subsequent chromatography on 2′,5′-ADP Sepharose 4B. Omission on the Procion Red step did not yield a pure product.

A final specific activity of 80 units per mg protein (pH 7.5, 25°C) was achieved with 40–50% recovery, representing a 380-fold purification relative to the crude cell-free homogenate. Gel electrophoresis of the purified enzyme indicated that the preparation was free of detectable non-enzymic protein and revealed the existence of two

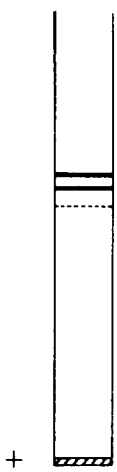

Fig. 2. Distribution of 6-phosphogluconate dehydrogenase after polyacrylamide gel electrophoresis. Purified 6-phosphogluconate dehydrogenase was applied to 7.5% polyacrylamide gels at pH 8.5 and subjected to electrophoresis at 2–3 mA per gel at 4°C for 1–2 h. The figure shows the pattern obtained by subsequent staining for either protein or 6-phosphogluconate dehydrogenase activity.

major species of 6-phosphogluconate dehydrogenase. These were estimated to occur in roughly equal amounts, as determined using either protein or enzyme-specific staining, with a minor component of higher mobility comprising less than 5% of the total protein (Fig. 2). In the presence of sodium dodecyl sulphate, two proteins with molecular weights of 45,000 and 49,000 were found in equal amounts. The existence in higher plants of isoenzymes of several reductive pentose phosphate enzymes is well documented [28–30]. Studies with both leaf[31] and non-photosynthetic tissues such as root [32,33] and endosperm[34] have shown that separate cytosolic and plastid forms of 6-phosphogluconate dehydrogenase occur. While further characterisation of the species identified by electrophoresis in the present work has not yet been performed, it is likely that the major proteins correspond to cytosolic and plastid forms of 6-phosphogluconate dehydrogenase in *Acer pseudoplatanus* L. The identity of the minor component also remains to be determined.

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Note

System for injection of continuously variable amounts of sample vapours for physico-chemical measurements by gas chromatography

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In previous publications[1–3], a computer-linked apparatus for precise measurements of gas chromatographic retention volume was described. With this equipment, the retention volume is directly measured by means of integration of the carrier gas flow-rate, without going through the concept of retention time. For such equipment, used exclusively for different types of physico-chemical measurement, special considerations must be given to the sample inlet system in comparison with a conventional analytical gas chromatograph. The sample must be injected without appreciably disturbing the carrier gas flow-rate and pressure, so that errors are not created in the retention volume measurements. The instant of injection, *i.e.* the starting point of the chromatogram, must be accurately known. Samples of widely differing sizes (down to the limit of detection) should be injected, without simultaneously introducing large volumes of solvent. It must be possible to automate the process of sample injection, to permit unattended operation and the collection of large amounts of data. It is also characteristic of the process that the samples studied are usually pure substances or simple mixtures of pure substances.

With the sample inlet system, originally designed for our apparatus[4], injection of vapours of the sample substances was performed. The amount of sample injected could be controlled by variation of the time interval during which the sample vapours were fed to the chromatographic column, and by variation of the concentration of the vapour by diluting the liquid to be vapourized with non-volatile solvents. The combination of these techniques permitted a variation of sample size over more than four decades, with some time-consuming manual operations. As it is of great interest to study the variation of the retention volume in this wide interval[5], and as the rest of the equipment is highly automatic, a pneumatic system was developed which can automatically provide the desired variation of vapour concentration, under computer control. The aim of this note is to present the technical solution of this problem.

APPARATUS

Fig. 1 shows a schematic diagram of the carrier gas system and the sample inlet system. The components for sample size variation are NV1, FR3, RV, and SC (see the legend to Fig. 1 for descriptions). The other components are present in approximately the same configuration as in previous descriptions of this apparatus (Fig. 1 in ref. 2;

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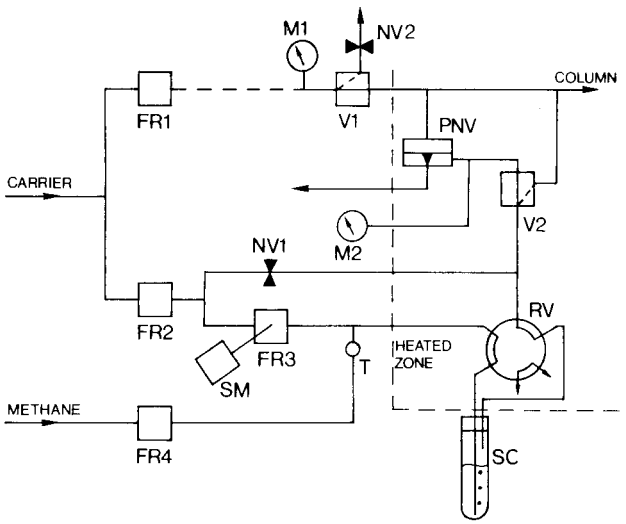

Fig. 1. Schematic diagram of the apparatus. FR1–FR4 = flow controllers (Brooks, Model 8743); M1 and M2 = manometers, 0.6 atm (Sv. Manometerfabriken, Stockholm, Sweden); NV1 and NV2 = needle valves (Brooks Model 8503); PNV = pressure-controlled needle valve (see text); RV = rotary valve (Valco, Model V-6-HPa); SC = sample chamber; SM = stepper motor (Philips, Model 9904 112 27001); T = toggle valve (Hoke, Model 1521); V1 and V2 = solenoid valves (Bürkert, Model 329, modified).

Fig. 1 in ref. 4) and are included here for completeness and ease of understanding.

In normal operation, the carrier gas passes via the flow regulator FR1 and the valve V1 to the column. Between FR1 and V1, the gas passes through various valves, a flow sensor and, optionally, a pre-column (see Fig. 1 in ref. 2). These components are irrelevant in this context. Another stream of the same gas is regulated by FR2 and afterwards split into two streams. One stream (the sample stream) is passed through a third flow regulator, FR3, a six-port valve, RV, a sample container, SC, (which is a test-tube with a ground-glass joint), to the solenoid valve V2. In front of the latter it is joined by the other stream (the diluting stream), which has passed through the needle valve NV1. The combined gas streams further pass through the pressure-controlled needle valve PNV, described below and in ref. 4.

The sample gas stream bubbles through the sample in SC and becomes approximately saturated with sample vapour. The sample concentration when the combined gas streams have reached V2 is obviously determined by the relation between the flow-rates in the diluting stream and the sample stream. This relation is adjusted by FR3 in co-operation with NV1, which is always kept in a fixed position, open about one turn. When FR3 is fully open, practically all the gas passes through the sample stream, giving maximum sample concentration. As FR3 is gradually closed, an increasing proportion of the total gas flow passes through NV1, diluting the sample. This can be continued until FR3 is completely closed. Essential for this simple arrangement to work is FR2, which ensures that the sum of the flows in the two gas streams is constant and independent of the variation in total flow resistance, which is created by FR3. By means of the valve RV, the sample container SC can be isolated from the system, permitting easy sample change, without appreciably disturbing the pressure and flow conditions in the system.

The gas stream containing the sample is directed to the column ("injected") by the simultaneous activation of V1 and V2. The carrier gas is vented to the atmosphere through the needle valve NV2. After a fixed time interval (typically 200 ms), V1 and V2 return to their original states. It is essential that the switching of the flows occurs without pressure and flow disturbances, otherwise the accuracy of the retention volume measurements is impaired. Within the present configuration the flows are easily balanced by the following procedure. With the valves V1 and V2 energized, the needle valve NV2 is adjusted until the manometer M1 shows the normal value. After this adjustment, which is not critical, the flow resistance of NV2 is approximately the same as that of the column. Secondly, still with V1 and V2 operationg, FR2 is adjusted so that the pressure drop over the column (measured by the computer system[2] is the same as normal, ensuring that the flow-rate through V2 is the same as that of the carrier gas. After these adjustments and after restoring normal gas paths, the PNV automatically adjusts its pneumatic resistance closely to that of the column, and the switching of flows can be performed with negligible disturbances.

