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ADVANCES IN SEPARATION TECHNIQUES IN SEQUENCE ANALYSIS OF PROTEINS AND PEPTIDES

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CONTENTS

1. INTRODUCTION

In spite of numerous efforts to overcome the time-consuming operations in sequence analysis, either by automation of current procedures or by outlining new methods suitable for computerization, chromatographic methods continue to be the keystone in the multi-step procedures used for the determination of the primary structure of proteins and peptides.

During the past 30 years, numerous methods have appeared that are suitable for the determination of the liberated N-terminal amino acid after applying Edman's classical single-step procedure. About four years ago, together with the late Jan Rosmus, we tried to summarize the data available on chromatographic separations of different

derivatives used in sequence analysis and found that there were about 41 types of derivative (Rosmus and Deyl[1,2]). At first sight it was obvious that the individual methods were developed to considerably different degrees and also that the separation techniques used for individual types of derivative differed substantially in respect to the quality of separations obtained. Some of these techniques were very popular and necessitated the division of the review into two parts, one of which was devoted solely to the separation of dinitrophenyl (DNP) derivatives and phenylthiohydantoin (PTH) derivatives, while the other was devoted to the remaining 39 methods.

Now, four years later, it is, perhaps, reasonable to re-examine this topic. The above division of the problem into two parts according to the popularity of individual techniques seems justified even today; in the meantime, however, the interests of most research workers have changed. The separation of dinitrophenyl derivatives appears nowadays to be only of historical interest and these were replaced by fluorescent derivatives, mainly the 1-dimethylaminonaphthalene-5-sulphonyl (dansyl; Dns) compounds, which offer much higher sensitivity and are capable of giving very good separations. Unlike DNP-amino acid derivatives, hydantoins, phenylthiohydantoins and other derivatives of this type withstood the fashionable trends over the years and interest in their use in facilitating separations remains high. This latter fact is obviously due to the use of Edman's procedure, which, except for being automated, has not changed substantially. Currently, progress in chromatographic techniques in the area of sequence analysis appears to be intensive rather than extensive. Since the first steps taken in the chromatography of Dns-amino acids about 10 years ago (Seiler and Wiechman[3] and Deyl and Rosmus[4]), the technique for their separation has been highly developed and today offers a wide selection of all types of separation, including the flat-bed arrangement, classical liquid-column separation and high-speed, high-resolution chromatography; the same considerations hold for different organic isocyanates, especially PTH-amino acids.

As mentioned earlier, the newly developed methods are few, and most are directed towards slight modifications of aromatic sulphonyl chlorides, which would offer higher yields of fluorescence and thus increase the sensitivity of the method beyond the level of Dns derivatives. Among the really new procedures, the use of pivalyl and benzoyl chloride for the gas chromatographic separation and determination of N-terminal amino acids should be emphasized (Cavadore *et al.*[5]).

It is not within the scope of this paper to present an exhaustive review on the chromatographic separations of N-terminal amino acid derivatives and those readers who require more detailed information should be directed to previously published reviews, many of which have lost little of their value even after the many years that have elapsed since they appeared (Fox[6], Rosmus and Deyl[1,2], Meloun[7], Holeyšovský[8], Pataki[9], Bailey[10], Seiler[11], Gray[12], Deyl and Juřicová[13], Deyl[14]). It is therefore desirable to concentrate on the progress that has been made within the last four years respecting the effects of the new progressive chromatographic techniques, which involve not only the quality of separation but equally the problems of quantitation.

In the formal presentation of this survey, repetition of the mechanisms of the reactions examined in the two previous reviews (Rosmus and Deyl[1,2]) is avoided. Thus only the reaction schemes of the newly introduced types of reagents are presented here. Reaction schemes of analogous reagents, which have generally the same structural skeleton but differring only in one or two substituents have also been omitted.

2. DNS DERIVATIVES

(A) Derivatization

In principle, the technique of dansylation has not changed and most workers recommend that the reaction be carried out in 0.1 *M* $NaHCO_3$. In addition to the techniques described in our previous reports, it would perhaps be reasonable to refer here to the method of Varga and Richards[15], which, when carefully carried out, allows the quantitation of Dns-amino acids after polyamide thin-layer chromatography (TLC) at the picomole level.

To 3.6×10^{-11} mole of the protein to be analysed, contained in a test tube, 5 μl of 0.1 *M* $NaHCO_3$ are added and made to cover the base of the tube by vibrating it on a Vortex mixer; 5 μl (approx. 500 molar excess) of Dns chloride (Dns-Cl) solution (2 mg/ml) in acetone are then added and, after briefly mixing it, the reaction mixture is allowed to react for 12 h at 20°. The mixture is hydrolysed with 6 *N* HCl, the residue obtained after evaporation dried *in vacuo* and the Dns-amino acids and side products of the reaction are then extracted by vibrating the test tube with 25 μl of ethyl acetate saturated with water. The extract is spotted on two polyamide layers (2 μl each) such that the spots on the starting line are less than 2 mm in diameter. Simultaneously, a standard mixture of Dns-amino acids is run in parallel, thus providing an internal control for accurate quantitation, as it eliminates differences in TLC plate batch behaviour, humidity, solvents and minor discrepancies in the chromatographic process itself.

For hydrolysis under the above conditions, 25 μl of 6 *N* HCl are used and the evaporated residue after hydrolysis taken up in pyridine–acetic acid (1:1). Different amino acid derivatives exhibit different levels of stability towards hydrolysis, as summarized by Casola *et al.*[16] (Table 1).

