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CHROMATOGRAPHIC AND ELECTROPHORETIC BEHAVIOUR OF AMINO ACIDS ARISING FROM POST-TRANSLATIONAL REACTIONS IN PROTEINS

M. HORÁKOVÁ and Z. DEYL
Physiological Institute, Czechoslovak Academy of Sciences, Prague-Krč (Czechoslovakia)
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INTRODUCTION

Although the separation procedures applied to the identification and quantitation of the "classical twenty" amino acids have generally become routine in recent years, the chromatographic and electrophoretic properties of the less common amino acids have not yet been surveyed. This, perhaps, reflects the fact that rare amino acids occur mostly in highly specific proteins, where they contribute substantially to the specificity, and therefore naturally attract the attention of a limited number of research workers. These rather rare amino acids originate in post-translational reactions and are, in fact, the result of *in vivo* modifications of proteins. This means also that uncommon amino acids that arise during protein catabolism, whether in plants or in animal materials such as biological fluids, are omitted from this review. For similar reasons, other compounds related to amino acids that can be called unusual amino acids, *e.g.*, hydroxamic acids, are not considered here.

In this review we have attempted to survey only the separational properties of uncommon amino acids. As far as the history, chemistry and biological aspects are concerned, we refer readers to more qualified authors (for a review, see Uy and Wold[179]).

Over 140 compounds arising from post-translational covalent modifications of proteins can be traced in the literature. Perhaps the following list of parent amino acids together with the products arising from the post-translational modifications will help the reader to understand the limits of this review, bearing in mind that we have considered only those products which preserve the character of an amino acid. The existence of some of the amino acids summarized below is based on indirect evidence and therefore appropriate reliable separational procedures are lacking. For more details, see the review by Uy and Wold[179].

Primary amino acids	*Secondary derived amino acids*
Alanine	N-Acetylalanine, N-methylalanine
Arginine	N^{ω}-Methylarginine, N^{ω},N^{ω}-dimethylarginine, N^{ω},$N^{\omega'}$-dimethylarginine, ADP-ribosylarginine, citrulline, ornithine, arginyl-protein
Asparagine	Aspartic acid, N^{4}-(N-acetylglucosaminyl)asparagine, N^{ε}-(β-aspartyl)-lysine
Aspartic acid	Aspartic α-amide, N-acetylaspartic acid, O^{4}-phosphonoaspartic acid
Cysteine	Cystine, S-mercaptocysteine, S-galactosylcysteine, S-glucosylcysteine, S-cysteinyl-haeme, 8α-(S-cysteinyl)flavin thiohemiacetal, dehydroalanine
Glutamic acid	Glutamic α-amide, γ-carboxyglutamic acid, γ-methylglutamic acid

Glutamine	Glutamic acid, glutaminamide, pyroglutamic acid, N^{ε}-(γ-glutamyl)-lysine
Glycine	Glycinamide, N-acetylglycine, N-formylglycine, N-glucuronylglycine
Histidine	Histidinamide, π-methylhistidine, π-phosphonohistidine, τ-phosphonohistidine, 4-iodohistidine, 8α-(π-histidyl)flavin, 8α-(τ-histidyl)-flavin
Isoleucine	
Leucine	Leucyl-protein
Lysine	N^{ε}-Methyllysine, N^{ε}-dimethyllysine, N^{ε}-trimethyllysine, N^{ε}-phosphonolysine, N^{ε}-acetyllysine, N^{ε}-(phosphopyridoxyl)lysine, N^{ε}-lipoyllysine, N^{ε}-biotinyllysine, N^{α}-mureinlysine, allysine, dehydrolysinonorleucine, lysinonorleucine, allysine aldol, dehydroallysine aldol, dehydromerodesmosine, merodesmosine, dihydrodesmosines, desmosines, tetrahydrodesmosines, "compound 285", (allysine aldol), histidine δ-Hydroxylysine (Hyl); the following compounds are derivatives of Hyl and the δ has been omitted: N-trimethylhydroxylysine, O^{δ}-(β-D-galactosyl)hydroxylysine, hydroxyallysine, (dehydrohydroxylysino)-norleucine, hydroxylysinonorleucine, (dehydrohydroxylysino)hydroxynorleucine, (hydroxylysino)hydroxynorleucine, syndesine, dehydrohydroxymerodesmosine, (dehydrohistidino)hydroxymerodesmosine, (hydroxyallysinealdol)histidine
Methionine	Methioninamide, N-acetylmethionine, N-formylmethionine
Penylalanine	Phenylalaninamide, β-hydroxyphenylalanine, O^{β}-glycosyl-β-hydroxyphenylalanine, phenylalanyl-protein
Proline	Prolinamide, 3,4-dihydroxyproline, 4-hydroxyproline (4Hyp), 3-hydroxyproline (3Hyp), O^{4}-arabinosylhydroxyproline, O^{4}-galactosylhydroxyproline
Serine	Pyruvate, N-acetylserine, O^{β}-phosphonoserine, O^{β}-(ADP-ribosylphosphono)serine, O^{β}-methylserine, O^{β}-(4′-phosphonopantetheinyl)-serine, O^{β}-xylosylserine, O^{β}-mannosylserine, O^{β}-(N-acetylgalactosaminyl)serine, O^{β}-galactoserine
Threonine	α-Ketobutyrate, N-acetylthreonine, O^{β}-phosphonothreonine, O^{β}-methylthreonine, O^{β}-fucosylthreonine, O^{β}-mannosylthreonine, O^{β}-(N-acetylgalactosaminyl)threonine, O^{β}-galactosylthreonine
Tyrosine	Tyrosinamide, tyrosine O^{4}-sulphate, 3-iodotyrosine, 3,5-diiodotyrosine, 3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine, 3,5-dibromotyrosine, 5-bromo-3-chlorotyrosine, 3,5,3′-triiodothyronine, 3,5,3′,5′-tetraiodothyronine, 3,3′-bityrosine, 3,3′,5′,3″-tertyrosine, O^{4}-adenylyltyrosine, O^{4}-uridylyltyrosine, β-hydroxytyrosine, O^{β}-glycosyl-β-hydroxytyrosine, dihydroxyphenylalanine, proteinyltyrosine
Valine	Valinamide, N-acetylvaline

In general, it is necessary to stress the possibility of artefact formation during handling of the protein, *e.g.*, with naturally occurring lysine-derived cross-links. A new unusual amino acid must always be judged carefully before stating the final verdict. Thus, Patchornik and Sokolovsky[127] reported that alkaline treatment of a

number of cysteine-containing proteins results in the presence of an addition product originating from the reaction of lysine with dehydroalanine residues. Such reactions do not occur in a living body but they are of practical importance as long they contribute to the insolubility of alkali-treated wool and are mentioned in this review.

Like every other, this review is based on literature data available to us at the time of writing the manuscript. We are aware, of course, that in the meantime some new unusual amino acids may have been observed or some of those claimed as an integral part of a protein may be proved to be artefacts.

1. METHYLATED AMINO ACIDS

This section deals with methylated lysines, arginines and histidines. In addition, several methylprolines have been isolated from natural sources, mainly from antibiotics, but none of them has so far been isolated from a protein. These include *cis*-3-methyl-L-proline, *trans*-4-methyl-L-proline, *cis*-4-methyl-L-proline and *cis*-5-methylproline. Their behaviour during amino acid analysis, electrophoresis and paper and gas chromatography was described by Mauger *et al.*[100].

1.1. Methylated lysines

1.1.1. Ion-exchange chromatography and behaviour during amino acid analysis

One of the first separations of methylated amino acids was achieved by Tallan *et al.*[167] in 1954, using a 46 × 0.9 cm column of Dowex 50 and phosphate buffer (pH 6.6–6.8) (6.2 g of Na_2PO_4 + 7.85 g of $NaH_2PO_4 \cdot H_2O$ per litre). With urine as

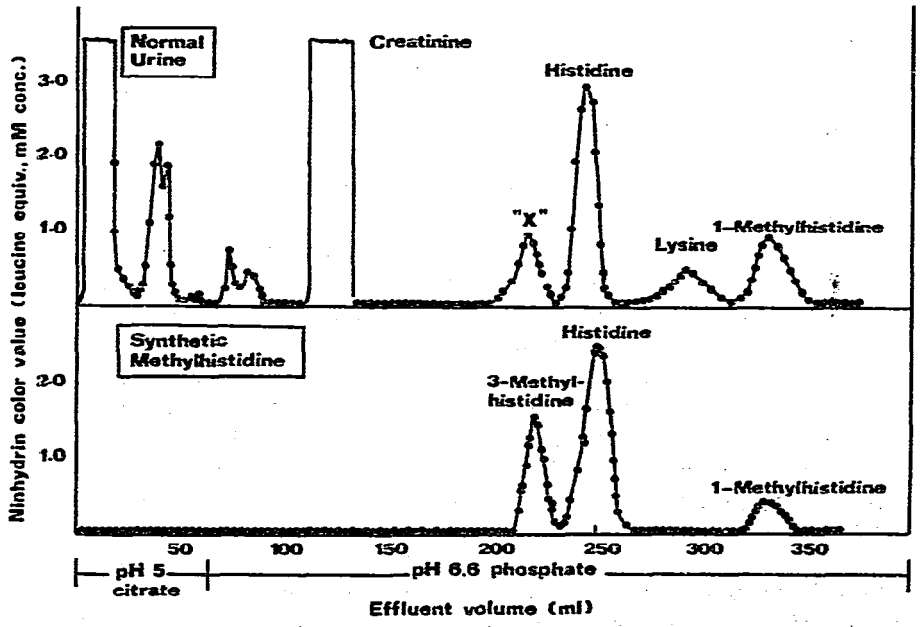


Fig. 1 (a) Separation of the basic ninhydrin-positive components of urine on a 46 × 0.9 cm column of Dowex 50. (b) Separation on a 46 × 0.9 cm column of Dowex 50 of the methylhistidines obtained by methylation of phthaloylhistidine.

the sample chromatography at room temperature gave the separation shown in Fig. 1.

At a very early stage in the analysis of N-methylated amino acids (Paik and Kim[125]), it was demonstrated that for the separation of ε-N-methyl- and ε-N-dimethyllysine a higher pH (5.84) at 28° is necessary with Beckman UR-30 resin. Later, when applied to muscle proteins, basically the same system offered the possibility of separating all of the naturally occurring methylated derivatives of lysine.

Soon the necessity for comparing the results obtained with respect to methylated lysines with those from various amino acid analysers arose. This was carried out by Kuehl and Adelstein[88], who exploited the differences in their chromatographic behaviour for the determination of ε-N-monomethyllysine and ε-N-trimethyllysine in rabbit myosin. Columns of dimensions 55 × 0.9 cm were used at a flow-rate of 68 ml/h with 0.35 *N* sodium citrate buffer at different pH values (Table 1). The results for system A were obtained with a Beckman 120 amino acid analyser using Beckman AA 15 resin, and another analyser was used to test the properties of Beckman UR 30 resin. Whereas with Beckman AA 15 resin no separation between the mono- and dimethyl derivatives was obtained, with Beckman UR 30 resin at pH 5.84 problems arose in the separation of 3-methylhistidine and ammonia, which was unsatisfactory if large amounts of the latter were present in the sample.

TABLE 1

RETENTION TIMES OF BASIC AMINO ACIDS WITH VARIOUS AMINO ACID ANALYSER SYSTEMS

In each instance a 55-cm column was used and elution at a flow-rate of 68 ml/h was carried out with 0.35 *N* sodium citrate buffer at the pH and temperature indicated. Pattern A was obtained on a Beckman 120 amino acid analyser, using Beckman AA15 resin; patterns B and C were obtained on another Beckman analyser, using Beckman UR-30 resin. MML, DML and TML = mono-, di- and trimethyllysine, respectively.

Residue	*System A: pH 5.28, 50°*	*System B: pH 5.84, 27°*	*System C: pH 5.28, 27°*
Ornithine	175	154	190
Lysine	182	163	204
MML	199	189	231
DML	199	203	244
TML	190	211	244
Histidine	231	225	323
Ammonia	284	245	297

Alternatively, the separation of methylated lysines and histidines can be effected with Phoenix XX 860-0 resin, and a 40 × 0.9 cm column has been recommended by Huszar and Elzinga[75] for this purpose. The chromatographic separation was carried out at 50.3°, using 0.35 *M* citrate buffer (pH 5.36) as the eluent at a flow-rate of 80 ml/h. Neutral and acidic amino acids were eluted by 30 min, tyrosine appeared at 36 min, phenylalanine at 40 min, lysine at 91 min, ε-N-methyllysine at 121 min, ammonia at 136 min, homoserine lactone at 165 min and arginine at 218 min.

With Durrum-type resins, the separation of methylated lysines is easy and

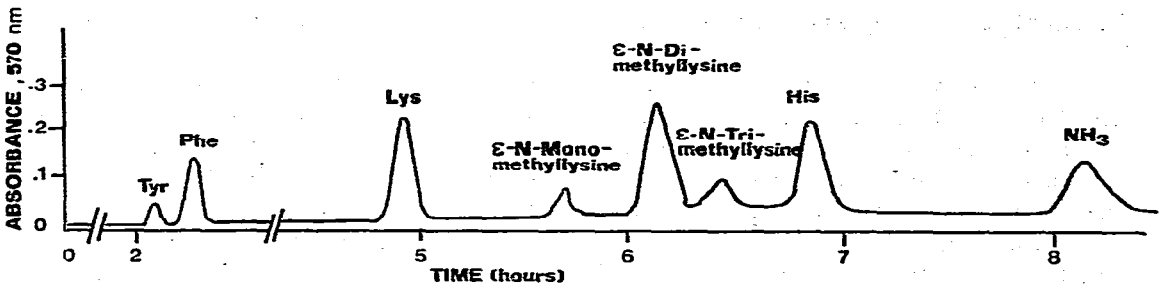


Fig. 2. Separation of ε-N-methyllysines on a column (48 × 0.9 cm) of Durrum DC-2 resin, eluted at a flow-rate of 28.2 ml/h with 0.35 *M* sodium citrate buffer (pH 5.84) at 28°. Different amounts of the various amino acids were used. Acidic and neutral amino acids elute before 1 h; arginine elutes at 18 h. (De Lange *et al.*[33].)

effective, as shown in Fig. 2; a 48 × 0.9 cm column was used in this instance at a flow-rate of 28.2 ml/h using citrate buffer (pH 5.84) at 28°. The bulk of acidic and neutral amino acids were eluted within 1 h and arginine emerged after 18 h.

A more rapid separation of methylated lysines on Aminex A-5 has been devised by Seely *et al.*[154]. Conditions for the separation and retention times are summarized in Table 2, together with comparative data for two other resins. It can be seen that though shorter runs can be carried out, the separation on Aminex A-5 is a reasonable compromise between the speed of separation and the resolution achieved. If the flow-rate is doubled to 68 ml/h, the separation of methylated lysines becomes incomplete, as shown in Fig. 3. On the other hand, for incomplete separations of methylated amino acids or for the determination of one of the whole group, routine procedures are frequently satisfactory. Thus, with the pH 5.28 buffer it is possible to separate ε-N-methyllysine at 50° on a 15-cm column. In this instance the N-methylated derivatives emerge between lysine and histidine, with retention volume 62 ml (Comb *et al.*[24]). Another method for the separation of ε-N-monomethyllysine was published by Beckerton *et al.*[10].

Occasionally, preparative procedures for methylated amino acids are required in order to isolate appropriate standards from natural sources. The procedure developed by De Lange *et al.*[32] for the isolation of ε-N-trimethyllysine can serve as a good example. A 108 × 0.9 cm column packed with Dowex 50-X8 (200–400 mesh) was recommended for this purpose. The column was washed with 500 ml of 1 *M* pyridine to remove acidic and neutral amino acids, then with 1 *M* pyridine–acetic acid (pH 5.2). The column was eluted at a flow-rate of 31 ml/h and fractions of 5.2 ml were collected. Histidine and ε-N-trimethyllysine were eluted in fractions 15–19, lysine in fractions 27–33 and arginine in fractions 95–105.

ε-N-Trimethyl-L-δ-hydroxylysine phosphate and ε-N-trimethyl-L-δ-hydroxylysine are also methylated lysines. They were reported to be constituents of diatomaceous cell-wall proteins and their separation was attempted by numerous techniques such as thin-layer chromatography, ion-exchange chromatography and paper electrophoresis. Their mutual separation on an amino acid analyser and by electrophoresis is good, but a poor separation is obtained by thin-layer chromatography. The results obtained by Nakajima and Volcani[114] are summarized in Table 3.

TABLE 2

DATA OBTAINED FROM THE BECKMAN AMINO ACID ANALYSER RELATING TO THE SEPARATION OF BASIC AMINO ACIDS

Compound	*Eluting buffer, 0.35 N sodium citrate; ninhydrin flow-rate, 34 ml/h*					
	Eluent pH 5.28, 68 ml/h, 57°				*Eluent pH 6.48*, 34 ml/h, 25°*	
	50 × 0.9 cm AA-15 resin		*7 × 0.9 cm PA-35 resin*		*15 × 0.9 cm Aminex A-5 resin*	
	Time (min)	*Constant***	*Time (min)*	*Constant***	*Time (min)*	*Constant***
L-Lysine	164	22.06	25	23.80	84	43.5
ε-N-Methyl-L-lysine	178	20.15	25	21.02	97.5	39.7
ε-N-Dimethyl-L-lysine	178	19.48			105	39.2
ε-N-Trimethyl-L-lysine	159	18.48			111.5	35.5
D,L-Homolysine	228	22.77	33	24.77		

* Prepared by adding NaOH to pH 5.28 buffer.

** Mean of five determinations for different amounts. The reproducibility was within ±3%.

1.1.2. Paper chromatography

Several types of mobile phases have been used with varying success for the separation of methylated lysines. For example, *m*-cresol–phenol was suggested by Paik and Kim[125]. The R_F values are given in Table 4. Other systems that offer good separations of methylated lysines are those using phenol–cresol–borate mobile phases

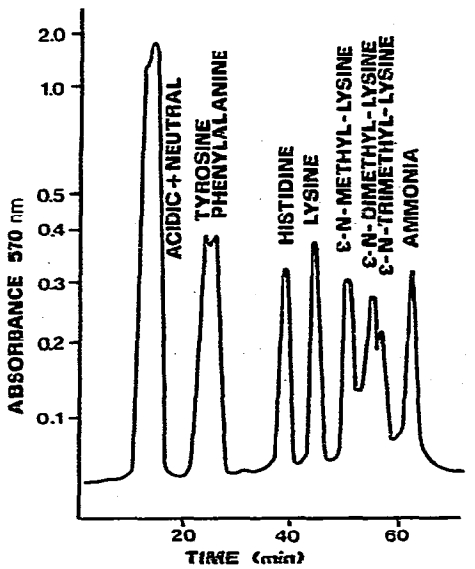


Fig. 3. Chromatography of ε-N-methyllysines in the presence of a calibration mixture on a 15 × 0.9 cm column of Aminex A-5 resin eluted with 0.35 *N* sodium citrate (pH 6.48) at 25°. Buffer flow-rate, 68 ml/h. The mixture contained, apart from ammonia, 0.25 μmole of each component.

TABLE 3

COMPARISON OF CHROMATOGRAPHIC AND ELECTROPHORETIC MIGRATIONS, ELUTION TIME AND CHEMICAL REACTION OF ε-N-TRIMETHYL-L-δ-HYDROXYLYSINE PHOSPHATE (THLP) AND ε-N-TRIMETHYL-L-δ-HYDROXYLYSINE (THL)

Procedure	*THLP*	*THL*
Thin-layer chromatography: R_F in solvents:*		
I	0.13	0.28
II	0.12	0.18
III	0.09	0.11
Electrophoretic mobility** in solvents:		
A, pH 1.9	−2.4 cm	−6.1 cm
B, pH 6.9	+2.6 cm	−7.1 cm
C, pH 9.0	—	−12.1 cm
Emergence of peak on amino acid analyser:		
Dus *et al.*[36] system	51 min	200 min
Hamilton[66] system	105 min	948 min
Colour reactions:***		
Ninhydrin	Blue-violet	Blue-violet
Iodoplatinate	Blue	Blue
Dragendorff	Orange	Orange
Ammonium molybdate	Blue	Negative

* Chromatograms run on Eastman cellulose chromatogram sheets (6064) without fluorescent indicator. Solvents: I = *n*-butanol–acetic acid–water (12:3:5); II = *n*-butanol–acetic acid–water (4:1:5); III = *sec.*-butanol–*tert.*-butanol–butanone–water (4:4:8:5) + 0.5% diethylamine.

** Carried out in the electrophoresis apparatus described by Crestfield and Allen[27]; Whatman paper No. 1 was used with a voltage of 33.3 V/cm in the following solvents: A, 0.6 *N* formic acid–2 *N* acetic acid buffer (pH 1.9) for 20 min; B, 0.02 *M* sodium phosphate buffer (pH 6.9) for 10 min; C, 0.05 *M* borate buffer (pH 9.0) for 50 min.

*** Ninhydrin solution, 0.2% in *n*-butanol. Iodoplatinate: after Jackson and Moss[78]. Dragendorff: after Katiyone and Hashimoto[84]. Ammonium molybdate after Burrows *et al.*[19].

(De Lange *et al.*[32]) or propanol–ammonia–water (8:1:1) on paper (Kuehl and Adelstein[88]).

A good separation of ε-N-dimethyllysine, ε-N-monomethyllysine and ε-N-trimethyllysine was obtained by two-dimensional paper chromatography using pyridine–acetone–3 *M* ammonia solution (50:30:25) in the first direction followed by propanol-2–formic acid–water (4:1:1) in the second direction. In the same system, as indicated in Fig. 4, both dimethylarginines are separated from residual amino acids, but their mutual separation is incomplete and further identification of the position of the methyl group is difficult (Kakimoto and Akazawa[83]).

TABLE 4

R_F values of methylated lysines

Amino acid	R_F
Lysine	0.11–0.12
ε-N-Monomethyllysine	0.33–0.36
ε-N-Dimethyllysine	0.70–0.73

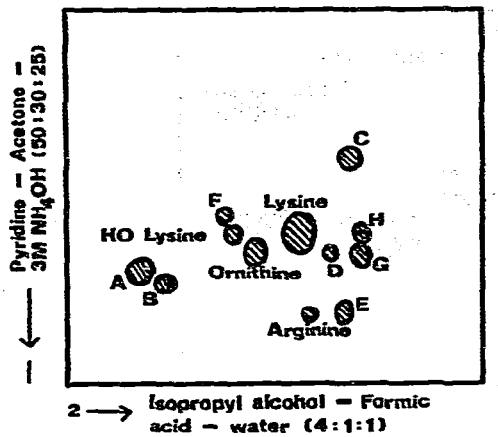


Fig. 4. Two-dimensional paper chromatography of the aliphatic basic amino acid fraction of human urine. A, glucosyl-galactosyl-δ-hydroxylysine; B, galactosyl-δ-hydroxylysine; C, N^{ε},N^{ε}-dimethyllysine; D, N^{ε}-methyllysine; E, N^{ε},N^{ε},N^{ε}-trimethyllysine; F, unidentified; G, N^{G},N'^{G}-dimethylarginine; H, N^{G},N^{G}-dimethylarginine.

Another mobile phase for the separation of methylated lysines is butanol-1–pyridine–acetic acid–water (15:10:3:12). The mobilities relative to lysine are ε-N-monomethyl- and ε-N-dimethyllysine 1.18 and arginine 1.38. Trimethyllysine is not separated from lysine. Other butanol-based systems do not give even a partial resolution of methylated lysines.

1.1.3. Electrophoretic separations

Electrophoretic separation of methylated amino acids was used by Kakimoto and Akazawa[83] for monitoring column effluents. An Amberlite IR-120 column (114 × 6 cm) was eluted with ammonia solution of increasing concentration and 100-ml fractions were collected and subjected to electrophoresis as shown in Fig. 5. Electrophoresis was carried out on Toyo-Roshi No. 51 paper in a pH 3.6 buffer (pyridine–acetic acid–water, 5:50:945) using a potential gradient of 100 V/cm. The running time was 30 min. It is obvious that high-voltage electrophoresis is not a convenient method for separating these amino acid derivatives, as all methylated lysines, for instance, merge into a single zone together with lysine.

The separation of histidine and ε-N-trimethyllysine can be achieved by paper electrophoresis for 35 min at pH 1.9 and 3000 V. Under these conditions trimethyllysine moves slightly ahead of histidine. Also, methylated lysines migrate as a single zone and in a mixture they cannot be distinguished from arginine.

For additional attempts to carry out electrophoretic separations, see p. 237.

1.2. Methylated arginines

1.2.1. Behaviour during amino acid analysis

Sometimes it is difficult to follow only a single type of methylated amino acid, as in practice methylated amino acids derived from different parent compounds occur together. Thus, on several occasions the separation properties of methylated arginines,

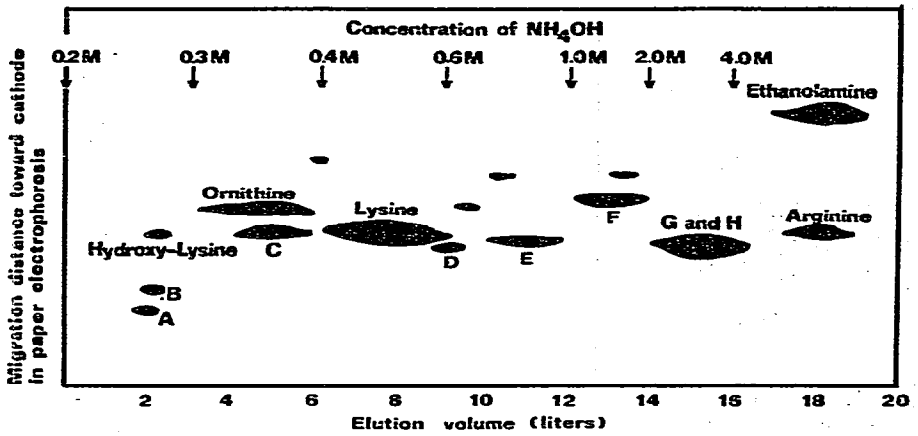


Fig. 5. Elution pattern of the aliphatic basic amino acids of human urine. The fraction obtained from 120 l of urine was chromatographed on a 114 × 6 cm column of Amberlite IR-120 (NH_4^+) with increasing concentrations of ammonia solution as indicated. Fractions of about 100 ml were collected. Aliquots were examined by paper electrophoresis at pH 3.6 and compounds were rendered visible with ninhydrin. Elution volumes are shown on the abscissa and the migration distance of each substance in electrophoresis on the ordinate. The identities of substances A–H are given in Fig. 4.

especially with regard to the separation of methylated lysines, have already been mentioned (*cf.*, p. 232).

A good separation of N^G,N^G-dimethylarginine but only a partial resolution of N^G-monomethylarginine from arginine was reported by Deibler and Martenson[31]. Using a 30 × 0.9 cm column packed with Durrum DC-2A resin they were able to obtain the chromatogram shown in Fig. 6. The starting buffer was 0.35 *N* sodium citrate (pH 5.8 or 5.84) and the temperature 28°. After 200 min the buffer was changed to 0.35 *N* sodium citrate (pH 4.7) and the temperature was increased to 55.5°. The resulting change in absorbance of the eluent occurred at a time when no amino acids were being eluted. The flow-rate applied was 45 ml/h with an initial back-pressure of 300 psi.

In a flat-bed arrangement *n*-butanol–acetic acid–water (12:3:5) on paper gives

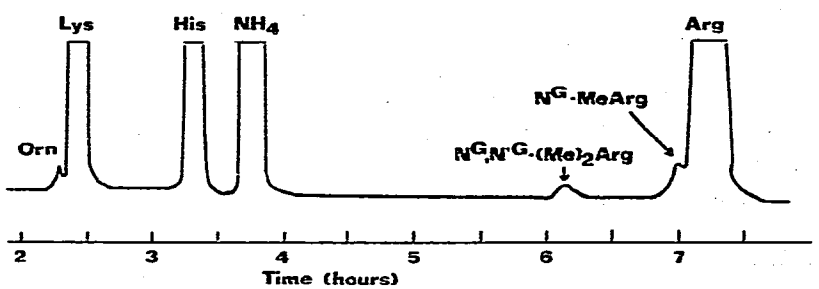


Fig. 6. Chromatography of basic amino acids present in the acid hydrolysate of basic protein extracted from purified guinea-pig myelin. The sample of hydrolysate (equivalent to 0.6 mg of lyophilised protein) contained 0.88 μmole of arginine. The pH of the starting buffer was 5.80. N^G,N'^G-$(Me)_2$Arg and N^G-MeArg = N^G,N^G-dimethyl- and N^G-monomethylarginine, respectively. N^G,N^G-Dimethylarginine, if present, would have been eluted 20 min before N^G,N'^G-dimethylarginine.

the following R_F values for methylated arginines and their neighbours: monomethylarginine 0.12, dimethylarginine 0.17, monomethylhistidine 0.13 and lysine 0.10. Acetic acid–formic acid buffer (pH 2.1) can be used in electrophoresis for the separation of methylated arginines (Baldwin and Carnegie[6]).

1.3. Methylated histidines

1.3.1. Behaviour during amino acid analysis

For the ion-exchange separation of 3-methylhistidine from histidine, the Beckman analyser (15 × 0.6 cm column, pH 5.28 citrate buffer, 0.35 *M* with respect to Na^+, temperature 52.5°) can be used (Johnson *et al.*[81]).

An elution profile of methylated histidines and ε-N-dimethyllysine is presented in Fig. 7 (Gershey *et al.*[53]). The separation was carried out with a Beckman 120 B amino acid analyser, using a 50 × 0.9 cm column operated at 28° and eluted with sodium citrate buffer (pH 5.84) (0.35 *M* citrate). Under these conditions, a clear separation was achieved; dimethyllysine emerged from the column at 333 min, preceded by monomethyllysine, while 3-methylhistidine did not emerge until 422 min after histidine and before 1-methylhistidine. It should be pointed out that if the temperature is kept at 50°, the methyllysines are not separated from lysine and methylhistidines are not separated from histidine. Another type of separation is shown in Fig. 8.

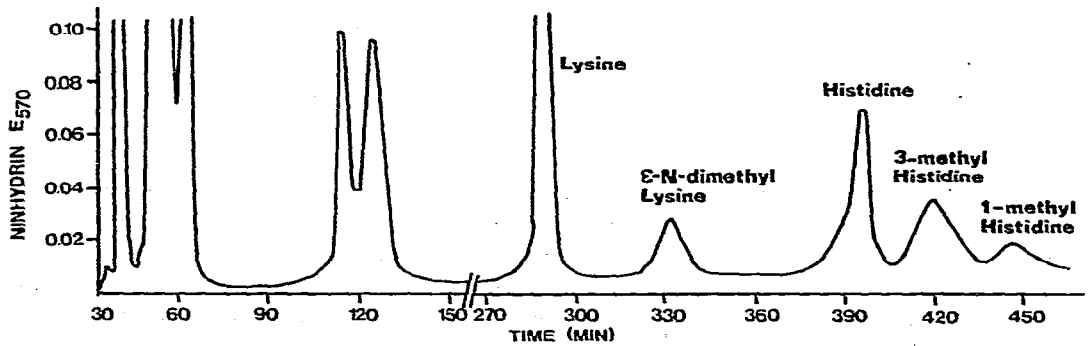


Fig. 7. Elution profile of a standard amino acid mixture containing the methylated derivatives of lysine and histidine and separated on the amino acid analyser column. The ninhydrin colour intensity is plotted against the time of elution. Note the clear separation of 3-methylhistidine from histidine and 1-methylhistidine and of lysine from N^{ε}-dimethyllysine.

1.3.2. Electrophoretic separations

Methylated histidines, unlike methylated lysines, can be separated from the parent amino acids by paper electrophoresis, using Whatman No. 3MM paper, pH 6.5, pyridine (5%, v/v)–acetic acid (0.2%, v/v) buffer and a potential gradient of 100 V/in. 3-Methylhistidine moves slightly behind histidine (towards the cathode) whereas 1-methylhistidine is faster. Also, the separation from lysine does not cause any problems, the distance between lysine and 1-methylhistidine being sufficiently long to allow a clear separation. The potential can be increased to 300 V without any effect on the result of the separation. As reported by Hardy and Perry[69], lysine and ε-N-monomethyllysine form the fastest zone towards the cathode, followed by

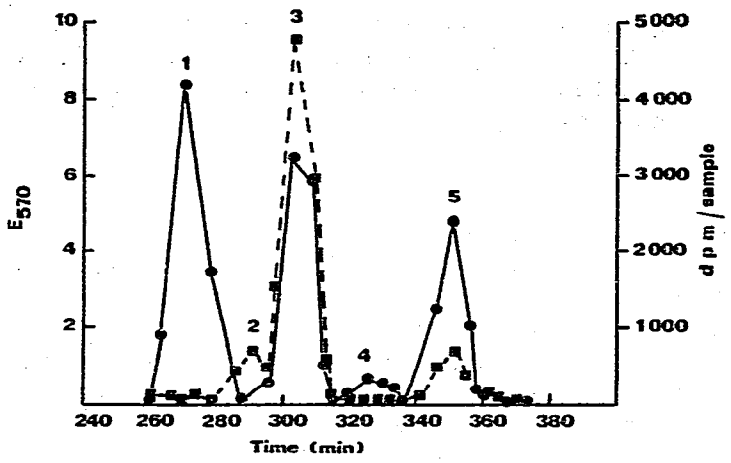


Fig. 8. Column chromatography of the acid hydrolysate of the myofibrillar fraction isolated from muscle homogenate incubated with [^{14}C]methyl-labelled S-adenosyl-L-methionine; 5.0 μmole each of N^{ε}-methyllysine and 3-methylhistidine were added to the hydrolysate and the basic amino acids were eluted with 0.35 *M* citrate buffer (pH 5.2) using the Beckman Unichrom analyser. Column dimensions, 53 × 0.9 cm; flow-rate, 40 ml/h. Acidic and neutral amino acids eluted in early fractions not shown. Peaks: 1 = lysine; 3 = ε-N-methyllysine; 4 = histidine; 5 = 3-methylhistidine. ■, Radioactivity, dpm per sample; ●, ninhydrin colour.

a triple zone of 1-methylhistidine, histidine and 3-methylhistidine. 1-Methyl- and 3-methylhistidine can be also separated at 2500 V/cm in pyridine–acetic acid (pH 6.1) using Whatman No. 3MM paper, as reported by Asatoor and Armstrong[3].