Dead volumes usually are measured by injection of methane, introduced into the system as shown in Fig. 1. It is most convenient to measure the retention of methane separately, so RV should be turned to isolate SC from the rest of the system. All parts of the system that conduct sample vapour are heated to *ca.* 100 C to prevent condensation and adsorption. The tubings connecting SC and RV are made of glass, and those connecting RV and V2 are made of glass-lined stainless steel (SGE, Australia).

AUTOMATION

The valves V1 and V2 are controlled as described in ref. 2; they are directly connected to the computer interface and they are simultaneously energized or released by two separate computer commands, decoded in the control unit (Fig. 3 in ref. 2). The automatic variation of sample concentration is accomplished by a stepper motor, which by means of a simple gear operates the flow regulator FR3. One revolution of the system of FR3 corresponds to 96 steps by the stepper motor, so the total resolution over the fifteen possible turns is 1440 steps. The stepper motor is powered by a drive unit, built around the integrated circuit SAA 1027. The drive unit is connected to the control unit in the computer interface, and the computer can command the stepper motor to move one step in either direction. The counting of steps and the choice of direction is made by software.

RESULTS

Fig. 2 shows a typical dependence of peak area on the position of FR3. The shape of such curves are nearly independent of the nature of the sample. It is normally desired to inject samples, the sizes of which are approximately equally distributed over the available range, in such a way that the number of moles in successive injections forms a geometric series. As can be seen from Fig. 2, it is necessary to operate FR3 in a strongly non-linear way; between two of the smallest samples it should be turned only few steps, while for the largest samples several complete turns are necessary to produce the same relative change in concentration. Neither is the necessary

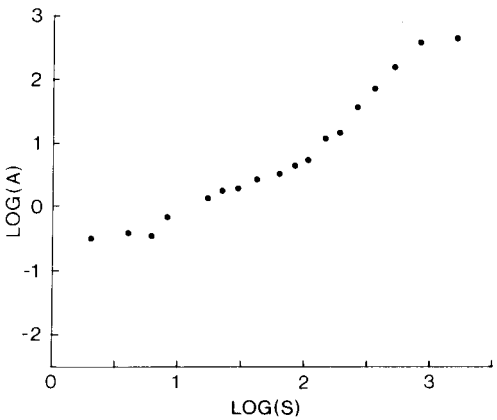

Fig. 2. The logarithm of peak area (A) as a function of the logarithm of the number of steps (S) of the stepper motor from the closed position of the flow regulator FR3. Solute: diisopropyl ether.

function linear when expressed logarithmically (as in Fig. 2). It would undoubtedly be possible to find a function that well enough approximates the curvature, but we found it more convenient to use 40 fixed positions (arranged in a table), dividing the range of possible concentrations into 40 approximately equal (in the geometrical sense) intervals. The "language" GASIC, described in ref. 3, has been extended with commands to control this process.

Usually, FR3 is initially nearly closed, giving the minimum concentration. Then for each injection (or, say, every second injection), it is successively opened to the next (or second next. . .) position, until the maximum concentration is reached, whereafter it is successively closed again in a similar manner. The flexibility of the software system permits many variations on this pattern. When the minimum concentration is reached, a turning (manual) of RV further decreases the concentrations to very low, soon undetectable values. During this time, further measurements can be made, if desired. After this, the system is clean and ready for a new sample.

This sample inlet system has been used in several investigations[6–8]. It makes possible the coverage of a wide range of solute concentrations, and thus the measurement of complete isotherms, with great convenience. This offers possibilities for detailed studies of adsorption phenomena in gas chromatography.

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Note

Determination of 5-ethyl-5-(1-ethylpropyl)barbituric acid in pentobarbital by high-performance liquid chromatography

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5-Ethyl-5-(1-ethylpropyl)barbituric acid (I) can occur in pentobarbital, 5-ethyl-5-(1-methylbutyl)barbituric acid (II). Its presence is related to the use of 2-bromopentane, containing 3-bromopentane, in the synthesis of the malonate ester. 3-Bromopentane is a frequently occurring impurity of 2-bromopentane[1,2].

Several pharmacopoeias prescribe a limit test for I and II[3–6]. The test consists in the determination of the melting range of the 4-nitrobenzyl derivative. The Ph. Eur.[3] and the Ph. Helv. VI[4] prescribe a range of 136–148°C, the BP 1973[5] 136–155°C and the USP XX[6] 136–146 C. The PH. Nord. 1963[7] uses the 4-nitrobenzyl derivative for the identification and mentions a melting range of 139–157° C. The determination of melting points of 4-nitrobenzyl derivatives of barbiturates has often been applied for identification purposes[8–10]. Their use in the determination of isomer content has been discussed in the literature[11,12]. The melting point of underivatized pentobarbital cannot be used for the determination of the isomer content[13]. Jerslev *et al.* published the melting range of the 4-nitrobenzyl derivatives of a series of mixtures of I and II[12]. From these results it can be deduced that the Ph. Eur. and Ph. Helv. VI allow *ca.* 6% of I, the BP 1973 *ca.* 12% and the USP XX *ca.* 5%. The melting range in the Ph. Nord. 1963 corresponds to *ca.* 14% of I. The pharmacopoeial test for the content of I in II is not precise and is tedious to perform. We therefore examined analysis by high-performance liquid chromatography (HPLC), which is fast, easy to perform, and more precise. The same method also allows the detection of impurities other than I, and is suitable for the identification of barbiturates. HPLC has already been mentioned in recent literature as a suitable technique for the analysis of barbituric acid derivatives[14–16]. Gas–liquid chromatography (GLC), another powerful analytical technique, usually needs derivatization of barbiturates, although capillary GLC of free barbiturates has been described[17].

EXPERIMENTAL

Samples

Pure I was kindly donated by Professor B. Jerslev, Royal Danish School of Pharmacy, Copenhagen, Denmark. Samples of pentobarbital were provided by Professor J. Bosly, Institut de Pharmacie, Liège, Belgium and by Mr. Dooms, Apotheek

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Academisch Ziekenhuis St. Rafaël, Leuven, Belgium. For some samples the manufacturer was unknown, other samples were from Abbott (Chicago, IL, U.S.A.), Rhône-Poulenc (Paris, France) and Siegfried (Zofingen, Switzerland).

Apparatus

The HPLC apparatus consisted of a Waters pump Model 6000 A (Waters Assoc., Milford, MA, U.S.A.), a Valco injector Model CV-6-UHPa-N60 equipped with a 10-μl loop (Valco, Houston, Texas), a Pye Unicam detector model LC3UV (Pye Unicam, Cambridge, Great Britain) and a Kipp & Zonen recorder model BD40 (Kipp & Zonen, Delft, The Netherlands). Columns (25 cm × 4.6 mm I.D.) were packed with SAS-Hypersil (Shandon Southern, Cheshire, Great Britain) or with Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.).

Reagents, mobile phases and operating conditions

Methanol 99+ % (Aldrich Europe, Beerse, Belgium) and distilled water were glass-distilled before use. Potassium monohydrogen phosphate and potassium dihydrogen phosphate pro analysi (E. Merck, Darmstadt, G.F.R.) were used to prepare a 0.2 *M* buffer of pH 6.0. Mobile phases used for the determination of isomer content consisted of methanol–water–0.2 *M* phosphate buffer pH 6.0 mixture in a ratio of 37:58:5 for the Hypersil column and 55:40:5 for the Zorbax column. (See figures for composition of other mobile phases.) Mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and the paper speed at 5 mm/min. All separations were carried out at room temperature (*ca.* 20°C). For the determination of the isomer content the detector was set at 240 nm and 0.04 a.u.f.s. (See figures for detector setting of other chromatograms.)