TABLE 1

RECOVERY OF Dns-AMINO ACIDS AFTER TREATMENT WITH 6 *N* HYDROCHLORIC ACID AT 110° FOR 16 h

Amino acid	*Recovery (%)*
Lys	92
Phe	93
Leu	92
Ile	86
Val	93
Pro	14
Ser	65
Thr	97
Asp	93
Glu	100
Gly	64
Ala	87

(B) Flat-bed separations

As mentioned in many earlier papers, one of the considerable problems in Dns-amino acid chromatography is that the unreacted Dns-Cl hydrolyses to the corre-

sponding acid, which interferes with the chromatographic separation if the choice of the solvent is such that the free acid has a high R_F value. Varga and Richards[15] recommend overcoming this problem by multiple development with the solvents systems used by Woods and Wang[17], *i.e.*, benzene–acetic acid (9:1), formic acid–water (1.5:98.5 or 15:100) and later ethyl acetate–methanol–acetic acid (20:1:1). With the first system the Dns acid remains on the starting line and does not interfere in the subsequent step.

Most of the Dns-amino acids are resolved by running the sample in parallel in the first two solvent systems. Dns-arginine (Arg) is separated from Dns-histidine (His), and Dns-aspartic acid (Asp) from Dns-glutamic acid (Glu), by running the plate developed previously in benzene–acetic acid (9:1) in the last solvent system, *i.e.*

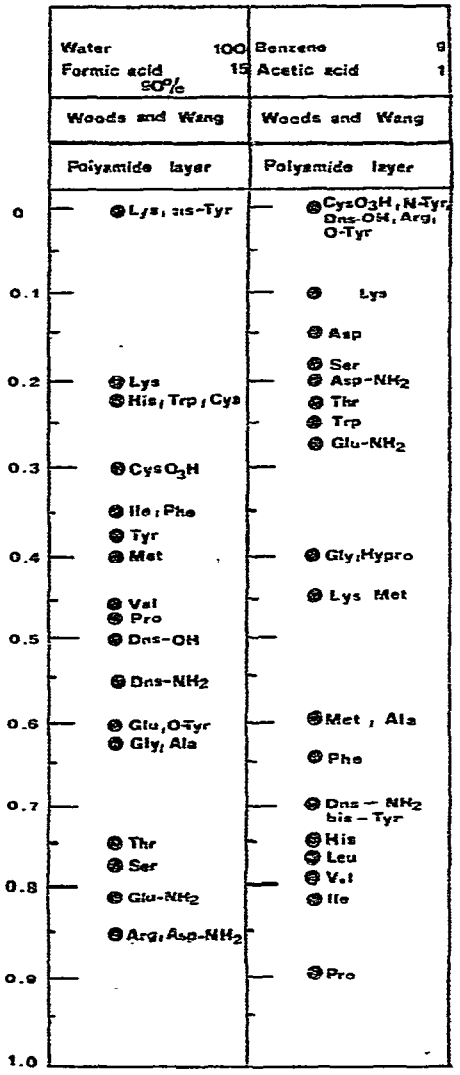


Fig. 1. Schematic representation of spots of Dns derivatives of amino acids on polyamide layer (Woods and Wang[17]). These data are used for the first run separation using the technique of Varga and Richards[15] (for details see text).

ethyl acetate–methanol–acetic acid (20:1:1). In order to separate Dns-Arg, Dns-serine (Ser) and Dns-threonine (Thr), the ethyl acetate–methanol–acetic acid mobile phase is run over the plate developed previously in formic acid–water (1.5:98.5 or 15:100). Positions of individual spots are summarized in Fig. 1. Perhaps the most unique feature of this procedure lies in the use of polyamide layers that are covered on both sides with the sorbent. Usually, the reference mixture is spotted on the reverse side of the layer. This arrangement is necessary for subsequent quantitation, as will be described later.

The only disadvantage of the above procedure is that it constantly obscures the spot of Dns-cysteine (Cys), which is indistinguishable from that of the free acid. It is therefore recommended that some of the solvent systems that offer almost complete separation of all Dns-amino acids, as evolved by Deyl and Rosmus[4] and Arnott and Ward[18], be applied.

Quantitation of the spots can be achieved by direct scanning of emitted fluorescence as reported by Seiler[11] for silica gel plates. The fluorescence depends, however, on the wetness of the plate, and apparently some decomposition or evaporation of the Dns derivatives occurs even if the plates are stored in a completely dark place. In the recently reported method of Varga and Richards[15], suitable for polyamide layers, the heating procedure of Boulton *et al.*[19], which increases the sensitivity of detection, is omitted. Essentially, the procedure of Varga and Richards[15] is a more precise application of the original trials of Bruton and Hartley[20], Weiner *et al.*[21] and Spivak *et al.*[22] for quantitative microanalysis.