1.4. Diverse methylated amino acids

δ-N-Methyl-L-ornithine is another rarely seen amino acid in the brain. Of practical importance is its separation from methylated lysines, for which paper chromatography or electrophoresis can be used, and pyridine–acetone–3 *M* ammonia solution (50:30:25) or propanol-2–formic acid–water (4:1:1) were recommended as mobile phases by Matsuoka *et al.*[99]. They also applied a paper electrophoretic separation at 100 V/cm in pyridine–acetic acid–water (33:17:950) (pH 5.3) with slightly poorer results than those obtained by paper chromatography. The results are summarized in Table 5.

For the separation of 4-N-trimethylaminobutyric acid and 6-N-trimethyllysine, the following procedure was devised by Cox and Hoppel[26]. A column of AG 50-X8 (50 × 1 cm) was eluted with a citrate buffer–sodium hydroxide gradient. The starting citrate buffer was 0.25 *M* with respect to Na^+ (pH 4.1) and was replaced with 0.25 *M* sodium hydroxide solution. The elution profile is shown in Fig. 9.

Alternatively, the 50 × 1 cm AG 50-X8 column can be used with an increasing hydrochloric acid gradient (150 ml each of 1.5 *M* and 4 *M* hydrochloric acid). Detection was effected by radioactivity[26], but obviously ninhydrin detection could also be used. The flow-rate was not specified in the original paper, 12-ml fractions were collected. Besides 4-trimethylaminobutyric acid it is possible to separate also 5-trimethylaminopentanoic acid and 6-trimethylaminohexanoic acid. The results are shown in Fig. 10.

TABLE 5

PAPER CHROMATOGRAPHY AND HIGH-VOLTAGE ELECTROPHORESIS DATA OF ORNITHINE, LYSINE AND THEIR ω-N-METHYL DERIVATIVES

The high-voltage electrophoresis migration is the distance travelled towards the cathode after 30 min of electrophoresis with a potential gradient of 100 V/cm in pyridine–acetic acid–water (33:17:950; pH 5.3).

Compound	R_F *values*		*High-voltage electrophoretic migration (cm)*
	Pyridine–acetone–3 M ammonia solution (50:30:25)	*Propanol-2–formic acid–water (4:1:1)*	
L-Ornithine	0.37	0.32	22.0
L-Lysine	0.41	0.41	21.0
δ-N-Methyl-L-ornithine	0.33	0.44	21.2
ε-N-Methyl-L-lysine	0.35	0.53	20.1

1.5. Complex mixtures and complete separations of methylated amino acids

Muscle proteins contain considerable amounts of methylated derivatives and have been frequently studied. On a Beckman Unichrom analyser with 0.35 *M* sodium citrate buffer (pH 5.79) at 28°, the best resolutions achieved were reportedly of the type depicted in Fig. 11. The separation of ε-N-mono-, ε-N-di- and ε-N-trimethyllysines is very good and no problems arise from merging with the lysine and histidine peaks. Alternatively, elution with pH 5.28 buffer at 55° can be used (De Lange *et al.*[32]), but the separation is poorer than that when using the buffer of higher pH. In the pH 5.28 system it is possible to separate ε-N-monomethyllysine from lysine and 3-methylhistidine from histidine, but the separation of tri-N-ε-methyllysine from

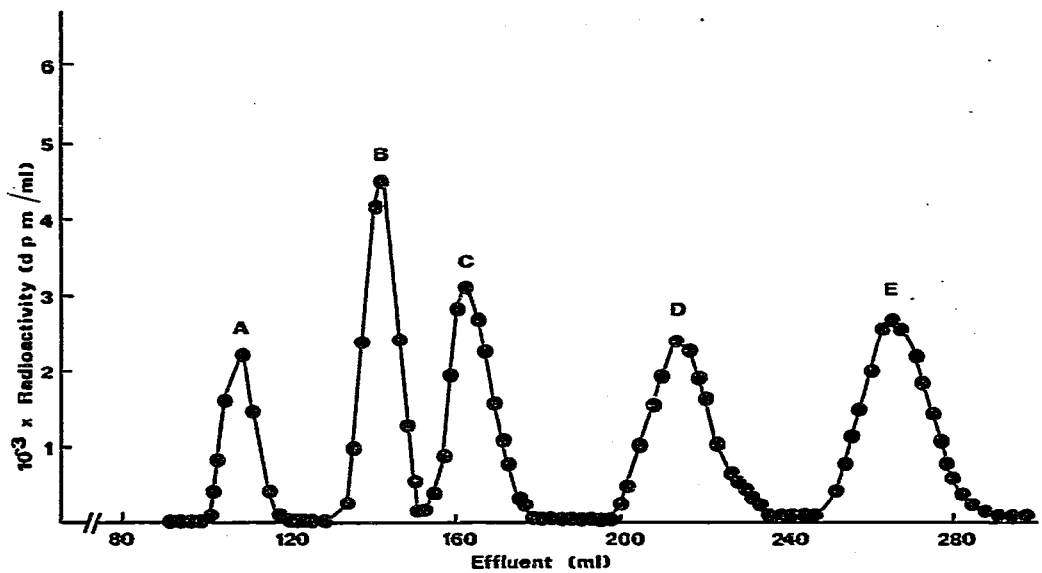


Fig. 9. Chromatography of carnitine (A), 4-trimethylaminobutyrate (B), 5-trimethylaminopentanoate (C), 6-trimethylaminohexanoate (D) and 6-trimethyllysine (E) on AG-50 ion-exchange resin.

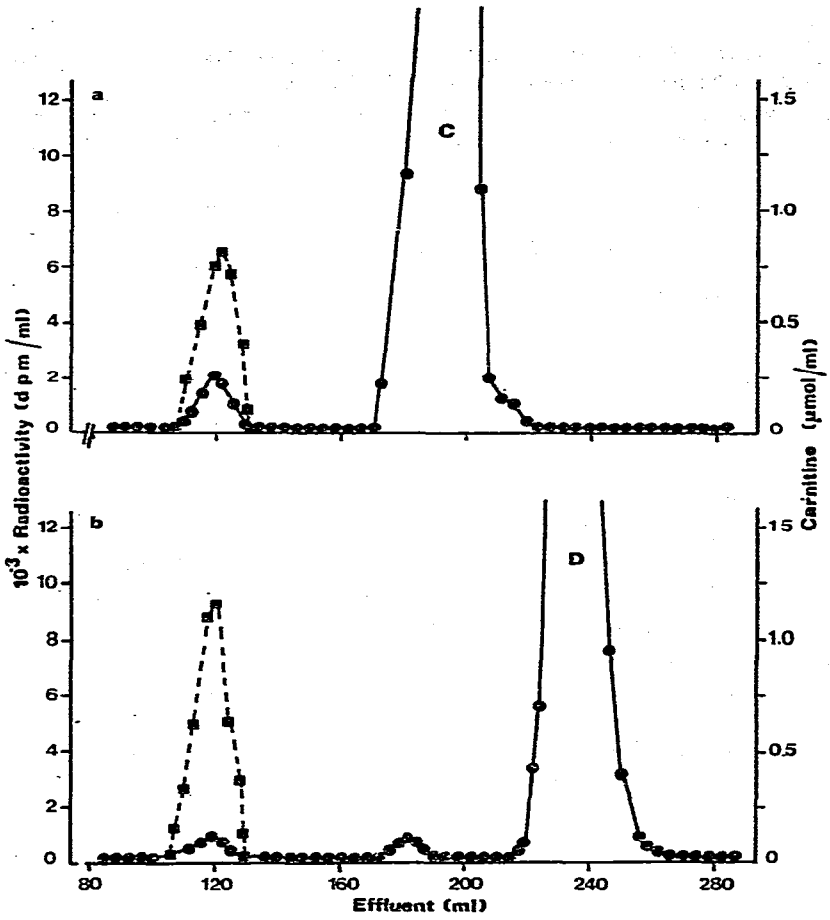


Fig. 10. Chromatography of carcass extracts from rats injected with labelled 5-trimethylaminopentanoic acid or 6-trimethylaminohexanoic acid. C = 5-Trimethylaminopentanoate; D = 6-trimethylaminohexanoate.

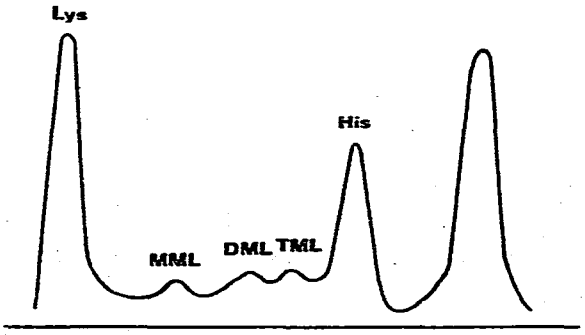


Fig. 11. Elution profile of subfragment 1 on the Beckman Unichrom analyser in 0.35 *M* sodium citrate buffer (pH 5.79) at 28°. Subfragment 1 (3 mg) + 0.02 µmole of di-N^{ε}-methyllysine (DML).

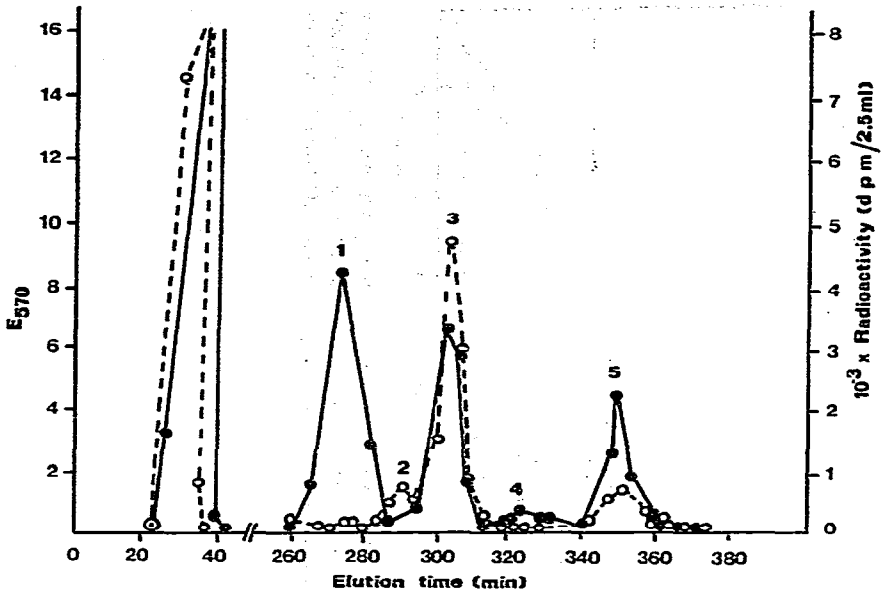


Fig. 12. Column chromatography at pH 5.28 of the acid hydrolysate of the myofibrillar fraction isolated from muscle homogenate incubated with ^{14}C-labelled S-adenosyl-L-methionine. Manno-N$^{\epsilon}$-methyllysine (5.0 μmole) and 3-methylhistidine (5.0 μmole) were added to the hydrolysate and the amino acids were eluted with 0.35 *M* sodium citrate buffer (pH 5.28) at 55°. Fractions (2.5 ml) were collected. Peaks: 1 = lysine; 2 = tri-N-methyllysine; 3 = mono-N$^{\epsilon}$-methyllysine; 4 = histidine; 5 = 3-methylhistidine. ●, Amino acid determination by the ninhydrin method (E_{570}); ○, radioactivity (dpm per 2.5 ml).

lysine and from the monomethyl derivative is inadequate (Fig. 12). Buffer of pH 5.28 has also been applied to the quantitation of ε-N-methyllysine in histones (Murray[111]).

A practical problem, apart from structural studies, is the determination of methylated amino acids in urine. This determination has been carried out with an advanced procedure by Kakimoto and Akazawa[83]. A 30 × 0.9 cm column of the Yanagimoto Type LC-3 amino acid analyser was used and amino acids were eluted with 0.51 *M* sodium chloride in 0.2 *M* sodium citrate buffer (pH 3.24). The amount of urine used for analysis corresponded to 5 mg of creatinine. The flow-rate used was 60 ml/h (30 ml/h in the ninhydrin line). The analysis was started at 35° and after 70 min was increased at the rate of 1° per 3 min up to 58°. The results of such a separation are shown in Fig. 13. ε-N-Trimethyllysine is well separated from ornithine and ε-N-dimethyllysine, but ε-N-monomethyllysine forms a broad shoulder at the beginning of the lysine peak. Of the methylated arginines, N^{G},N^{G}-dimethylarginine and N^{G},N$'^{G}$-dimethylarginine elute at 250 and 270 min, respectively, and are well separated from the other amino acids, *e.g.*, lysine and unmodified arginine.

Complete separations of methylated amino acids have been achieved only recently. Numerous systems using the two-column arrangement of the amino acid analyser have been devised but mostly proved too complex to operate. By using Durrum DC-6A resin, Zarkadis[192] was able to use a single-column analysis, at a single

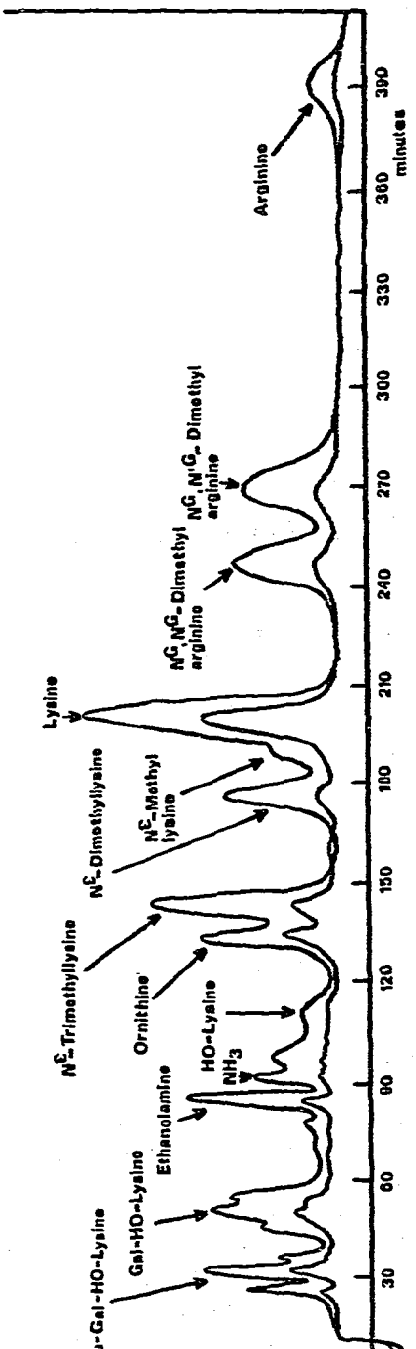


Fig. 13. Ion-exchange chromatography of the aliphatic basic amino acid fraction of human urine. Chromatography was carried out on a 30 × 0.9 cm column with 0.51 *M* NaCl in 0.2 *M* sodium citrate buffer (pH 3.24) at a flow-rate of 60 ml/h. The temperature was increased from 35° to 58° after 70 min at the rate of 1° per 3 min. Glu-Gal-HO-lysine = glucosyl-galactosyl-δ-hydroxylysine; Gal-HO-lysine = galactosyl-γ-hydroxylysine; HO-lysine = δ-hydroxylysine.

pH value and with no temperature change, to separate all members of the methylated series. The analysis was carried out with the standard Spinco Model 120 B analyser using a 60 × 0.9 cm column. The eluting buffer was 0.35 *N* sodium citrate containing octanoic acid adjusted to either pH 5.734 or 5.657 ± 0.002 at 25°. Samples were dissolved in the same buffer and run at 28°. The flow-rate in the sample line was maintained at 30 ml/h and in the ninhydrin line at 15 ml/h. The results of the separations obtained are shown in Fig. 14.

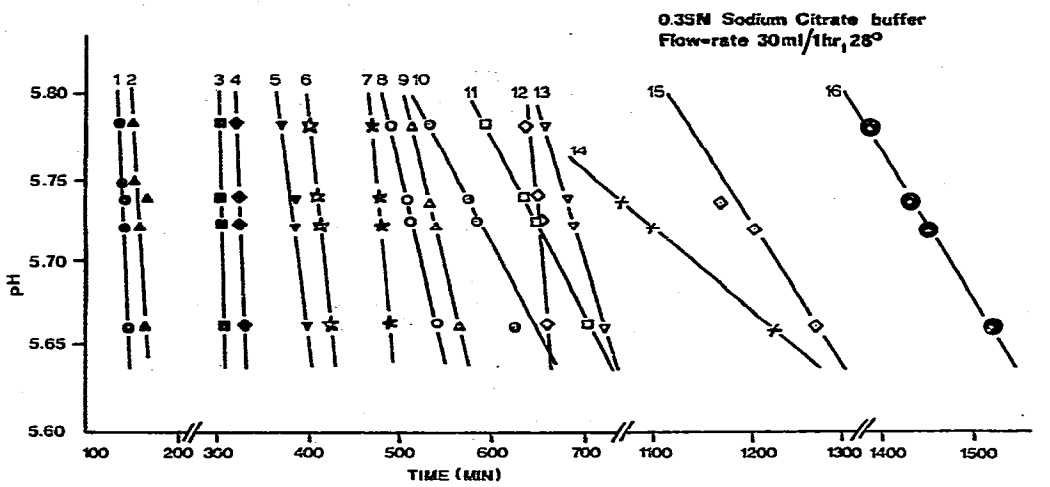


Fig. 14. Resolution of methylated basic amino acids and related compounds as functions of pH. 1, Tyrosine; 2, phenylalanine; 3, δ-hydroxy-D,L-lysine; 4, δ-*allo*-hydroxy-D,L-lysine; 5, D,L-ornithine; 6, lysine; 7, N^{ε}-monomethyl-L-lysine; 8, $N^{\varepsilon},N^{\varepsilon}$-dimethyl-L-lysine; 9, [U-^{14}C]-$N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-trimethyl-L-lysine: 10, histidine; 11, 3-methyl-L-histidine; 12, ammonia; 13, 1-methyl-L-histidine; 14, N^{G},N^{G}-dimethylarginine; 15, $N^{G},N^{G'}$-dimethylarginine; 16, arginine.

Very rigid control of the pH value is necessary as the positions of ε-N-trimethyllysine, 1- and 3-methylhistidine and histidine change considerably with the slightest change in the pH value of the eluting buffer. Zarkadis[192] specifies two optimal pH conditions (Fig. 15) for the separation of basic amino acids that can be recommended for naturally occurring biological systems. At pH 5.734 the ε-N-trimethyllysine peak is completely separated from histidine and ammonia occurs midway between 3- and 1-methylhistidines. At pH 5.657 the histidines are more retarded and emerge after the ammonia peak. In both buffer systems methylated arginines are also well separated. The elution times are summarized in Table 6.

In the flat-bed arrangement, a complex mixture of "minor basic amino acids", *e.g.*, methylated derivatives, can be separated successfully on ion-exchange plates [Fixion 50-X8, Dowex 50-X8 or Ionex 25 (Macherey, Nagel & Düren Co., G.F.R.) can be used]. R_F values are presented in Table 7. The plates were equilibrated with sodium citrate buffer (pH 3.28, 0.02 *N* Na^+) and developed in the mobile phases specified. The procedure was described by Tyihák *et al.*[177]. On the other hand, N-methylleucine cannot be separated from N-methylisoleucine by ion-exchange chromatography, but can be separated with *tert.*-butanol–4.25 *N* ammonia solution (4:1), *n*-butanol–acetic acid–water (4:1:5) or *tert.*-amyl alcohol–acetic acid–water (20:1:20)

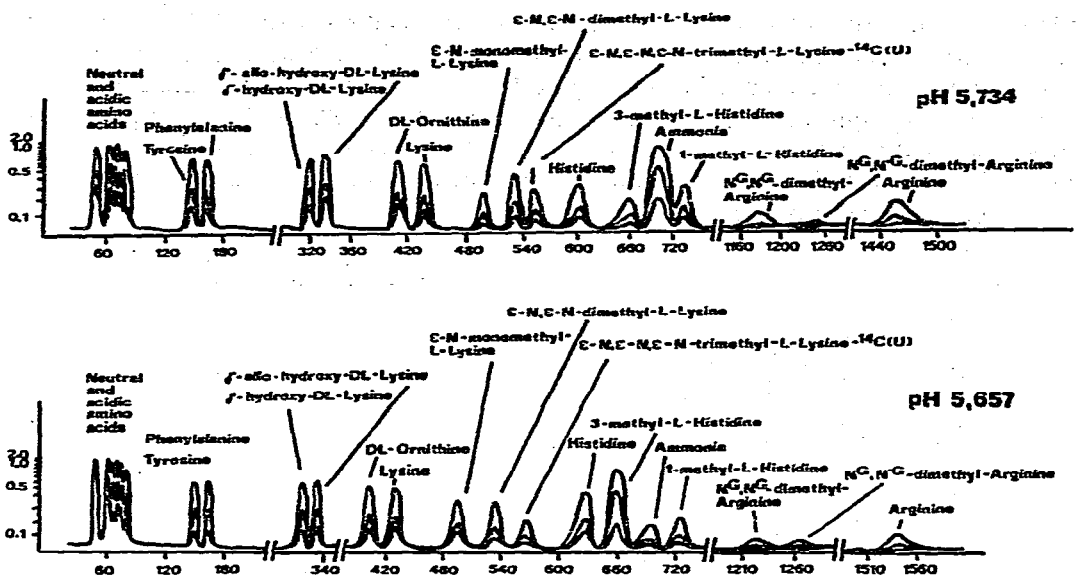


Fig. 15. Separation of a synthetic mixture of 16 methylated basic amino acids and related compounds on a 60 × 0.9 cm column of Durrum DC-6A spherical resin. The column was operated at 28° using 0.35 *N* sodium citrate buffer adjusted to either pH 5.734 (A) or 5.657 ± 0.002 (B) at a flow-rate of 30 ml/h. The upper two curves show absorbance at 570 nm, using two different cell depths, and the lower curve that at 440 nm.

TABLE 6

ELUTION TIMES OF METHYLATED BASIC AMINO ACIDS AND RELATED COMPOUNDS

Compound	*Elution times (min)*	
	pH 5.657	*pH 5.734*
Tyrosine	151	148
Phenylalanine	165	162
δ-Hydroxy-D,L-lysine	315	312
δ-allo-Hydroxy-D,L-lysine	330	328
D,L-Ornithine	405	401
Lysine	433	427
N^{ε}-Monomethyllysine	496	489
$N^{\varepsilon},N^{\varepsilon}$-Dimethyllysine	536	525
$N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-Trimethyllysine	567	552
Histidine	629	576
Ammonia	660	682
3-Methylhistidine	696	653
1-Methylhistidine	725	707
N^{G},N^{G}-Dimethylarginine	1226	1178
N^{G},N^{G}-Dimethylarginine	1271	1235
Arginine	1534	1460

TABLE 7

$R_F \times 100$ VALUES OF 30 AMINO ACIDS SO FAR IDENTIFIED IN PROTEINS ON A FIXION 50-X8 CHROMOPLATE WITH SPECIAL REFERENCE TO METHYLATED AMINO ACIDS DERIVATIVES

*Amino acid**	*Developing buffer***		
	A	*B*	*C*
Asp	80	79	82
Thr	79	78	80
Ser	80	80	81
Glu	80	79	80
Gly	78	64	79
Ala	72	60	72
Pro	51	49	51
Val	65	61	65
Met	58	50	57
Ile	52	49	52
Leu	53	50	53
Tyr	51	41	50
Phe	54	50	55
Try	10	—	11
Asn	69	—	70
Gln	61	—	60
Cys	77	—	79
Cys_2	60	—	62
H_4Pro	75	—	78
His	47	16	48
1-MeHis	37	15	36
3-MeHis	26	12	24
Lys	59	25	58
MML	38	19	39
DML	23	16	21
TML	14	12	13
Arg	29	8	28
MMA	21	8	20
DMA	14	9	13
DMA′	15	8	16

* MML = N^{ε}-monomethyl-D,L-lysine hydrochloride; DML = N^{ε},N^{ε}-dimethyl-D,L-lysine hydrochloride; TML = N^{ε},N^{ε},N^{ε}-trimethyl-D,L-lysine dihydrochloride. The guanidino-methylated arginines are: MMA = N^{G}-monomethyl-L-arginine; DMA = N^{G},N^{G}-dimethyl-L-arginine; DMA′ = N^{G},N'^{G}-dimethyl-L-arginine.

** The compositions of the developing buffers are as follows:

Component	*Buffer solution*		
	A (pH 6.0)	*B (pH 5.28)*	*C (pH 6.0)*
Hydrated citric acid (g)	100.0	24.6	105.0
Hydrochloric acid (ml)	14.0	6.5	—
Sodium hydroxide (g)	60.0	14.0	60.0
Sodium chloride (g)	—	—	58.5
Sodium ions (*N*)	1.5	0.35	2.5

(Audhya and Russel[5]) using Whatman No. 3 MM paper. A mass fragmentographic assay of N-methylated amino acids in muscle proteins was developed by Barbier *et al.*[7].

1.6. Fluorimetric detection

N-Monomethyl amino acids, used for testing the influence of the N-methylamino groups on fluorescamine detection, have been shown to be important constituents of peptide antibiotics. The procedure described by Felix and Terkelsen[43,44] can, however, also be used for those which occur in protein structures and therefore it is discussed here in more detail.

The single-column fluorimetric methylamino acid analyser is shown schematically in Fig. 16. It is based on a similar instrument devised by Udenfriend *et al.*[178] for the detection of proline and hydroxyproline. Four pumps were used to introduce the appropriate reagents. Pump 1 introduced the eluting buffers by means of a rotary valve, which permitted selection of the appropriate buffer (buffer 1, sodium citrate, pH 3.28, 0.2 *M* Na^+; buffer 2, sodium citrate, pH 4.24, 0.2 *M* Na^+; flow-rate, 9.2 ml/h). Pump 2 was used for the introduction of 10^{-3} *M* N-chlorosuccinimide in 0.05

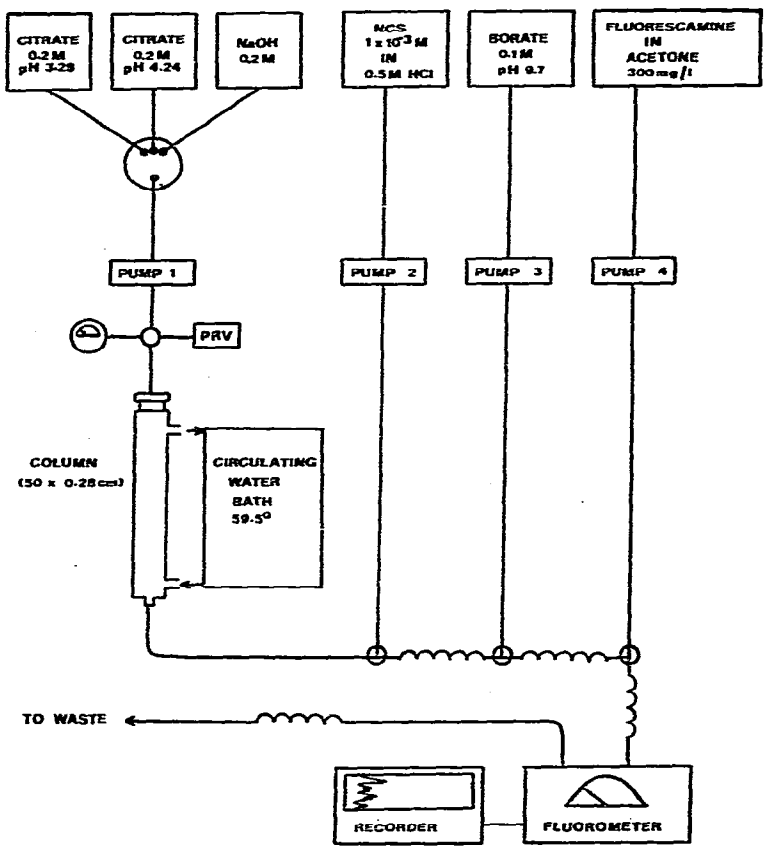


Fig. 16. Schematic representation of the fluorimetric methylamino acid analyser.

M hydrochloric acid. This pump was kept on throughout the analysis, and the pH of the eluent was set at 2–2.4 by adjusting the flow-rate from pump 2 (6.6 ml/h). Pump 3 was used for the introduction of the borate buffer (pH 9.7, 0.10 *M*), and the pH of the eluent was set at 8.5–9.0 by adjusting the flow-rate from the pump (25.2 ml/h). Pump 4 was used for the introduction of fluorescamine (300 mg/l in acetone; flow-rate, 19.6 ml/h). The fluorescent mixtures were detected in an Aminco Fluoro-Microphotometer equipped with an 85-W mercury vapour lamp assembly, 2 mm I.D., a high-pressure flow cell, a Corning No. 7-51 primary filter and a Wratten No. 4 secondary filter (American Instrument). The photomultiplier output was connected to a Kontron Model 1100 recorder operating at a speed of 12 cm/h. The jacketed ion-exchange column (50 × 0.28 cm) was heated to 59.5° by means of a Lauda K-2 circulator (Brinkmann Instrument) and packed with Durrum DC-4A resin. A height of 1 cm of Jeol AR-15 resin was placed at the top and bottom of the column and fitted with a stainless-steel screen to prevent leakage of the Durrum resin. Samples were introduced on to the column by means of a 10-μl slider valve. The column was regenerated by pump 1 for a minimum of 30 min with 0.2 *M* odium hydroxide solution and equilibrated for a minimum of 30 min with buffer 1 prior to sample application.

1.7. Isolation of methylated amino acids (as standards) from urine

For this purpose, chromatography on Amberlite CG-50 or Dowex 50-X2 can be recommended. Individual fractions are isolated from the amino acid analyser first (step 1, Table 8), followed by with re-chromatography as specified in Table 9, as step 2. For final purification, a third step is sometimes required. The procedures lead to standards that otherwise may be difficult to obtain. Kakimoto and Akazawa[83], however, also isolated amino acid derivatives other than the methylated derivatives and thus demonstrated the general applicability of their procedure. For another preparative procedure, see p. 239.

TABLE 8

PURIFICATION OF METHYLATED AMINO ACIDS FROM URINE BY ION-EXCHANGE CHROMATOGRAPHY

Substance	*Elution volume (ml)* *
Glucosylgalactosyl-δ-hydroxylysine Galactosyl-δ-hydroxylysine	1710–2680
N^{ε},N^{ε}-Dimethyllysine	4210–5450
N^{ε}-Methyllysine	8445–9675
N^{ε},N^{ε},N^{ε}-Trimethyllysine	10225–11775
Unidentified	12275–13890
N^{G},N'^{G}-Dimethylarginine N^{G},N^{G}-Dimethylarginine	13890–16465

* Chromatography of the aliphatic basic amino acids fraction was carried out on a column (114 × 6.0 cm) of Amberlite IR-120 (NH_4^+) (100–200 mesh) by elution with an increasing concentration of ammonia solution.

TABLE 9

FURTHER PURIFICATION OF METHYLATED AMINO ACIDS FROM URINE ISOLATED BY THE PROCEDURE IN TABLE 8

Substance	*Step 2**			*Step 3**		
	*Resin** and size of column*	*Concentration of ammonia solution for elution*	*Elution volume (ml)*	*Resin** and size of column*	*Concentration of ammonia solution for elution*	*Elution volume (ml)*
Glucosylgalactosyl--δ-hydroxylysine	Amberlite CG-50, 46 × 2 cm	Water (180 ml), 0.05 *M* (590 ml) and 0.1 *M* successively	770–850	Dowex 50-X8, 20 × 2 cm	0.05 *M* (200 ml) and 0.1 *M* successively	138–226
Galactosyl-δ--hydroxylysine						
N^{ε},N^{ε}-Dimethyllysine	Amberlite CG-50, 46 × 2 cm	0.1 *M*	250–370			
N^{ε}-Methyllysine	Amberlite CG-50, 46 × 2 cm	0.15 *M*	210–350			
N^{ε},N^{ε},N^{ε}-Trimethyllysine	Dowex 50-X2, 66 × 3 cm	0.5 *M*	1300–1860			
Unidentified	Amberlite CG-50, 46 × 2 cm	0.3 *M*	510–850	Dowex 50-X2, 66 × 3 cm	0.8 *M*	400–560
N^{G},N'^{G}-Dimethylarginine					0.5 *M*	306–384***
N^{G},N^{G}-Dimethylarginine	Dowex 50-X2, 35 × 4 cm	1 *M*	930–1490	Dowex 1, 135 × 3 cm		340–476***

* Procedure characterized in Table 8 is to be considered as Step 1.