Preparation of samples, reproducibility of the method

A 0.040% m/v solution of barbital in methanol–water (1:1) was used as the internal standard solution (IS). For the samples examined it was checked that no sample peak eluted together with this IS. The sample (25.0 mg) was dissolved in 5.0 ml of IS and the volume was made up to 10.0 ml with methanol–water (1:1). In order to prepare a calibration curve, 20.0 mg of I was dissolved in 20.0 ml of methanol–water (1:1). Aliquots (3.0, 2.0 and 1.0 ml) of this solution were diluted with 5.0 ml of IS and the volume was made up to 10.0 ml with methanol–water (1:1). Samples (10 μl) were repeatedly injected on the Hypersil solumn and peak height ratios were determined. A calibration curve for the ratio peak height/peak height IS *versus* concentration in mg/ml was obtained with a linear regression $y = 2.732x - 0.0012$ and a correlation coefficient of 0.999. When pure II was used to prepare a calibration curve, a linear regression $y = 2.014x - 0.0015$ and a correlation coefficient of 0.999 were obtained. From this it is deduced that if II were used to prepare a calibration curve to determine I, the results were to be multiplied by a conversion factor of 0.736. The reproducibility of the method was checked by injecting six times a sample of II containing I. A mean value of 4.73% of I with a standard deviation of 0.09 was obtained. The limit of detection (three times the baseline noise) was *ca.* 0.1%.

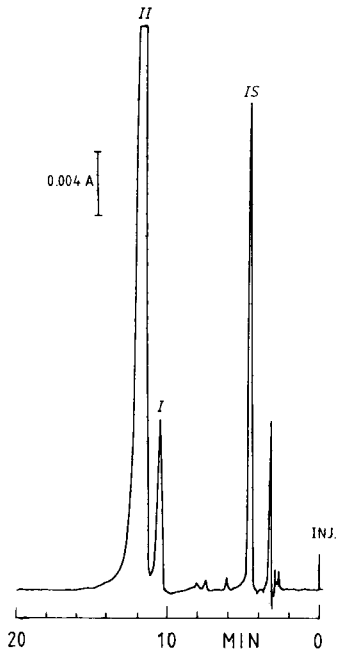

Fig. 1. Separation of 5-ethyl-5-(1-ethylpropyl)barbituric acid (I) and pentobarbital (II) in sample A3. IS (internal standard), barbital; column, SAS-Hypersil, 25 cm × 4.6 mm I.D.; mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (37:48:5); flow-rate 1.0 ml/min; detection: 240 nm, 0.04 a.u.f.s.; paper speed, 5 mm/min.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained with the Hypersil column. I and II are well separated. The Zorbax column gives similar separations. Impurities other than I were also observed in the pentobarbital samples. The results are compiled in Table I. They were obtained with the Hypersil column. The unknown impurities are indicated by their relative retention times, the retention time of barbital (IS) being 1.0. Since the structures of these impurities were unknown, their content was expressed as isomer I. This procedure gives an estimation of the purity of the different samples.

All the samples from manufacturer C are free of isomer I. The samples from manufacturers A and B contain *ca.* 4–6% of I. Sample 3, of unknown origin, contains 5.9% of I. The melting point of the 4-nitrobenzyl derivative of this sample was 142°C, which is lower than 148°C, the value derived from literature results for an isomer content of *ca.* 6%[12]. The melting point of the nitrobenzyl derivative of sample 5, of unknown origin, was 136°C. No sample contained more than a total of 3% of unknown impurities, calculated as I.

Fig. 2 shows the separation of a series of barbiturates, obtained on the Hypersil column and on the Zorbax column. The elution on Hypersil, having a particle size of 5 μm and a shorter aliphatic chain, is faster. The aliphatic chain is probably trimethylsilyl[18]. The twelve barbiturates are not all separated. Decreasing the methanol content of the mobile phase does not improve the separation of the pairs allobarbital–

TABLE I
IMPURITY CONTENT OF PENTOBARBITAL

	Sample	Impurity content (%, m/m)*						
		Isomer I	Retention time of unknown impurities relative to barbital (IS) = 1.0					
			1.27	1.35	1.67	1.81	1.87	2.33	4.07
Manufacturer A	1	5.2	0.3	0.4					
	2	4.9	0.3	0.4		0.5			
	3	5.0		0.3	0.3	0.2			
	4	4.8		0.4	0.3	1.8	1.1		
Manufacturer B	1	4.7	0.3	0.3					
	2	6.1		0.4		0.5			
Manufacturer C	1	ND**		0.5			1.1		
	2	ND		0.3					
	3	ND		0.9		0.6			
	4	ND		0.3		0.3			
	5	ND		0.4	0.4	0.4			0.7
	6	ND		0.4	0.5		0.5		0.6
Manufacturer unknown	1	ND		0.3					
	2	ND		0.5			0.7		
	3	5.9		0.3			0.4		
	4	ND		0.3			0.4	0.4	
	5	ND		0.2	0.4		0.4		0.5

* Impurities other than isomer I are calculated as I.
** ND = Not detectable (less than 0.1 %).

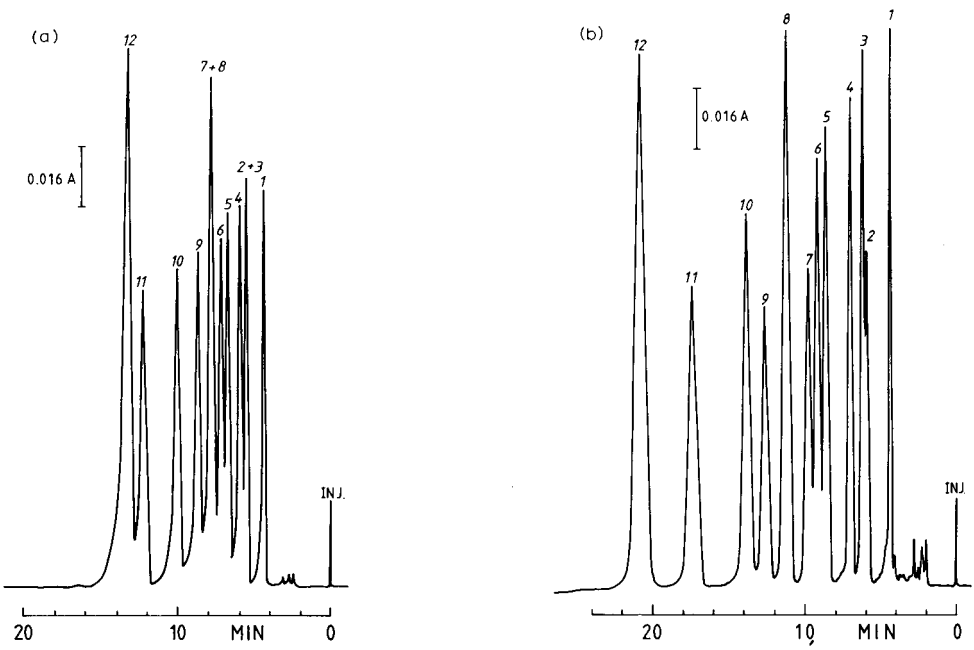

Fig. 2. Separation of barbital (1), allobarbital (2), phenobarbital (3), aprobarbital (4), secbutabarbital (5), butobarbital (6), butalbital (7), methylphenobarbital (8), 5-*sec*.-butyl-5-ethyl-2-thiobarbituric acid (9), pentobarbital (10), secobarbital (11) and thiopental (12). Column, 25 cm × 4.6 mm I.D.; flow-rate, 1.0 ml/min; detection, 254 nm, 0.16 a.u.f.s.; paper speed, 5 mm/min. (a) SAS-Hypersil. Mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (40:55:5). (b) Zorbax C_8. Mobile phase, methanol–water–0.2 *M* phosphate buffer pH 6.0 (55:40:5).

phenobarbital and butalbarbital–methylphenobarbital. The elution on the Zorbax column, with 7-μm particles and a octylsilyl chain, is slower but the separation is more complete.