In addition to direct *in situ* spectrofluorimetry, Varga and Richards[15] described the possibility of quantitation of fluorescent spots by photocopying densitometry. This method is somewhat more laborious than direct scanning; however, it offers the advantage that permanent records can be kept. In principle, a sandwich is made by using contrast film on one side of a gelatin filter (Kodak Wratten No. 93) and a double-sided polyamide thin-layer plate on the other, with the layer containing the Dns-amino acid spots on the outside, thus directly facing the ultraviolet (UV) source. The plate transmits the visible light but prevents penetration by UV radiation. At the concentrations used for quantitation, the Dns-amino acids on the inside layer of the double-sided polyamide plate do not receive enough UV light to interfere with quantitation. The interference limit of these spots is a 1000-fold excess compared with the quantitated amount on the outside layer. The sandwich, held firmly together with appropriate clamps, is exposed to the UV lamp (UVS-11 mineralight) for 10 sec from a distance 17.5 cm. The sandwich is then taken apart and the film developed and scanned in an appropriate densitometer. Normally, quantitation is carried out at the 5–20 pmole range; however, the sensitivity extends down to the fentomole level. A further increase in sensitivity can perhaps be achieved by varying the excitation wavelength of fluorescence. The corresponding fluorescence intensities are now available, as indicated in Table 2.

In sequence analysis, not only quantitation but also identification may cause considerable problems. To facilitate identification the recent suggestion by Lederer that layers with different sorbent paths should be used with a single-solvent system seemed promising and resulted in the data reported by Deyl and Rosmus[23]. The spreading device has been divided into three equal parts and silica gel (silica gel. Woelm 210, neutral, 67 g per 100 ml of water), aluminium oxide (aluminium oxide

TABLE 2

FLUORESCENCE EMISSION MAXIMA AND RELATIVE FLUORESCENCE INTENSITY OF Dns-AMINO ACIDS ON POLYAMIDE PLATES

Amino acid	*Maximum (nm)*	*Intensity*	
		(μA)	*(%)*
Ala	470	0.50	100
Asn	470	0.59	118
Asp	468	0.44	88
Arg	468	0.48	96
Gln	466	0.45	90
Glu	469	0.54	108
Gly	470	0.43	86
Ile	465	0.47	94
Leu	468	0.54	108
Lys (ε-Dns)	470	0.42	84
Lys (ε-Dns)	469	0.53	106
Lys (bis-Dns)	468	0.70	140
Met	468	0.46	92
Phe	468	0.51	102
Pro	465	0.40	80
Ser	468	0.45	90
Thr	468	0.43	86
Tyr (α-Dns)	468	0.46	92
Tyr (bis-Dns)	405	0.33	118
	485	0.26	
Trp	466	0.47	94
Val	466	0.52	104
Dns–NH_2	395	0.40	80
Dns–OH	426	0.89	178

G, Woelm 113, 67 g per 100 g of water) and polyamide (polyamide, Woelm 410, 11 g per 100 ml of methanol) were used and spread in the individual parts of the device. The layers of silica gel and alumina were spread together in the first run of the spreader. The plates were then heated to 105° for 3 h and the second movement of the spreader was used to prepare the layer of polyamide. After being spread with polyamide, the layer was allowed to stand at room temperature for at least 15 min and finally dried again at 60° in a ventilated oven. The layers were approximately 250 μm thick and were stored in a desiccator over silica gel. Cellulose was not used for layer preparation as Dns derivatives tend to stick to the starting line and tail badly in all of the solvent systems used.

Among the solvent systems tested, the following two proved suitable for the identification procedure: chloroform–benzyl alcohol–acetic acid (70:3:3) (Fig. 2a); and *n*-butanol–pyridine–acetic acid–water (30:20:6:24) (Fig. 2b). As shown in the figures both systems allow complete identification of eighteen common acids in the form of their Dns derivatives. The usefulness of this technique becomes clear from some examples: in the solvent system based on chloroform the application of the third sorbent (polyamide) permits the identification of Asp, Arg and His in the presence of each other, a combination that is otherwise very difficult to separate. Valine (Val), and isoleucine (Ile) is another combination in which, without using the third sorbent

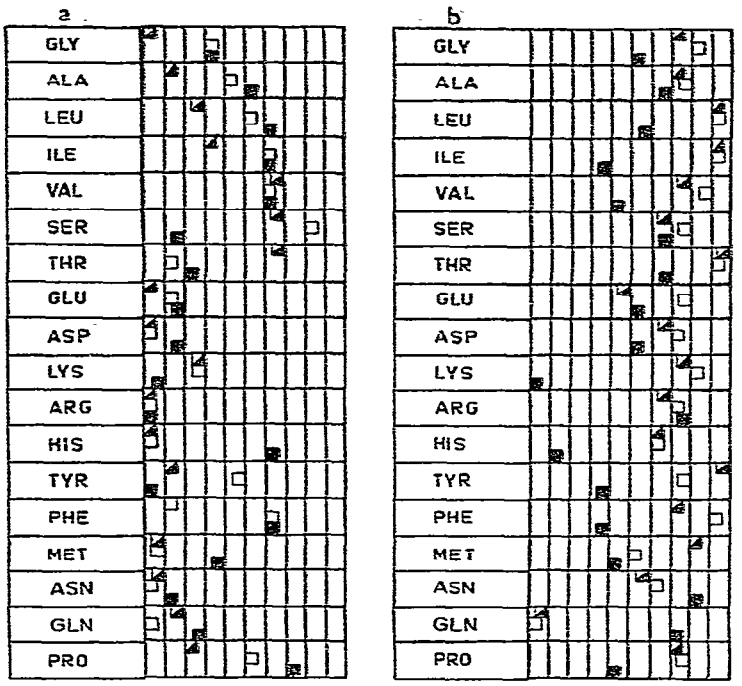


Fig. 2. Identification scheme for eighteen common amino acids as their Dns derivatives. Solvents: (a) chloroform–benzyl alcohol–acetic acid (70:30:3) and (b) *n*-butanol–pyridine–acetic acid–water (30:20:6:24). Sorbents used: aluminium oxide (hatched squares), silica gel (open squares), and polyamide (filled squares).