** Amberlite CG-50 (NH^+, 100–200 mesh), Dowex 50-X2 (NH^+, 100–120 mesh), Dowex 50-X8 (NH^+, 200–400 mesh), or Dowex 1-X8 (OH^-, 100–200 mesh).

*** As the resolution of the two compounds was incomplete, a portion in which both compounds were eluted concomitantly was re-chromatographed twice, resulting in almost complete separation of the two.

2. IODINATED AMINO ACIDS*

A tremendous amount of work has been carried out on the paper and thin-layer chromatography of these compounds and the applications of these techniques are vast. Although these amino acids cannot be considered as rare, they are produced by post-translational iodination of aromatic residues and hence they can be included in this review.

2.1. Sorption and chromatography on Sephadex gels

The separation of T_4 and T_3 on Sephadex has been attempted many times with different results, and such separations represent one of the earliest approaches to the separation of iodinated amino acids. A wide variety of solutions have been employed to elute T_4 and T_3 after their adsorption on Sephadex. With neutral buffers very large volumes were required, but alkaline solutions such as 0.015 *N* sodium hydroxide solution reduced the elution volumes substantially and frequently, but not always, good separations were obtained, T_3 being eluted ahead of T_4 (Green[58]). The irregularities that occur in these separations can be explained on the basis of strong adsorption of sodium hydroxide on the Sephadex matrix. As reported by Gelotte[51], sodium hydroxide solutions, on account of the already mentioned adsorptivity, migrate less rapidly than neutral salt solutions. Hence, when sodium hydroxide solution follows a neutral salt solution, there will be a zone between the tail of the salt solution and the retarded sodium hydroxide front in which the ionic strength is extremely low. If buffers of low ionic strength are used for the elution of T_3 and T_4 from Sephadex G-25 columns, their order of elution is reversed compared with elution with alkaline buffers, *e.g.*, T_4 migrates more rapidly than T_3. This may result in acidic compounds being excluded from the gel grains and eluted more rapidly at low ionic strength. Thyroxine, with its lower pH for hydroxyl ionization, would be accelerated more by such effects. In order to avoid this effect, small amounts of sodium chloride can be added to the sodium hydroxide eluent solution (Gelotte[51]). The best results were obtained with 0.015 *N* sodium hydroxide–0.5 *N* sodium chloride as the equilibration solution, 1 *N* sodium chloride–0.2 *N* phosphate buffer (pH 6.5) as the sample diluent and 0.1 *N* sodium hydroxide–0.005 *N* sodium chloride as the eluent. Columns of dimensions 17 × 2.2 cm at a flow-rate of 1.7 ml/min were used.

With an alkaline solution only and with Sephadex G-25, reasonable separations of 3,5-diiodotyrosine, 3,5,3′-triiodothyronine and thyroxine can be obtained, as reported by Mougey and Mason[110]. Columns of dimensions 40 × 1.5 cm were used with 0.01 *N* sodium hydroxide solution (pH 11.5) as the mobile phase. The separation obtained is shown in Fig. 17. This procedure, however, does not separate iodide and monoiodotyrosine, which merge with the diiodotyrosine peak.

In another early attempt to achieve the column separation of iodinated amino acids (T_3 and T_4), Makowetz *et al.*[95] used a Sephadex G-25 column (110 × 0.2 cm) with a slightly more concentrated mobile phase (0.015 *N* sodium hydroxide solution) at a flow-rate of 1 ml per 25 min. The results are shown in Fig. 18. The procedure

* The following abbreviations are used throughout this section: T_1 = 3-monoiodothyronine, T_2 = 3,5-diiodothyronine, T_3 = 3,5,3′-triiodothyronine, T_4 = thyroxine.

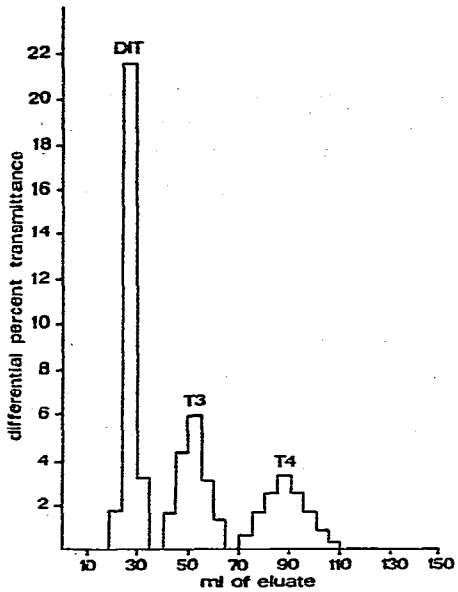


Fig. 17. Separation of 3,5-diiodotyrosine (0.07 μg), 3,5,3′-triiodothyronine (0.10 μg) and thyroxine (0.10 μg) on an 8-cm Sephadex G-25 column using 0.01 *N* NaOH as eluent. The $\%T$ records at 400 nm is corrected [$\%T$ (iodine tube) minus $\%T$ (blank tube)]. Fractions of 5 ml were collected.

was reported to give good separations of T_3 and T_4, together with inorganic iodide.

The method itself is obviously a slight modification of the procedure of Mougey and Mason[110], directed towards overcoming the problems outlined at the beginning of this section. Instead of inorganic bases, buffers containing pyridine (Osborn and Simpson[122]) also offer good separations of iodinated amino acids. Thus, monoiodo-

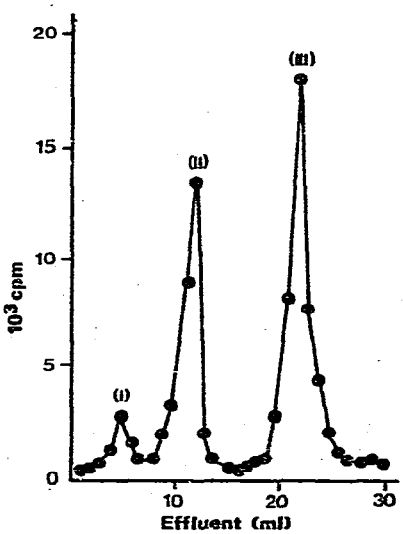


Fig. 18. Separation of ^{131}I-labelled standard samples of triiodothyronine (6 mg of T3) (peak II) and thyroxine (50 mg of T4) (peak III); inorganic iodide as contaminant (peak I).

tyrosine and iodide are well separated in pyridine–acetic acid–water (45:11.5:1943.5) (pH 5.6). This mobile phase is, however, not capable of eluting T_3 and T_4, which can be separated by changing the mobile phase abruptly to *tert.*-amyl alcohol saturated with 2 *N* ammonium solution. An incomplete separation of T_3 and T_4 can be achieved, as shown in Fig. 19.

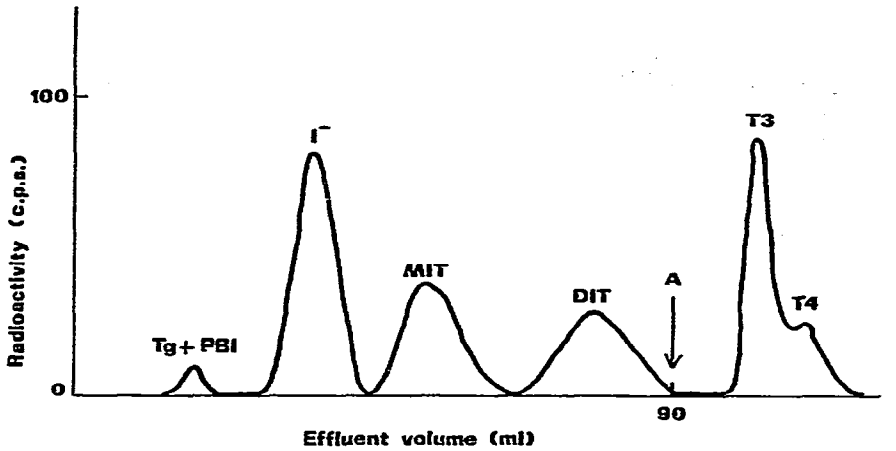


Fig. 19. Separation of iodoamino acids on Sephadex G-25. Tg = thyroglobulin; PBI = protein-bound iodine; I = iodide; MIT = monoiodotyrosine; DIT = diiodotyrosine; T3 = triiodothyroxine; T4 = thyroxine. A indicates the introduction of *tert.*-amyl alcohol saturated with 2 *N* ammonia solution.

The separation of iodopeptides, iodide, 3-monoiodotyrosine, 3,5-diiodotyrosine and iodothyronines is frequently required in the analysis of thyroid dialysates and hydrolysates. Peyron and Simon[129] carried out extensive studies with Sephadex G-10 in addition to the commonly used Sephadex G-25, and devised a two-step single-column procedure using the former. Columns of dimensions 40 × 1 cm can be recommended for this purpose, with *n*-butanol–water–acetic acid (78:17:5), 0.15 *M* with respect to sodium chloride, followed by *n*-butanol–water–acetic acid in an abrupt gradient as mobile phases. An example of the separation of a natural sample is presented in Fig. 20.

In addition to unmodified Sephadex, the separation properties of hydrophobic Sephadex LH-20 can also be used (Williams *et al.*[186]). For instance, separations can be carried out in 60 × 0.8 cm columns using ethyl acetate–methanol–ammonia solution (400:100:40) as the mobile phase. The results of the separation are shown in Fig. 21. During the separation, the flow-rate was kept at 0.5 ml/min and the complete separation (as shown in Fig. 21) required the collection of 100–110 fractions of 4-ml volume. For detection wet ashing with chloric acid was used in order to determine the iodine content. The samples must be completely dry before this operation as methanol reacts violently with hot chloric acid. Alternatively, detection can be carried out simply by measuring the absorbance at 297 nm, bearing in mind the differences in extinction coefficients and the positions of the absorption maxima of individual iodinated amino acids (Table 10).

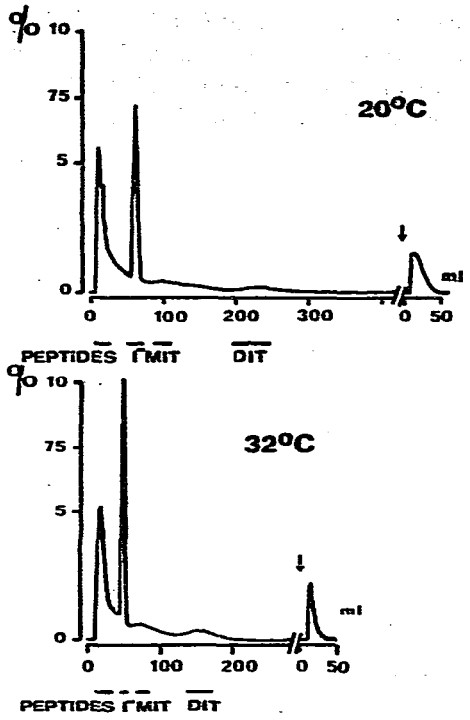


Fig. 20. Chromatography of rat thyroid dialysates on Sephadex G-10 at 20 and 32°. Column, 40 × 1 cm; sample, 1 ml; flow-rate, 0.33 ml/min.

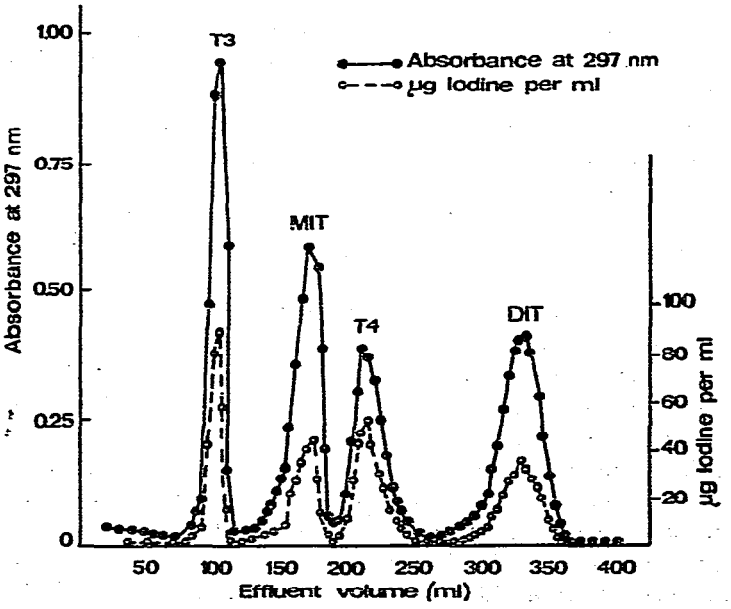


Fig. 21. Elution pattern from a Sephadex LH-20 gel filtration column of a mixture of pure iodoamino acids. Solid line = absorbance at 297 nm. Broken line = iodine concentration (µg/ml).

TABLE 10

SPECTROPHOTOMETRIC CHARACTERISTICS OF PURE IODOAMINO ACIDS

Molar extinction coefficient (1-cm light path) at 297 nm in ethyl acetate–methanol–ammonia eluting solvent.

Compound	*Extinction*	*Ratio, 297:310 nm*
Thyroxine	3900	0.956
Triiodothyronine	4253	1.276
Diiodotyrosine	2720	0.744
Monoiodotyrosine	2053	9.626

2.2. Chromatography on polyacrylamide gel

A rarely used method for the complete separation of most naturally occurring iodinated amino acids is the procedure suggested by Thomopoulos[172], involving the use of Bio-Gel P-2 for the separation of monoiodohistidine, diiodohistidine, monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine. Bio-Gel P-2 (polyacrylamide gel, 200–400 mesh) was allowed to swell at 20° for 16 h and a 50 × 0.9 cm column was packed with the swollen gel (32 ml). The column was then equilibrated with at least three column volumes of the buffer to be used for elution. The mobile phase 0.5 *M* Tris–maleic acid (pH 5.3, 5.6 and 7.4) gave a complete separation of iodinated amino acids; alternatively, reasonable results, especially a good separation of T_4 and T_3, were obtained with Tris–hydrochloric acid buffer at pH 9.0. All of the buffers used contained 0.1% of sodium azide. The flow-rate was kept at 6.0 ml/h. The results are shown in Fig. 22.

A useful procedure for the separation of T_3 and T_4, in addition to other iodinated amino acids, is to use Tris–maleic acid–sodium hydroxide buffer (pH 6.0) and to separate monoiodohistidine, diiodohistidine, monoiodotyrosine and diiodotyrosine in this first step. Then, on changing the eluting buffer abruptly to Tris–hydrochloric acid buffer (pH 9.0), a further separation of T_4 and T_3 can be achieved, as shown in Fig. 23.

2.3. Ion-exchange chromatography

Dowex 1-X2 and 50-X4 can be used as ion exchangers. With Dowex 1-X2, a 100 × 0.9 cm column was used (Wynn *et al.*[188]). Vessels necessary to set up the gradient were arranged as follows. At the top of the column was a jar with a stirring device (100 ml), and a 250-ml dropping-funnel reservoir was joined at the top of the stirred vessel, which was initially filled with 70 ml of 5% (w/v) formic acid. The dropping funnel was filled with 160 ml of 45% (w/v) formic acid. Fractions of 5 ml were collected. After the collection of the thirtieth fraction, the remaining 45% formic acid in the funnel was removed. The residual formic acid in the stirred jar was made 70% (w/v) and 80 ml of 88% (w/v) formic acid were added to the dropping funnel. At this stage the column temperature, which had been held at 45° from the beginning of the separation, was increased to 55°. The solvents were allowed to pass through

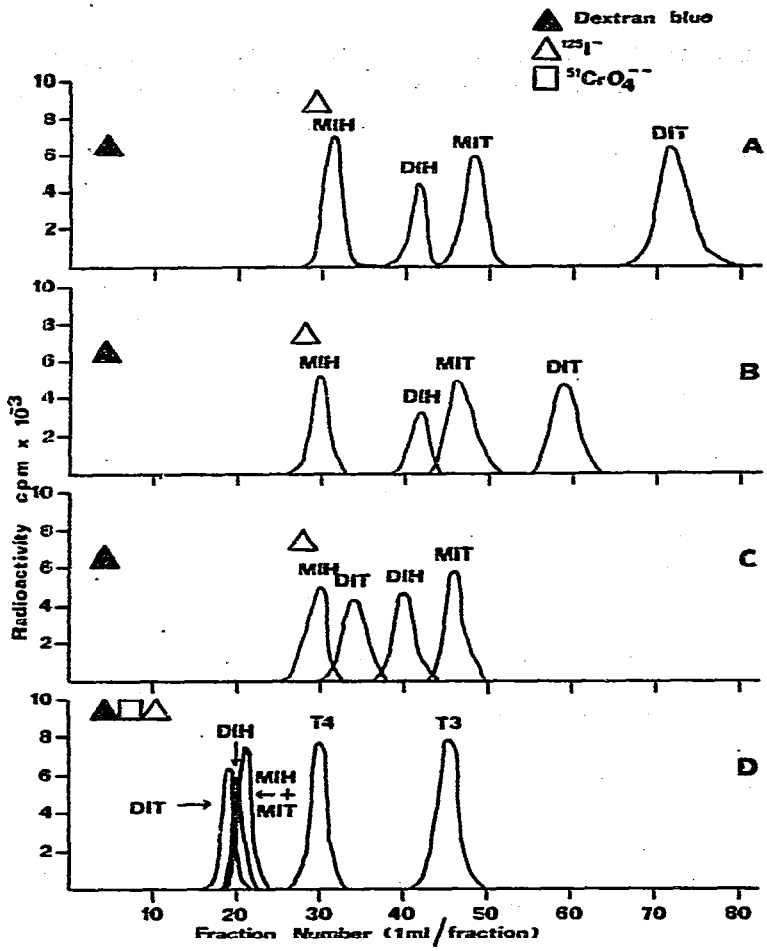


Fig. 22. Chromatography of radioactive iodoamino acids on a Bio-Gel P-2 column at 20° (50 × 0.9 cm gel bed). Samples volume, 0.4 ml; flow-rate, 6 ml/h. A, Elution pattern with 0.05 *M* Tris–maleic acid–NaOH at pH 5.3; B, same buffer at pH 6; C, same buffer at pH 7.4; D, elution pattern with 0.05 *M* Tris–HCl at pH 9. The elution patterns of dextran blue (▲), iodide (△) and chromate (□) are also indicated.

the column until exhaustion, which allowed 65 fractions to be collected. The results of radioactivity counting of individual fractions are shown in Fig. 24.

Another procedure using the same ion exchanger was described by Galton and Pitt-Rivers[50]. The separation was carried out in small (3 × 1 cm) columns. Prior to operation, the column was equilibrated with acetate buffer (pH 5.6) and, after the sample has been applied, solutions of progressively decreasing pH were passed through the column, the solution being changed only when no further radioactivity could be detected. Finally, acetic acid followed by 3 *N* sodium bromide solution was passed through the column in order to elute the iodide. Fractions of 3 ml were collected and assayed in a scintillation counter. As indicated in Table 11, monoiodotyrosine, diiodotyrosine, thyroxine and thyroglobulin (if present in natural samples)

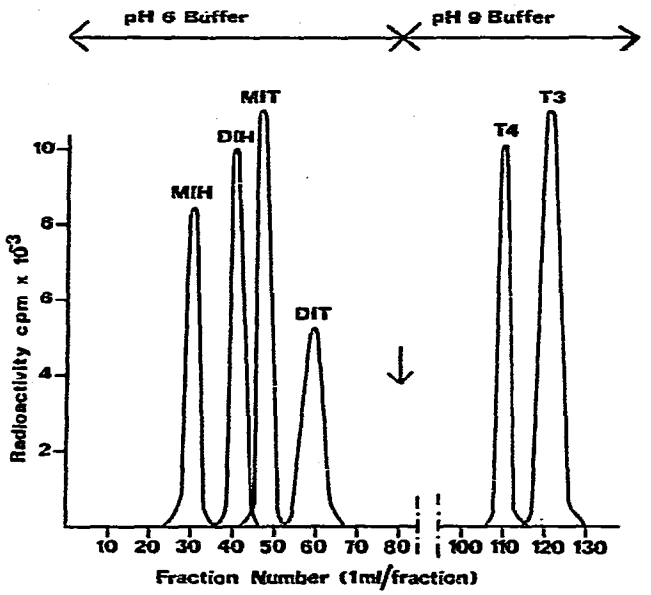


Fig. 23. Elution pattern of radioactive iodoamino acids on a Bio-Gel P-2 column equilibrated and eluted with 0.05 *M* Tris–maleic acid–NaOH (pH 6) and subsequently (arrow) with 0.05 *M* Tris–HCl at pH 9. Gel bed size, sample volume, flow-rate and temperature as in Fig. 22.

emerge as a single peak at the beginning of the chromatogram. Most of the thyroglobulin occurs in the second fraction, and mono- and diiodotyrosine are well separated. The bulk of iodide remains in the last fraction. Some iodide migrates with the first fraction as a contaminant.

It is unlikely that separations of this type will gain in popularity; from the separation point of view it is, however, interesting to see that ion-exchange chromatography can be applied to some extent to this type of amino acid derivative. Other workers, such as Blanquet *et al.*[14], Meyniel *et al.*[104] and Ingbar *et al.*[76], have carried out similar studies.

The results obtained with Dowex 50-X4 are better than those with Dowex 1-X2 (Lerner[91]). Columns of dimensions 15 × 0.9 cm are sufficient for the separation. Elution with ammonia solution (0.2 *N*, containing 30% of ethanol) was started after the column had been packed and the resin was cycled through a pH 4.5–7.5–9.1–0.2 *N* ammonia solution–pH 4.5 sequence. The material was applied in buffer of pH 3.5. After the sample had drained into the column, the automatic programmer was set to deliver buffer of pH 4.5 for 5 h, buffer of pH 7.5 for the next 4 h, buffer of pH 9.1 for 8 h and 0.2 *N* ammonia solution (containing 30% of ethanol) for 2 h, then returning to buffer of pH 4.5. All of the buffers used were ammonium formate (0.2 *N*) containing 30% of ethanol. Arsenite + cerium(IV) detection and radioactivity counting were used to locate the peaks (Fig. 25).

Using 0.04 *M* ammonium acetate buffer (pH 4.7), containing 30% (v/v) of ethanol, at 50° and a gradient of increasing pH prepared from 100 ml of the starting buffer and 100 ml of 0.65 *N* ammonia solution, Sorimachi and Ui[157] achieved the

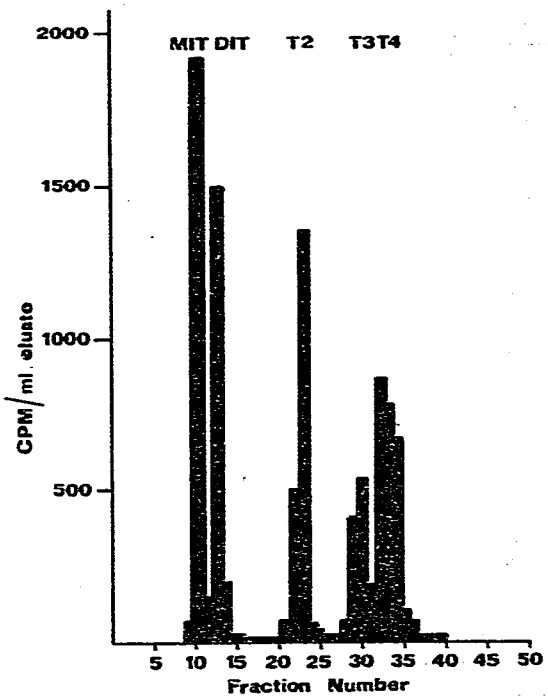


Fig. 24. Chromatography of labelled iodoamino acid fractions on Dowex 1-X2.

TABLE 11

SEPARATION OF IODINATED AMINO ACIDS IN A THYROID HYDROLYSATE WITH DOWEX-1

Fraction	*pH or solvent*	*Counts/min*	*^{131}I-labelled compounds detected by chromatography*
Thyroid hydrolysate	8.6	30,420	Thyroglobulin, monoiodotyrosine, diiodotyrosine, thyroxine, iodide
1–5	5.6	—	
6–10	3.6	800	Thyroglobulin
11–16	3.4–3.2	—	—
17–24	3.0	20,447	Monoiodotyrosine
25–35	2.8–2.4	—	
36–40	2.2	7371	Diiodotyrosine
41–100	1.6–1.4	300	Thyroxine(?)
101–120	3 *N* NaBr	1102	Iodide
Total eluted material		30,020	

separation of iodothyronines, as shown in Fig. 26. The elution volumes and relative peak areas (sulphate–arsenous acid detection) are summarized in Table 12.

2.4. Controlled-pore glass separations

Williams *et al.*[186,187] reported the possibility of using controlled-pore glass for

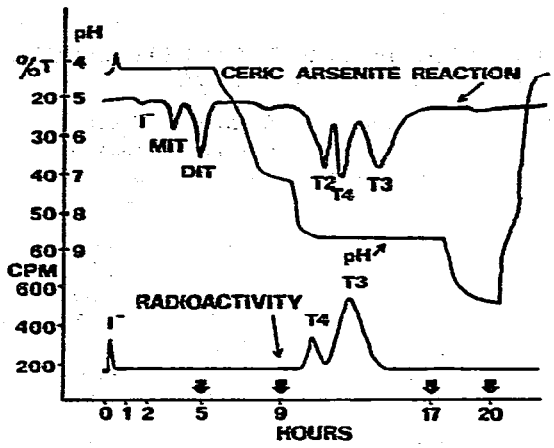


Fig. 25. Chromatographic separation of a synthetic mixture of iodinated amino acids labelled with ^{131}I in the 3′- or 3′,5′-positions, showing small iodide contaminant; 2.5 nmole of MIT, DIT and T4 and 7.5 nmole of T2 and T3 were applied to the column. The cerium(IV)–arsenite reaction results are printed out about 1.5 h later then the radioactivity measurement.

this type of separation. The main aim of their work was to overcome the problems due to poor reproducibility of Sephadex G-25 separations and fragmentation of gel particles when using operating conditions that caused clogging of the column. CPG 10-75 and 10-240 were used as column packings; 50 × 0.8 cm columns gave the separations shown in Figs. 27 and 28. Ethyl acetate–methanol–2 *N* ammonia solution (400:100:40) and ethyl acetate–methanol–4 *N* ammonia solution (400:60:30) are suitable mobile phases.

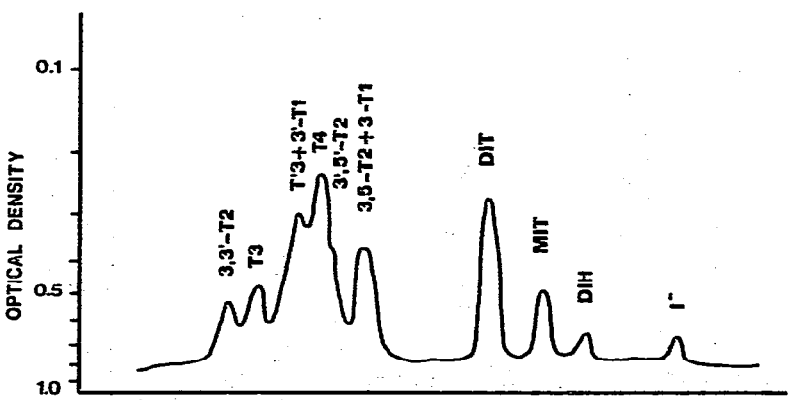


Fig. 26. Elution profiles in the cation-exchange chromatography of iodoamino acids. A mixture of authentic iodoamino acids containing 2 nmole of each compound except for DIH (20 nmole) was used. Peaks: MIT = monoiodotyrosine; DIT = diiodotyrosine; 3-T_1 = 3-monoiodothyronine; 3′-T_1 = 3′-monoiodothyronine; 3,5-T_2 = 3,5-diiodothyronine; 3,3′-T_2 = 3,3′-diiodothyronine; T_3 = 3,3′,5-triiodothyronine; T′$_3$ = 3,3′,5′-triiodothyronine; T_4 = thyroxine; DIH = diiodohistidine; MIH = monoiodohistidine.

TABLE 12

ELUTION VOLUMES AND RELATIVE PEAK AREAS IN THE CATION-EXCHANGER CHROMATOGRAPHIC ANALYSIS OF IODOAMINO ACIDS AND IODIDE

Compound	*Abbreviation*	*Approx. elution volume (ml)*	*Peak area measured (cm^2/nmole)*	*Relative peak area per mole*	*Relative peak area per iodine atom*
Monoiodotyrosine	MIT	37	1.52	1.00	1.00
Diiodotyrosine	DIT	50	3.82	2.50	1.25
3-Monoiodothyronine	3-T_1	80	1.53	1.00	1.00
3′-Monoiodothyronine	3′-T_1	96	0.50	0.33	0.33
3,5-Diiodothyronine	3,5-T_2	80	2.56	1.68	0.84
3,3′-Diiodothyronine	3,3′-T_2	113	2.32	1.53	0.76
3′,5′-Diiodothyronine	3′,5′-T_2	88	2.04	1.34	0.67
3,3′,5-Triiodothyronine	T_3	106	2.94	1.93	0.64
3,3′,5′-Triiodothyronine	$T_{3'}$	96	3.27	2.15	0.72
Thyroxine	T_4	91	5.72	3.75	0.94
Monoiodohistidine	MIH	—	0.005	<0.01	<0.01
Diiodohistidine	DIH	27	0.07	0.04	0.02
Iodide	I^-	6*	2.96	1.94	1.94

* Iodide was not retarded in this chromatogram.

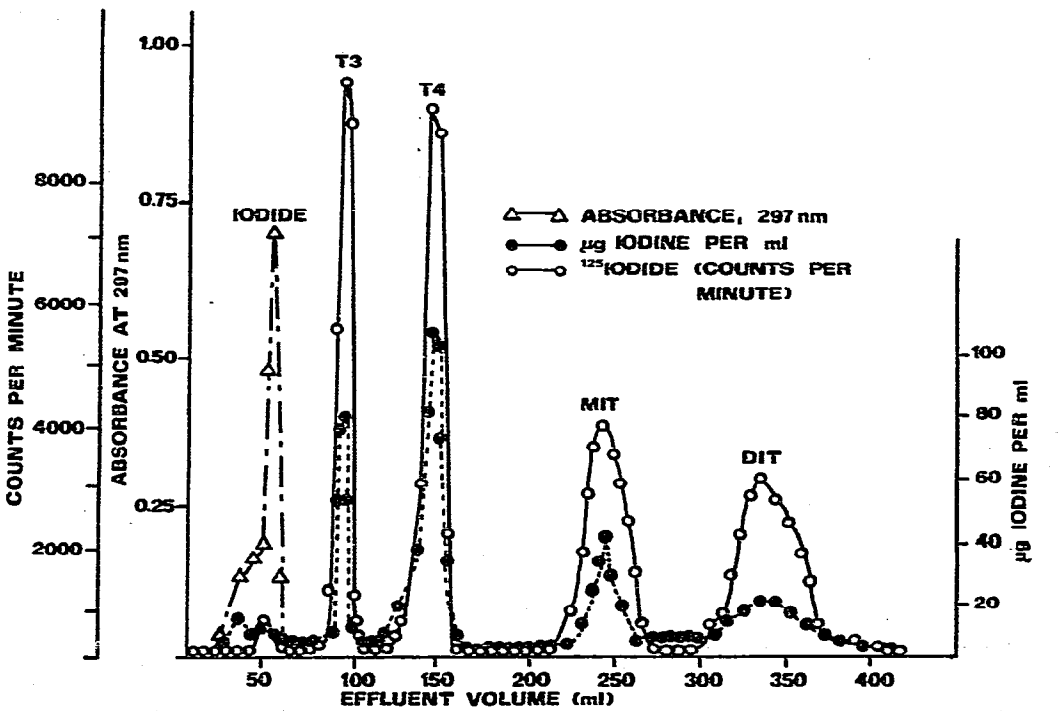


Fig. 27. Elution pattern from a CPG 10-75 column of a mixture of pure iodoamino acids and $Na^{125}I$. Solid line and open circles = absorbance at 297 nm. Dotted line and closed circles = iodine concentration (µg/ml). Broken line and triangles = $Na^{125}I$ (counts/min/ml). Eluting mixture = ethyl acetate–methanol–2 *N* ammonia solution (400:100:40).