The use of a pH 6.0 buffer for the preparation of the mobile phase has several advantages. The column packing material will have a longer lifetime than with more alkaline mobile phases, containing for example ammonium carbonate, which has been proposed for use in HPLC of barbiturates[16]. Furthermore, since it is well known that the absorbance of barbiturates is influenced by the pH of the solution, the peak height can change when different qualities of distilled water and methanol are used for the preparation of the mobile phase. This was observed in the routine use of HPLC of barbiturates during practical exercises by students.

The results show that the isomer content of pentobarbital, as well as the content of other impurities, can easily be determined by HPLC on reversed-phase materials of different chain lengths, using buffered methanol–water mixtures as the mobile phase. With some adjustment of the methanol content in the mobile phase, the same system has also been used for the analysis of other barbiturates.

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Note

Chromatofocusing of pyridoxalated and polymerized human haemoglobin

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(Received July 31st, 1981)

Chromatofocusing offers a new approach to analytical and preparative fractionation of heterogeneous protein samples according to the different p*I* values of their individual components[1,2]. It combines classical isoelectric focusing with column chromatography. In the present paper we describe results obtained for pyridoxalated and polymerized stroma-free haemoglobin (SHF-P and SFH-PG, respectively), which are being investigated as one of the potential blood substitutes for infusion therapy[3]. The results were compared to those obtained by means of thin-layer analytical isoelectric focusing, where it was possible to detect up to about 23 protein fractions[4].

MATERIALS AND METHODS

Pyridoxalated human haemoglobin SFH-P and its polymerized derivative SFH-PG were prepared according to refs. [3–5]. Before application to the chromatofocusing column, the samples were equilibrated with the eluent [Polybuffer™ 96 (Pharmacia, Uppsala, Sweden) diluted 1:9 with degassed distilled water and adjusted with acetic acid to pH 6.0] by using gel filtration on Sephadex G-25. Chromatofocusing was done according to the manufacturer's instruction manual[6]. The Polybuffer exchanger PBE™ 94 gel (Pharmacia) was settled and equilibrated with the starting buffer, 0.025 mol/l Tris–acetic acid, pH 8.3, at a relatively low linear flow-rate in two different columns. The bed dimensions were 15 × 0.9 cm and 27 × 1.6 cm, respectively. All buffers were degassed before use. The samples were applied by first running 5 ml of eluent, followed by 5 ml of SFH-P or SFH-PG. The applied sample was then eluted with Polybuffer, pH 6.0. The absorbances of the eluted proteins were measured at 280 nm using a Unicam spectrophotometer. The pH gradient was established by measuring the pH in each collected fraction using a Radiometer PHM 64 pH meter (Radiometer, Copenhagen, Denmark).

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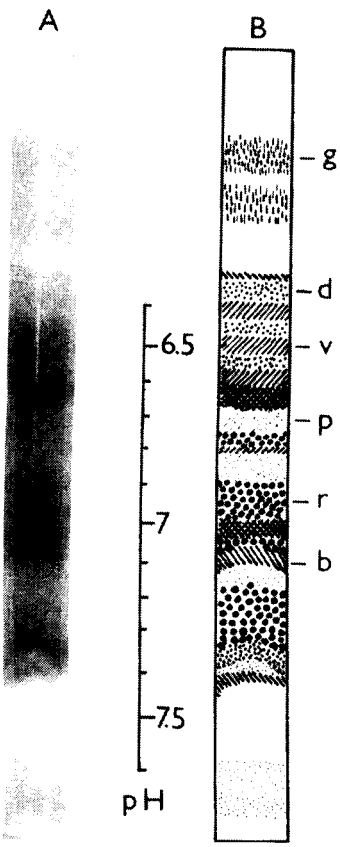

Fig. 1. Chromatofocusing of pyridoxalated human haemoglobin (SFH-P). A, Photograph of the column (panchromatic negative) 5.5 h after the start; B, scheme of A with zone colours (g = grey-green; d = deep purple; v = violet-purple; p = pink; r = red; b = brown). Bed dimensions: 15 × 0.9 cm. Flow-rate: 2.5 ml cm^2 h^{-1}.

RESULTS AND DISCUSSION

Fig. 1 shows the development of a SFH-P pattern 5.5 h after the start, just before the first haemoglobin fraction began to leave the column (15 × 0.9 cm). About 21–25 haemoglobin bands were usually observed, having different tints of red, deep purple, pink, brown and violet. (The black and white photograph is a poor guide to the degree of separation achieved.) The comparison of the positions and sequence of the main bands with their p*I* values (estimated from Fig. 2, curve 1) enabled the construction of an approximate pH scale valid for the column at this time. The assumed pH gradient was relatively smooth and linear. The two grey-green zones near to the start remained at that position even after all other fractions had been eluted. Their elution was difficult and usually incomplete, and they cannot unambiguously be assigned.

The large number of differently coloured zones was due chiefly to the simultaneous presence of the oxygenated and deoxygenated forms of various pyridoxa-

lated and native haemoglobin subfractions, as well as of their ferro and ferri forms which mutually differ in their p*I* values. After elution from the column into open test-tubes, all fractions (except the brown-red ferrihaemoglobins) rapidly gained the typical bright red coloration of oxyhaemoglobin. In our previous flat bed isoelectric focusing (IEF) study of SFH-P and SFH-PG in the presence of atmospheric oxygen[4] all pyridoxalated fractions with p*I* values less than 6.8 also had a bright red colour. Evidently, the deep purple and violet bands in the column correspond to the deoxy forms of haemoglobin subfractions and derivatives.

The column had been equilibrated and eluted by deaerated (*i.e.*, deoxygenated) Polybuffer, while the SFH-P samples were not deoxygenated before and during application to the column. Thus, the respective oxy–deoxy equilibria were established on the column during chromatofocusing. Under such "partly anaerobic" conditions there is a rough but important analogy between the oxy- and deoxyhaemoglobins in the column and those present in the periphery of the blood stream. In contrast to IEF, chromatofocusing may give more reliable information on the expected heterogeneity of SFH-P and SFH-PG "*in vivo*". This heterogeneity involves not only the proteins "as such" but also the state of their haem groups. On the other hand, the different p*I* values of oxy- and deoxyderivatives make it difficult to compare the patterns achieved by chromatofocusing under anaerobic and by IEF under aerobic conditions.