(aluminium oxide), the components cannot be distinguished. In the aqueous system (see Fig. 2b), similar combinations are Asp–lysine (Lys), Lys–Arg–proline (Pro) or glycine (Gly)–alanine (Ala). In order to facilitate identification, a simple system for describing the three spots relating to a particular amino acid derivative has been used: assuming that the accuracy of an R_F determination lies in the range of 0.05 R_F, each spot on a particular sorbent is characterized by the nearest R_F value ending in 0 or 5 (0.05, 0.10, 0.15, etc.). These help to make the system handy without using many layers for record purposes.

An important way to increase the sensitivity of an N-terminal amino acid determination is to make use of ^{14}C-Dns-Cl. This technique, developed by Casola *et al.*[16], offers a 50- to 100-fold increase in sensitivity over the conventional procedure. In combination with the precise separation procedure itself, it is possible to approach the picomole level. Quantitation is carried out with cut-out spots and the separation itself is two-dimensional with two subsequent runs in the second direction carried out with different solvent systems. A polyamide layer serves as sorbent. Aqueous formic acid (1.5%) has been used in the first dimension while benzene–acetic acid (90:10) served as the mobile phase for the first run in the second direction. Ethyl acetate–methanol–acetic acid (20:1:1) was used for re-running the chromatogram in the second dimension. This last run is sometimes referred to as a third dimension run.

Last, but not least, is the flat-bed procedure, which has been developed recently in the field of Dns derivatives in the so-called TLC on pre-loaded silica gel sheets. This technique is based on the original finding of Geiss *et al.*[24] that interactions between the mobile phase and ambient atmosphere may considerably influence final separation. Reasonably good separations were achieved with a formic acid gradient used for pre-loading (5–40%), provided that 10% of methanol was added to the pre-loading mixture. In some other cases the pre-loading gradient was extended so that it covered 0–40% of formic acid, but the presence of methanol was always necessary in order to depress tailing. Jänchen[25], who introduced this technique, stressed its theoretical value more than the practical application in sequence analysis, so that at present this procedure indicates only a perspective for structural studies and can hardly be exploited for routine work.

Although used only for the determination of the N-terminal amino acid in a single step, the method of Kato *et al.*[26] should be mentioned here, as it offers the possibility of estimating N-terminal amino acids in a mixture of proteins and peptides and, moreover, makes it possible to check small amounts of a contaminant in an oligopeptide.

In principle, a mixture of peptides labelled with Dns-Cl is separated by electrophoresis by using a 12.5% gel in 0.1 *M* Tris–acetate buffer (pH 8.2) that is 8 *M* in urea. After electrophoresis, gels are removed from the tubes by the usual procedure and fluorescent zones are cut off, homogenized and extracted with 5 ml of water at 37° for 16 h under stirring. In some instances the removal of the Dns peptides is incomplete under these conditions, in which event the following procedure is recommended: the gel section, containing labelled peptide is placed on the bottom of a new casting tube and the electrode buffer is layered on the gel. An electric current is then applied in the direction opposite to that used during separation. Usually electrophoresis for 90 min at 5 mA per gel is sufficient to transfer the sample completely to the inner buffer. The extract obtained by either of the two methods is lyophilized and washed three times with 1 ml of acetone in order to remove urea and sodium dodecyl sulphate (SDS). Under these conditions the Dns peptide remains precipitated without much loss. The precipitate is spun off, dried in a stream of nitrogen, dissolved in 0.5–1.0 ml of 5.7 *N* HCl and transferred to a hydrolysis tube. Hydrolysis is carried out at 110° for 4–6 h, the hydrolyzate is dried *in vacuo* within 30 min and the residue taken into solution with 0.5 ml of acetone. The extract is concentrated in a stream of nitrogen, if necessary, and subjected to TLC in the system adopted by Woods and Wang[17] using polyamide sheets. Dns–Tris occurs as a specific by-product during this procedure, and is characterized by having an R_F value of 0.95 in 1.5% aqueous formic acid on polyamide sheet.

(C) Liquid column chromatography and high-speed separations

In the renaissance of liquid column chromatography it would be rather surprising if a series of compounds such as the Dns derivatives of amino acids were not subjected to this type of separation. A procedure, using a polyamide column, was evolved by Deyl and Rosmus[27] and can briefly be characterized as follows. The column used had dimensions of 100 × 1 cm and was adjusted for constant-temperature operation (35°); it was filled with Woelm polyamide (15 g). As the degree of separation

obtained is considerably influenced by the method of column packing used, a special device developed by Kesner[28] for uniform column filling was adopted.

After the column had been filled, benzene was pumped through it for about 1 h in order to pack it. During the packing procedure, the flow-rate was maintained at 2.5 ml/min and while operating the column the flow-rate was decreased to 0.1 ml/min. Also during the packing procedure, the thermostat was set at 35° and the column, the mixing chamber and the reservoirs were adjusted to this temperature. The outlet of the column was connected to an adapted Farrand spectrofluorimeter cell; as in most instances the fluorescence intensity was much too high for the recorder scale, a proportionating pump was inserted. The excess of outflow from the column was either discarded or retained in a fraction collector for further investigation by flat-bed techniques. The fluorescence wavelengths were set to 340 and 500 nm for excitation and luminescence, respectively. The Farrand spectrofluorimeter was alternatively set for a decreased sensitivity (1.0 position on the sensitivity scale), and the proportionating pump was by-passed. The outlet flow was diluted with acetone or methylcellosolve from an additional reservoir. The individual parts used for the split-stream procedure and all the tubing used were parts of the Technicon amino acid analyzer. The overall asembly of the apparatus is presented in Fig. 3.