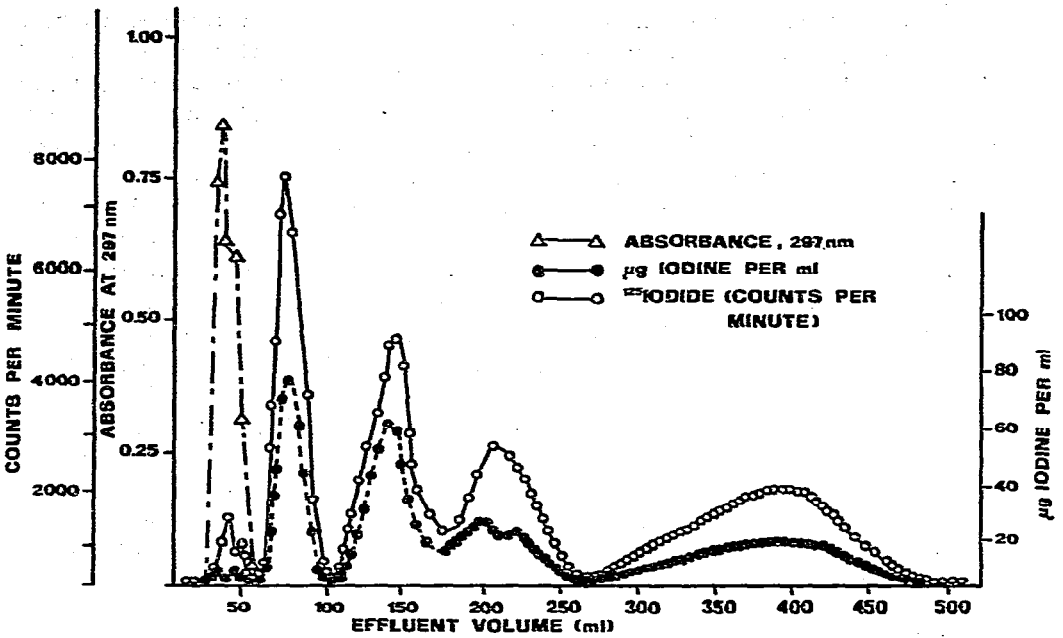


Fig. 28. Elution pattern from a CPG 10-240 column of a mixture of pure iodoamino acids and $Na^{125}I$. Lines and symbols as in Fig. 27. Eluting mixture: ethyl acetate–methanol–4 *N* ammonia solution (400:60:30).

2.5. Paper and thin-layer chromatography

Several outstanding reviews have been published that give much more detailed information on flat-bed separations of iodinated amino acids (Plaskett[132]; Stahl[158]; Pataki[126]; Cahnmann[20]; Hais[64]) and the reader is directed to these for more than introductory data regarding this type of compounds. In general, mobile phases suitable for paper chromatography are equally suitable for thin-layer chromatography (TLC). Of course, when comparing different TLC methods with paper chromatography, the relative positions of the spots and their separation may differ with the same solvent. Extensive lists of suitable mobile phases have been presented by Roche *et al.*[144], Michel[105], Schorn and Winkler[153] and Cahnmann[20].

The paper chromatography of iodinated amino acids has been reviewed by Plaskett[132].

A variety of mobile phases have been used for the separation of iodinated amino acids. Ammonia-containing systems are used in combination with phenol or collidine and are more effective in the separation of iodothyronines than iodotyrosines. Phenol-containing systems are used relatively rarely, because they are slow in drying and are capable of separating iodide, diiodotyrosine and thyroxine only with a considerable residue at the starting position. Collidine satured with water in an ammoniacal atmosphere is an excellent system for separating iodide, monoiodotyrosine, diiodotyrosine, thyroxine and 3,5,3′-triiodothyronine. A variation using both collidine and lutidine has been reported by Gross *et al.*[60].

It would perhaps be surprising if in this category of derivatives aliphatic alcohol-containing systems could not be used. Mobile phases such as *n*-butanol–2 *N* ammonia solution (1:1), *n*-butanol–3 *N* ammonia solution (1:1), *n*-butanol–6 *N* ammonia solution (1:1), *n*-butanol–9 *N* ammonia solution (1:1) and *n*-butanol–water–ammonia solution (250:178:72) can be recommended. At lower ammonia concentrations thyroxine is separated from 3,5,3′-triiodothyronine and 3,5-diiodothyronine, which form overlapping spots, while at higher ammonia concentrations 3,5-diiodothyronine is obtained separately at the expense of a mixed spot of thyroxine and 3,5,3′-triiodothyronine. Obviously, the separation capacity of a particular mobile phase depends on the proportion of ammonia, as has been demonstrated by Plaskett[132] (Table 13). Maximal resolution between thyroxine and 3,5,3′-triiodothyronine occurs at ammonia concentrations less than 2 *N*; concentrations between 0.75 and 1 *N* are to be preferred. With increasing ammonia concentration the resolution decreases as the mobility of both components increases. One of these mobile phases, namely *tert.*-pentanol–2 *N* ammonia solution (1:1), is particularly useful because of its clear separation of thyroxine from 3,5,3′-triiodothyronine and of tetraiodothyroacetic acid from 3,5,3′-triiodothyroacetic acid. It should be borne in mind that in the pentanol-containing systems there may be large differences in published R_F values (Plaskett[132], Wilkinson and Bowden[185]) and that, although applicable within one laboratory, owing to the difficulty of ensuring standard conditions these data cannot be transferred between different laboratories.

TABLE 13

R_F VALUES OF FIVE MAJOR IODINE-CONTAINING CONSTITUENTS OF THYROID HYDROLYSATES IN THE SOLVENT SYSTEM *n*-BUTANOL–AQUEOUS AMMONIA (1:1) WITH VARIOUS AMMONIA CONCENTRATIONS

Compound	*Ammonia concentration (N)*						
	0.25	*0.50*	*0.75*	*1.00*	*1.50*	*2.25*	*5.00*
3-Monoiodotyrosine	0.15	0.10	0.10	0.10	0.07	0.07	0.09
3,5-Diiodotyrosine	0.07	0.07	0.07	0.07	0.06	0.07	0.12
Thyroxine	0.62	0.44	0.37	0.40	0.48	0.51	0.68
3,5,3′-Triiodothyronine	0.81	0.62	0.60	0.65	0.59	0.60	0.70
Iodide	0.20	0.20	0.21	0.21	0.21	0.23	0.27
$R_F(T_3)/R_F(T_4)$	1.31	1.41	1.62	1.63	1.23	1.18	1.03

Within this category of mobile phases the following can also be included: isopentanol saturated with 2 *N* ammonia solution (Maclagan *et al.*[94]), isopentanol saturated with 6 *N* ammonia solution (Maclagan *et al.*[94]), *tert.*-pentanol–ethanol–2 *N* ammonia solution (5:1:2) (Taurog *et al.*[169,170]) and *n*-butanol–ethanol–2 *N* ammonia solution (5:1:2) (Taurog *et al.*[170]; De Groot and Davis[29]).

Neutral (frequently buffered) systems are represented by the following mobile phases: *sec.*-butanol–*tert.*-butanol–water (4:1:4.5) (Fink and Fink[45]), *n*-butanol–ethanol–water (4:1:5) (Fletcher[46]), methanol–0.2 *M* ammonium acetate (1:2.5)(pH 6.1) (Roche and co-workers[142,143,146]), *n*-butanol saturated with phosphate buffer (pH 7.2) (Gross and Leblond[61]), methanol–2 *M* ammonium acetate (pH 6.2) (1:0.5) at

40° and 9.5% ethanol–0.2 *M* ammonium carbonate (pH 7.8) (1:0.5) (Robins *et al.*[141]). This category of mobile phases does not offer the possibility of separating individual iodothyronines, but the separation of iodide, monoiodotyrosine, diiodotyrosine and iodothyronines is good.

Another category of mobile phases is that characterized by the presence of acetic or formic acid, *e.g.*, *n*-butanol saturated with 2 *N* formic acid (Gross *et al.*[60]), *n*-butanol–2 *N* acetic acid (1:1) and *n*-butanol–acetic acid–water (78:5:17) (Roche *et al.*[145]; Etling and Barker[40]).

With the trend towards rapid separation systems devised by West *et al.*[182,183] upper phases of ethyl acetate–methanol–2 *M* ammonia solution (5:2:3) and ethyl acetate–methanol–0.2 *M* acetic acid (5:2:3) were introduced. A pictorial survey of R_F values is presented in Fig. 29.

The separation of 3,5-diiodothyronines and 3,5,3′-triiodothyronine is difficult. According to Faircloth *et al.*[41], they can be separated by two-dimensional chromatography on silica gel using formic acid–water (1:5) in the first direction followed by *n*-butanol–ammonia solution–chloroform (188:35:30) in the second direction. The latter system has the advantage of separating all of the major iodinated amino acids, as shown by Milstein and Thomas[106]. Alternatively, Ouelette and Balcius[124] used ethanol–methyl ethyl ketone–2 *M* ammonia solution (1:4:1) in conjunction with an *n*-pentanol-containing system. Acetic acid (95%)–benzene or xylene is another example of a fast-running system (Schneider and Schneider[152]).

The following list of systems provides a further illustration of the wide range of aliphatic alcohol-containing mobile phases that can be used: *sec.*-butanol–concentrated ammonia solution (3:1) (Block *et al.*[15]), *sec.* butanol–3% ammonia solution (3:1) (Grinberg *et al.*[59]), *n*-butanol–*n*-pentanol–2 *N* ammonia solution (1:1:2) (Grinberg *et al.*[59]), *n*-butanol–*n*-pentanol–2 *N* ammonia solution (4:1:5) (Cameron[21]), *n*-butanol–*n*-pentanol–2 *N* ammonia solution (7:3:10) (Robins and Rall[140]), *tert.*-pentanol–2 *N* ammonia solution (1:1) (Gleason[55]), *tert.*-pentanol–2 *N* ammonia solution (163:37) Wilkinson and Bowden[185]), *tert.*-pentanol–water–ammonia solution (50:40:10) Etling and Barker[40]), *n*-butanol–acetic acid–water (120:30:50) (Wilkinson and Bowden[185]), *n*-butanol–acetic acid–water (40:6:15) (Taurog *et al.*[171]), *n*-butanol–acetic acid–water (78:10:12) (Pitt-Rivers and Tata[130]), *n*-pentanol–propionic acid–water (20:3:15) (McQuillan *et al.*[101]), *sec.*-butanol–acetic acid–water (33:2:22) (Taurog *et al.*[170]), *n*-propanol–acetic acid–0.001 *M* $Na_2S_2O_3$ (10:1:60) (Ljunggren[92]).

Gross *et al.*[60] reported the use of *n*-butanol–dioxan–2 *N* ammonia solution (4:1:5) and *n*-butanol–dioxan–0.6 *N* ammonia solution (4:1:5). These two (especially the former) are probably the most widely applied mobile phases in the paper chromatography of iodinated amino acids today. Spots of iodotyrosines, iodide, thyroxine and 3,5,3′-triiodothyronine are clearly separated in these mobile phases. In order to increase the difference between thyroxine and triiodothyronine, lower concentrations of ammonia should be used in the mobile phase (Benua and Dobyns[13]). The application of dioxan-containing mobile phases may suffer from the presence of peroxides, which result in the formation of multiple spots of thyroxine. Also, careful investigation by radioactive techniques revealed that the separation of both main thyroid hormones is incomplete with the system *n*-butanol–dioxan–2 *N* ammonia solution (4:1:5). Some workers believe (Plaskett[132]) that the inclusion of dioxan in the mobile phase is not essential and that with simpler *n*-butanol–ammonia systems

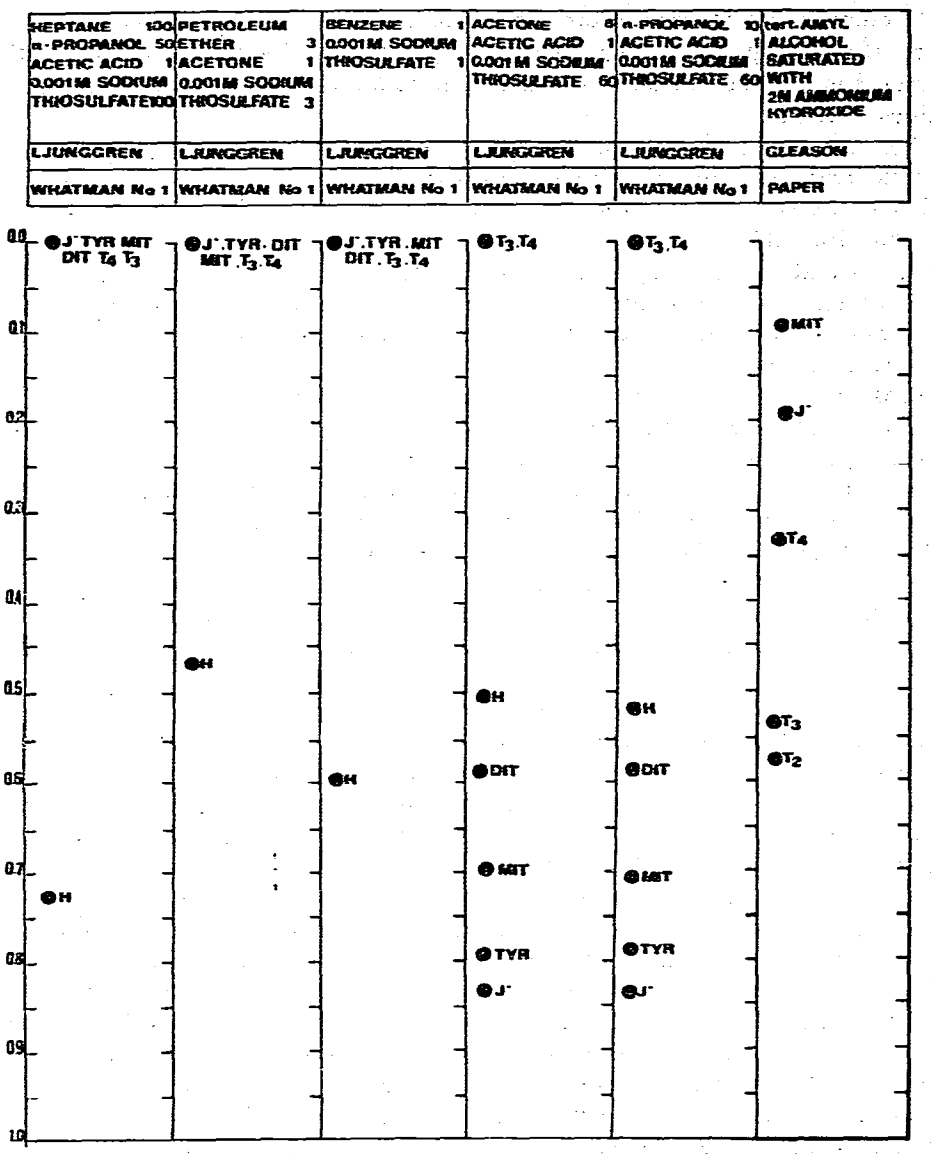


Fig. 29

even better results can be achieved, provided that an appropriate choice of ammonia concentration is made.

Some additional observations seem worth mentioning here. In general, R_F values decrease with an increase in the number of iodine atoms introduced into the aromatic residues in alkaline mobile phases, whereas the same substitution leads to increased R_F values in acidic mobile phases. Acidic mobile phases are more suitable for the separation of iodotyrosines, but better separations of iodothyronines are ob-

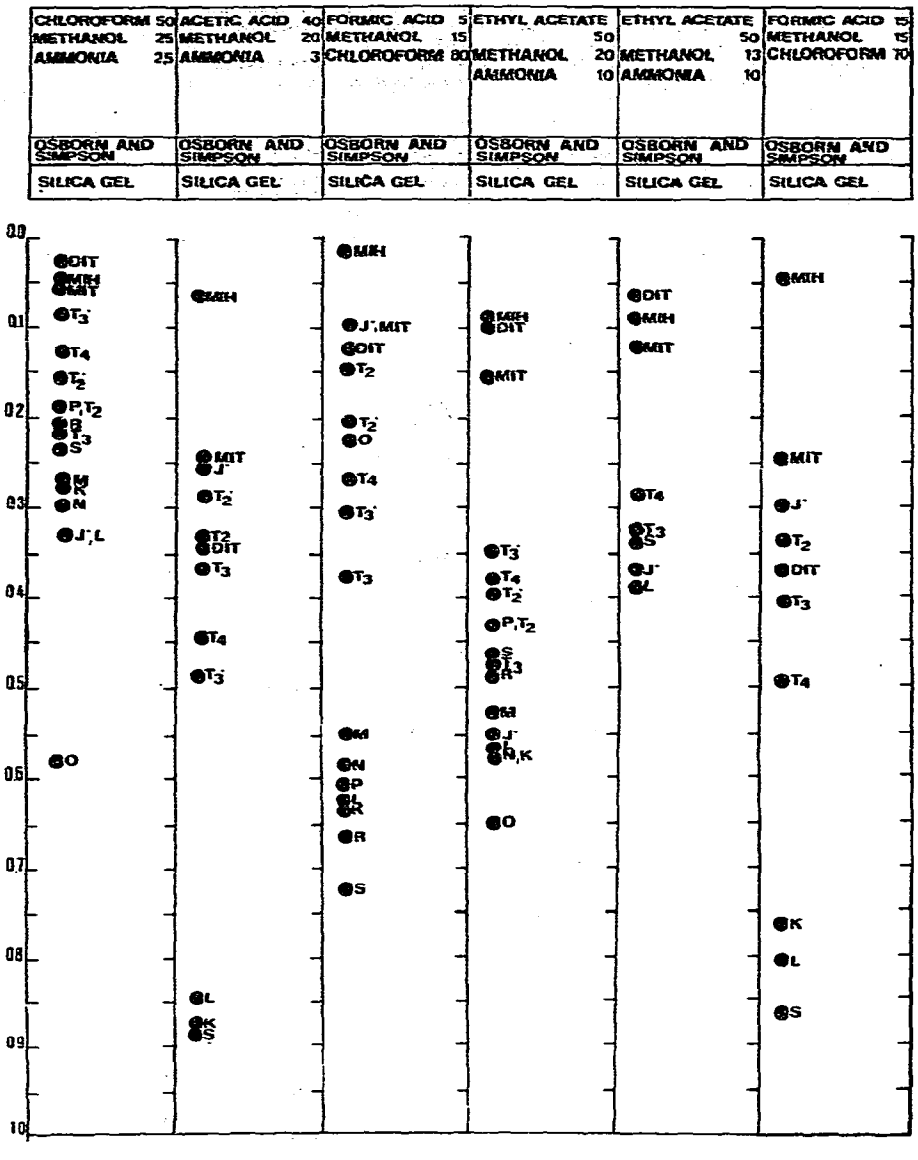


Fig. 29 *(continued)*

tained with alkaline solvents. Care should be taken to avoid overloading, which frequently occurs and results in tailing.

Among the artefacts that occur in the chromatography of iodinated amino acids, one should mention ester formation when T_3 or T_4 is extracted with alcohols (Osborn and Simpson[123]; Bellabarba and Sterling[12]). In order to prevent this undesirable reaction, extraction must be carried out very rapidly and the solution must be made alkaline as rapidly as possible. However, when eluting T_4 from silica gel

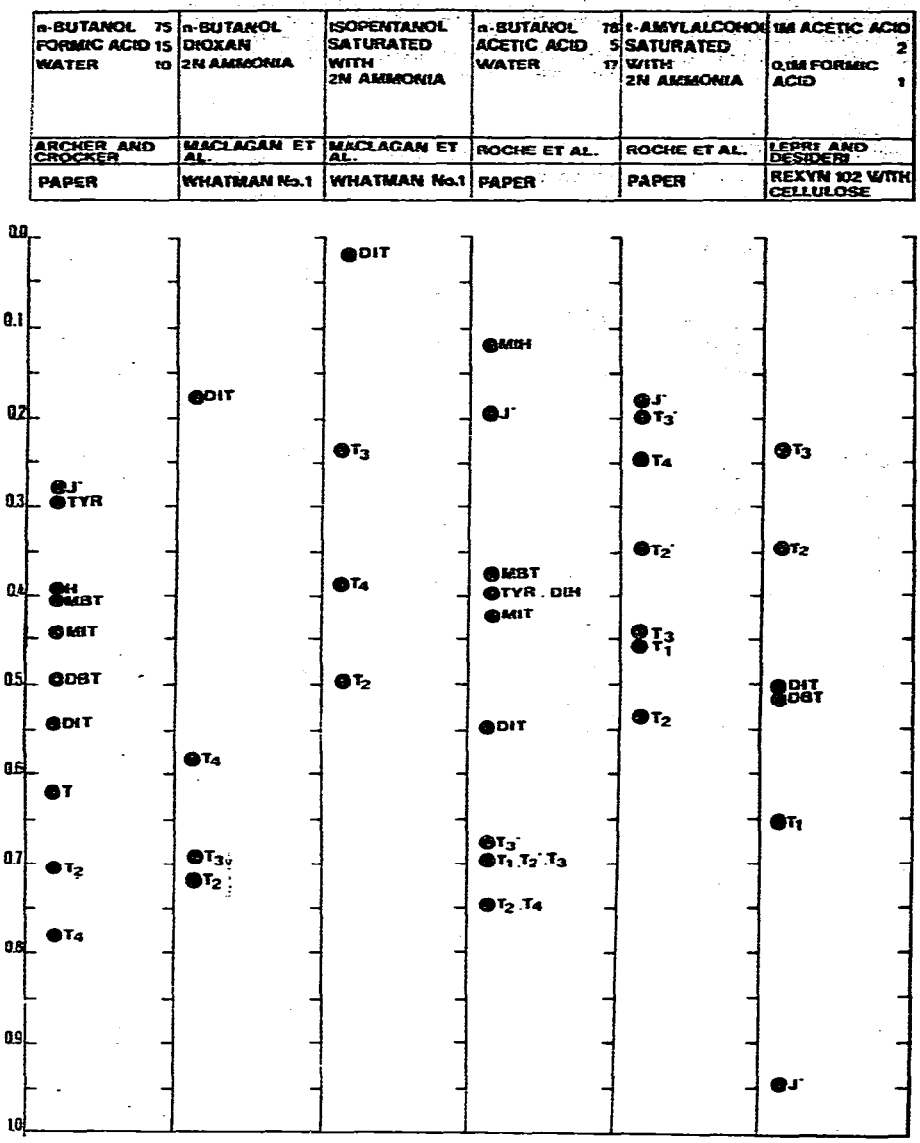


Fig. 29

with 0.2 *M* sulphuric acid in methanol or when T_3 is determined in serum by shaking the serum with methanol–chloroform in 0.025 *M* sulphuric acid, no ester formation occurs (West *et al.*[102]; Radichevich and Werner[134]; Cahnmann[20]). On the other hand, artefacts arising from deiodination are favoured in acidic solvents, whereas those arising from oxidation are favoured in alkaline media. Sometimes it is therefore recommended to work in an inert atmosphere (Cahnmann[20]).

In the chromatography of 3,3',5-triiodothyronine, formation of double spots was observed by Stanford and Golder[159].

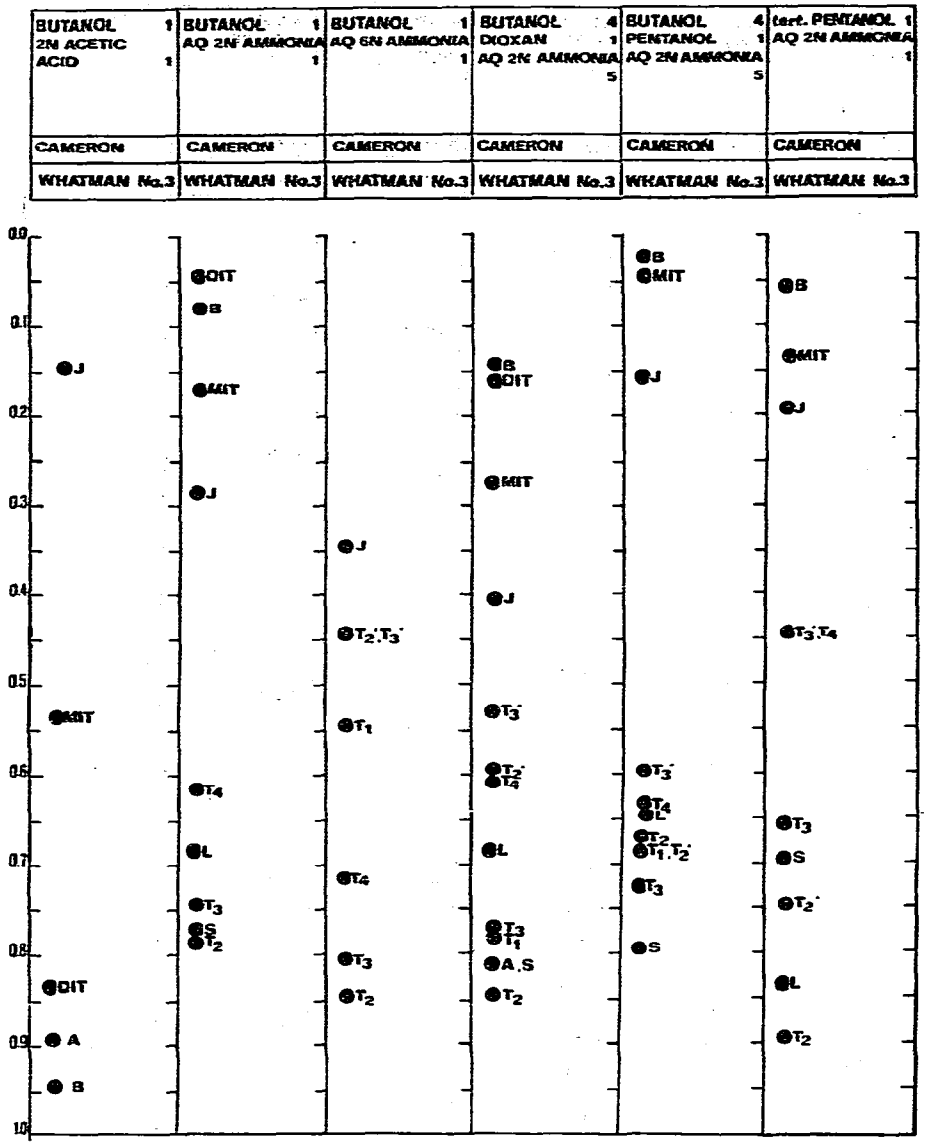


Fig. 29 *(continued)*

Iodinated amino acids can also be well separated by thin-layer chromatography using cellulose as the sorbent, as demonstrated by Sofianides *et al.*[156]. Thyroxine, triiodothyronine, 3,5-diiodotyrosine, 3-monoiodotyrosine, 3,5-diiodothyronine and tetraiodothyroacetic acid were separated by two-dimensional chromatography (20 × 20 cm plates) using *tert.*-butanol–3% ammonia solution (3:1) in the first direction and *n*-butanol–glacial acetic acid–water (4:1:1) in the second direction. The quality of separation can be seen from Fig. 30. R_F values in the recommended solvent systems are given in Table 14.

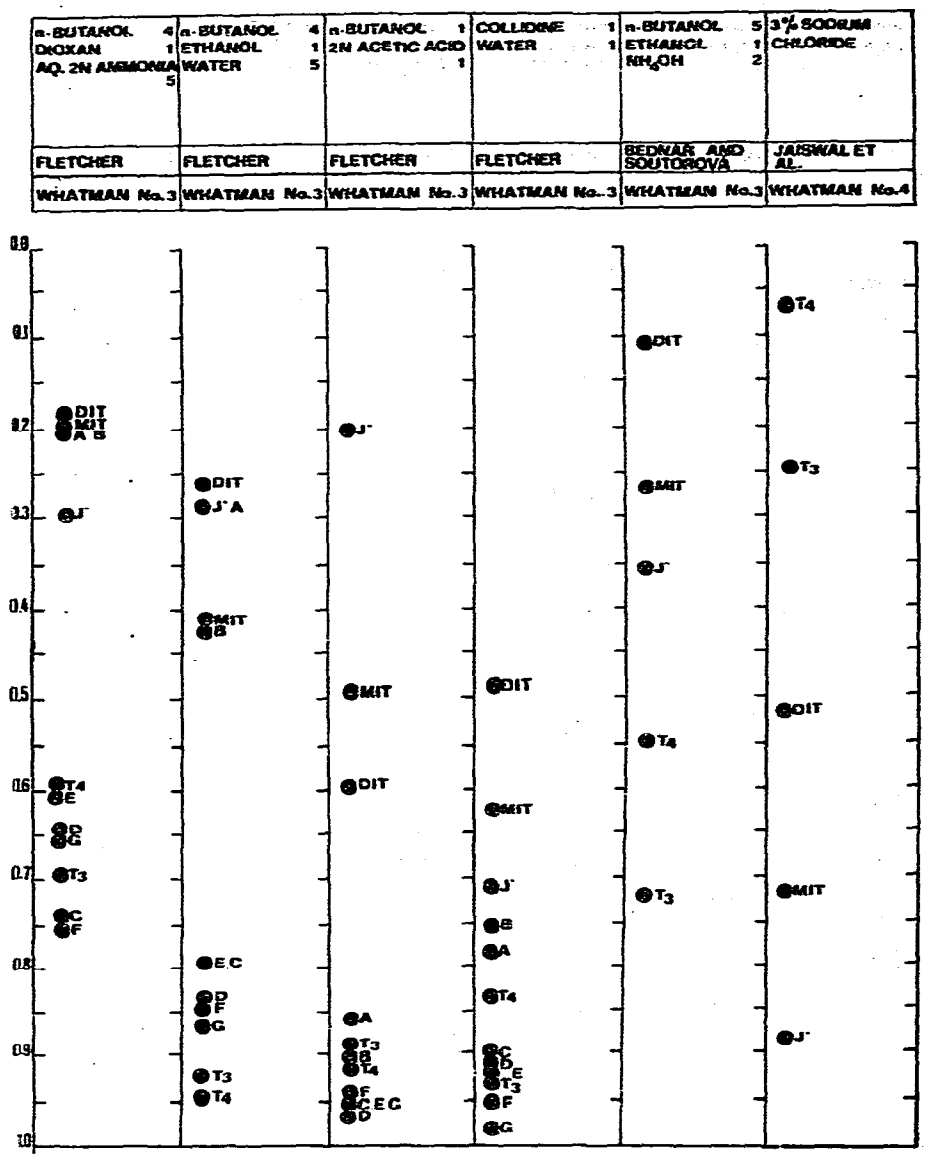


Fig. 29

For identification purposes, the chromatographic behaviour of acyl derivatives of iodo compounds and their methyl esters and methyl ethers in TLC in a number of mobile phases was described by Osborn and Simpson[123] (Tables 15 and 16).

For the separation of methyl esters of iodinated amino acids on silica gel layers (Stouffer *et al.*[166]), chloroform–methanol–formic acid (80:15:5) can be used. N,O-Dipivalyl methyl esters of iodinated amino acids can be separated inisooctane–chloroform–formic acid (10:20:1). R_F values of these derivatives are summarized in Table 17).

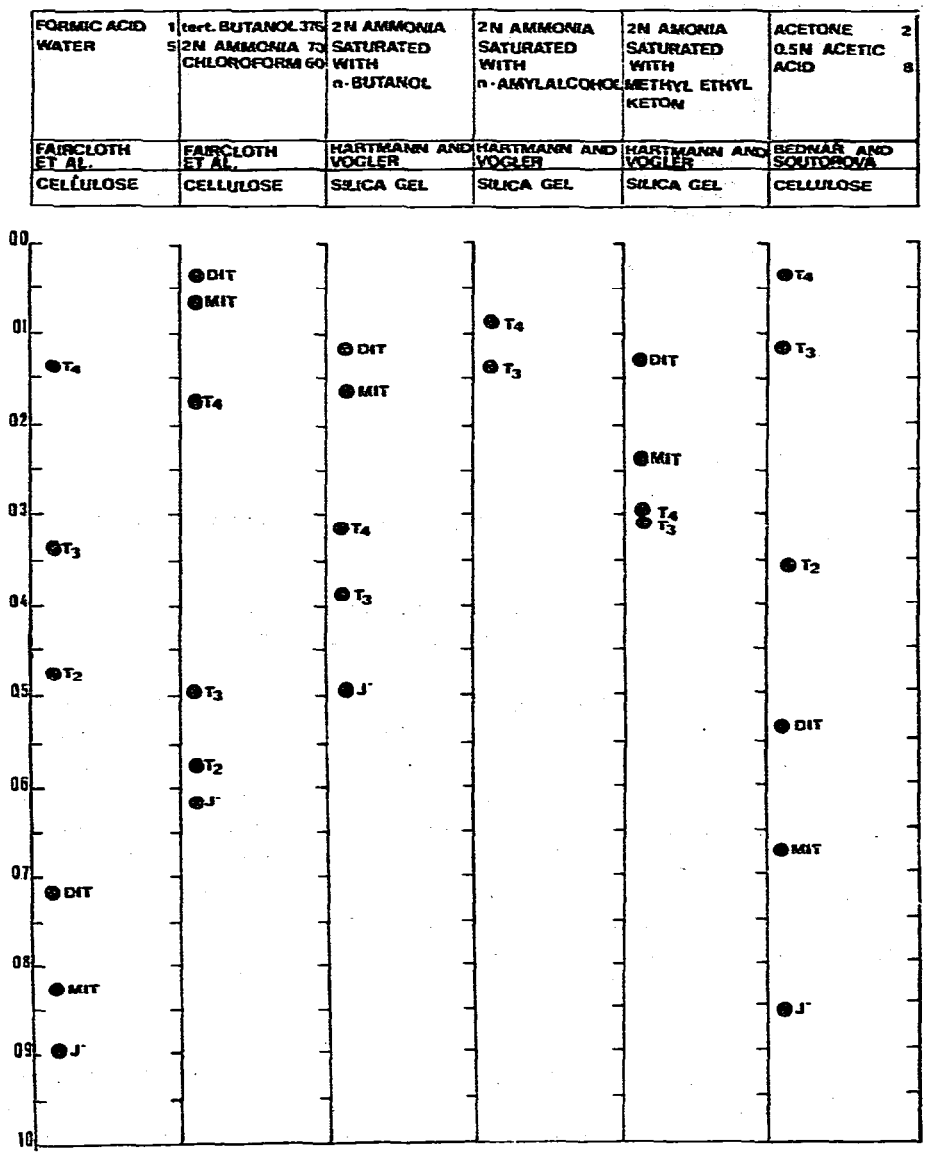


Fig. 29. Survey of R_F values of iodinated amino acids. T = Thyronine; T_1 = 3-monoiodothyronine; T_2 = 3,5-diiodothyronine; T_3 = 3,5,3′-triiodothyronine; T_4 = thyroxine; J^- = iodide; MIT = monoiodotyrosine; DIT = diiodotyrosine; MIH = monoiodohistidine; DIH = diiodohistidine; A = 3,5-diiodo-4-hydroxyphenylpyruvic acid; B = 3,5-diiodo-4-hydroxyphenyllactic acid; C = 3,5-diiodo-4-(3-iodo-4-hydroxyphenoxy)phenylacetic acid; D = 3,5-diiodo-4-(3,5-diiodo-4-hydroxyphenoxy)phenylacetic acid; E = 3,5-diiodo-4-(3,5-diiodo-4-hydroxyphenoxy)phenyllactic acid; F = 3,5-dioido-4-(3-iodo-4-hydroxypjenoxy)phenylpyruvic acid; G = 3,5-diiodo-4-(3,5-diiodo-4-hydroxyphenoxy)phenylpyruvic acid, H = 2,6-diiodohydroquinone; K = 3,5-diiodothyroacetic acid; L = 3,5,3′-triiodothyroacetic acid; M = 3,5,3′-triiodothyrolactic acid; N = 3,5,3′-triiodothyropyruvic acid; O = 3,5,3′-triiodothyronamine; P = 3,5,3′,5′-tetraiodothyrolactic acid; R = 3,5,3′,5′-tetraiodothyropyruvic acid; S = 3,5,3′,5′-tetraiodothyroacetic acid; T_3G = triiodothyronine-glucuronide; T_4G = thyroxine glucuronide; TG = thyroeglobulin; MBT = monobromotyrosine; DBT = dibromotyrosine. References cited: Ljunggren[92]; Gleason[55]. Osborn and Simpson[122,123]. Archer and Crocker[1]; Maclagan *et al.*[94]; Roche *et al.*[147,148]; Lepri and Desiden[90]; Cameron[21]; Fletcher[46]; Bednář and Soutorová[11]; Jaiswal *et al.*[29]; Faircloth *et al.*[41]; Hartmann and Vogler[70].