Fig. 2 shows that the general shapes of the elution patterns of two batches of SFH-P were similar, although differences in details were found. A smooth pH gradient was formed during chromatofocusing on both columns. A better separation of the SFH-P subfractions was achieved on the larger column, where the flow-rate per cm^2 was 3.2 times slower. A slow flow-rate was important also during sample application in order to prevent irregularities and distortions of the horizontal positions of bands. Direct observation of the fractionation of SFH-P on the column gave markedly more qualitative information than the elution curves alone (when measured at 280 nm). There was a discrepancy between chromatofocusing and thin-layer analyti-

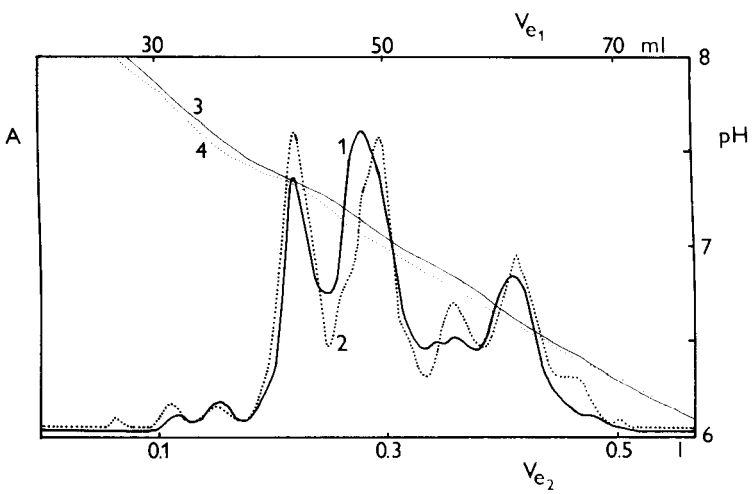

Fig. 2. Elution curves of pyridoxalated haemoglobin (SFH-P). 1 = SFH-P60 (the same batch and run as in Fig. 1), elution volumes V_{e_1} (upper scale); 2 = SFH-P61, run on a 27 × 1.6 cm bed at a flow-rate of 8 ml cm^{-2} h^{-1}, V_{e_2} values; 3 and 4 = pH gradients corresponding to curves 1 and 2, respectively.

cal IEF in the content of native and pyridoxalated haemoglobin subfractions in SFH-P. Thus, with IEF[4] more than 60% of haemoglobin was modified into acid derivatives of $pI < 6.8$; chromatofocusing suggested only about 40%. This difference might be due to the loss of pyridoxalated protein bound in the dark g zones in the column (Fig. 1). Generally similar patterns were achieved also during chromatofocusing of polymerized SFH-PG which had been treated with 10 mg glutaraldehyde per gram of haemoglobin[4], lyophilized with sucrose[7] and stored for 4 weeks at 20°C (batch No. SFH 56 PG/10).

It can be concluded that column chromatofocusing gives very good results when investigating the heterogeneity of chemically modified (pyridoxalated and polymerized) haemoglobins of new variants of blood substitutes. Even though there is a complication with SFH-P and SFH-PG in that the column is contaminated by an unidentified coloured material, the informational output of chromatofocusing is similar and in certain respects even higher than that of isoelectric focusing. However, thin-layer analytical IEF has the advantage of being suitable for the simultaneous analysis of large numbers of very small samples. The two methods could therefore be used in parallel.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. R. Berglund and L. Nyström (Pharmacia Fine Chemicals, Uppsala, Sweden) for helpful instructions, and to A. Velechovská and J. Štěrbíková for their skilful technical assistance.

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Errata

J. Chromatogr., 208 (1981) 209–216
Page 210, section *Capillary tubing preparation*, 2nd line, "hydrochloric acid, acetone, and methylene chloride" should read "hydrochloric acid, distilled water, acetone, and methylene chloride".

Page 211, section *Superox-4 pretreatment and deactivation*, last line, "300°C" should read 330°C".

J. Chromatogr., 211 (1981) 201–212
Page 205, the 5th line should read:

$$x_2 = 10^{V_{\text{methanol}}/(V_{\text{methanol}}+V_{\text{water}})} \text{ (where } V = \text{volume)}$$

J. Chromatogr., 214 (1981) 307–315
Page 313, Figs. 4 and 5 should be interchanged. (Please note that the legends are correct as printed.)

Vol. 219, No. 3

journal of chromatography news section

SYMPOSIUM PROGRAM

SEVENTEENTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '82"

The Seventeenth International Symposium on Advances in Chromatography will be held April 5–8, 1982, at Caesars Palace in Las Vegas, Nevada. This meeting directly follows the one held by the American Chemical Society in Las Vegas.

A total of 75 papers will be presented at the Symposium representing contributions from 18 countries. A special feature of this meeting will be a symposium on Biomedical Chromatography honoring Professor E.C. Horning. There will also be an exposition of the latest instrumentation and books. Two-day intensive short courses (in the following areas: (a) capillary gas chromatography, (b) reversed-phase liquid chromatography, (c) gas chromatography–mass spectrometry, and (d) high-performance thin-layer chromatography) will be presented on the Saturday and Sunday preceeding the meeting.

Registration should be made in advance. The programs, short course information, registration forms, and hotel reservation forms can be obtained from:

Professor Albert Zlatkis
Chemistry Department
University of Houston
Houston, TX 77004, U.S.A.
Tel. (713) 749-2623

The detailed program of the Symposium is given below.

MONDAY, APRIL 5, 1982

L.S. Ettre, presiding

9:00 Welcome to Symposium
9:15 Presentation of Tswett Chromatography Medal

CONTEMPORARY CHROMATOGRAPHY

9:30 B. Holmstedt (Karolinska Institutet, Stockholm, Sweden) – Use of gas phase analysis in studies of ethnobotanical and archaeological materials.
10:00 E.sz. Kováts and F. Riedo (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) – Adsorption from liquid mixtures and liquid chromatography.
10:30 Intermission
10:45 E. Jellum and A.K. Thorsrud (University of Oslo, Oslo, Norway) – Multicomponent analyses of human tissues and body fluids using capillary GC–MS and high resolution two-dimensional electrophoresis.

11:15 J.C. Giddings, M.N. Myers, F.-S. Yang, J.-P. Chang and K.D. Caldwell (The University of Utah, Salt Lake City, UT, U.S.A.) – A study of the transition from normal to steric field-flow fractionation.

11:40 H. Engelhardt and H. Miller (Universität des Saarlandes, Saarbrücken, G.F.R.) – Irregular and spherical silica – Does geometry influence selectivity.

Monday Afternoon

CAPILLARY COLUMN GAS CHROMATOGRAPHY

M. Novotny, presiding

2:00 R.E. Kaiser (Institute of Chromatography, Bad Dürkheim, G.F.R.) – Optimization in capillary gas chromatography.

2:20 D.F. Ingraham (Phillip Morris, Richmond, VA, U.S.A.) and C.F. Shoemaker and W. Jennings (University of California, Davis, CA, U.S.A.) – Optimization of liquid phase mixtures.

2:40 L. Blomberg, J. Buijten, K. Markides and Th. Wännman (University of Stockholm, Stockholm, Sweden) – Peroxide initiated *in situ* curing of some silicone gums for capillary columns. II.

3:00 S.R. Lipsky and W.J. McMurray (Yale University, New Haven, CT, U.S.A.) – Cross linking of silicone stationary phases on fused-silica glass capillary columns in gas chromatography: a preliminary assessment of techniques.

3:30 Intermission

W. Jennings, presiding

3:40 J.A. Rijks and C. Tilburg (Eindhoven University of Technology, Eindhoven, The Netherlands), P. Sandra and M. Roelenbosch (University of Ghent, Belgium) and G. Schomburg and H. Usmann (Max-Planck-Institut für Kohlenforschung, Mulheim/Ruhr, G.F.R.) – Testing of capillary column performance. Part I. Introduction and results of a round-robin experiment.

4:00 R.C. Kong, M.L. Lee, Y. Tominaga, R. Pratap, M. Iwao and R.M. Castle (Brigham Young University, Provo, UT, U.S.A.) and S.A. Wise (National Bureau of Standards, Washington, DC, U.S.A.) – Capillary gas chromatographic resolution of polycyclic aromatic sulfur heterocycles in coal liquids using mixed liquid crystal–silicone gum phases.

4:20 R.G. Mathews, J. Torres and R.D. Schwartz (Pennzoil Products Company, Shreveport, LA, U.S.A.) – Acetylene treatment of glass capillary columns.

4:40 F.I. Onuska (National Water Research Institute, Burlington, Ontario, Canada) – Novel technique for static coating of fused-silica wall-coated open tubular columns.

TUESDAY, APRIL 6, 1982

BIOMEDICAL CHROMATOGRAPHY – A TRIBUTE TO E.C. HORNING

W.J.A. VandenHeuvel, presiding

9:00 S.E. Barrow, K.A. Waddell, M. Ennis, C.T. Dollery and I.A. Blair (University of London, London, England) – Analysis of picomolar concentrations of 6-oxo-prostaglandin $F_{1\alpha}$ in biological fluids.