The measuring cuvette was adapted from a 5-mm round-shaped quartz tube, and the spectrofluorimeter gear box adapted to give a lower speed (20 cm/h).

The amount of sample analyzed varied from 50 to 500 μl. As the solvent system used by Woods and Wang[17] in thin-layer chromatography did not result in complete resolution of all the amino acid peaks, different proportions of benzene and acetic acid were examined. The most generally applicable mixture was that of benzene–acetic acid (90:5), in which, however, the fast-moving peaks of leucine (Leu) and isoleucine were not separated. In order to improve this situation, elution was started with a benzene/benzene–acetic acid (9:1) gradient, composed of two 200 ml mixed reservoirs. After 300 min, the inlet was switched automatically to the 9:1 benzene–acetic acid mixture and elution was carried out for the next 800 min without a gradient. In the final stage, this eluent was suddenly changed to a benzene–acetic acid (6:4) mixture, which made it possible to elute asparagine (Asn), hydroxyproline, arginine, cysteine and cysteic acid. The bluish band of Dns-amide remained uneluted and was removed during the regeneration procedure.

Before use, the column was washed with dry acetone (drying for 1.5 h was satisfactory), the flow-rate of the washing fluid being 1.5 ml/min. Acetone was then replaced with benzene, which was passed through the column for an additional 2 h. After this period, the column was ready for use for a fresh separation.

The gradient-elution system exhibits several advantages compared with the widely used flat-bed techniques. Firstly, it minimizes the possibility of inducing errors, as the separation is very precise and can easily be completed with an additional flat-bed check by using the same material, which is therefore not lost, and the demands on the amount to be analyzed are consequently very reasonable. Another advantage is based on the fact that the column technique gives a good possibility of recovering unusual amino acids or hydrolysis-resistant peptides, which may be of considerable importance in special situations such as in the analysis of complex peptide mixtures. As indicated in Fig. 4, this technique offers the possibility of separating almost all common amino acids in one run, and, under standard conditions, the technique can

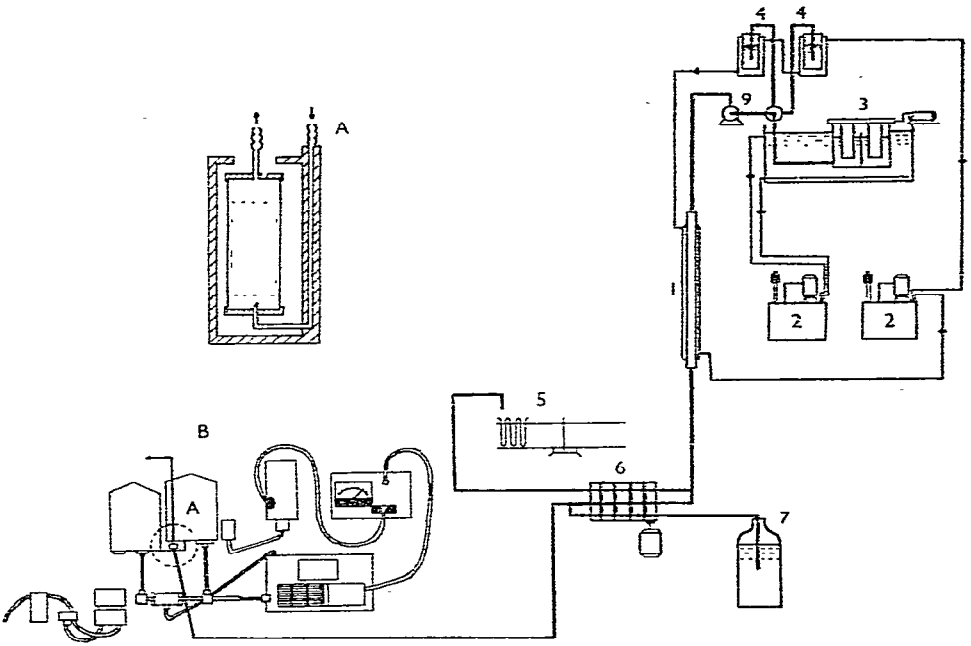


Fig. 3. The overall assembly of the chromatographic equipment. 1 = Separation column; 2 = thermostats; 3 = gradient device and reservoirs (benzene–acetic acid gradient); 4 = reservoirs for benzene–acetic acid (9:1 and 6:4 systems); 5 = fraction collector; 6 = proportionating pump; 7 = acetone reservoir; 8 = Farrand spectrofluorimeter (detail A, flow-through cuvette); 9 = programmed three-way tap.

also be used for quantitative determinations. These advantages are, of course, obtained at the cost of using more complicated equipment and slightly larger samples for analysis (at least twice as much as in the flat-bed technique).