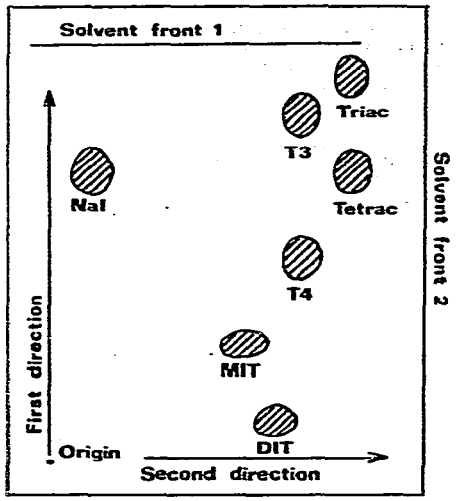


Fig. 30. Chromatogram stained with modified CAMB reagent demonstrating the separation of T4, T3, Tetrac, Triac, MIT, DIT and NaI.

The separation of derivatives does not serve for the flat-bed separation process itself but these methods were developed for identification purposes in connection with gas chromatography (see p. 272).

In order to achieve maximal and approximately equal resolutions of iodide, 5-monoiodotyrosine, 3,5-diiodotyrosine, 3,5,3′-triiodothyronine and 3,5,3′,5′-tetraiodothyronine, which is difficult on silica gel or cellulose (*cf.*, the survey of R_F values), the observations of Carlton and Bradbury[23] on competitive sorption in mixed beds was made use of by Ouelette and Balcius[124]. Cellulose and silica gel G were mixed in the ratios shown in Table 18 and the following mobile phases were used:

(1) ethanol–methyl ethyl ketone–2 *N* ammonia solution (1:4:1);
(2) *tert.*-amyl alcohol–dioxan–1 *N* ammonia solution (2:2:1);
(3) acetone–*n*-butanol–1 *N* ammonia solution (4:1:1);
(4) ethylene dichloride–*n*-butanol–1 *N* ammonia solution (1:8:1).

TABLE 14

R_F VALUES OF IODINATED AMINO ACIDS ON CELLULOSE THIN LAYERS IN TWO DIFFERENT MOBILE PHASES

Mobile phase 1 = *tert.*-butanol–3% ammonia solution (3:1). Mobile phase 2 = *n*-butanol–glacial acetic acid–water (4:1:1).

Compound	*Mobile phase 1*		*Mobile phase 2*	
	Average R_F	*Range*	*Average* R_F	*Range*
Triiodoacetic acid	0.90	0.81–0.98	0.93	0.87–0.96
Diiodothyroxine	0.90	0.83–0.98	0.78	0.71–0.95
Triiodothyronine	0.83	0.70–0.95	0.80	0.73–0.87
Tetraiodoacetic acid	0.72	0.69–0.79	0.94	0.89–0.96
Thyroxine	0.67	0.50–0.79	0.81	0.75–0.89
Sodium iodide	0.68	0.60–0.78	0.14	0.08–0.25
Diiodotyrosine	0.10	0.06–0.16	0.69	0.64–0.75
Monoiodotyrosine	0.28	0.22–0.33	0.64	0.56–0.73

Iodide served as an internal standard as its mobility changes only slightly in the above solvents.

It can be seen from the above tables that R_{Iodide} values decrease with decreasing concentration of silica gel for thyronines, whereas they are relatively constant or increase slightly for iodinated tyrosines. As one would expect in alkaline mobile

TABLE 15

R_F VALUES OF ACYL DERIVATIVES OF IODINATED AMINO ACIDS

Compound	*System**				
	a	*b*	*c*	*d*	*e*
N-Acetylthyroxine	0.21	0.82	0.52	0.45	—
N-Acetyl-3,5,3′-triiodothyronine	0.32	0.77	0.48	0.55	—
N-Acetyl-3,3′,5′-triiodothyronine	0.14	0.85	0.55	0.41	—
N-Acetyl-3,5-diiodothyronine	0.31	0.77	0.45	0.53	—
N-Acetyl-3,3′-diiodothyronine	0.27	0.79	0.43	0.47	—
N-Acetyl-3-monoiodotyrosine	0.13	0.72	—	—	0.21
N-Acetyl-3,5-diiodotyrosine	0.08	0.77	—	—	0.13
N-Acetyl-3-monoiodohistidine	0.11	0.33	—	—	0.15
O,N-Diacetylthyroxine	0.54	0.85	0.63	0.77	—
O,N-Diacetyl-3,5,3′-triiodothyronine	0.70	0.82	0.59	0.89	—
O,N-Diacetyl-3,3′,5′-triiodothyronine	0.40	0.86	0.63	0.67	—
O,N-Diacetyl-3,5-diiodothyronine	0.68	0.83	0.57	0.85	—
O,N-Diacetyl-3,3′-diiodothyronine	0.63	0.82	0.58	0.80	—
N-Carbobenzoxythyroxine	0.35	0.92	0.72	0.60	—
N-Carbobenzoxy-3,5,3′-triiodothyronine	0.49	0.88	0.63	0.67	—
N-Carbobenzoxy-3,3′,5′-triiodothyronine	0.32	0.92	0.75	0.57	—
N-Carbobenzoxy-3,5-diiodothyronine	0.45	0.87	0.61	0.66	—
N-Carbobenzoxy-3,3′-diiodothyronine	0.43	0.89	0.66	0.64	—
N-Carbobenzoxy-3-monoiodotyrosine	0.33	0.83	—	—	0.38
N-Carbobenzoxy-3,5-diiodotyrosine	0.26	0.89	—	—	0.30
N-Carbobenzoxy-3-monoiodohistidine	0.30	0.57	—	—	0.32
O,N-Dicarbobenzoxy-3,5-diiodotyrosine	0.66	—	—	—	0.56
O,N-Dicarbobenzoxythyroxine	0.65	—	0.80	0.82	—
O,N-Dicarbobenzoxy-3,5,3′-triiodothyronine	0.68	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′,5′-triiodothyronine	0.63	—	0.80	0.81	—
O,N-Dicarbobenzoxy-3,5-diiodothyronine	0.67	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′-diiodothyronine	0.67	—	0.76	0.83	—
N-Acetyl-O-methylthyroxine	0.33	—	—	0.57	—
N-Acetyl-O-methyl-3,5,3′-triiodothyronine	0.40	—	—	0.63	—
N-Acetyl-O-methyl-3,3,5′-triiodothyronine	0.30	—	—	0.54	—
N-Acetyl-O-methyl-3,5-diiodothyronine	0.37	—	—	0.60	—
N-Acetyl-O-methyl-3,3′-diiodothyronine	0.35	—	—	0.58	—
O-Acetyltetraiodothyropyruvic acid	0.49	—	0.77	0.62	—
O-Acetyl-3,5-3′-triiodothyropyruvic acid	0.50	—	0.69	0.69	—
O-Acetyltetraiodothyrolactic acid	0.25	—	0.75	0.45	—
O-Acetyl-3,5,3′-triiodothyrolactic acid	0.32	—	0.32	0.54	—
O,O-Diacetyltetraiodothyrolactic acid	0.50	—	0.81	0.60	—
O,O-Diacetyl-3,5,3′-triiodothyrolactic acid	0.51	—	0.51	0.66	—
N-Acetyl-3,5,3′-triiodothyronamine	0.85	—	0.63	—	—
O,N-Diacetylthyroxamine	0.90	—	0.78	—	—

* Systems: a = chloroform–methanol–ammonia (50:25:2.5); b = acetic acid–methanol–ammonia (40:20:3); c = formic acid–methanol–chloroform (5:15:80); d = ethyl acetate–methanol–ammonia (diluted 1:5); e = ethyl acetate–methanol–ammonia (50:20:10).

TABLE 16

R_F VALUES OF METHYL ESTERS AND METHYL ETHERS OF IODINATED AMINO ACIDS

Compound	*System**		
	a	*c*	*e*
Thyroxine methyl ester	0.77	0.40	—
3,5,3′-Triiodothyronine methyl ester	0.86	0.30	—
3,3′,5′-Triiodothyronine methyl ester	0.70	0.46	—
3,5-Diiodothyronine methyl ester	0.82	0.26	—
3,3′-Diiodothyronine methyl ester	0.79	0.36	—
3-Monoiodotyrosine methyl ester	0.75	0.24	—
3,5-Diiodotyrosine methyl ester	0.57	0.27	—
3-Monoiodohistidine methyl ester	0.88	0.85	—
3,5,3′-Triiodothyroacetic acid methyl ester	0.91	0.85	—
Tetraiodothyroacetic acid methyl ester	0.88	0.85	—
3,5-Diiodothyroacetic acid methyl ester	0.90	0.78	—
Tetraiodothyropyruvic acid methyl ester	0.90	0.82	—
3,5,3′-Triiodothyropyruvic acid methyl ester	0.93	0.75	—
Tetraiodothyrolactic acid methyl ester	0.85	0.77	—
3,5,3′-Triiodothyrolactic acid methyl ester	0.90	0.72	—
O-Methylthyroxine	0.32	0.32	0.47
O-Methyl-3,5,3′-triiodothyronine	0.33	0.27	0.50
O-Methyl-3,5-diiodothyronine	0.31	0.28	0.49
O-Methyltetraiodothyroacetic acid	0.51	0.87	0.58
O-Methyl-3,5,3′-triiodothyroacetic acid	0.48	0.85	0.63
O-Methyl-3,5-diiodothyroacetic acid	0.49	0.85	0.63

* Systems as in Table 15.

phases, the mobility decreases with increasing number of iodide atoms in the molecule. On the other hand, the mobility increases with increasing molecular weight.

2.6. Detection

Iodinated amino acids can be detected by several methods after flat-bed separation. As all of these compounds absorb UV light, their spots can be observed directly by using a mineral light lamp. In clinical analysis, thyroid hormones are labelled by administering radioiodide and hence radioactivity detection is also possible. The application of radioactivity has a limitation with regard to the actual status of the protein (for a review, see Zappi[191]).

In considering chemical detection, one has to bear in mind that compounds in this category do not have a common characteristic chemical function that would permit specific detection. Therefore, non-specific detection making use of the phenolic group, amino acid reactions and iodine detection has been employed. One such detection is the cerium(IV) sulphate–arsenious acid reagent (Kolthoff and Sandell's reagent), which has been applied in paper chromatography by Bowden and MacLagan[18]. The reagent consists of two solutions and yields white spots of iodinated amino acids on a yellow background:

Solution A: 10 g of $Ce(SO_4)_2 \cdot 4H_2O$ are added to 100 ml of 1 *N* H_2SO_4 previously cooled to 0°. The solution is centrifuged.

TABLE 17

THIN-LAYER CHROMATOGRAPHY OF THYROID HORMONES AND DERIVATIVES ON SILICA GEL

Compound	R_F		
	*Amino acids**	*Methyl ester***	*N,O-Dipivalyl methyl ester****
Thyroxine	0.58	0.41	0.54
Triiodothyronine	0.49	0.37	0.45
Diiodothyronine	0.30	0.28	0.32
Diiodotyrosine	0.38	—	—
Monoiodotyrosine	0.18	0.23	0.29
Thyronine	0.27	—	—
Tyrosine	0.12	—	0.28

* Chloroform–methanol–formic acid (70:15:15).
** Chloroform–methanol–formic acid (80:15:5).
*** Isooctane–methanol–formic acid (10:20:1).

TABLE 18

$R_{Iodide} \times 100$ VALUES OF IODINE-CONTAINING COMPOUNDS AT DIFFERENT SILICA GEL CONCENTRATIONS IN DIFFERENT SOLVENT SYSTEMS

Combined beads of silica gel G and cellulose.

*Solvent system**	*Compound*	*Silica gel G (%)*										
		0	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
A	MIT	49	72	67	60	56	51	50	49	51	46	47
	DIT	12	18	15	19	20	22	24	25	30	31	32
	T_3	200	190	176	157	140	130	120	112	108	96	88
	T_4	128	140	128	114	102	95	90	85	81	73	71
B	MIT	35	37	39	42	39	40	41	42	43	43	45
	DIT	8	14	17	20	22	22	24	26	27	29	30
	T_3	142	120	118	113	109	100	95	87	82	80	73
	T_4	92	92	89	89	87	83	81	77	73	68	63
C	MIT	36	37	39	40	41	42	45	47	47	47	53
	DIT	9	10	13	14	16	20	26	28	35	39	42
	T_3	139	113	110	103	99	94	86	80	78	74	71
	T_4	82	77	86	85	83	81	76	71	66	67	68
D	MIT	50	48	56	53	52	44	45	42	40	38	29
	DIT	15	22	20	20	20	17	21	22	20	26	27
	T_3	475	332	280	233	222	178	132	120	102	95	62
	T_4	261	200	189	169	141	106	105	96	96	89	61

* Solvent systems:
A = *tert.*-amyl alcohol–dioxan–1 *N* ammonia solution (2:2:1).
B = ethanol–methyl ethyl ketone–2 *N* ammonia solution (1:4:1).
C = acetone–*n*-butanol–1 *N* ammonia solution (4:1:1).
D = ethylene dichloride–*n*-butanol–1 *N* ammonia solution (1:8:1).

Solution B: 5 g of $NaAsO_2$ are dissolved with vigorous stirring in 100 ml of 1 *N* H_2SO_4 cooled to 0°.

Both solutions have to be kept in the cold and mixed in the ratio 1:1 before spraying the chromatogram.

The other widely used chemical detection is that applied by Gmelin and Virtanen[56]. In principle, this detection is based on the ability of organic iodides to catalyse the reduction of iron(III) ions by arsenious acid. As a result of concomitant oxidation and reduction, the insoluble pigment of Prussian blue appears at positions where iodinated amino acids are located. The reagent consists of the following solutions:

Solution A: 2.7 g of $FeCl_3 \cdot 6H_2O$ dissolved in 100 ml of 2 *N* HCl.

Solution B: 3.5 g of $K_3Fe(CN)_6$ dissolved in 100 ml of distilled water.

Solution C: 5.0 g of $NaAsO_2$ dissolved in 30 ml of 1 *N* NaOH at 0° and mixed with vigorous stirring with 65 ml of 2 *N* HCl.

Before being sprayed, the individual solutions are mixed (5 parts of solution A, 5 parts of solution B and 1 part of solution C). The excess of reagent is removed by washing the paper with water, which also removes the acid present and prevents the chromatogram from destroying.

A problem is that compounds such as phenols, vitamin C, tyrosine and other reducing substances give positive reactions with Gmelin and Virtanen's reagent. These artefacts can be eliminated by spraying the chromatogram with a mixture of solutions A and B (without arsenite). This reagent gives positive spots with interfering substances, but does not react with iodinated amino acids. Thus, by comparing two parallel chromatograms, one sprayed with the reagent containing arsenite and the other with the reagent in which arsenite is absent, there is the possibility of identifying iodo compounds.

Both of these reagents have also been applied for quantitation. The usual procedures of comparing spot areas and intensities with those of a standard series have been widely applied. It is also worth mentioning that other non-specific detection reactions such as that of palladium(II) chloride or silver nitrate together with diazotized sulphanilic acid or ninhydrin can be used.

When using natural materials, extraction procedures have to be applied before chromatographic separation. The scheme in Table 19 can be used as an example (Zappi[191]).

2.7. Isoelectric focusing and electrophoresis

For the analysis of biological samples representing multi-component protein mixtures, it is sometimes necessary to know the properties of unliganded T_3 and T_4. It therefore seems worthwhile mentioning that in isoelectric focusing (LKB 8101 analytical column at 5°, 4 W for 72 h) in an ampholyte–sucrose gradient both of these amino acids occur at different pHs. Whereas T_4 focuses at pH 3.8–4.3 (peak 4.3), the corresponding values for T_3 are 4.6–5.3 (peak 5.1) (Handwerger *et al.*[67]).

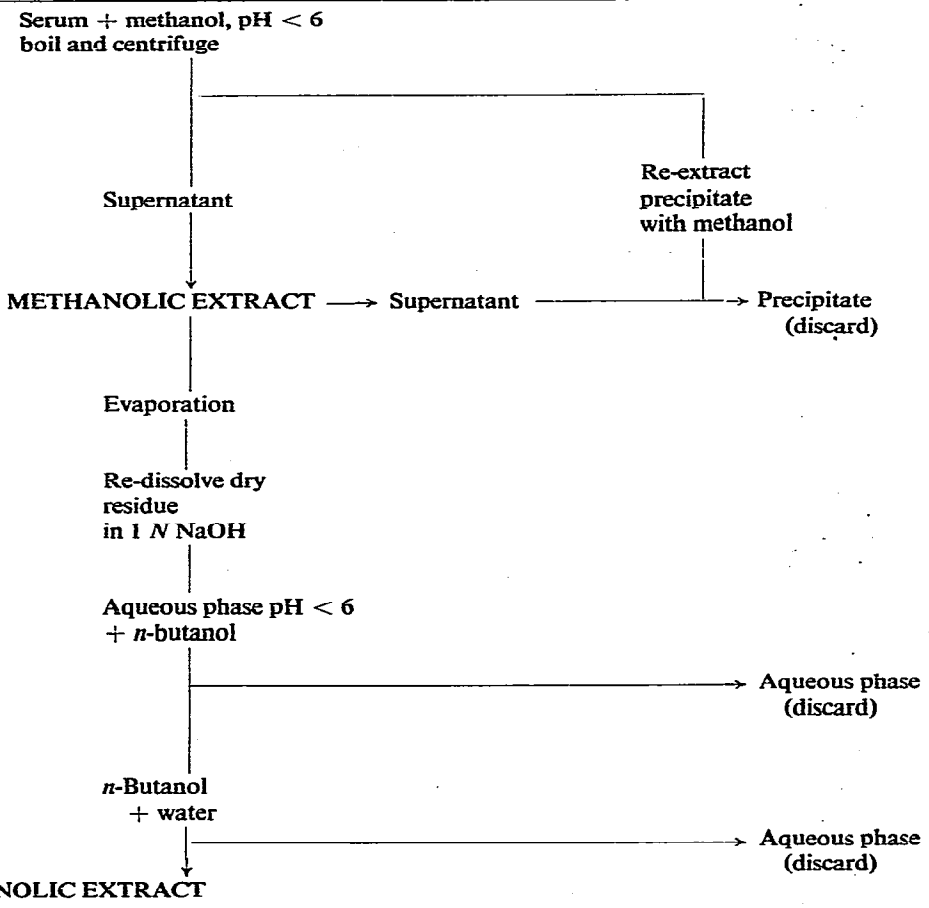
Electrophoresis of iodinated amino acids can be run on Sephadex G-25 thin layers, as reported by Reith and Brown[135]. These workers used a combination of thin-layer gel filtration and electrophoresis to separate dinitrophenyl derivatives of DNP-diiodotyrosine and DNP-monoiodotyrosine.

2.8. Gas chromatography

N,O-Bistrifluoroacetyl methyl esters were used for the gas chromatography

TABLE 19

COMBINED METHANOL–*n*-BUTANOL EXTRACTION OF PHENOLIC IODOAMINO ACIDS FROM PLASMA



of iodinated amino acids by Richards and Mason[136]. Chromatography was performed in a 4-ft. glass column packed with 3% SE on 80–100-mesh Diatoport S. Helium was used as the carrier gas at a flow-rate of 75 ml/min. The column was maintained at 250° and a flame-ionization detector was used. An example of the separation is presented in Fig. 31.

The gas chromatographic separation of iodinated amino acids (T_3, T_4, DIT, MIT) on OV-17-impregnated diatomite was recommended by Heinl *et al.*[72]. At a column temperature of 28° and a carrier gas (nitrogen) flow-rate of 40 ml/min the results shown in Fig. 32 were obtained.

The gas chromatography of iodinated amino acids can also be carried out with their trimethylsilyl derivatives, using a mixture of 5.00 ml of tetrahydrofuran, 2.00 ml of bis(trimethylsilyl)acetamide and 5 drops of chlorotrimethylsilane as the reagent. Squalene was used as the internal standard. The amino acid standards were

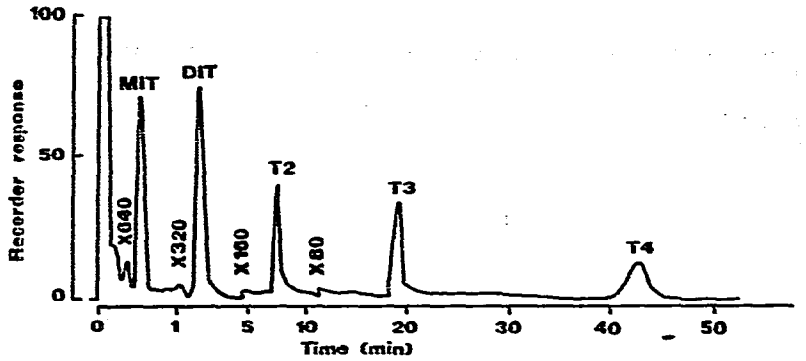


Fig. 31. Chromatogram of iodinated tyrosine and thyronine derivatives.

prepared at a concentration of 6 μmole per 5 ml in ammoniacal methanol. These standards were evaporated to dryness before derivatization, wetted with 100 μl of the reagent, sealed with a foil, heated under a hood on the edge of a boiling water-bath for 1 min, then cooled. The separation was carried out on a 2 ft. × 3 mm I.D. column packed with 2% SE-33 on Chrom Q. The flow-rate of the carrier gas (nitrogen) was 50 ml/min at 18 psi. A flame-ionization and an electron-capture detector were used. The column temperature was programmed from 150° to 180° at a rate of 10°/min. The results of a typical run are shown in Fig. 33.

Alternatively, an OV-1 column (4 ft. × 3 mm I.D.) can be used for the trimethylsilylated derivatives of iodinated amino acids. Isothermal conditions are used for the first 3 min of the separation, followed by temperature programming at a rate of 10°/min (Alexander and Scheig[2]). The separation is good, as can be seen from Fig. 34. The same workers have also applied another silylation procedure, in which a mixture of amino acids (1–3 mg of each) was suspended in 0.2 ml of acetonitrile and 0.2 ml of N,O-bis(trimethylsilyl)acetamide was added. The suspension was heated to 50° for 10 min and 2-μl aliquots of this solution were taken for analysis.

Another category of volatile derivatives of iodinated amino acids suitable for gas chromatography is N,O-dipivalyl methyl ester derivatives. The column used (4

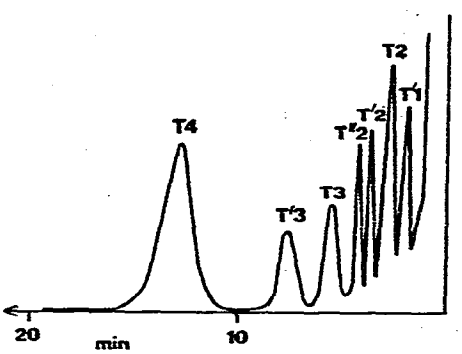


Fig. 32. Gas chromatographic separation of iodinated amino acids: flame-ionization detector, 300°, H_2 at 40 ml/h, air at 600 ml/min, column 500 × 3 mm packed with OV-17 on Diatomite CQ (80–100 mesh). Column temperature, 285°; carrier gass, N_2 at 40 ml/min.

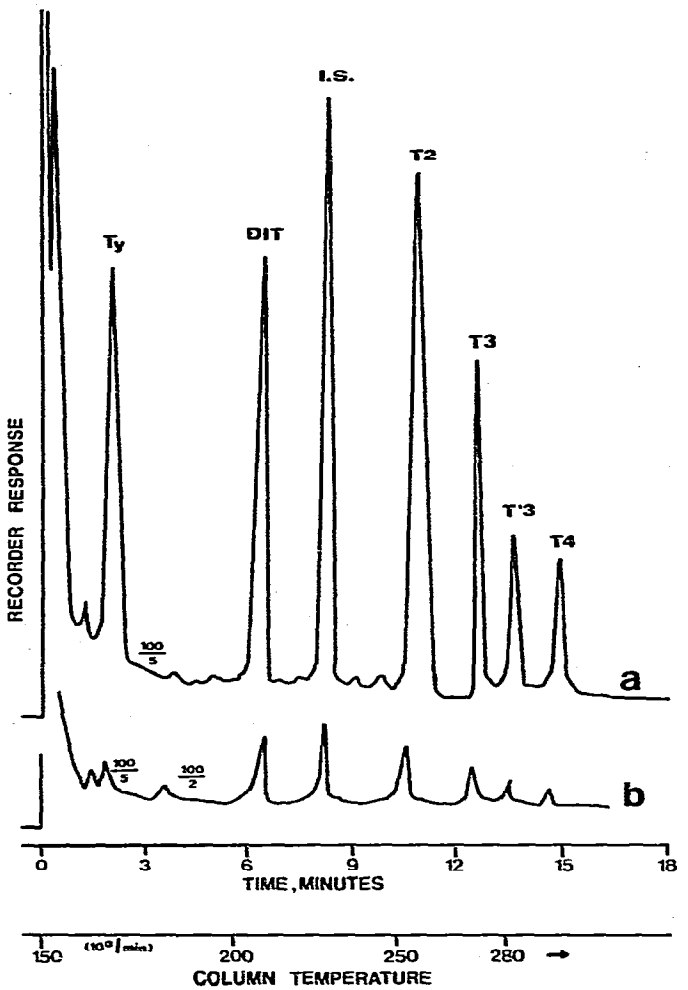


Fig. 33. Chromatograms of thyroid hormone standard mixture with a flame-ionization detector. (Hansen[68])

ft. × 2 mm I.D.) contained 5% OV-17 as the stationary phase and the temperature was programmed from 225° to 325° at a rate of 5°/min. A flame-ionization detector was used and the results of the separation are presented in Fig. 35 (Stouffer[165]).

3. OTHER HALOGENATED AMINO ACIDS

Halogenated amino acids (other than iodo compounds) do occur as constituents of proteins, although they are rather rare. Mono- and dibromotyrosine as well as monochloromonobromotyrosine are formed in invertebrate scleroproteins. It should be mentioned here that iodinated amino acids are also formed as integral parts of scleroproteins in invertebrates. For the purpose of identification, Sephadex chromatography is used. Thus, on a Sephadex G-15 column (55 × 22 cm) eluted with 0.02 *M* acetic acid, tyrosine is eluted after 210 ml of eluate have passed through the

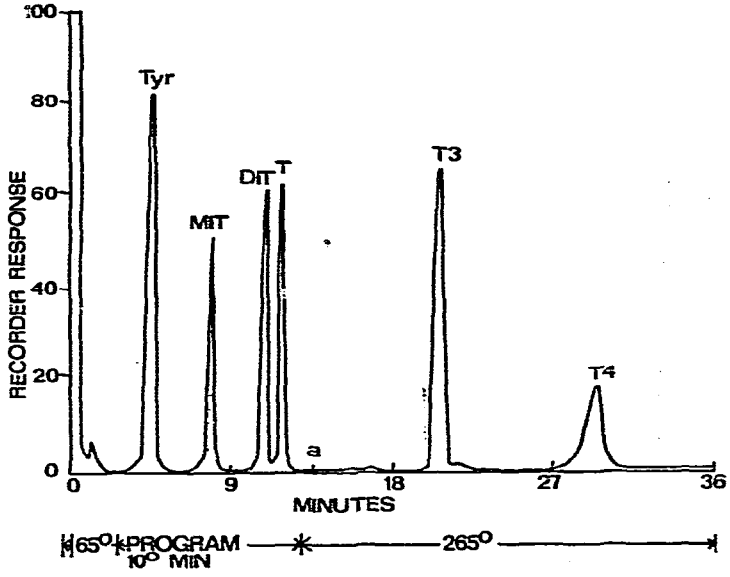


Fig. 34. Gas chromatogram of the trimethylsilyl derivatives of tyrosine, thyroxine, iodotyrosines and iodothyronines. The separation was carried out on a 4 ft. × 3 mm glass U-column containing OV-1. An isothermal temperature of 165° was maintained for 3 min and then programmed at 10°/min as indicated. Nitrogen was the carrier gas at 30 psig (60 ml/min at 165°). Injector temperature, 280°; detector, 285°. Maximum recorder response was $3 \cdot 10^{-9}$ A up to point (a), when it was changed to $9 \cdot 10^{-10}$ A. The amounts of each compound injected were Tyr 3.8, MIT 4.5, DIT 13.2, T 4.5, T3 13.2 and T4 14.6 μg.

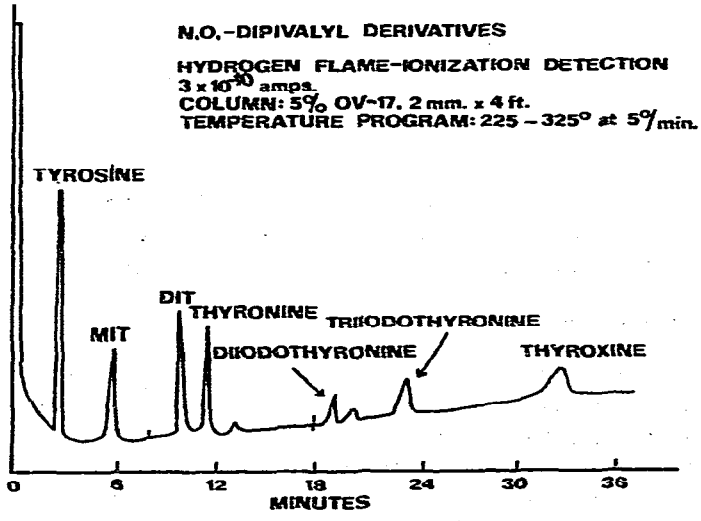


Fig. 35. Temperature-programmed separation of thyroid hormones as their N,O-dipivalyl methyl ester derivatives using a flame-ionization detector

TABLE 20

R_F VALUES OF HALOGENATED AMINO ACIDS ON PAPER

Solvent system: *n*-butanol–acetic acid–water (3:1:1).

Compound	R_F
Monobromotyrosine	0.56
Dibromotyrosine	0.69
Tyrosine	0.47

column. Monobromotyrosine is eluted at 360 ml, monochloromonobromotyrosine at 510 ml and dibromotyrosine at 600 ml. For paper chromatography, *n*-butanol–acetic acid–water (3:1:1) can be used. For R_F values, see Table 20 (Hunt and Breuer[74]). Diverse chlorinated amino acids, most of which are synthetic products, were chromatographed on paper by Zaima *et al.*[190].

4. HYDROXYLATED AMINO ACIDS

In accordance with the scope of this review, this section does not deal with hydroxylated derivatives of amino acids that are directly incorporated during proteosynthesis (*cf.*, tyrosine, serine, threonine).

4.1. Hydroxyproline

4.1.1. Ion-exchange chromatography and problems with ninhydrin detection

For routine 4-hydroxyproline (hypro) analyses, unlike for most other amino acids, specific colour reactions can be used, including those of Stegemann[163], and Neuman and Logan[116] or kits such as the Hypronosticon (Goverde and Veenkamp[57]). However, when molar proportions in relation to other amino acids are required, separation procedures are necessary.

For single-column amino acid analysis, capable of resolving hypro from other amino acids, the procedure of Goverde and Veenkamp[57] can be recommended. The samples are pre-treated and analysed as follows.

(a) Pre-treatment of samples.

(1) Apply a 5.0-ml urine sample to a 150 × 5 mm I.D. Dowex 50W-X8 (neutral H^+) column.