9:20 J.C. Craig, L.D. Gruenke and T.-L. Nguyen (University of California, San Francisco, CA, U.S.A.) – Simultaneous analysis of imipramine and its metabolite desipramine in biological fluids

9:40 H. Brandenberger and F.B.Ch. West (University of Zürich, Zürich, Switzerland) – Chromatographic detection by dual mass spectrometry.

10:00 D.M. Desiderio, S. Yamada, F.S. Tanzer and G. Fridland (University of Tennessee, Memphis, TN, U.S.A.) – High-performance liquid chromatography and field desorption mass spectrometric measurement of endogenous neuropeptides in biologic tissue at the picomole level.

10:20 Intermission

A. Karmen, presiding

10:30 P. Capella, G. Lercker and L.S. Conte (Della Universita Degli Studi, Bologna, Italy) – Study of fatty acid oxidation products and their application in sunflower seed oil selection.

10:50 K. Nakatsu, J.F. Brien, H. Taub, W.J. Racz and G.S. Marks (Queens University, Kingston, Ontario, Canada) – 3,3′,4,4′-Tetrachlorobiphenyl: gram quantity synthesis and chromatographic assessment.

11:10 E. Wetzel, Th. Kuster and H.-Ch. Curtius (Kinderspital Zürich, Zürich, Switzerland) – A split system applicable as GC- MS interface and as eluent splitter for specific GC detectors.

11:30 R.E. Finnigan, M.S. Story and T.Z. Chu (Finnigan Corporation, Sunnyvale, CA, U.S.A.) – Instrument development through cooperative efforts with users.

Tuesday Morning

ENVIRONMENTAL PROBLEMS: GAS CHROMATOGRAPHY

R.D. Schwartz, presiding

9:00 P.D. Goldan, F.C. Fehsenfeld and M.P. Phillips (National Oceanic and Atmospheric Administration, Boulder, CO, U.S.A.) – Detection of carbon monoxide at ambient levels with an N_2O sensitized electron-capture detector.

9:20 K.W.M. Siu and W. Auc (Dalhousie University, Halifax, Canada) – Studies on the mechanism of the electron-capture detector.

9:40 K. Jacob, G. Schnabl, C. Krauss and W. Vogt (Universität München, München, G.F.R.) – Mono- and bifunctional, P-containing reagents for GLC determination of amines.

10:00 R.M. Barkely, S. Singhawangcha, M.A. Wizner, R.S. Hutte, M.K. Conditt, E.J. Williams and R.E. Sievers (University of Colorado, Boulder, CO, U.S.A.) – New gas chromatographic techniques applied to environmental problems.

10:20 Intermission

D. Nurok, presiding

10:30 B.S. Middleditch (University of Houston, Houston, TX, U.S.A.) – Volatile constituents of the produced water effluent from the Buccaneer gas and oil field.

10:50 F.W. Karasek, R.E. Clement and A.C. Viau (Guelph-Waterloo Centre for Graduate Work in Chemistry, Waterloo, Ontario, Canada) – Distribution of PCDD and other toxic compounds generated on fly ash particulates in municipal incinerators.

11:10 F.F. Andrawes (American Cyanamid, Stamford, CT, U.S.A.) – Analysis of formaldehyde by gas chromatography using helium ionization detection.

11:30 F. Shunbo, F. Abdilly and P. Literathy (Kuwait Institute of Scientific Research, Kuwait, Kuwait) – Baseline assessment of marine pollution along the coastline waters of Kuwait.

11:50 S.A. Estes, P.C. Uden and R.M. Barnes (University of Massachusetts, Amherst, MA, U.S.A.) – Plasma emission spectral detection for high resolution gas chromatographic study of group IV organometallic compounds.

Tuesday Afternoon

BIOMEDICAL CHROMATOGRAPHY - A TRIBUTE TO E.C. HORNING

R.N. Stillwell, presiding

2:00 C.J.W. Brooks, W.J. Cole, J.H. Borthwick and G.M. Brown (University of Glasgow, Glasgow, Scotland) – Characterization of dihydroarenediols and related compounds by GC–MS: comparison of derivatives.

2:20 C.D. Pfaffenberger and A.J. Peoples (University of Miami School of Medicine, Miami, FL, U.S.A.) – Long-term variation study of blood plasma levels of chloroform and related purgeable compounds.

2:40 R.J. Hamilton, S.F. Mitchell and P.A. Sewell (Liverpool Polytechnic, Liverpool, England) – Detection of lipids in high-performance liquid chromatography.

3:00 W.A. König and I. Benecke (Universität Hamburg, Hamburg, G.F.R.) – A new procedure for gas chromatographic enantiomer separation of chiral alcohols.

3:20 Intermission

B. Holmstedt, presiding

3:30 H. Frank (Universität Tübingen, Tübingen, G.F.R.) and T. Bailey (University of Washington, Seattle, WA, U.S.A.) – Determination of the enantiomers of 3-methoxy-4-hydroxy phenylethylene glycol by GC–MS: application to study stereoselectivity of metabolism.

3:50 S. Takatsuto, B. Ying, M. Morisaki and N. Ikekawa (Tokyo Institute of Technology, Tokyo, Japan) – Microanalysis of brassinolide and its analogs by gas chromatography and GC–MS.

4:10 K.D. Haegele, R.G. Alken, J. Grove and J. Bartholeyns (Merrell International Research Center, Strasbourg, France) – Pharmacokinetics and metabolism of some irreversible enzyme inhibitors as determined by HPLC and combined GC–MS.

4:30 D.J. Harvey, B.R. Martin, J.T. Leuschner and W.D.M. Paton (Oxford University, Oxford, England) – Gas chromatographic and mass spectrometric studies on the metabolism and pharmacokinetics of the cannabinoids from marihuana.

4:50 C. Chiabrando, A. Noè, A. Noseda, R. Reginato and R. Fanelli (Istituto di Richerche Farmacologiche "Mario Negri", Milan, Italy) – Metabolic profiles of prostaglandins and related compounds by high resolution gas chromatography–mass spectrometry.

Tuesday Afternoon

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

R.E. Kaiser, presiding

2:00 R.M. Becker, W.B. Gorman and D. Nurok (Purdue University at Indianapolis, Indianapolis, IN, U.S.A.) – Aspects of binary solvents optimization in thin-layer chromatography.

2:20 S.A. Schuette and C.F. Poole (Wayne State University, Detroit, MI, U.S.A.) – Unidimensional, sequential separation of PTH-amino acids by high-performance thin-layer chromatography.

2:40 R. Segura and X. Navarro (Autonomous University of Barcelona, Bellaterra, Spain) – Determination of lipid classes using submicroliter amounts of plasma, by thin-layer chromatography and "*in situ*" spectrofluorometry.

3:00 L. Zhou, H. Shanfield and A. Zlatkis (University of Houston, Houston, TX, U.S.A.) – Quantitative determination of lecithin and sphingomyelin at nanogram levels by HPTLC using fluorescence.

WEDNESDAY, APRIL 7, 1982

BIOMEDICAL CHROMATOGRAPHY – A TRIBUTE TO E.C. HORNING

A. Frigerio, presiding

9:00 J.J. Vrbanac, W.E. Braselton, J.F. Holland and C.C. Sweeley (Michigan State University, East Lansing, MI, U.S.A.) – Automated qualitative and quantitative metabolic profiling analysis of urinary steroids by a gas chromatography–mass spectrometry data system.

9:20 H. Miyazaki, M. Ishibashi, G. Idzu, Y. Hashimoto and Y. Furuta (Nippon Kayaku Co., Tokyo, Japan) – Simultaneous determination of glyceryl trinitrate and its principle metabolites, glyceryl 1,2- and 1,3-dinitrates in human plasma by gas chromatography–negative ion chemical ionization–selected ion monitoring.