As in every separation of a complex mixture there are pairs of Dns derivatives which are difficult to separate, such as phenylalanine (Phe) and a number of others with high chromatographic mobilities. In order to achieve adequate separations, which may be subjected to quantitation by using the technique common in non-derivatized amino acid analysis, one has to work in the region of *ca.* 2000 theoretical plates. An improved separation has been obtained by introducing a gradient system at the beginning of the chromatographic run. The operating times and solvent

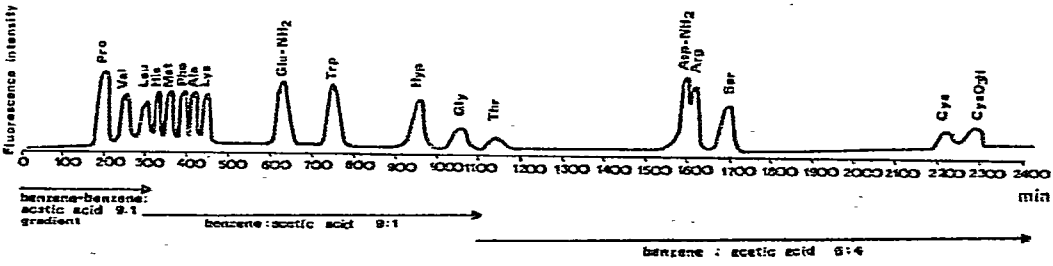


Fig. 4. Typical elution profile of Dns-amino acids on a polyamide column.

TABLE 3

RETENTION VOLUMES AND RELATIVE RETENTION VOLUMES OF Dns-AMINO ACIDS

Amino acid	*Benzene–acetic acid (9:1)*		*Benzene–acetic acid (6:4)*	
	V_e*	V_e/V_{pro}**	V_e	V_e/V_{pro}
Leu	138	1.53	80	1.02
Val	124	1.35	82.5	1.05
His	143	1.64	—	—
Met	152	1.69	81	1.04
Ala	163	1.81	92	1.08
Lys	196	2.18	108	1.38
Gly	765	8.39	117	1.50
Trp	500	5.55	134	1.71
Thr	865	9.60	142	1.82
Ser	2540	28.21	485	6.21
Cys	—	—	980	12.57
Arg	—	—	384	4.92
Hyp	700	7.76	—	—
Pro	90	1	78	—
Phe	155	1.72	85	1.09
Gln	370	4.12	126	1.61
Asn	1350	15.00	370	4.74

* V_e = Elution volume.

** V_e/V_{pro} = Elution volume relative to proline.

systems used are as follows: 0–300 min, gradient of benzene/benzene–acetic acid (9:1), 200 ml of each solvent; 300–1100 min, benzene–acetic acid (9:1); and 1100–2500 min, benzene–acetic acid (6:4). The chromatographic properties of the individual solvent systems used are summarized in Table 3.

Although no precise rules for predicting chromatographic mobility can be formulated, there are general features which, for an unknown derivative, may serve as a guide. An increase in the number of carbon atoms in the amino acid side-chain decreases the retention time. Compared with a straight chain, the difference due to a $-CH_2$ group in a branched side-chain has a much smaller effect in decreasing the retention time. Hydroxylation, however, shifts retention times to much higher values and the differences in a homologous series are increased in hydroxylated amino acids. While the presence of a second amino group causes the amino acid to move with a low retention time, guanidylation considerably retards the chromatographic mobility.

High-speed liquid column chromatography has been introduced by Engelhardt *et al.*[29] for the separation of Dns-amino acids. Silica gel particles of size ranging between 5 and 8 μm were packed into a 50 cm × 4.2 mm stainless-steel column. The sorbent was dynamically equilibrated with water using water-saturated dichloromethane. In the final stage, the sorbent contained 0.4 g of stationary liquid per gram of silica. Separation was run under 255 atp with a flow-rate of 3.2 ml/min. A very good separation, as exemplified in Fig. 5, was achieved within 22 min with water-saturated dichloromethane as the mobile phase. However, only less polar amino acid derivatives were eluted in this step. Therefore, in the next separation step, a more polar mobile phase was used. If the column is eluted with dichloromethane con-

taining 10% of water, the elution pattern depicted in Fig. 6 is obtained. It appears that with high-speed liquid column chromatography it is possible to resolve in two successive steps most of the common Dns-amino acid derivatives. A continuous gradient elution would presumably give even better results; however, the detection method of choice (not specified clearly in the paper, but presumably with a differential refractometer) in the above procedure was difficult to balance with respect to the baseline shift under gradient elution.

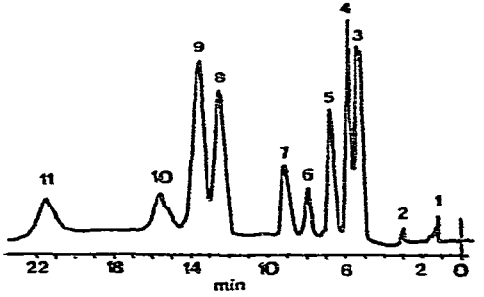


Fig. 5. High-speed separation of Dns-amino acids. (Engelhardt *et al.*[29]). Column: 50 cm (4.2 mm I.D., drilled); spherical silica (Professor Unger, Technische Hochschule, Darmstadt, G.F.R.) dynamically coated with 0.4 g of stationary liquid per gram of silica; particle size 5–8 μm; mobile phase, dichloromethane, water saturated (1500 ppm H_2O) + 1% acetic acid + 1% 2-chloroethanol; pressure, 255 atm; linear velocity, 0.6 cm/sec; flow-rate, 3.2 ml/min. 1 = Inert ($k' = 0$); 2 = unknown (1.4); 3 = Dns-Ile (2.9); 4 = Dns-Val (3.25); 5 = Dns-Leu (3.9); 6 = Dns-Tyr (4.7); 7 = Dns-Ala (6.5); 8 = Dns-Trp (8.0); 9 = Dns-Gly (8.8); 10 = Dns-His (10.1); 11 = Dns-Lys (14.4).