(2) Wash with 50 ml of distilled water.

(3) Elute with 4 *N* ammonia solution until peptide-free (15 ml).

(4) Dry the eluate quantitatively in a rotating evaporator.

(5) Hydrolyse the residue for 24 h with 6 *N* hydrochloric acid at 110° in a vacuum.

(6) Add S-carboxymethylcysteine (SCM) as an internal standard, centrifuge and dry three times in a rotating evaporator as usual in amino acid analysis.

(7) Dissolve the residue in 0.1 *N* hydrochloric acid.

(b) Procedure.

(8) Apply sufficient sample (equal to about 0.1 μmole of SCM) to a 135 × 6 mm I.D. Chromobeads B column equilibrated with 0.2 *N* citrate buffer (pH 2.85).

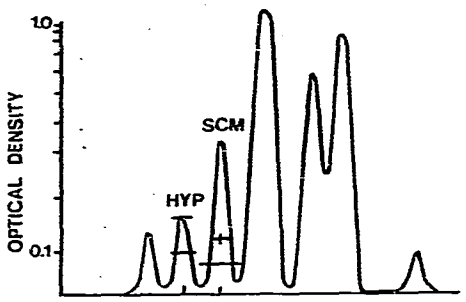


Fig. 36. Determination of hydroxyproline (Hyp) on a single-column amino acid analyser using S-carboxymethylcysteine (SCM) as an internal standard (I.S.). The 440-nm record was used for calculation; the tangents to the tops and the half-height lines of the curves have been drawn in full. Eluent, sodium citrate, 0.2 *N* (pH 2.85); temperature, 45°.

(9) Elute with the same buffer at 45° at a flow-rate of 34 ml/h until asp, tur and ser have been recorded.

The results of the separation are summarized in Fig. 36.

Proteoglycans of connective tissue, when analysed for amino acids, frequently show peaks faster than that of aspartic acid during routine automated ion-exchange chromatography. As it has been pointed out by Murray and Milstein[112] that hydroxylated amino acids may react with the formation of C-sulphates when heated with trace amounts of inorganic sulphate, it could be postulated that these unknown peaks may be artefacts arising upon hydrolysis by a similar reaction of bound sulphate. Indeed, Dziewiatkowski *et al.*[37] were able to prove that C-sulphate esters of hydroxyproline, serine and threonine are present in such hydrolysates [for elution times on Beckman UR-30 resin eluted by a stepwise gradient of citrate buffer (pH 3.25) which was changed to pH 4.3 after a running time of 85 min, see Table 21].

TABLE 21

COMPARISON OF ELUTION TIME OF O-SULPHATE ESTERS AND CORRESPONDING HYDROXYAMINO ACIDS

Analyses were carried out as suggested in the Beckman "Procedures Manual" (1966) for analysis of protein hydrolysates, except that as soon as a sample was delivered on to the column of UR-30 resin the recorder was started. Citrate buffer of pH 3.25 was changed to citrate buffer of pH 4.30 at 85 min.

Amino acid	*Elution time (min)*	*o-Sulphate ester*	*Elution time (min)*
Hydroxyproline	44.5	Hydroxyproline O-SO_4	17.0
Serine	56.5	Serine O-SO_4	18.0
Threonine	52.5	Threonine O-SO_4	18.0
Tyrosine	160.0	Tyrosine O-SO_4	21.5
Cysteic acid	18.5		

It is therefore advisable to take precautions against artefact formation in the analysis of materials that contain sulphated heteropolysaccharides; hydroxyproline, serine, threonine and perhaps tyrosine estimates may be too low.

Hydroxyproline (and proline), when detected by the ninhydrin procedure, requires the colorimeter to be set to an absorbance of 570 nm in order to obtain peaks

suitable for quantitation. On the other hand, the spectra of amino acid– and imino acid–ninhydrin reaction products, as shown in Fig. 37, represent a rationale for quantifying ninhydrin chromogens of imino acids, amino acids and closely related compounds with a single recording system operating at 405 nm (Table 22). As reported by Ellis and Garcia[38], single wavelength detection gives satisfactory results.

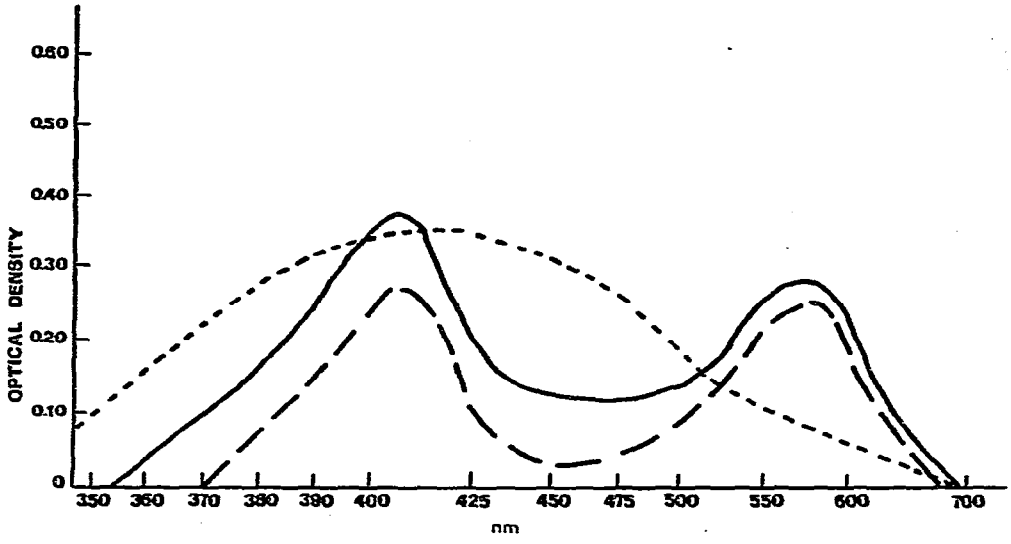


Fig. 37. Adsorption spectra of the ninhydrin products of cystine (———), threonine (— — —) and proline (· · · ·).

Alternatively, the detection problems can be solved by using a modified detection manifold (Ellis and Prescott[39]) in the routine Technicon procedure. First, the effluent is split into two almost equal streams, one of which is mixed with conventional ninhydrin reagent (Nin. I). The second modification consists in mixing a new ninhydrin reagent (Nin. II) with the other half of the effluent. The preparation of Nin. II is identical with that of Nin. I except that hydrindantin is omitted. The third modification involves the application of three colorimeters. As shown in Fig. 38, two of the colorimeters are equipped in such a way to permit absorbance measurements at 440 and 570 nm. The stream passing these two results from mixing Nin. I with the first half of the column effluent. The third instrument allowed readings to be made at 440 nm and was inserted in such a way as to allow absorbance measurement of the reaction products with the hydrindantin-free reagent. The main achievement of the system is the elimination the interference of the aspartic acid peak with hydroxyproline.

It must also be emphasized here that in the *in vitro* oxidation of protein-bound proline, numerous unusual peaks occur during amino acid analysis. Some of these were identified as 3-hydroxyproline, β-alanine and 4-aminobutyric acid (Gruber and Mellon[62]).

A fluorescamine-based amino acid analyser has been used with considerable success for the detection of hydroxyproline (Udenfriend *et al.*[178]), the detection limits

TABLE 22

RELATIVE ABSORBANCE OF NINHYDRIN CHROMOGEN WITH SPECIAL REFERENCE TO HYDROXYPROLINE DETERMINATION

Compound	*Relative absorbance**
Ammonium sulphate	1.00
Leucine	1.01
Isoleucine	1.02
Arginine	1.03
Threonine	1.03
Glycine	1.04
Alanine	1.04
Methionine	1.04
Tyrosine	1.04
Phenylalanine	1.04
Serine	1.05
Tryptophane	1.05
Aspartic acid	1.05
Valine	1.06
Histidine	1.11
Ornithine	1.15
Glutamic acid	1.21
Lysine	1.24
Cystine	1.36
Proline	1.06
Hydroxyproline	1.09

* Values given are $A_{405nm}:A_{570nm}$ for all compounds except proline and hydroxyproline, which are $A_{450nm}:A_{440nm}$.

being decreased beyond those of the ninhydrin procedure. A more advanced version of the manifold is described on p. 246.

About 20 years ago Piez and Gross[131] observed that in hydrolysates of vertebrate and invertebrate collagen an unknown peak occurs in front of hydroxyproline, adjacent to methionine sulphoxides. This was later identified as 3-hydroxy-L-proline and can be easily separated by ion-exchange chromatography using IR-120 resin. Dowex 50-X4 can also be used for this purpose (Ogle *et al.*[120]). A recommended method (Gryder *et al.*[63]) is as follows.

A 60 × 0.6 cm column packed with Durrum-type resins, operated at 52° and a flow-rated of 0.58 ml/min can be used. To separate 3- and 4-hydroxyproline and hydroxylysine, the Durrum Pico buffer system II can be used with slight modifications. A fourth buffer (A; pH 2.95), prepared by acidifying Pico buffer A with hydrochloric acid, was used as the first eluent before Pico buffer A. Pico buffer C was modified by reducing its sodium chloride concentration to 58 g/l and increasing the pH to 4.6 with sodium hydroxide. The buffer was changed after 45, 105 and 165 min. *trans*-3-Hydroxyproline was eluted at 60 min and *trans*-4-hydroxyproline at 80 min.

4.1.2. Paper and thin-layer chromatography

Numerous solvent systems are suitable for resolving hydroxyproline from the "classical twenty" amino acids on both paper and thin layers. The separation of 3-

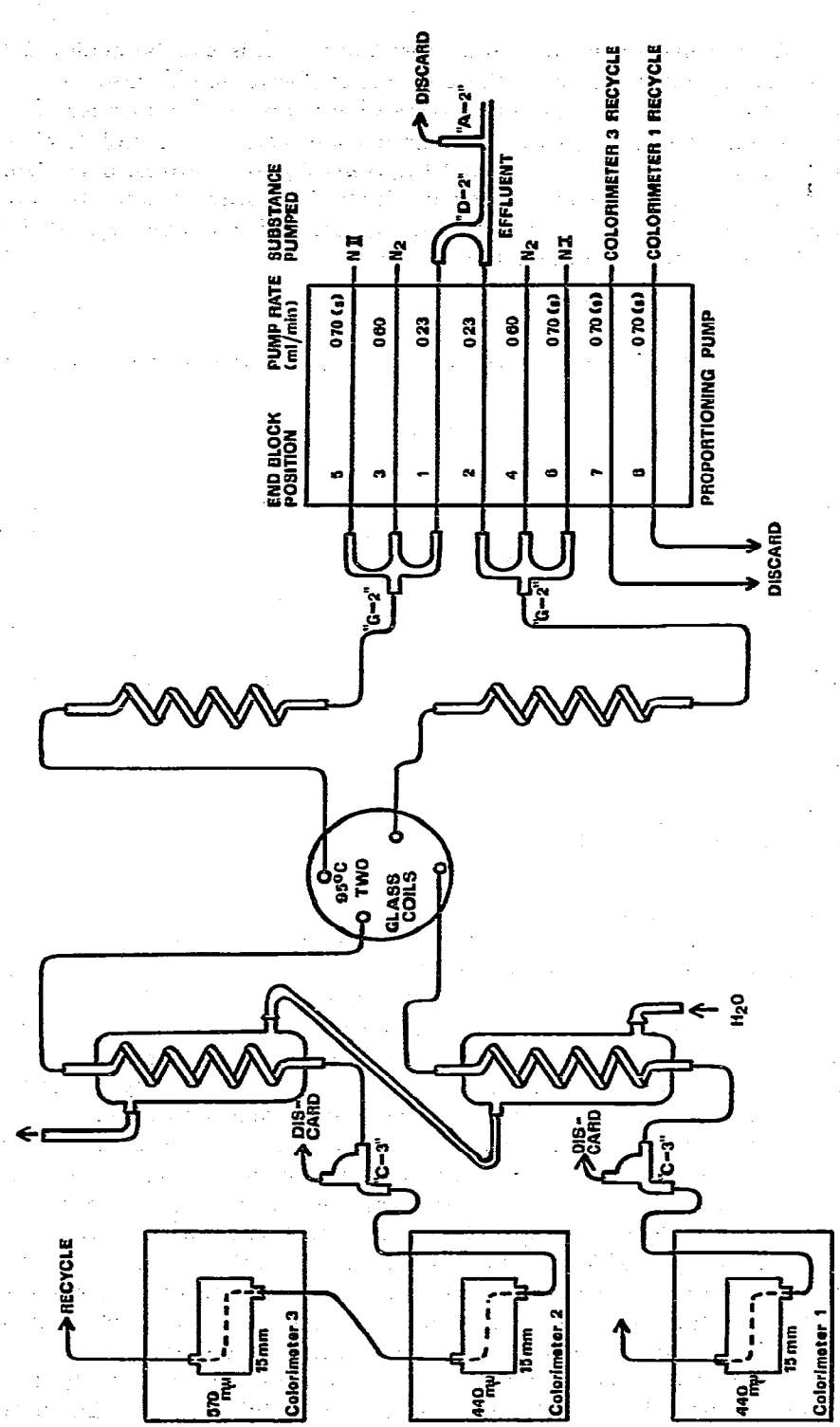


Fig. 38. Flow scheme of the experimental system used to study the analysis of imino and amino acids. The letters and digits enclosed in quotation marks (*e.g.*, "A-2") refer to Technicon glass connections, and the notation (S) under the heading PUMP RATE indicates the use of Solvaflex manifold tubing. Nin-I denotes the unmodified ninhydrin reagent; Nin-II is the hydrindantin-free reagent.

hydroxyproline from 4-hydroxyproline and other amino acids can be achieved by using phenol–water (8:2) as the mobile phase on paper (Oeriu and Tanase[119]).

The separation of L- and D-*allo*-hydroxyproline is also of importance. Good results can be obtained on silica gel with methanol–water (7:3) as the mobile phase: the R_F value for hydroxyproline is 0.54 and for D-*allo*-hydroxyproline 0.45. Other mobile phases can be used, as summarized in Table 23 (Drawert and Barton[35]) Various hydroxyproline derivatives (mainly nitroso derivatives) were separated by Marucci and Mussini[97] and Myhill and Jackson[113].

TABLE 23

R_F VALUES OF HYDROXYPROLINE ON SILICA GEL IN DIFFERENT SOLVENT SYSTEMS

Compound	*Solvent system**				
	A	*B*	*C*	*D*	*E*
L-Hydroxyproline	0.54	0.42	0.58	0.37	0.20
D-*allo*-Hydroxyproline	0.45	0.34	0.51	0.31	0.14
4-Hydroxy-1-pyrrolin-2-carboxylic acid	0.54(?)			0.48(?)	0
Pyrrol-2-carboxylic acid	0.78	0.64	0.73	0.58	0.87
2-Oxyglutaric acid	0.70	0.58	0.70	0.18	
L-Glutamine	0.64	0.45	0.61	0.15	0.17
Oxoproline	0.58	0.34	0.50	0.10	0.18
L-Proline	0.55	0.39	0.54	0.46	0.20

* Solvent systems:
A = methanol–water (7:3)
B = ethanol–water (7:3)
C = ethanol–water (55:45)
D = *n*-propanol–33% ammonia solution (67:33)
E = *n*-butanol–acetic acid–water (4:1:1)

4.1.3. Gas chromatography

Various systems have been devised for the routine determination of hydroxyproline by gas chromatography. That of Mee[103] uses a 6 ft. × 1/4 in. column packed with 0.325% (w/w) EGA on 80–100-mesh AWHT Chromosorb G. Trifluoroacetyl-amino acid *n*-butyl esters were used for the separation, with helium as the carrier gas at a flow-rate of 60 ml/min. The column temperature was programmed from 140 to 230° at the rate of 5°/min. The oven temperature was 210°, the pyrolyser temperature was 820° and a Coulson electrolytic conductivity detector was used.

Gibbs *et al.*[54] applied trimethylsilyl esters (for derivative preparation, see Cardinale *et al.*[22]) to the separation of hydroxyproline in urine. A column of Dowex 50-X8 (30 × 1 cm) was used for the purification of the hydrolysate before injecting the sample into the gas chromatograph. The acid hydrolysate was adjusted to pH 3.0 with 40% sodium hydroxide solution, applied to the column and eluted with 0.1 *M* citrate buffer (pH 2.9). The fraction in the eluate between 45 and 60 ml was collected, evaporated and subjected to derivatization and chromatographic separation. A 6-ft. OV-1 column and a flame-ionization detector were used.

For the simultaneous determination of hydroxyproline and hydroxylysine, in addition to other amino acids, a technique was developed by Moss and Lambert[108]

using a 244 × 4.1 mm U-shaped column packed with 15% Dexsil 300GC coated on 80–100-mesh acid-washed, dimethyldichlorosilane-treated Chromosorb W. The column was operated at 140° initially, followed by temperature programming to 270° at a rate of 5°/min. Amino acids were separated in the form of N-heptafluorobutyryl *n*-propyl esters, prepared by the procedure proposed by Coulter and Hann[25]. After propylation, 0.1 ml of ethyl acetate was added together with 0.1 ml of heptafluorobutyric anhydride, then the reaction mixture was sealed in a vial and heated at 150° in an oil-bath for 10 min. The tube was subsequently cooled and opened, the contents were evaporated to dryness under nitrogen, the residue was re-dissolved in 0.2 ml of chloroform and excess of heptafluorobutyric acid removed by washing with distilled water. The chloroform layer was evaporated to dryness, re-dissolved in 0.1 ml ethyl acetate and used for analysis.

The advantage of this procedure, the result of which is shown in Fig. 39, is the good separation of lysOH from lys. These amino acids co-elute on OV-1 over a wide range of conditions, although by appropriate adjustment of the chromatograph they can be separated (Moss *et al.*[109]). On the other hand, the separation of hydroxyproline from residual amino acids is excellent on this sorbent.

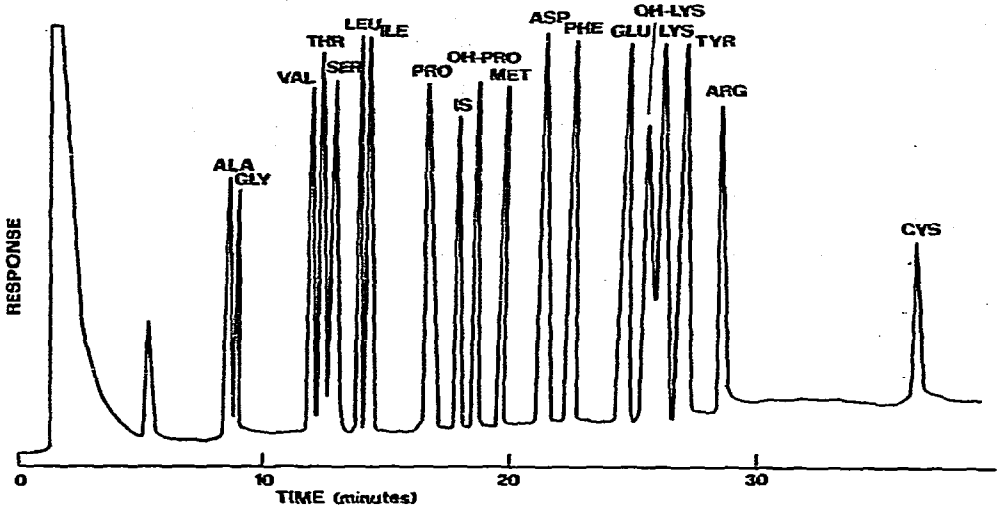


Fig. 39. Gas chromatogram of hydroxyproline (Hyp), hydroxylysine (Hyl), 2,4-diaminobutyric acid (2,4-DABA) and other amino acids run on a 15% Dexsil 300-GC column.

The procedure for the gas chromatographic separation of hydroxylated protein amino acids as developed by Moss and Lambert[108] does not give a good separation of serine and valine. A separate run would be required if serine, valine, hydroxyproline and hydroxylysine are to be assayed at the same time. MacKenzie and Tenaschuk[93] modified the above method in order to obtain the results in a single run. N-Heptafluorobutyryl isobutyl esters of the analysed amino acids were used (for the method of preparation, see MacKenzie and Tenaschuk[93]). Pyrex columns (11–12 ft. × 2.5 mm I.D., thin walled) packed with 3% SE-30 on Gas-Chrom Q (100–200 mesh) were applied, with nitrogen as the carrier gas at a flow-rate of 30 ml/min,

temperature programming from 90 to 240° at 4 or 6°/min, an injector temperature of 250°, a detector temperature of 280°, an air flow-rate of 300 ml/min and a nitrogen flow-rate of 25 ml/min. A typical chromatogram is shown in Fig. 40.

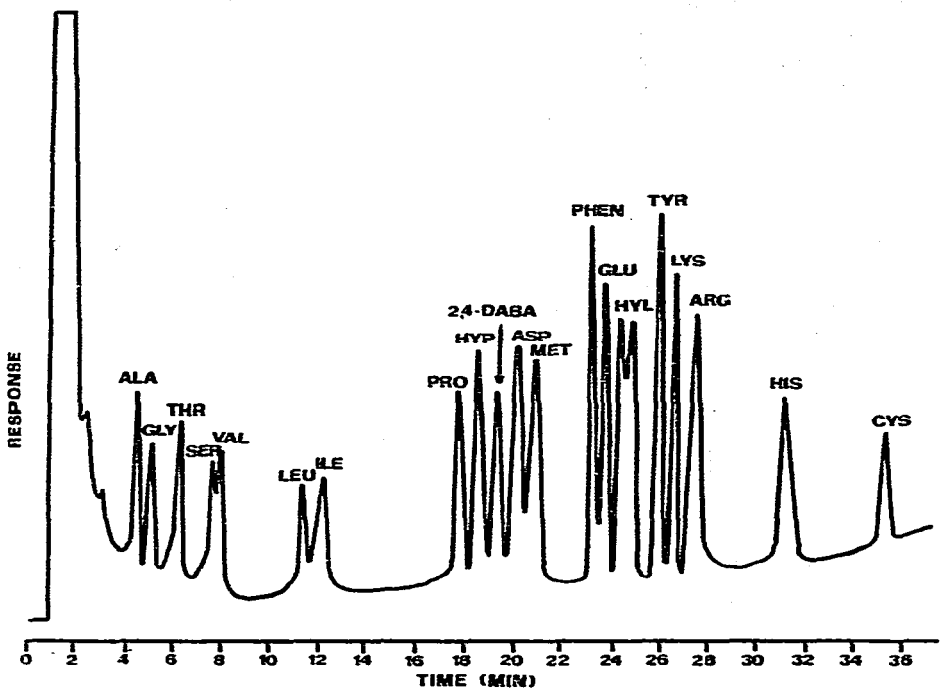


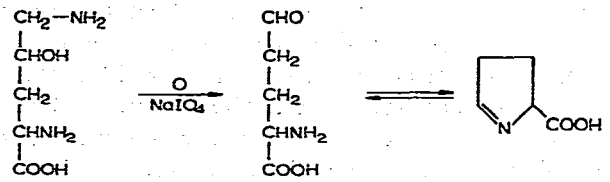
Fig. 40. Gas chromatogram showing separation of hydroxyproline and hydroxylusine from other protein amino acids. IS = Internal standard; temperature programming rate, 4°/min.

4.2. Hydroxylysine

Hydroxylysine is one of the few unusual amino acids that does not require specific precautions in order to separate it from the "classical twenty" amino acids by routine procedures. Problems may arise when hydroxylysine is to be separated from the cross-linking amino acids (see the relevant section). For information regarding the separation of hydroxylysine and *allo*-hydroxylysine, the reader is directed to general reviews on amino acid analysis (Zmrhal *et al.*[194]).

The gas chromatographic analysis of hydroxylysine (together with hydroxyproline) is described on p. 283.

An unusual procedure for the determination of hydroxylysine was developed by Blumenkrantz and Prockop[16]. Hydroxylysine is oxidized by periodate to glutamic acid semialdehyde, which is probably in equilibration with Δ^1-pyrroline-5-carboxylic acid:



The products of the reaction give a colour reaction with *p*-dimethylaminobenzaldehyde. As long as proline gives the same colour, separation is necessary. This can be done, for example, on silica gel plates with propanol–water (7:3) as the mobile phase.

4.3. δ-Hydroxyornithine

δ-N-Hydroxyornithine, a constituent of ferrichromes, albomycin, fusarinines and rhodotorulic acid, can be separated in a similar manner to hydroxylysine using standard conditions for amino acid analysis with sodium citrate buffer of pH 5.28 (0.35 *N*) (Tomlinson and Viswanatha[175]). A comparison with data obtained by paper chromatography and electrophoresis is presented in Table 24.

TABLE 24

CHROMATOGRAPHIC MOBILITIES AND SENSITIVITY TO SOME COLORIMETRIC TESTS OF δ-N-HYDROXYORNITHINE AND ITS CYCLIC LACTAM (ORNITHINE AS REFERENCE)

Compound	R_F	*Cathodic mobility* (cm)*	*Column chromatographic mobility*** (ml)*
δ-N-Hydroxyornithine	0.19	7.5	16
1-Hydroxy-3-amino-2-piperidone	0.27	12.0	72
Ornithine	0.075	10.0	26

* Paper chromatography; *n*-butanol–acetic acid–water (4:1:5).

** Paper chromatography; pyridine–acetic acid–water (7:5:465), pH 5.0, 10 V/cm, 3 h. All components ninhydrin-positive, the lactase yielding a characteristic yellow colour which changes to red on further heating. δ-N-Hydroxyornithine gives a red colour with alkaline triphenyltetrazolium chloride (TPTZ).

*** Analysis performed under conditions used for basic amino acids.

5. GLUTAMIC ACID DERIVATIVES

5.1. γ-Carboxyglutamic acid

γ-Carboxyglutamic acid is present in a limited number of proteins such as thrombin and some glycoproteins from the connective tissue (Nelsestuen and Suttie[115]), where it is responsible for binding Ca^{2+} ions and, perhaps, for the formation of nucleation centres at the beginning of calcification. The γ-carboxy group is extremely labile and, under the routine conditions for amino acid analysis in acidic media, the compound is decarboxylated to glutamic acid. This is obviously the reason why no adequate method existed until recently for the direct determination of this amino acid (Hauschka[71]).

γ-Carboxyglutamic acid withstands alkaline hydrolysis in 2 *N* sodium hy-

droxide solution in a nitrogen atmosphere. The test-tubes in which samples to be hydrolysed are placed are transferred into a desiccator filled with potassium hydroxide. The desiccator is evacuated and left in a ventilated oven at 110° for 22 h. Beckman AA-20 resin was used by the above workers for separation. The column of the amino acid analyser was operated at 51° with a stepped series of 0.2 *N* citrate buffers, ranging from pH 3.1 to 7.13. The timing of the changes of buffers etc. was as follows: pH 3.1 buffer, 0–30.6 min; pH 3.44 buffer, 50.6–74.1 min; pH 4.1 buffer, 74.1–84.1 min; pH 7.13 buffer, 84.1–157.1 min; washing with 0.2 *N* NaOH–0.1% EDTA, 157.1–162.1 min; cycling with pH 3.1 buffer, 162.1–182.2 min. Retention data are summarized in Table 25 (Hauschka[71]).

TABLE 25

RETENTION TEMPERATURES OF BTFA-AMINO ACIDS (N-TRIFLUOROACETYL-*n*-BUTYL ESTERS)

Class	*Amino acid*	*Retention temperature* (°C)*	
		OV-17	*Dexsil 300-GC*
Monoaminomonocarboxylic			
Non-sulphur-containing	Alanine	114.7	119.1
	Norvaline	124.8	134.6
	Norleucine	132.9	144.0
	2-Amino-*n*-octanoic acid	151.0	164.6
S-Alkylcysteines	S-Methylcysteine	148.4	153.0
	S-Ethylcysteine	153.7	160.8
	S-*n*-Propylcysteine	161.8	169.2
	S-*n*-Butylcysteine	170.1	179.6
S-Alkylhomocysteines	Methionine	160.8	169.2
	Ethionine	167.0	176.2
Monoaminodicarboxylic			
Non-sulphur-containing	Aspartic acid	169.8	177.2
	Glutamic acid	184.6	192.8
	2-Aminopimelic acid	204.2	214.4
S-Carboxyalkylcysteines	S-Carboxymethylcysteine	208.5	215.0
	S-β-Carboxyethylcysteine	218.1	225.6
	S-γ-Carboxypropylcysteine	227.0	235.4
S-Carboxyalkylhomocysteines	S-Carboxymethylhomocysteine	220.0	225.6
	S-β-Carboxyethylhomocysteine	230.0	237.8
	S-γ-Carboxypropylhomocysteine	237.6	247.2
Diaminomonocarboxylic			
Non-sulphur-containing	2,4-Diaminobutyric acid	156.2	166.9
	Ornithine	176.6	184.4
	Lysine	188.2	198.8
S-Aminoalkylcysteines	S-Aminoethylcysteine	200.2	205.8
Diaminodicarboxylic			
Non-sulphur-containing	2,6-Diaminopimelic acid	212.2	230.2
Sulphur-containing	Lanthionine	221.6	235.4
	Cysteine	240.6	254.4
	Homocysteine	260.6	276.4
(Biscysteines)	S,S′-Methylenebiscysteine (djenkolic acid)	253.8	266.8
	S,S′-Ethylenebiscysteine	263.2	276.4

* Initial temperature, 100°; temperature programming rate, 8°/min.

The position of the γ-carboxyglutamic acid on the amino acid analyser can be identified by means of alkaline hydrolysates of prothrombin. Further verification of the peak designated as γ-carboxyglutamic acid which appears before the peak of aspartic acid can be effected by subjecting the sample to routine acid hydrolyris with 6 *N* hydrochloric acid, after which a shift of the retention time of this peak to that of glutamic acid is observed.

*5.2. Pyrrolidonecarboxylic acid**

Pyrrolidonecarboxylate is more studied in the free form as the acid is the metabolic intermediate related to the so-called γ-glutamyl cycle. On the other hand, it is also present at the N-terminus of a number of proteins and peptides (for a review, see Orlowski and Meister[121]).

For the gas chromatographic separation, 3% OV-17 on Gas-Chrom Q (60–80 mesh) and 3% DC-200 coated on Gas-Chrom Q (100–200 mesh) were recommended by Wilk and Orlowski[184]. Both columns were operated isothermally at 125° and the flow-rates for the OV-17 and DC-200 columns were 20 and 25 ml/min, respectively. Pyrrolidonecarboxylic acid was chromatographed as theperfluoroderivative: derivatization was carried out with 20% 2,2,3,3,3-pentafluoro-1-propanol in pentafluoropropionic anhydride in stoppered tubes at 75°, the reaction time being 15 min. After evaporation under nitrogen the reaction was completed by the addition of a further portion of pentafluoropropionic anhydride and heating for an additional 5 min at 75°. Before analysis the evaporated sample was diluted with ethyl acetate.

Quantitation is usually based on the ratio of the peak heights of prorylidonecarboxylate to piperidonecarboxylate (an internal standard being derivatized in the same way)in an unknown sample. For the identification of pyrrolidonecarboxylic acid, Jones[82] used paper chromatography in water-saturated phenol or paper electrophoresis in pyridine–acetate buffer (pH 6.5 or 3.5) at 3000 V/cm. Although data on migration were given by Jones, there is no information available on the behaviour of pyrrolidonecarboxylic acid with respect to the "classical twenty" amino acids for the following reasons. Liberation of pyrrolidonecarboxylic acid can be effected only by pyrrolidonyl peptidase digestion (Jones[82]). This is carried out in 50 m*M* phosphate buffer (pH 7.3) containing 30 m*M* 2-mercaptoethanol and 1 m*M* EDTA. Digestion is carried out at 32° for 12 h with the addition of 0.6 mg of the enzyme after 6 h. Digested protein samples are lyophilized and pyrrolidonecarboxylic acid is identified in the peptide mixture.

6. SULPHUR- AND SELENIUM-CONTAINING AMINO ACIDS AND ARTIFACTS RELATED TO DEHYDROALANINE

The chromatographic separation of rarely occurring sulphur amino acids such as lanthionine and cystathionine usually does not present any problems and can be carried out by the standard Stein and Moore procedure (Newton *et al.*[117]; Stein and Moore[164]). Further, lanthionine, β-methyllanthionine, dehydroalanine and β-methyldehydroalanine can be separated by electrophoresis at pH 2.0 (Ingram[77]).

The chromatographic separation of methionine sulphoxide and its identifica-

* In the literature this compound is occassionally referred to as pyroglutamic acid.

tion among the members of the "classical twenty" amino acids can be achieved by both column and paper chromatography. The following mobile phases can be used on paper: (1) *tert.*-butanol–methyl ethyl ketone–water–diethylamine (10:10:5:1); (2) *n*-butanol–acetic acid–water (12:3:5); (3) *n*-butanol–pyridine–water (1:1:1); and (4) isobutyric acid–ammonia solution–water (66:1:33). Iodoplatinate solution (Toenniss and Kolb[174]) is used for detection.

For column separation it is possible to use Dowex 1 with a linear gradient of formic acid from 0 to 1.0 *M* or to elute the components of the mixture stepwise with 0.01, 0.1 and 1.0 *M* formic acid.

N-Formyl- and N-acetylmethionine* occur in honey bee thorax proteins. Both formyl- and acetylmethionine can be easily separated by paper electrophoresis at pH 4.8 in pyridine–acetate buffer. Paper chromatography in *n*-butanol–acetic acid–water (4:1:5) can also be recommended.