9:40 M. Tetsuo, H. Eriksson and J. Sjövall (Karolinska Institutet, Stockholm, Sweden) – GC–MS analysis of endogenous estradiol in plasma and uterine cytosol of rats.

10:00 J. Szafranek, J. Kusmierz and W. Czerwiec (University of Gdańsk, Gdańsk, Poland) – GC–MS investigations of high boiling crude oil alkane fractions.

10:20 Intermission

B.S. Middleditch, presiding

10:30 K. Tanaka (Yale University School of Medicine, New Haven, CT, U.S.A.) – Compilation of gas chromatographic retention indices of 155 metabolically important organic acids, and their use in detection of patients with organic acidemia.

10:50 L. Johansson and J. Vessman (AB Hässle, Mölndal, Sweden) – Determination of tocainide in human plasma and urine by gas chromatography and nitrogen selective detection after Schiff base formation.

11:10 Y. Hasegawa, M. Kunihara and Y. Maruyama (Japan Upjohn Research Laboratories, Takasaki-City, Japan) – Determination of choline and acetylcholine in blood by newly developed pyrolysis gas chromatography–mass spectrometry.

11:30 H.M. Liebich, H.J. Buelow and R. Kallmayer (Medizinische Universitätsklinik, Tübingen, G.F.R.) – Quantification of endogenous aliphatic alcohols in serum and urine.

Wednesday Morning

NEW DEVELOPMENTS IN LIQUID CHROMATOGRAPHY

Cs. Horváth, presiding

9:00 T. Crispin and I. Halász (Universität des Saarlandes, Saarbrücken, G.F.R.) – Determination of the pore size distribution, by exclusion chromatography, of ion-exchange polymers which swell in water.

9:20 E. Grushka, I. Atamna and C. Gilon (Hebrew University, Jerusalem, Israel) – Separation and detection of N-methylated amino acids using reversed-phase HPLC.

9:40 B. Bidlingmeyer (Waters Associates, Milford, MA, U.S.A.) – Investigation of the retention mechanism for reverse-phase ion-pair liquid chromatography using UV-absorbing eluent modifiers.

10:00 A. Karmen, S.K. Lam and G. Malikin (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) – Therapeutic drug monitoring by chromatography.

10:20 Intermission

V. Berry, presiding

10:30 E. Katz, K. Ogan and R.P.W. Scott (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.) – Dispersion in packed beds – Fact and theory.

10:50 M. Novotny and V.L. McGuffin (Indiana University, Bloomington, IN, U.S.A.) – Element-specific thermionic detection in micro-HPLC.

11:10 A. Aszalos (Food and Drug Administration, Rockville, MD, U.S.A.) – The use of high-pressure liquid chromatography in the analysis of antibiotics.

11:30 J.R. Chrétien (Université Paris VII, Paris, France) – Experiment design in chromatography: original contribution of the DARC topological system with EURECAS data-base and CHROMATO-DATA bank.

Wednesday Afternoon

GENERAL CHROMATOGRAPHY

C.F. Poole, presiding

2:00 R. Brazell (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) – Analysis of volatile constituents in physiological fluids from tobacco smoke exposed animals.

2:20 C.L. Guillemin (Rhone-Poulenc, Paris, France) – Computerized auto-control of on-line process or laboratory gas and liquid chromatographs.

2:40 J.K. Haken and J.A. Obita (University of New South Wales, Kensington, Australia) – The chromatographic analysis of aromatic polyhydrazides, oxalyl arylene polyhydrazides and aromatic poly(amidehydrazides) after alkali fusion.

3:00 M. Koel, M. Kaljurand and E. Küllik (Academy of Sciences of the Estonian SSR, Tallinn, U.S.S.R.) – Correlation chromatography: calibration, the method by the exponential dilution.

3:20 Intermission

R.S. Juvet, presiding

3:30 T. Hanai, K.C. Tran and J. Hubert (Université de Montréal, Montréal, Quebec, Canada) – Prediction of the retention times for aromatic acids in liquid chromatography.

3:50 E.P. Grimsrud and M.J. Connolly (Montana State University, Bozeman, MT, U.S.A.) – Spacial distribution of ion densities within ^{63}Ni ionization cells.

4:10 F.S. Hsu, B.W. Good and M.E. Parrish (Phillip Morris, Inc., Richmond, VA, U.S.A.) – Pattern recognition for gas phase of cigarette smoke by glass capillary gas chromatography.

4:30 J.F. Parcher, M.I. Selim and P.J. Lin (University of Mississippi, University, MS, U.S.A.) – Adsorption of polar solutes on liquid-modified supports.

THURSDAY, APRIL 8, 1982

LIQUID CHROMATOGRAPHY

E. Grushka, presiding

9:00 H. Kalász and Cs. Horváth (Yale University, New Haven, CT, U.S.A.) – High-performance displacement chromatography of corticosteroids; scouting for displacer and analysis of the effluent by thin-layer chromatography.

9:20 V.V. Berry and R.E. Shansky (Polaroid Corporation, Cambridge, MA, U.S.A.) – Universal liquid chromatography methods. III. New techniques for sensitive, low-wave wavelength, full-gradient runs.

9:40 K. Klotter, T. Schlabach, R. Cunico and R.E. Majors (Varian Associates, Walnut Creek, CA, U.S.A.) – Improved post-column detection of nitrogenous-based compounds in HPLC.

10:00 N.J. D'Allura and R.S. Juvet, Jr. (Arizona State University, Tempe, AZ, U.S.A.) – Quantitative determination of completely unresolved HPLC peaks.

10:20 Intermission

10:30 S.K. Lam and A. Karmen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) – Stereoselectivity of histidine and histidine derivatives in the resolution of optical isomers of dansyl amino acids.

10:50 P. Kucera and H. Umagat (Hoffman-La Roche, Inc., Nutley, NJ, U.S.A.) – Total amino acid analysis using pre-column fluorescence derivatization.

11:10 G. Schill (University of Uppsala, Uppsala, Sweden) – Reversed-phase HPLC with detection by ion pairing agent.

11:30 W. Voelter (Universität Tübingen, Tübingen, G.F.R.) – Application of a precolumn technique for HPLC investigations of drug metabolism.

NEW BOOKS

Liquid chromatography of polymers and related materials III, edited by J. Cazes, Marcel Dekker, New York, Basel, 1981, VIII + 299 pp., price SFr. 108.00, ISBN 0-8247-1514-4.

Steroid analysis by HPLC: Recent applications, edited by M.P. Kautsky, Marcel Dekker, New York, Basel, 1981, XIV + 397 pp., price SFr. 135.00, ISBN 0-8247-1324-9.

Liquid–liquid equilibrium and extraction – A literature source book, by J. Wisniak and A. Tamir, Elsevier, Amsterdam, Oxford, New York, 1981, XXII + 1438 pp., price Dfl. 450.00, US$ 191.50, ISBN 0-444-42023-1.

Chromatography of environmental hazards, Vol. 4, Drugs of abuse, by L. Fishbein, Elsevier, Amsterdam, Oxford, New York, 1982, X + 490 pp., price Dfl. 225.00, US$ 95.75, ISBN 0-444-42024-X.

Handbook of laboratory distillation – with an introduction into the pilot plant distillation, by E. Krell, Elsevier, Amsterdam, Oxford, New York, 1981, *ca.* 576 pp., price Dfl. 225.00, US$ 95.75, ISBN 0-444-99723-7.

Analytical methods in geochemical prospecting (Handbook of Exploration Geochemistry, Vol. 1), by W.K. Fletcher, Elsevier, Amsterdam, Oxford, New York, 1981, XII + 256 pp., price Dfl. 140.00, US$ 59.50, ISBN 0-444-41930-6.