For laboratories that do not possess the complex high-speed chromatographic equipment, the above technique is still of value as it can also be used with lower pressures according to the size of sorbent particles used.

Very recently another technique, which can be classified as high-speed liquid column chromatography has been published by Yamabe *et al.*[30]. TSK-gel LS-140, a

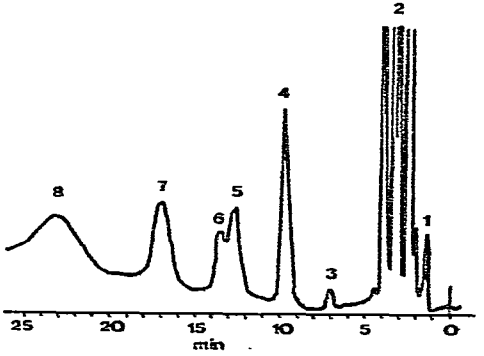


Fig. 6. High-speed separation of Dns-amino acids. (Engelhardt *et al.*[29]). Conditions as for Fig. 5, except 10% 2-chlorethanol in the mobile phase. 1 = Inert; 2 = mixture separated in Fig. 5; 3 = unknown; 4 = Dns-Thr ($k' = 5.9$); 5 = Dns-Ser (8.0); 6 = Dns-Glu (8.5); 7 = Dns-Asp (11.0); 8 = Dns-Cys (15.5).

macroreticular poly(vinyl acetate) gel (average grain size 10 μm), served as sorbent; *n*-hexane–ethanol–acetic acid–triethylamine (90:10:1:1) was used as mobile phase at a flow-rate of 2.2 ml/min and at a pressure of 83 atm. The column size was 50 cm × 3 mm and running time only 60 min; however, no baseline separations were obtained with a standard mixture of amino acids, as indicated in Fig. 7.

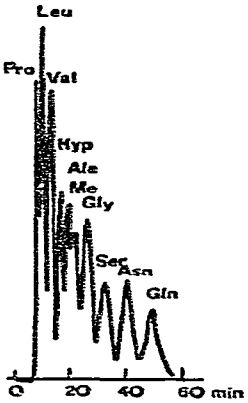


Fig. 7. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–ethanol–acetic acid–triethylamine (90:10:1:1). Flow-rate, 2.2 ml/min; pressure, 83 kg/cm^2.

3. BANSYL DERIVATIVES

The replacement of the dimethylamino group of Dns-Cl with a di-*n*-butylamino group offers some advantages over Dns derivatives, the resulting compounds being less polar and easily separated in non-polar solvents (Seiler *et al.*[31]). The derivatization is carried out at room temperature in acetone–water (3:1) saturated with sodium carbonate, in a similar way to that with Dns-Cl. Thin layers of silica or polyamide can be used for the separation. With polyamide, benzene–acetic acid (9:1) may serve as mobile phase. Spot positions are obvious from the scheme shown in Fig. 8. The sensitivity range is in the order of tenths of a nanomole, which appears to be another important advantage of these derivatives.

4. DABSYL DERIVATIVES

Recently, a new, coloured type of derivative for the N-terminal labelling, 4-N,N-dimethylaminoazobenzene-4′-sulphonyl (dabsyl) chloride, has been synthesized by causing methyl orange to react with phosphorus pentachloride. The sulphonyl groups react readily with primary and secondary amino groups. According to Chang and Creaser[32], the intense chromophoric dabsyl amino acids formed permit the detection of amino acids as coloured spots in the range 10^{-10}–10^{-11} mole.

The silica gel plates originally suggested for thin-layer separation of these derivatives suffer from diffusion effects, which in some instances may obscure the results. Therefore, in later work performed by Chang and Creaser[32] polyamide sheets were used instead. The whole procedure is carried out on 5 × 5-cm sheets and optimum detection is observed with 10–20 pmole of each amino acid.

The practical procedure for preparing dabsyl derivatives is as follows. The requisite amount of each amino acid (50 nmole) is dissolved in 50 nmole of 0.2*M* $NaHCO_3$ and allowed to react with an equal amount of dabsyl chloride in 50 μl of acetone at 70° for 5–10 min; 10–15 pmole are then applied to the 5 × 5-cm polyamide sheet. It has been stressed that the size of the starting spot should not exceed 1.0–1.3 mm. In general, the solvent systems of Woods and Wang[17] originally proposed for Dns-amino acids are recommended. Thus in the first dimension the plate is developed in water–2-chloromethanol–formic acid (100:60:3.5) while in the second dimension the plate, after being carefully dried, is developed in benzene–acetic acid (6:1). A schematic representation of the separation is presented in Fig. 9. The spots can be intensified by exposure to HCl vapour.