Hydrolysates for analysis must be prepared by enzyme treatment as N-formyl and N-acetyl residues are easily cleaved in acidic media (1 *M* hydrochloric acid).

Carboxymethylation is a suitable procedure for detecting protein-bound selenocysteine, according to Rinaldi *et al.*[139]. This compound can be separated on a long column of an amino acid analyser (54 × 9 cm) packed with Aminex A-6. The elution buffer applied is 0.2 *M* sodium citrate (pH 3.25). Carboxymethylcysteine is eluted ahead of aspartic acid, followed by carboxymethylselenocysteine and threonine.

S-Carboxymethylcysteine occurs as the integral part of some proteins (immunoglobulin G) or, as mentioned, it may arise during structural studies by the reaction of the terminal residue with iodoacetamide. In both instances the necessity for identifying this amino acid occurs in the laboratory. Ion-exchange chromatography is the most suitable procedure as it gives a direct result without additional modification of the routinely applied programme. Thus, with pH 3.25 buffer at 62° on a 55-cm column packed with Amberlite IR-120 (flow-rate 70 ml/h), a good separation can be obtained. Carboxyethylcysteine can be analysed in a similar manner.

S-Carboxymethylcysteine can be also separated by paper chromatography in *n*-butanol–acetic acid–water (4:1:1). The R_F values of the nearest neighbours are: asp 0.22, S-carboxymethylcysteine 0.25 and glu 0.27. In high-voltage electrophoresis the mobility relative to aspartic acid is 1.87.

The chromatographic properties of selenium-containing analogues of amino acids, such as selenolysine, have been extensively studied (De Marco *et al.*[34]), but they are not included into protein structures and are not dealt with here.

Wool fibres are a source of unusual amino acids. A number of artefacts arise that are produced by the reactions of side-groups during wool treatment. In addition to compounds already discussed, S-*n*-allyl-, S-*ω*-carboxyallyl and S-*β*-aminoethylcysteine belong to this category of compounds, and also such amino acids as S,S-methylene- and S,S-ethylenebiscysteines. Sakamoto *et al.*[149] devised a gas chromatographic procedure for separating these compounds, using OV-17 and Dexsil 300 GC as column packings on Chromosorb G (1 m × 3 mm I.D. column). The temperature was programmed from 100 to 280° at a rate of 8°/min. The flow-rate of the carrier gas was about 70 ml/min. Retention data of a number of unusual amino acids in both systems are summarized in Table 26.

* For other acyl derivatives of amino acids, see p. 303.

TABLE 26

ELUTION TIMES OF AMINO ACIDS ON BECKMAN AA-20 RESIN WITH RESPECT TO THE BEHAVIOUR OF γ-CARBOXYGLUTAMIC ACID (GLA)

Amino acid	*Elution time (min)*	*Amino acid*	*Elution time (min)*
Cysteic acid	12.4	Methionine	82.2
Gla and taurine	17.2	*allo*-Isoleucine	83.2
Methionine sulfoxide	30.3, 31.3	Isoleucine	84.6
4-Hydroxyproline	33.0	Leucine	86.3
Aspartic acid	35.6	Ttrosine	93.0
Threonine and *allo*-4-hydroxyproline	41.2	Phenylalanine	95.5
Serine	43.5	Histidine	99.3
Homoserine	49.9	Hydroxylysine	102.3
Glutamic acid	54.4	Ornitihine	107.2
Proline	58.2	Lysine	108.8
Glycine	66.1	Ammonia	116.2
Alanine	68.3	Tyrypthophan	145.2
Cysteine	74.6	Arginine	151.1
Valine	77.7		

TABLE 27

R_F VALUES OF SULPHUR AMINO ACIDS ON WHATMAN NO. 3MM PAPER

*Amino acid**	*Solvent***			
	1	*2*	*3*	*4*
Cystine	0.14***	0.08	0.05	0.08
Cysteine	0.16***	0.09	0.35	0.07
Methylcysteine	0.48	0.41	0.33	0.53
Methylcysteine sulphoxide	0.30	0.20	0.17	0.24
Methylcysteine sulphone	0.38	0.19	0.15	0.20
Ethylcysteine	0.58	0.50	0.43	0.63
Butylcysteine	0.73	0.63	0.69	0.75
Cysteic acid	0.27	0.13	0.11	0.12
Cysteinesulphinic acid	0.28	0.19	0.12	0.22
Homocysteine	0.27	0.24	0.09	0.24
Homocysteic acid	0.30	0.10	0.18	0.16
Taurine	0.41	0.14	0.24	0.18
Cystathionine	0.17	0.05	0.04	0.13
Methionine	0.55	0.49	0.42	0.66
Methionine sulphoxide	0.31	0.24	0.20	0.35
Methionine sulphone	0.39	0.25	0.19	0.36
Methionine sulphoximine	0.26	0.18	0.14	0.21
Methylmethionine sulphonium chloride	0.19	0.23	0.12	0.14
Djenkolic acid	0.13	0.14	0.04	0.14

* 20 μg of each amino acid applied to the paper.

** Solvents:

1 = *n*-butanol–pyridinewater (1:1:1);

2 = *n*-butanol–acetic acid–water (25:6:25);

3 = *n*-butanol–ethanol–water (2:2:1);

4 = *tert.*-butanol–formic acid–water (14:3:3).

*** Streaks at high amino acid concentration.

S-Methylcysteine and its derivatives are mostly present in the free form and are not discussed in detail here. For their separation it is possible to use paper chromatography in *n*-butanol–acetic acid–water or various phenol–ammonia mobile phases that are generally applicable to the separation of methylated sulphur-containing amino acids (Barna and Bhuyan[8]; Peterson and Butler[128]) (Table 27).

The dehydroalanyl residues can undergo additional reactions with amino and thiol groups in the protein with concommitant formation of lysinoalanine, β-aminoalanine and lanthionine. Other compounds of this type were studied by Asquith and Carthew[4] in order to elucidate the mechanism that occurs during treatment of wool with ammonia. Most of these compounds are best separated by high-voltage electrophoresis at pH 1.85 or 1.1. At pH 1.85 it is possible to resolve β-ethylaminoalanine and β-propylaminoalanine, and at pH 1.1 β-butyl- and β-amylaminoalanines are separated. The relative mobilities with respect to glycine and alanine are presented in Table 28.

TABLE 28

CONDITIONS FOR THE SEPARATION OF ALANINE DERIVATIVES (A) AT pH 1.85, 1.75 h AT 75 V/cm, 18°, AND (B) AT pH 1.1, 2.25 h AT 50 V/cm, 18°

Conditions	*Amino acid*	*R_m relative to glycine*	*R_m relative to alanine*
A	β-Ethylaminoalanine	1.13	1.3
	β-Propylaminoalanine	1.07	1.21
B	β-Butylaminoalanine	0.85	0.962
	β-Amylaminoalanine	0.82	0.83

Among the artefacts that arise during the treatment of proteins, another that is chromatographically well characterized is N^{ε}-(D,L-2-amino-2-carboxyethyl)lysine. This amino acid is formed in proteins exposed to alkali (ribonuclease) and is believed to arise from the addition to the ε-amino group of lysine to the double bond of the dehydroalanyl residue. This amino acid moves ahead of lysine during routine two-column amino acid analysis.

Electrophoretic separations on DEAE-paper in pyridine–acetic acid buffer (pH 6.0) are also possible (Table 29).

7. CROSS-LINKING AMINO ACIDS

7.1. Desmosine, isodesmosine, lysinonorleucine, "aldol" and related compounds

7.1.1. Automated amino acid analysis

Collagen and elastin contain several types of cross-linking elements and corresponding intermediates in cross-link formation that determine to a considerable extent the properties of the tissue and are today accessible with some difficulty by ion-exchange chromatography (Masuda *et al.*[98]).

In contrast to compounds considered in other sections of this review, cross-linking amino acids are frequently referred to by trivial names that give little idea

TABLE 29

ELECTROPHORETIC MIGRATION DISTANCES AND R_F VALUES ON DEAE-PAPER OF SULPHUR AMINO ACIDS

*Amino acid**	*Electrophoretic migration, pH 2.7 (cm)***	*DEAE, pH 4.7*	
		R_F	*Ninhydrin colour*
Cystine	− 3.3	0.14	Brown
Cysteine	− 3.1	0.11	Brown
Methylcysteine	− 2.1	0.36	Brown-grey
Methylcysteine sulphoxide	− 1.8	0.25	Yellow-brown
Methylcysteine sulphone	− 1.7	0.26	Yellow-brown
Ethylcysteine	− 2.0	0.38	Brown-grey
Butylcysteine	− 2.1	0.40	Brown-grey
Cysteic acid	+15.5	0.05	Grey-blue
Cysteinesulphinic acid	+14.0	0.10	Blue
Homocystine	− 4.8	0.15	Blue
Homocysteic acid	+13.0	0.10	Blue
Taurine	− 1.9	0.33	Grey-blue
Cystathione	− 1.2	0.18	Blue
Methionine	− 4.0	0.43	Purple
Methionine sulphoxide	− 2.7	0.34	Purple
Methionine sulphone	− 2.6	0.29	Purple
Methionine sulphoximine	− 3.0	0.28	Purple
Methylmethionine sulphonium chloride	−19.0	0.80	Purple
Djenkolic acid	− 3.2	0.15	Grey

* 20 μg of each amino acid applied to the paper.
** Distance migrated (cm) towards anode or cathode in 4 h.

of their chemical nature. Therefore, the lysine-derived cross-links, their chemical formulae and their names are listed in Table 30.

Using a 52 × 0.9 cm column, good separations of desmosine and isodesmosine were achieved by Gerber and Kemp[52] on Beckman Custom Research Resin AA15. The buffers used were 0.2 *N* sodium citrate (pH 3.25) and 0.38 *N* sodium citrate (pH 4.26) at a flow-rate of 70 ml/h. The column was first equilibrated with 0.2 *N* sodium citrate buffer and eluted with this buffer for 2 h at 31°. This also permitted the separation of hydroxyproline and α-amino acid adipic. The buffer change to 0.38 *N* sodium citrate was accompanied by an increase in temperature to 50°. The results of the separation are shown in Fig. 41.

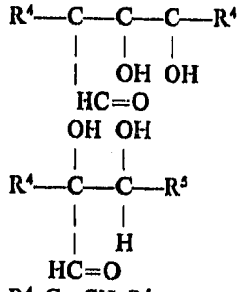
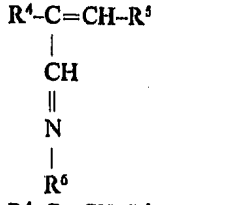
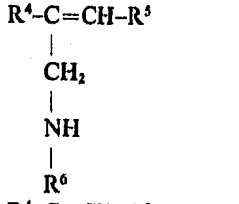
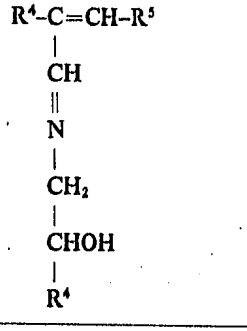
Hebkel *et al.*[73] recommended the use of Beckmann M 82 resin packed into a 52 × 0.9 cm column for the separation of cross-linking amino acids. The analyser was operated with citrate buffers of pH 4.25, 5.25 and 6.20. In a typical run, the instrument was equipped with a stream-splitting device and monitored for tritium in a scintillation counter in order to detect $NaBT_3$-reduced cross-linking amino acids that otherwise are unstable under the conditions of acid hydrolysis.

For the routine separation of cross-linking amino acids that occur in elastin, automated amino acid analysis using two columns packed with JLC-R-2 resin was developed by Volpin and Michelotto[180]. Basic amino acids, excluding desmosine, isodesmosine, merodesmosine and lysinonorleucine, are resolved on a short column (15 × 0.8 cm) using 0.35 *N* sodium citrate buffer (pH 5.28). Acidic and neutral amino

TABLE 30

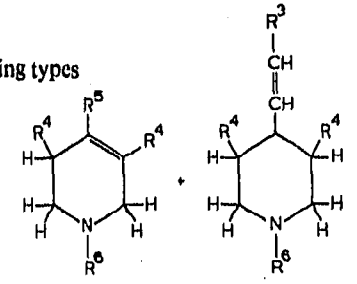
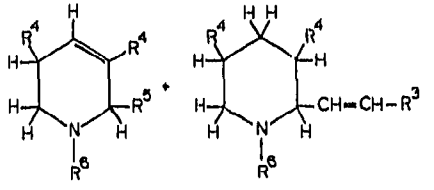
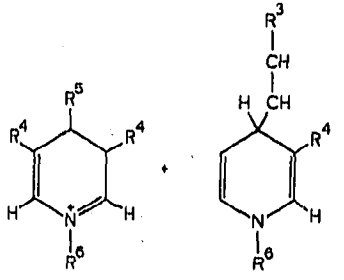
COMPOUNDS WHOSE RESIDUES ARE INVOLVED IN COLLAGEN AND ELASTIN CROSS-LINKS

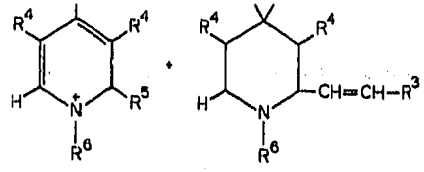
No.	*Abbreviation*	*Trivial name*	*Formula*	*O/L**	*Derived from*
1	L	Lysine	$R^4-CH_2-CH_2-NH_2$**	0/1	–
2	HL	δ-Hydroxylysine	$R^4-CH(OH)-CH_2-NH_2$	1/1	L
3	AL	α-Aminoadipic acid δ-semialdehyde; allysine	$R^4-CH_2-C(H)=O$	1/1	L
4	HAL	δ-Hydroxy-α-aminoadipic acid δ-semialdehyde; hydroxyallysine	$R^4-CH(OH)-C(H)=O$	2/1	HL or AL
5	deLNL	$\Delta^{6,7}$-Dehydrolysinonorleucine	$R^4-CH_2-CHDN-CH_2-R^5$	1/2	L+AL−H_2O
6	LNL	Lysinonorleucine	$R^4-CH_2-CH_2-NH-CH_2-R^5$	0/2	L+AL−H_2O+2H
7a		"Aldol"; "allysine aldol"	$R^4-CH(HC=O)-CH(OH)-R^5$		AL+AL
7b	$ALAL_d$	Dehydrated "aldol"	$R^4-C(HC=O)=CH-R^5$	2/2	AL+AL−H_2O
8a	$deHLNL_a$	Dehydrohydroxylysinonorleucine	$R^5-CHDN-CH_2-CH(OH)-R^4$	2/2	AL+HL−H_2O
8b	$deHLNL_b$		$R^4-CH(OH)-CHDN-CH_2-R^5$		HAL+L−H_2O
9	HLNL	δ-Hydroxylysinonorleucine	$R^5-CH_2-NH-CH_2-CH(OH)-R^4$	1/2	AL+HL−H_2O+2H or HAL+L−H_2O+2H
10	deHLHNL	Dehydrohydroxylysinohydroxynorleucine	$R^4-CH(OH)-CHDN-CH_2-CH(OH)-R^4$	3/2	HAL+HL−H_2O
11	HLHNL	Hydroxylysinohydroxynorleucine	$R^4-CH(OH)-CH_2-NH-CH_2-CH(OH)-R^4$ H H H	2/2	HAL+HL−H_2O+2H

12	HALAL	Syndensine; hydroxy "aldol"		3/2	HAL+AL
13	deM	Dehydromerodesmosine		2/3	$ALAL_d+L-H_2O$ or $deLNL+AL-H_2O$
14	M	Merodesmosine		1/3	$ALAL_d+L-H_2O+2H$
15	deHM	Dehydrohydroxymerodesmosine		3/3	$ALAL_d+HL-H_2O$ $deHLNL+AL-H_2O$ or

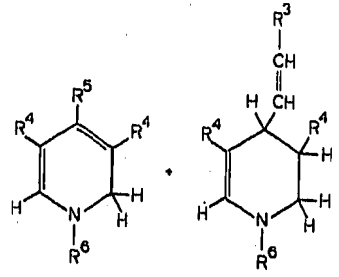
(Continued on p. 294)

TABLE 30 *(continued)*

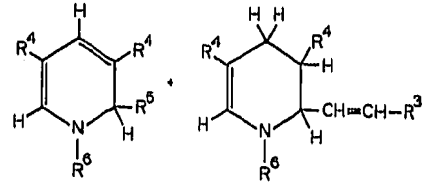
No.	*Abbreviation*	*Trivial name*	*Formula*	*O/L**	*Derived from*
16	dihydroD	Dihydrodesmosines; $\Delta^{3,4}$, $\Delta^{5,6}$-dehydrodesmopiperidines and $\Delta^{5,6}$, $\Delta^{14,15}$-dehydrodesmopiperidines; various ring types	 (Type A)  (Type D)	3/4	3AL+L−2H_2O or deLNL+ALAL$_d$−H_2O or deM+AL−H_2O
17	D	Desmosines (including isodesmosine) $\Delta^{1,2}$, $\Delta^{5,6}$-dehydrodesmopiperidines and $\Delta^{2,3}$, $\Delta^{5,6}$, $\Delta^{14,15}$-dehydrodesmopiperidines; various ring types		4/4	dehydroD−(H or 2H) or 3AL+L−2H_2O−(H or 2H) or deLNL+ALAL$_d$−H_2O−(H or 2H) or deM+AL−H_2O−(H or 2H)



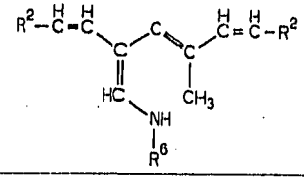
(Type D)

18	Tetrahydro-D	Tetrahydrodesmosines; $\Delta^{3,4}$-dehydro-desmopiperidines and $\Delta^{14,15}$-dehydro desmopiperidines; various ring types		2/4	dihydroD+2H or 3AL+L−2H_2O+2H or deLNL+ALAL$_d$−H_2O+2H or deM+AL−H_2O+2H

(Type A)

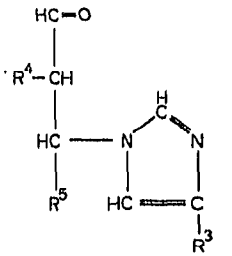
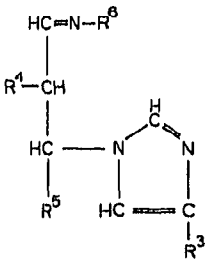
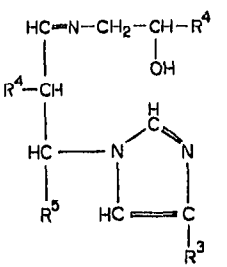


(Type D)

19	285	Compound 285; (one possible isomer)		5/4	D−(2H or 2H)

(Continued on p. 296)

TABLE 30 *(continued)*

No.	*Abbreviation*	*Trivial name*	*Formula*	*O/L**	*Derived from*
20	–	Aldol-histidine (isolated after $NaBH_4$ reduction in the alcohol form and called Compound 16)		–	$ALAL_d$+NISTIDINE
21	–	Dehydromero-histidine (isolated as a reduced Shiff base, mero-histidine, after $NaBH_4$ reduction and called posthistidine II)		–	deM+histidine
22	–	Dehydrohydroxymero-histidine (isolated as hydroxymero-histidine after $NaBH_4$ reduction and called post-histidine)		–	deHM-histidine

* O/L is the ratio of the number of oxidations and/or dehydrogenations to the number of lysyl residues originally involved.

** $R_n = -CH_2-(CH_2)_nM_3-CH(NH_2)-COOH$

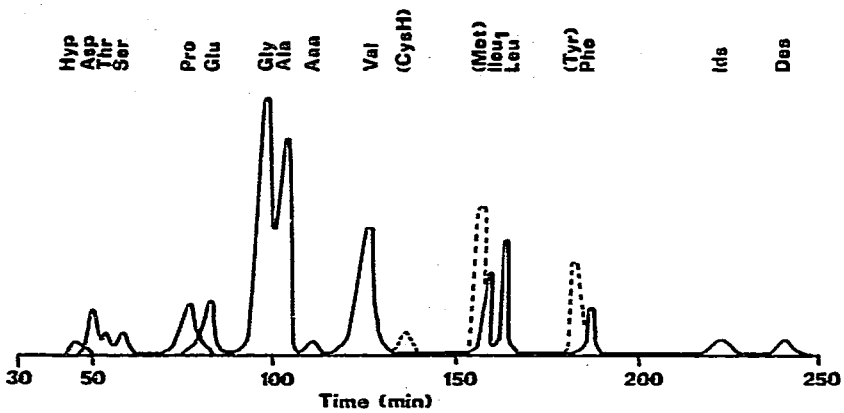


Fig. 41. Chromatogram from the hydrolysate of an elastin derivative with the position of cysteine, methionine and tyrosine, taken from a standard run, also shown. Aaa = α-aminoadipic acid; Ids = isodesmosine; Des = desmosine.

acids are separated on a long column (70 × 0.8 cm) by using a stepwise gradient starting with 0.2 *N* sodium citrate (pH 3.30) and changing to 0.2 *N* sodium citrate (pH 4.25) after 230 min. After the last of the acidic amino acids to be expected on the long column (phenylalanine) has emerged, the column is eluted with 0.35 *N* sodium citrate buffer (pH 5.28), which resolves isodesmosine, desmosine, merodesmosine and lysinonorleucine (Fig. 42).

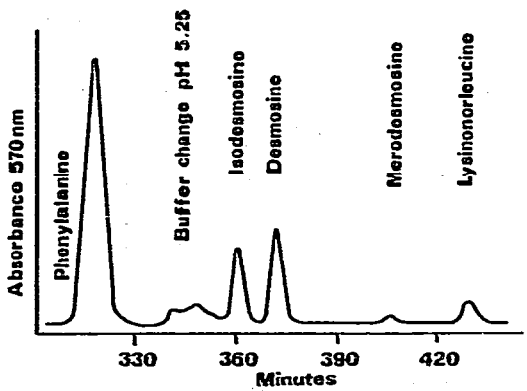


Fig. 42. Part of an amino acid chromatogram of a sample (0.5 mg) of adult bovine ligament elastin showing the separation of polyfunctional amino acids.

Similar results were obtained by Mechanic[102] using the same citrate buffer (pH 5.28). A spherical cation-exchange resin (Mark Instrument, Villanova, Pa., U.S.A.; 9–12 μm) was packed into a 30 × 0.9 cm column and 0.55% of benzyl alcohol was added to the eluting buffer. A radioactivity detector was used and the results of the separation are shown in Fig. 43.

Hydroxymerodesmosine, another member of the family of polyfunctional cross-linking amino acids, elutes together with aldolhistidine, dihydroxylysinonor-

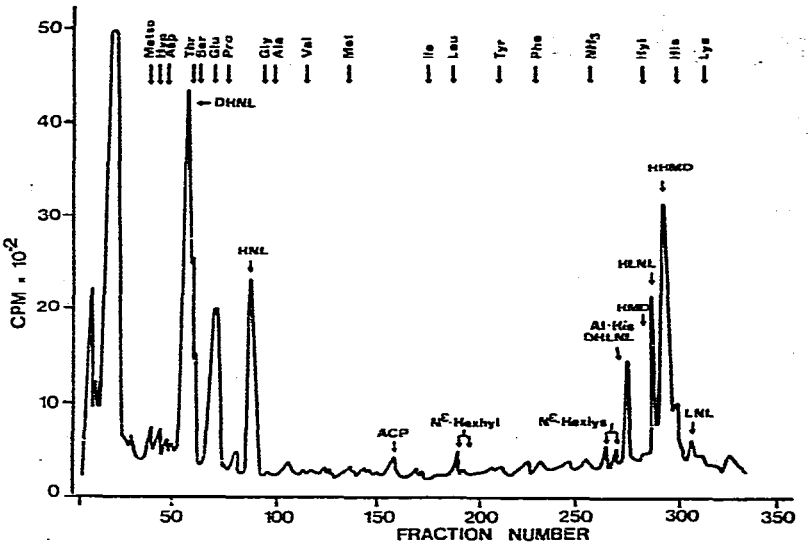


Fig. 43. Radioactive elution profile of a 3 *N p*-toluene sulphonic acid hydrolysate of [^{3}H]$NaBH_4$-reduced insoluble bovine dermal collagen fibrils. The column was packed with a cation-exchange resin, 8% cross-linked (9–12 μm) with the dimensions 58 × 0.9 cm. Elution was carried out with a complex gradient generated by a Phoenix Varipump with a flow-rate of 80 ml/h. The starting buffer was pH 2.91 sodium citrate 0.25 *M* in Na^+ and contained 6% propanol-2. The limit buffer was 0.25 *M* sodium citrate. The column temperature was 54°. Abbreviation for radioactive compounds: DHNL, dihydroxynorleucine; HNL, hydroxynorleucine; ACP, aldol condensation product; N^{ε}-Hexlys, N^{ε}-hexosylhydroxylysine; N^{ε}-hexlyl, N^{ε}-hexosyllysine; Al-His, aldol-histidine; DHLNL, dohydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; LNL, lysinonorleucine.

leucine and glycosylated lysines in most systems devised for amino acid analysis. Tanzer *et al.*[168] suggested the use of Spinco PA-35 resin packed in a 13 × 0.9 cm column with 0.35 *M* sodium citrate (pH 5.25) at a flow-rate of 80 ml/h and 50°. This system gave the complete separation of aldolhistidine, hydroxymerodesmosine, hydroxylysinonorleucine, lysinonorleucine and hydroxylysine. Other systems that can be used with varying success for the separation of hydroxymerodesmosine are summarized in Table 31.

TABLE 31

CHROMATOGRAPHIC PROPERTIES OF HYDROXYMERODESMOSINE

System A: Spinco PA-35 resin; 13 × 0.9 cm column; 0.35 *M* sodium citrate (pH 5.25); flow-rate 80 ml/h.
System B: Bio-Rad Aminex A-5 resin; 21 × 0.9 cm column; 0.2 *M* pyridine acetate (pH 3.5); linear gradient to 0.8 *M* pyridine acetate (pH 5.2); 100 ml in each gradient chamber; flow-rate 60 ml/h.
System C: Spinco UR-30 resin; 55 × 0.9 cm column; 0.25 *M* sodium citrate (pH 2.9); complex gradient to 0.4 *M* citric acid; total volume, 380 ml; flow-rate 80 ml/h. All systems run at 50°.

System	*Elution time (min)*
A	23
B	90
C	167

A complete separation of most of the rare amino acids in collagenous proteins can also be achieved on Spinco UR-30 resin (for conditions, see Table 31). A 55 × 0.9 cm column can be used. Elution is started with 0.25 *M* sodium citrate (pH 29) followed by a gradient to 0.4 *M* citric acid. The total volume passed through the column is 380 ml, the flow-rate applied being 80 ml/h. The system is operated at 50° and detection is effected by radioactivity counting, as the amounts used are usually outside the sensitivity range of the ninhydrin reaction. The results of the separation are shown in Fig. 44.

Hydrophobic chromatography using organic solvents and cellulose as the sorbent can be used for the large-scale preparation of desmosine and isodesmosine (Starcher and Galione[162]).

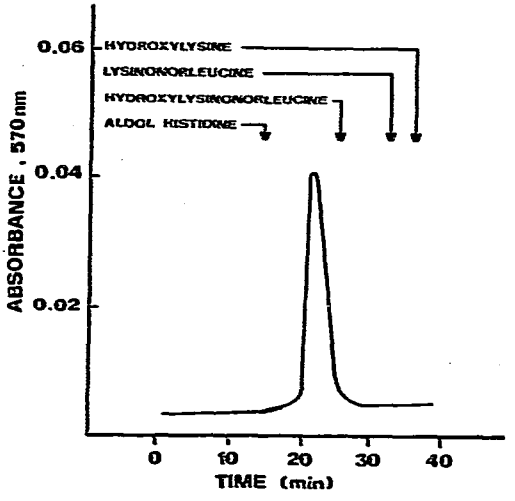


Fig. 44. Chromatographic elution of hydroxymerodesmosine. Conditions were those of system A in Table 31. The elution locations of several other compounds are noted.

7.1.2. Paper chromatography

Paper chromatography in general is not advisable for the separation of cross-linking amino acids as their R_F values in a variety of solvents are too low to allow good separations. Starcher[160] reported the data presented in Table 32.

TABLE 32

R_F VALUES OF LYSINE AND LYSINE-DERIVED CROSS-LINKS IN *n*-BUTANOL–PYRIDINE–9 *M* AQUEOUS AMMONIA (1:1:1) ON WHATMAN NO. 3 PAPER

Amino acid	R_F
Lysine	0.21
Lysinonorleucine	0.10
Merodesmosine	0.05
Desmosine + isodesmosine	0.02

On the other hand, the zero mobility of desmosine and isodesmosine in *n*-butanol–acetic acid–water (4:1:1) can be used for the micro-scale isolation of these amino acids and subsequent quantitation of elastin, using desmosines as marker amino acids (Starcher[161]). A sheet of Whatman No. 3MM paper or a layer of silica gel is developed with the above solvent, all amino acids except desmosines being moved from the starting lines. The desmosines are eluted from the chromatogram with water and quantitated by ion-exchange chromatography.

Starcher[160], in his search for merodesmosine, also reported some data regarding the electrophoretic behaviour of the lysine-derived polyfunctional amino acids. A 1% (w/v) ammonium carbonate solution (pH 8.9) with paper as the carrier and with a potential gradient of 43 V/cm were used. The mobilities can be seen in Fig. 45.

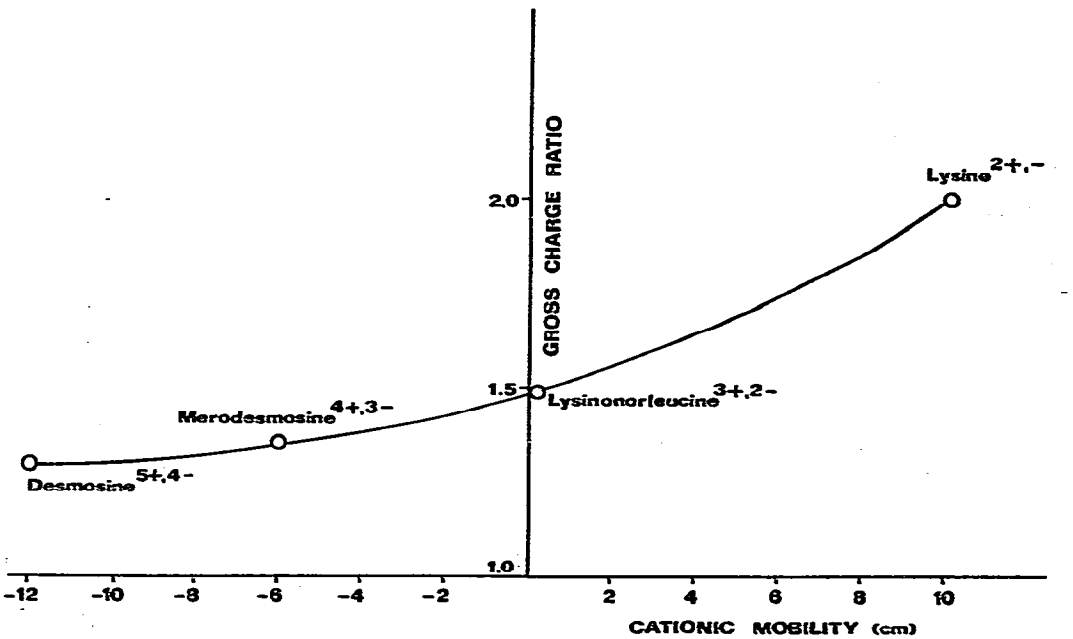


Fig. 45. Paper electrophoresis of α-amino acid derivatives of lysine. Buffer solution, ammonium carbonate (1%, w/v), pH 8.9. Field strength, 43 V/cm. The mobilities are corrected for endoosmotic flow by use of a glucose reference. The mobilities of lysine, lysinonorleucine and desmosine fall on a smooth curve when plotted against their gross charge ratios. From its corrected mobility, merodesmosine contains four potential positive and three potential negative charges (indicated by superior numbers).

7.2. Lysinoalanine

Lysinoalanine [N^{ε}-(D,L-2-amino-2-carboxyethyl)-L-lysine] occurs in alkali-treated proteins. Although not present in native structures, its analysis is of extreme importance. Most of the procedures described for this amino acid are based on the programmes used for the routine separation of natural basic amino acids (Bohak[17]; Ziegler *et al.*[193]; De Groot and Slump[30]). The expected disadvantage of these procedure is incomplete separation of lysinoalanine from other amino acids. With the

so-called special lysinoalanine programme (Slump[155]), the column used was packed with Bio-Rad Aminex A-4 into a 50 × 0.9 cm column. The operating temperature was 57° and the elution rate was 60 ml/h. Elution was carried out in a single step using sodium citrate buffer (0.61 *N* sodium + 0.2 *M* citrate, pH 4.5) containing 59.6 g of sodium citrate dihydrate, 19.7 ml of concentrated hydrochloric acid, 5.4 ml of 17% Brij 35 solution and 0.1 ml of *n*-caprylic acid per litre. For protein hydrolysates, a modified 3-h programme with stepwise elution was used; the buffer compositions and buffer change programmes suggested by Slump[155] are summarized in Table 33.