Geochemical exploration 1980 (Proc. 8th Int. Geochemical Exploration Symp., Hannover, April 10–15, 1980), edited by A.W. Rose and H. Gundlach, Elsevier, Amsterdam, Oxford, New York, 1981, XII + 724 pp., price Dfl. 360.00, US$ 153.25, ISBN 0-444-42012-6.

Reminiscense and reflections, by H. Krebs, Oxford University Press, Oxford, London, 1982, 250 pp., price *ca.* £ 10.00, ISBN 0-19-854702-1.

MEETINGS

3rd INTERNATIONAL SYMPOSIUM ON ISOTACHOPHORESIS

The 3rd International Symposium on Isotachophoresis (organised by the Hannover Isotachophoresis Group) will be held on June 1–4, 1982, in Goslar (near Hannover), G.F.R.

The symposium topics will include all aspects of isotachophoresis, from theoretical treatments of phenomena associated with this method, to instrumental developments and all types of applications in the field of analytical chemistry and biochemistry, as well as the uses of isotachophoresis in experimental biology and medicine, and industrial applications. Both applications on an analytical and on a preparative scale are relevant, either in free-flow systems, gels or other media.

Further details may be obtained from Dr. C.J. Holloway, Organising Chairman, ITP 82, Abteilung für klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. Tel.: (0511) 532 2838/2834.

DIOXIN 82: 3rd INTERNATIONAL SYMPOSIUM–WORKSHOP ON CHLORINATED DIOXINS AND RELATED COMPOUNDS

Dioxin 82: the 3rd International Symposium–Workshop on Chlorinated Dioxins and Related Compounds will be held at the International Conference Center in Salzburg, Austria, on October 12–14, 1982. The symposium is sponsored by the International Association of Environmental Analytical Chemistry and the International Society of Toxicological and Environmental Chemists in collaboration with different national and international agencies and groups. The preliminary organizing committee consists of R.W. Frei and O. Hutzinger (The Netherlands), E. Merian and G. Reggiani (Switzerland), F. Pocchiari (Italy) and B.M. Shepard and A.L. Young (U.S.A.).

The aim of this symposium is to bring together specialists from different fields of dioxin research, which will allow the problem to be treated in an interdisciplinary fashion. Similar to the first successful workshop in Rome and the second event in Washington, October 1981, emphasis will be on discussion and exchange of information. Symposium proceedings will be published. Topics will be: Incineration sources; analysis and standards; fate, distribution and levels; laboratory safety and disposal practices; toxicology and risk assessment; and legal and regulatory aspects.

For further information please contact Dr. E. Merian, Im Kirsgarten 22, CH - 4106 Therwil, Switzerland.

Electron Capture – Theory and Practice in Chromatography

edited by A. ZLATKIS, *Houston, TX, USA and* C.F. POOLE, *Detroit, MI, USA*

JOURNAL OF CHROMATOGRAPHY LIBRARY – Volume 20

Sept. 1981 xii + 418 pages
Price: US $76.50/ Dfl. 180.00
ISBN 0-444-41954-3

This book provides the first comprehensive coverage of all aspects of the theory, design, operation and applications of the electron capture detector (ECD) from the chromatographer's point of view. In addition, an up-to-date look at the ancillary techniques of selective electron-capture sensitization, atmospheric pressure ionization and plasma chromatography has been included. ECD users will find the solutions to instrumental and technical problems which arise during practice particularly valuable. These have been derived from the experiences of the internationally distinguished team of authors.

Each chapter has been prepared by experts in their field and provides an in-depth coverage of its topic. The basic theory of the mechanisms of electron capture detection is included. Practical sections form the bulk of the book and are devoted to such topics as the construction and operating principles of the detector, including the establishment of instrument design criteria, and the different methods of derivatization. A more personal touch is provided by the inventor of the ECD, J.E. Lovelock, in his review of the development of the technique. Other chapters illustrate the importance of ECD in trace analysis in environmental and biomedical research. A unique feature is the extensive tabulation of all the pertinent data concerning the use of ECD in gas and liquid chromatography.

For those analytical chemists who use chromatography in their research, this book should become a standard text.

CONTENTS: Chapter 1. The electron-capture detector – A personal odyssey *(J.E. Lovelock).* 2. The design and operation of the electron-capture detector *(C.F. Poole and A. Zlatkis).* 3. Theory of electron capture *(W.E. Wentworth and E.C.M. Chen).* 4. Selective electron-capture sensitization *(F.C. Fehsenfeld, P.D. Goldan, M.P. Phillips and R.E. Sievers).* 5. Oxygen-doping of the carrier gas in electron-capture detection *(E.P. Grimsrud).* 6. Wide-range calibration of electron-capture detectors *(R.E. Kaiser and R.I. Rieder).* 7. Response of the electron-capture detector to compounds with natural electrophores *(J. Vessman).* 8. Sensitive derivatives for the determination of organic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis).* 9. The detection of inorganic and organometallic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis).* 10. Environmental applications of the electron-capture detector – pesticides *(W.P. Cochrane and R.B. Maybury).* 11. Environmental applications of the electron-capture detector – dioxins *(F. Bruner).* 12. The electron-capture detector as a monitor of halocarbons in the atmosphere *(P.G. Simmonds).* 13. Biomedical applications of the electron-capture detector *(J. Vessman).* 14. Negative ion atmospheric pressure ionization mass spectrometry and the electron-capture detector *(E.C. Horning, D.I. Carroll, I. Dzidic and R.N. Stillwell).* 15. Electron-capture process and ion mobility spectra in plasma chromatography *(F.W. Karasek and G.E. Spangler).* 16. The electron-capture detector as a detector in liquid chromatography *(U.A. Th. Brinkman).* Subject index.

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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
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Chromatographic Reviews							220/1					220/2		220/3
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2	224/3	225/1	225/2	226/1	226/2

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

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

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 11.

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 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, crystal structure, surface structure, morphology and adsorption properties. For the first time the different groups of oxides are treated together in order to emphasise their similarities and differences.

This major reference work is mainly directed to electrochemists and those working on catalysis. It will also be useful to those in the fields of materials science, physical chemistry, surface and colloid chemistry and in areas where oxide surfaces may play a major role as in chromatography and photochemistry.

CONTENTS: Chapters. 1. Elec nic Band Structure of Oxides Metallic or Semiconducting Characteristics *(J. M. Honig).* 2. Chemisorption and Catalysi on Metal Oxides *(A. Cimino a S. Carrà).* 3. Oxide Growth and Oxygen Evolution on Noble Metals *(L. D. Burke).* 4. Electro chemistry of Lead Dioxide *(J. P. Pohl and H. Rickert).* 5. Pr perties of Spinel-Type Oxide Electrodes *(M. R. Tarasevich a B. N. Efremov).* 6. Physico-chemical and Electrochemical Properties of Perovskite Oxide *(H. Tamura, Y. Yoneyama and Y. Matsumoto).* 7. Properties of Conductive Transition Metal Oxides with Rutile-Type Structu *(S. Trasatti and G. Lodi).* 8. Fundamental Properties of t Oxide/Aqueous Solution Inter-face *(D. N. Furlong, D. E. Yates and T. W. Healy).* 9. Reactions o Hydrogen and Organic Substar ces with and at Anodic Oxide Films at Electrodes *(B. E. Conway).* 10. Oxygen and Chlorine Evolution at Conductiv Metallic Oxide Anodes *(S. Trasa and G. Lodi).* 11. Technological Impact of Metallic Oxides as Anodes *(A. Nidola).*

Part A:
1980 xvi + 366 pages.
US $72.25/Dfl. 170.00.
ISBN 0-444-41912-8.

Part B:
1981 xiv + 336 pages.
US $72.25/Dfl. 170.00.
ISBN 0-444-41988-8.

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