TABLE 33

BUFFER COMPOSITIONS AND BUFFER CHANGE PROGRAMMES USED FOR AMINO ACID ANALYSIS WITH SPECIAL REFERENCE TO LYSINOALAMINE

Parameter	*pH*				
	3.45 ± 0.01	*4.25 ± 0.02*	*6.28 ± 0.02*	*6.28 ± 0.02*	*>13*
Sodium ions (*N*)	0.2	0.2	1.03	1.42	0.2
Sodium citrate dihydrate (g)	196.1	196.1	619.6	619.6	—
Sodium chloride (g)	—	—	213.2	462.4	—
Sodium hydroxide (g)	—	—	—	—	80
Concentrated HCl (ml)	116.6	83.7	6.3	6.3	—
17% aq. solution of Brij 35 (ml)	54	54	54	54	—
Phenol (g)	—	10	10	10	—
n-Caprylic acid (ml)	1	—	—	—	—
Final volume (l)	10	10	10	10	10
Time schedule for buffers (minutes after starting the analysis)	0– 33 and 170–190	33– 78	78–114	114–160	160–170

Beckman M-82 resin was packed into a 50 × 0.9 cm column, the operating temperature was 56° and the elution rate was 100 ml/h. The results of the separation are shown in Fig. 46.

A number of lysine derivatives have been isolated from diverse sources (*e.g.*, octopus and yeast). Compounds such as octopine, lysopine, saccharopine, aspergillomarasmime A, aspergillomarasmime B and anhydroaspergillomarasmime can be separated by paper chromatography using mobile phases such as *n*-butanol–acetic acid–water (4:1:5), phenol–water (8:2), pyridine–acetic acid–water (10:7:3), *n*-butanol–pyridine–0.1 *N* hydrochloric acid (5:3:2), *tert.*-butanol-formic acid–water (69.5:1:29.5), propanol-2–ammonia solution–water (70:10:20) and pyridine–water (97.5–52.5).

7.3. Tyrosine-derived cross-linking amino acids

Dityrosine and trityrosine are present in resilin, an elastic protein from *Schistocerca gregaria* and in the elastin of vertebrates. These derivatives can also be formed in insoluble cow-skin collagen and soluble collagen treated with sodium peroxide and peracetic acid. For the separation of tyrosine, dityrosine and trityrosine, DEAE-cellulose column chromatography can be recommended (Waykole and Heide-

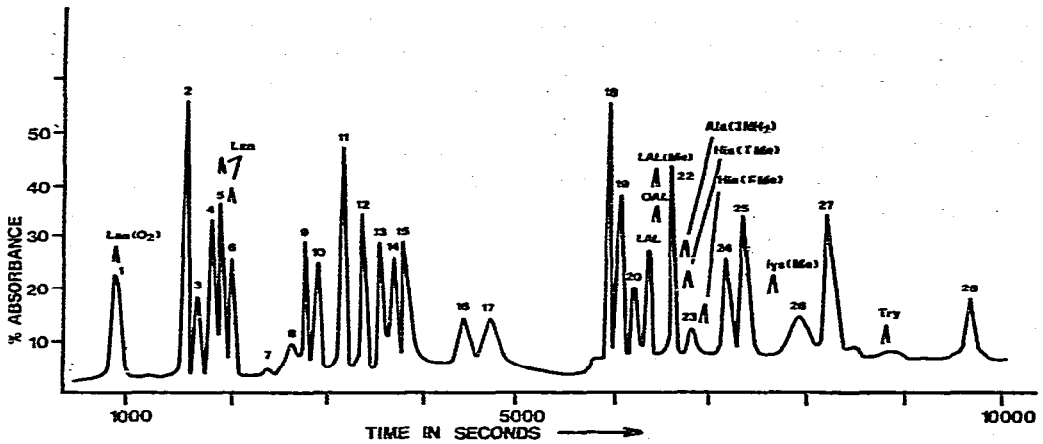


Fig. 46. Chromatography of a mixture of amino acids. Column (50 × 0.9) cm: Beckm·n M-82 resin. Buffers: see Table 33. Peaks: 1 = Cys O_3H; 2 = Asp; 3 = Met-O_2; 4 = Thr; 5 = Ser; 6 = Glu; 7 = Pro; 8 = Cys; 9 = Gly; 10 = Ala; 11 = Val; 12 = Met; 13 = Ile; 14 = Leu; 15 = Nle; 16 = Tyr; 17 = Phe; 18 = Nhb; 19 = GlcN; 20 = GalN; 21 = Lal; 22 = His; 23 = Hyl; 24 = Orn; 25 = Lys; 26 = Pec; 27 = Amm: 28 = Arg (Hyl = hydroxylysine, Lal = lysinoalanine, GalN = galactosamine). Solid line, absorbance at 570 nm; broken line, absorbance at 440 nm.

mann[181]). The ion exchanger is equilibrated first with 0.02 *M* Na_2PO_4 and the elution is carried out at room temperature. The gradient of the mobile phase is established by running 0.006 *M* Na_2PO_4 into 300 ml of 0.2 *M* Na_2HPO_4. The results of the separation are shown in Fig. 47.

Descending paper chromatography was attempted by Keeley and LaBella[85] for the separation of dityrosine from elastin hydrolysates. *n*-Butanol–acetic acid–

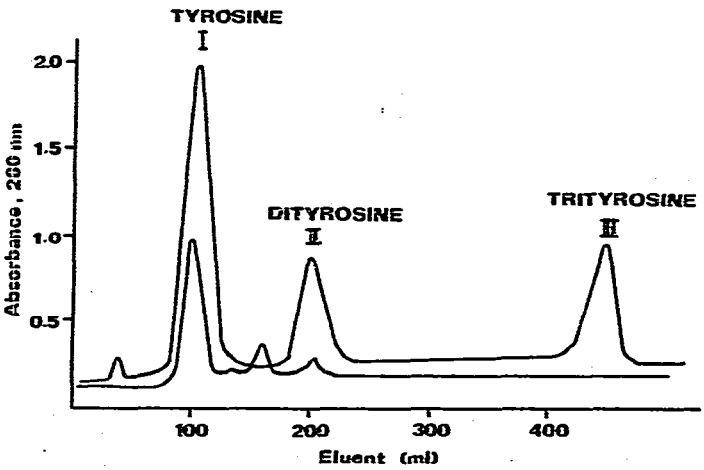


Fig. 47. Fractionation of 6 *N* HCl hydrolysate of insoluble collagen on DEAE-cellulose equilibrated with 0.02 *M* Na_2HPO_4. The elution was performed at room temperature and the gradient for the elution was established by running 0.006 *M* NaH_2PO_4 into 300 ml 0.02 *M* Na_2HPO_4.

water (55:15:30 or 35:30:35) was used as the mobile phase. Dityrosine always moved in the neighbourhood of glycine, from which it was incompletely separated.

At least three other tyrosine derivatives in this category were separated on Sephadex G-15 by Kimura and Kubota[87], but their nature remains obscure.

8. N-ACYL DERIVATIVES OF AMINO ACIDS

N-Acetylated amino acids that occur in proteins at their N-termini are accessible to indirect assay by flat-bed techniques or can be analysed directly by gas chromatography. The strategy is to split the acetylated N-terminal peptide with a suitable enzyme, *e.g.*, pronase, remove all free amino acids and peptides without a blocked N-terminus and subject the N-acetylated peptide to hydrazinolysis. Then chromatography is carried out in order to separate acyl hydrazides, free hydrazine and amino acid hydrazides. Satake *et al.*[151] recommended for this purpose paper chromatography in pyridine–aniline–water (9:1:4). In order to obtain a good resolution, electrophoretic separation in pyridine–acetic acid–water (pH 6.6) at 500 V/cm has to be carried out before chromatography (Fig. 48). The assignment of the acyl group to a particular amino acid present in the combined spot of amino acid hydrazides can be achieved by partial hydrolysis and by comparison of liberated N-termini.

The separation of N-acetylated peptide from the pronase digest of aprotein can be effected on a Dowex 50-X4 (H^+) column (30 × 2 cm). The column is eluted with water (about 600 ml) and the eluate is tested for acetylated peptides by running paper chromatograms in *n*-butanol containing 10% of acetic acid. The spots are rendered visible with bromocresol blue. The strong retention of acetylated amino acid

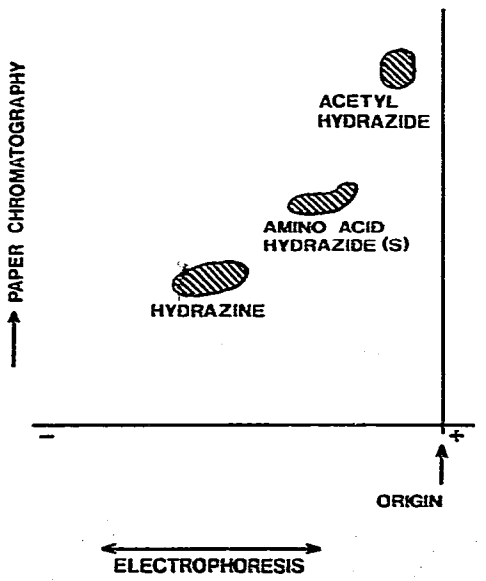


Fig. 48. Two-dimensional separation of the hydraxinolysate of a protein. Hydrazinolysate was resolved by electrophoresis (500 V for 1 h with the use of pyridine–acetic acid–water (pH 6.6) as the electrolyte, then by chromatography (pyridine–aniline–water, 9:1:4); 10% ammonia–silver nitrate was used for the detection.

derivatives on hydroxyalkylpropyl-Sephadex can also be used for separation (Prakash and Nandi[133]).

On the other hand, in gas chromatography the situation is much simpler as N-acyl amino acid esters have often been used in gas chromatography (Darbre and Aslam[28]; Lamkin and Gehrke[89]; Halasz and Bunnig[65]; Fales and Pisano[42]; Johnson *et al.*[80]; Saroff and Karmen[150]; Youngs[189]; Bayer[9]).

A detailed study of the separation of N-acetylated amino acids by gas chromatography was described by Fu and Mak[47,48]. N-Acyl derivatives of leucine were chromatographed as appropriate esters using two types of columns. Column A was 1.83 m × 32 mm O.D., packed with 10% Carbowax 20M Chromosorb W (60–70 mesh), acid washed and dimethylchlorosilane treated and pre-conditioned for 10 h at 225°. Column B was of the same dimensions, packed with 1% GE XE-60 silicone gum on Chromosorb W (60–70 mesh), acid washed, dimethylchlorosilane treated and pre-conditioned as for column A. The dependence of the retention time on the nature of the N-acyl residue and alcohol alkyl group is shown in Table 34. Retention times

TABLE 34

RETENTION TIMES (min) OF ACYLLEUCINE ALKYL ESTERS

Type	*N-Acyl group*	*Column A*			*Column B*			*Ester group*
		165°	*200°*	*225°*	*150°*	*165°*	*200°*	
N-Acylleucine methyl esters	Acetyl	25.2	8.2	3.0	4.4	2.4	0.9	Methyl
	Propionyl	25.2	8.2	3.0	5.0	2.8	1.1	Methyl
	n-Butyryl	32.6	10.0	3.6	7.4	3.8	1.3	Methyl
	n-Valeryl	46.6	13.4	4.6	11.6	5.6	1.7	Methyl
	n-Caproxyl	67.6	18.2	6.0	18.4	8.2	2.2	Methyl
	Enanthynyl	100.0	25.2	7.8	31.6	13.0	3.0	Methyl
N-Acetylleucine alkyl esters	Acetyl	25.2	8.2	3.9	4.4	2.4	0.9	Methyl
	Acetyl	25.4	8.1	3.1	5.0	2.8	1.1	Ethyl
	Acetyl	34.0	10.4	3.7	7.8	3.9	1.4	*n*-Propyl
	Acetyl	47.6	13.7	4.6	11.8	5.5	1.8	*n*-Butyl
	Acetyl	68.2	18.6	6.1	18.8	8.2	2.5	*n*-Amyl
	Acetyl	99.6	25.6	8.0	29.6	12.0	3.6	*n*-Hexyl
N-Acylleucine alkyl esters	Acetyl	26.2	8.2	3.0	4.4	2.4	0.9	Methyl
	Propionyl	30.2	9.4	3.1	5.5	2.5	1.1	Ethyl
	n-Butyryl	61.2	15.4	4.4	10.1	4.8	1.4	*n*-Propyl
	n-Valeryl	106.0	25.3	7.1	25.2	8.8	2.2	*n*-Butyl
	n-Caproyl		47.1	12.0	62.0	17.6	3.8	*n*-Amyl
	Enanthyl		9.4	20.4	164.0	35.2	7.0	*n*-Hexyl

for *n*-butyrylamino acid *n*-propyl esters and N-acylglycine methyl esters are given in Tables 35 and 36.

Esterification of the N-acylamino acid can be carried out as follows. To the dried N-acylamino acid residue, 2 ml of the appropriate alcohol and 0.5 ml of benzene are added in the presence of *ca.* 1 mg of Amberlite IR-120 (H^+), which has previously been thoroughly washed successively with absolute ethanol and benzene. This reaction mixture is then refluxed for 10 min. The resin is separated from the solution by filtration and washed three times with 1-ml portions of benzene. The excess of

TABLE 35

RETENTION TIMES (min) OF *n*-BUTYRYLAMINO ACID *n*-PROPYL ESTERS
Temperatures: injected port, 230°; detector, 210°.

Amino acid	*Isothermal, 165°*	*Programmed from 100 to 185° at 4°/min*	*Isothermal, 150°*	*Isothermal, 165°*	*Programmed from 100 to 180° at 4°/min*
Ala	36.6	30.0	6.3	2.6	13.9
But	40.6	31.3	6.8	3.1	14.5
Val	41.0	31.3	7.8	3.5	14.9
isoLeu	51.6	40.6	10.2	4.4	15.6
Leu	61.2	37.6	10.4	4.8	16.9
Gly	67.2	41.4	7.8	3.6	15.4
β-Ala	82.0	47.4	11.7	5.0	17.2
Pro	109.6	67.4	17.7	7.2	18.9
γ-But	— **	97.0	26.6	10.6	21.1
Thr	— **	140.4	38.0	14.0	24.4
Asp	— **	— **	41.6	17.0	26.6
Ser	— **	— **	47.2	18.2	25.6
Met			60.0		29.6
Phe			66.0		28.8
Cys			96.4		34.8
Glu			105.2		33.7
Hypro			149.6		46.0
Lys			— **		21.9
Thr			— **		22.2
His			— **		33.6***
Tyr			— **		53.6***
His			— **		— **
Try			— **		— **
Arg					
Orn					
Cit					

* Injection port 240° and detector 220°.
** No peak appeared after an additional 120 min.
*** Column temperature programmed from 100 to 230° at 4°/min; injection port 270° and detector 250°.

alcohol and benzene is removed under reduced pressure in a flash evaporator. The residual N-acylamino acid ester is dissolved in 1 ml of benzene or ethanol not used for chromatography.

9. GLYCOSYLATED AMINO ACIDS

Other constituents of connective tissue proteins that can be classified as rare amino acid derivatives are hydroxylysine galactoside and hydroxylysine glucoside galactoside. The preparation of the hydrolysates should be carried out carefully in order not to destroy the compounds that are being sought. Moozar and Moczar[107] recommended heating in 2 *N* sodium hydroxide solution at 105° for 24 h in sealed polypropylene tubes. The hydrolysate is acidified with 1 *N* acetic acid (22 volumes) and centrifuged. The supernatant is evaporated to dryness over potassium hydroxide

TABLE 36

RETENTION TIMES (min) OF GLYCINE ALKYL ESTERS

Temperature: injection port, 230°; detector, 210°.

Type	*N-Acyl group*	*Column A*		*Column B, 165°*	*Ester group*
		165°	200°	165°	
N-Acylglycine methyl esters	Acetyl	28.8	8.8	1.5	Methyl
	Propionyl	30.1	9.5	1.6	Methyl
	n-Butyryl	39.5	11.9	2.0	Methyl
	n-Valeryl	57.8	16.8	2.8	Methyl
	n-Caproyl	83.6	21.4	4.4	Methyl
	Enanthyl	117.4	31.0	6.4	Methyl
N-Acetylglycine alkyl esters	Acetyl	28.8	8.8	1.5	Methyl
	Acetyl	31.9	9.0	1.9	Ethyl
	Acetyl	44.6	12.2	2.3	*n*-Propyl
	Acetyl	66.4	16.8	3.2	*n*-Butyl
	Acetyl	96.0	23.4	4.4	*n*-Amyl
	Acetyl	148.0	35.0	6.2	*n*-Hexyl
N-Acylglycine alkyl esters	Acetyl	28.8	8.8	1.5	Methyl
	Propionyl	32.2	10.2	1.9	Ethyl
	n-Butyryl	67.2	18.1	3.6	*n*-Propyl
	n-Valeryl	145.4	34.8	7.2	*n*-Butyl
	n-Caproyl		58.8	14.0	*n*-Amyl
	Enanthyl		118.2	29.4	*n*-Hexyl

under vacuum and the residue is redissolved in water. The preparation of the hydrolysates in sealed glass tubes results in the quantitative recovery of glycosylated hydroxylysine derivatives.

9.1. Ion-exchange chromatography and automated amino acid analysis

Both single- and double-column systems can be used for the separation of glycosylated amino acids. In the two-column system the long column was equilibrated with 0.2 *N* sodium citrate buffer (pH 4.25) at 60° and eluted with the same buffer After eluting galactosyl hydroxylysine, hexosamines and free hydroxylysine were eluted with 0.35 *N* sodium citrate buffer (pH 5.28). The results of the separation are shown in Fig. 49 (Kimura[86]). When using type AA-15 resin (Odell *et al.*[118]), glucosylgalactosyl hydroxylysine precedes methionine and galactosylhydroxylysine preceeds tyrosine, provided that the elution programme indicated in Table 37 is applied. The buffer composition is specified in Table 38.

9.2. Electrophoretic separations

For electrophoretic separation, pyridine–acetic acid–water (1:10:89) (pH 3.8) has been recommended as a convenient buffer system. The separation was run at 4000–5000 V and took 1.5–2 h. The spot positions after electrophoresis are shown in Fig. 50.

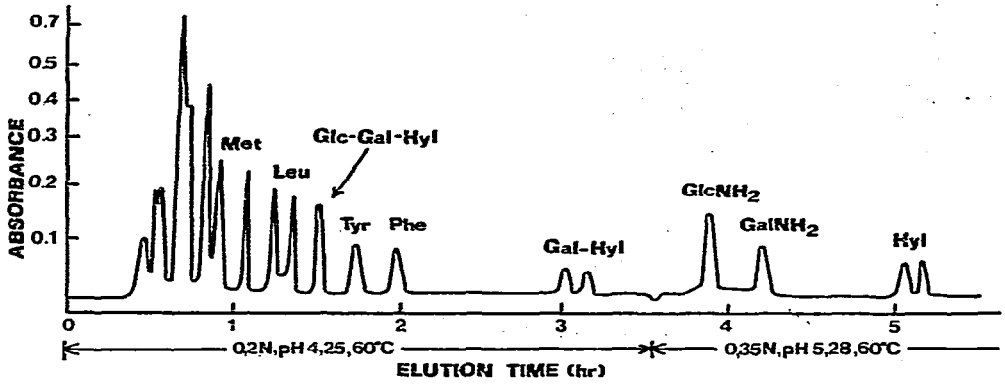


Fig. 49. Chromatographic separation of the standard mixture of amino acids, Glc-Gal-Hyl-Gal-Hyl, Glc-NH_2 and GalNH_2, on the long column of the amino acid analyser (JLC-3BC Liquid Chromatograph). The standard mixture contains each of these compounds dissolved in 0.01 *N* HCl and the rate of 0.05 μmole/ml and 1 ml of the mixture was applied on the column. The Hyl peak was doubled due to the presence of the diastereomer of hydroxylysine.

TABLE 37

ELUTION PROGRAMME FOR THE SEPARATION OF GLUCOSYLGALACTOSYL-HYDROXYLYSINE AND GALACTOSYLHYDROXYLYSINE

*Buffer**	*Pumping time (min)*	*Run time (min)*	*Column temperature (°C)*
A	40	0– 40	55
B	60	40–100	67
C	60	100–160	67
D	60	160–220	67
NaOH	11 (regeneration)	220–236	67
A	60 (equilibration)	236–286	55

* See Table 38.

** The column temperature reached 67° 35 min after buffer B has been started, and returned to the initial 55° after the regeneration step had been concluded.

TABLE 38

BUFFER COMPOSITION FOR THE AUTOMATED AMINO ACID ANALYSIS OF GLYCOSYLATED AMINO ACIDS

Sodium citrate Beckman concentrates were diluted 1:10. Pentachlorophenol (0.4 ml of stock solution) was added per 4 l of solution as an anti-mould agent. Sodium ion concentration was adjusted by the addition of crystalline NaCl. Methyl-Cellosolve and *n*-propanol were added as indicated and the final pH values were adjusted with either concentrated HCl of 50% NaOH as required.

Buffer	*Beckman concentrates*		*Final composition*		
	Na^+ concentration (N)	*pH (25°)*	*NaCl concentration*	*pH (25°)*	*Organic solvent (vol.-%)*
A	2.0	3.25 ± 0.01	—	2.83	2% methyl-Cellosolve
B	2.0	3.25 ± 0.01	7.0	3.32	
C	2.0	3.25 ± 0.01	70.5	3.89	
D	3.5	5.26 ± 0.02	183.2	5.09	16% *n*-propanol

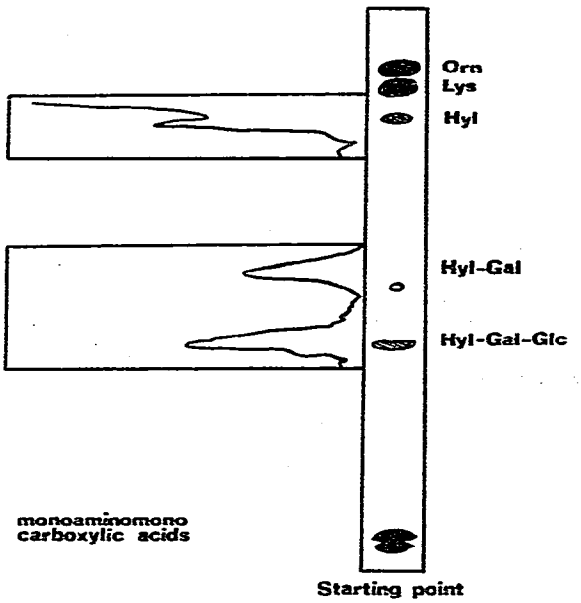


Fig. 50. Electropherogram and photodensitometric recording of hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine rendered visible by the ninhydrin–cadmium from an alkaline hydrolysate of 150 μg of calf corneal stroma.

10. COMPLEX MIXTURES AND MISCELLANEOUS

A wide variety of unusual amino acids present as admixtures in the "classical twenty" have been subjected to chromatographic separations[49], but it is beyond the scope of this review to cover all of these applications. Some, however, may serve as representative examples, especially when they are oriented more towards unusual derivatives than towards the "classical twenty".

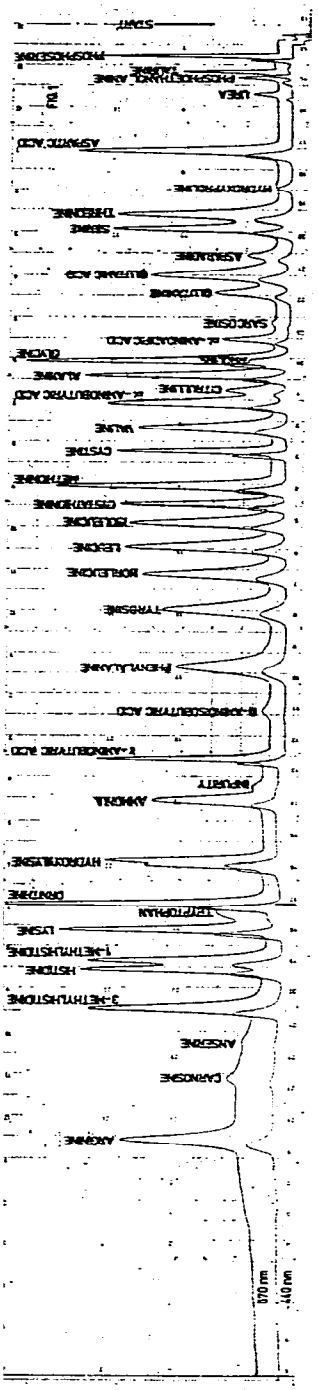
Thus, for gas chromatographic separations OV-17 on 80–100-mesh Chromosorb G or Dexsil (1.5%, w/w) on Chromosorb G can be used, preferably in 1 m × 3 mm I.D. columns. The applicability of such a system to the separation of sulphur-containing amino acids was shown on p. 288.

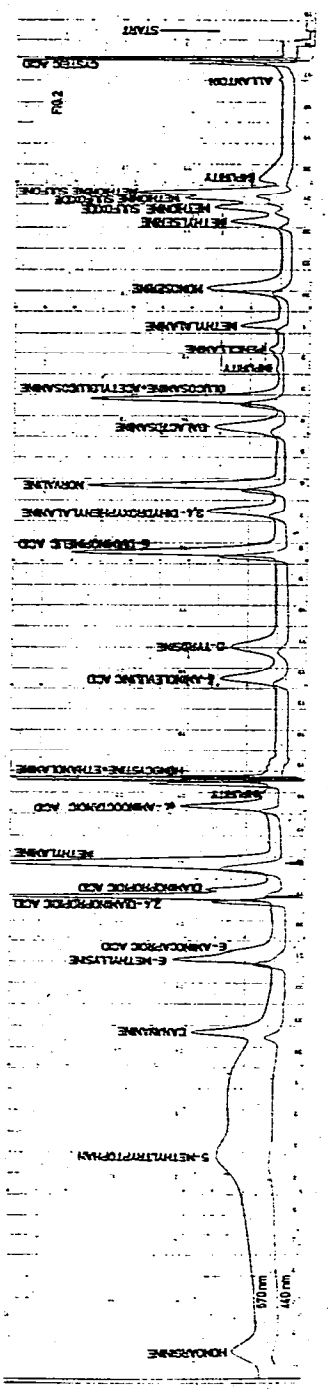
With ion-exchange chromatography, a three-temperature programme was recently devised with a Biotronic LC 6000 instrument (Tutschek *et al.*[176]), by means of which about 40 common and uncommon amino acids can be separated. A further

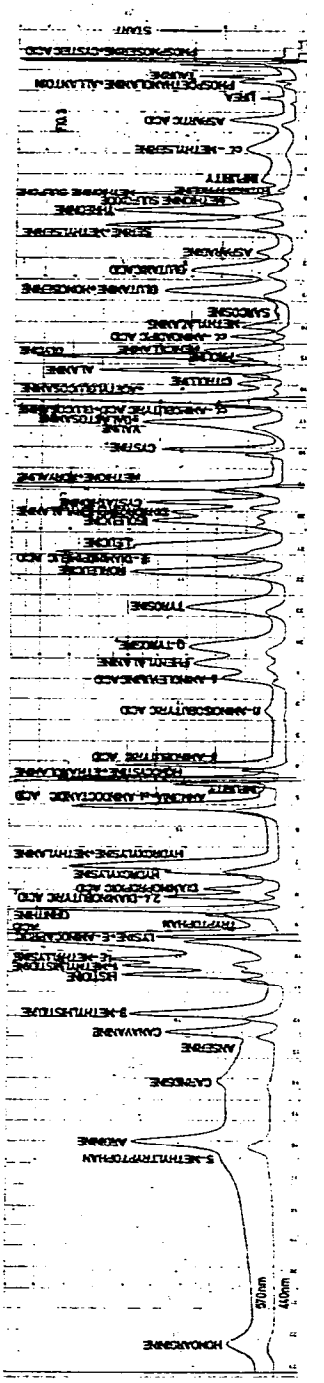
TABLE 39

pH VALUES AND LITHIUM CONCENTRATION RECOMMENDED FOR COMPLEX AMINO ACID MIXTURES ON DURRUM DC-LA RESIN

Parameter	*Buffer*				
	A	*B*	*C*	*D*	*E*
pH	2.72	3.04	3.14	4.33	3.37
Lithium concentration (*N*)	0.20	0.30	0.45	1.00	1.40


START
PHOSPHOSERINE
PHOSPHOETHANOLAMINE
UREA
ASPARTIC ACID
HYDROXYPROLINE
THREONINE
SERINE
ASPARAGINE
GLUTAMIC ACID
GLUTAMINE
SARCOSINE
α-AMINOBUTYRIC ACID
GLYCINE
ALANINE
CITRULLINE
α-AMINOBUTYRIC ACID
VALINE
CYSTINE
METHIONINE
CYSTATHIONINE
ISOLEUCINE
LEUCINE
HOMOLEUCINE
TYROSINE
PHENYLALANINE
β-AMINOISOBUTYRIC ACID
γ-AMINOBUTYRIC ACID
AMMONIA
HYDROXYLYSINE
ORNITHINE
TRYPTOPHAN
LYSINE
1-METHYLHISTIDINE
HISTIDINE
3-METHYLHISTIDINE
ANSERINE
CARNOSINE
ARGININE
570 nm
440 nm


START
CYSTEIC ACID
ALLANTOIN
FIG 2
METHIONINE SULFONE
METHIONINE SULFOXIDE
METHIONINE
HOMOSERINE
METHYLALANINE
PHENYLALANINE
GLUCOSAMINE-ACETYLGLUCOSAMINE
LACTOSAMINE
NORVALINE
α,γ-DIAMINOBUTYRIC ACID
β-UREIDOISOBUTYRIC ACID
β-TYROSINE
β-AMINOISOBUTYRIC ACID
HOMOCYSTINE+ETHANOLAMINE
α-AMINOADIPIC ACID
METHIONINE
α-AMINOPIMELIC ACID
α,γ-DIAMINOPROPIONIC ACID
α-METHYLLYSINE
α-AMINOCAPROIC ACID
CARNOSINE
5-METHYLTRYPTOPHAN
570 nm
440 nm
HOMOANSERINE


START
PHOSPHOSERINE-CYSTEIC ACID
PHOSPHOETHANOLAMINE-ALLANTOIN
UREA
ASPARTIC ACID
α-AMINOBUTYRIC ACID
METHIONINE SULFONE
METHIONINE SULFOXIDE
METHIONINE
HOMOSERINE
GLUTAMIC ACID
GLUTAMINE+HOMOSERINE
SARCOSINE
α-AMINOADIPIC ACID
GLYCINE
ALANINE
CITRULLINE
α-AMINOBUTYRIC ACID-GLUCOSAMINE
VALINE
CYSTINE
METHIONINE-NORVALINE
CYSTATHIONINE
ISOLEUCINE
LEUCINE
β-UREIDOISOBUTYRIC ACID
HOMOLEUCINE
TYROSINE
β-TYROSINE
PHENYLALANINE
β-AMINOISOBUTYRIC ACID
γ-AMINOBUTYRIC ACID
HOMOCYSTINE+ETHANOLAMINE
α-AMINOADIPIC ACID-α-AMINOCAPRIC ACID
AMMONIA
HOMOCITRULLINE+METHIONINE
HOMOCITRULLINE
DIAMINOPROPIONIC ACID
α,γ-DIAMINOBUTYRIC ACID
ORNITHINE
TRYPTOPHAN
LYSINE-ε-AMINOCAPROIC ACID
ε-METHYLLYSINE
HISTIDINE
3-METHYLHISTIDINE
CARNOSINE
ANSERINE
5-METHYLTRYPTOPHAN
570nm
440nm
HOMOANSERINE

modification of this programme was reported by Thönes *et al.*[173]. The composition and pH of the lithium buffers used are summarized in Table 39. The buffer flow-rate was 90 ml/h and that of ninhydrin 40 ml/h. Durrum DC-1A resin was used. An example of the separation of three different complex mixtures is shown in Fig. 51.

In addition to complex mixtures, the occurrence some additional unusual amino acids that arise during post-translational reactions that cannot be classified in the preceding sections should be briefly mentioned.

γ-Hydroxyarginine is a rarely occurring amino acid and its presence in the active site of some proteases has been reported. Ion-exchange chromatography, which, however, does not allow quantitation, can be carried out on a two-column amino acid analyser. Buffer of pH 5.28 yields a good resolution from ammonia (Rickert and Viswanatha[137]).

Analogous compounds that posess similar properties with respect to the construction of the active centre, such as canavanine, desaminocanavanine, N-amidinohomoserine, N-amidinohomoserine anhydride and 4-amino-2-amidinoperhydro-1,2-oxazin-3-one were studied by Rickert and Viswanatha[138] by using paper electrophoresis (pH 3.5, 0.05 *M* phthalate buffer, 3000 V/cm). Alternatively, paper chromatography can be used with *n*-butanol–acetic acid–water (4:1:5) as mobile phase.

Chromatography on Celite in the system *n*-butanol–*n*-propanol–0.1 *N* hydrochloric acid offers the possibility of isolating compounds such as pipecolic acid, α,β-diaminobutyric acid and α,β-methylaspartic acid (Martin and Hausmann[96]).

11. SUMMARY

Several categories of post-translational reactions, such as alkylation, halogenation, carboxylation, etc., occur in proteins. The biological importance of these reactions is enormous as they substantially influence the properties of the protein. However, information regarding the separational properties of these modified amino acids is still scattered throughout the literature. An attempt to survey present knowledge of their chromatographic and electrophoretic behaviour is presented here. Due to the diverse chemical nature of the arising amino acid derivatives the methods of choice for their separation also differ substantially and no general recommendations can be made in this respect.

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