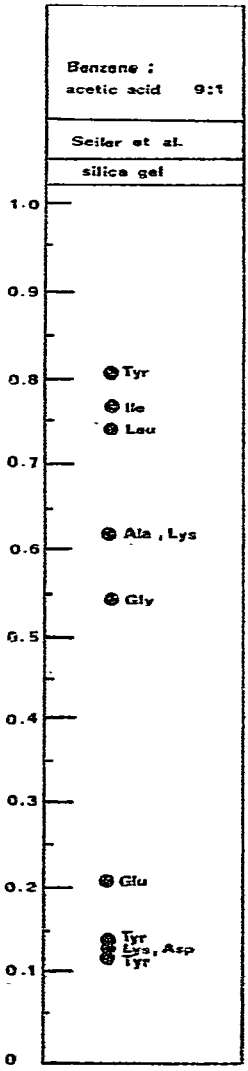


Fig. 8. Schematic representation of the separation of bansyl derivatives.

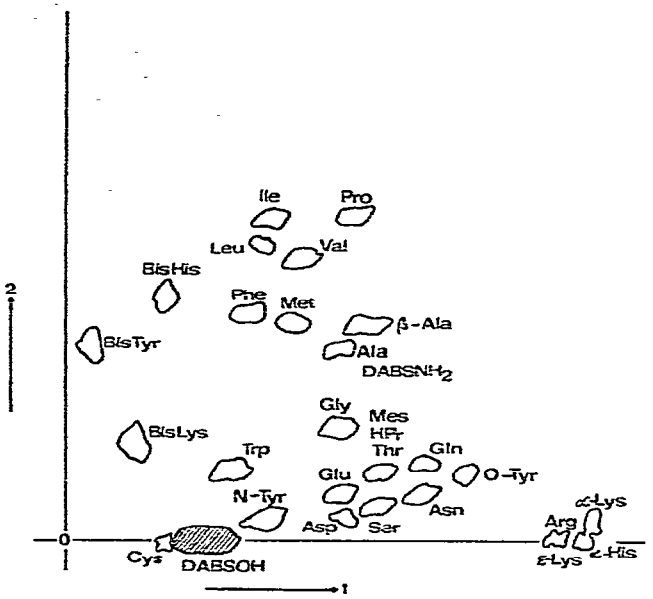


Fig. 9. Schematic representation of the separation of dabsyl derivatives.

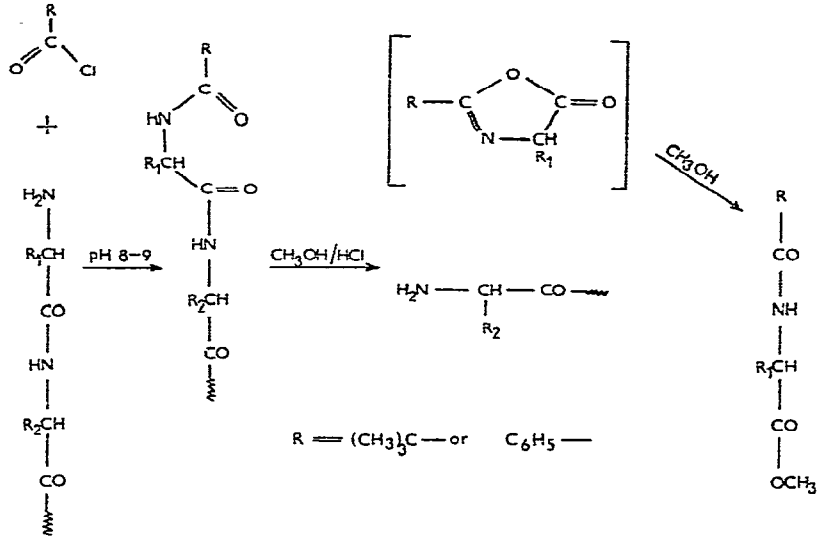
In the above system, several combinations of amino acids are not separated, thus the spot of cysteic acid is fused with that of dabsyl-OH, methionine (Met) sulphone is indistinguishable from hydroxyproline (Hyp) and Thr, and α-Lys derivative and α-His derivative form a combined spot; Arg and Lys are also difficult to separate. The use of other solvent systems that allow the separation of these overlaps was suggested. Thus dabsyl-OH and cysteic acid (corresponding derivative), dabsyl-NH_2 and Ala, methionine sulphone, Hyp and Thr, and α-monohistidine, α-monolysine and Arg, can be separated (always from the last-mentioned amino acid derivative) by developing the plate in water–pyridine–28% ammonia–formic acid (100:20:10:2). Arg can be separated from monosubstituted Lys and His by developing the plate in water–28% ammonia–ethanol (9:1:10). Very recently Creaser[33] used the isothiocyanate derivative of the dabsyl reagent for direct sequencing work.

5. PIVALYL AND BENZOYL DERIVATIVES

(A) Derivatization

Both pivalyl and benzoyl chloride are reagents that are very reactive towards the N-terminal amino group. Also, the introduction of a benzoyl or pivalyl group facilitates the hydrolysis of an N-terminal amino acid; both of these reagents were used for the first time by Cavadore *et al.*[5]. The final identification was achieved by gas chromatography after converting the liberated benzoyl and pivalyl derivatives into their corresponding methyl esters. Schematically, both reactions can be represented

as follows:



In practice, 10–15 μmole of the peptide to be analysed are dissolved in 100 μl of N-ethylmorpholine contained in a vial tube and 20 μl of pivalyl chloride or benzoyl chloride are added. The reaction is allowed to proceed for 30 min at room temperature and the solvent is then evaporated off in a stream of nitrogen. The residue is dissolved in 300 μl of methanol saturated with hydrogen chloride and heated to 70° for 30 min. The acidic methanol is evaporated off and the residue is dissolved in 200 μl of 0.1 *N* HCl and the solution extracted twice with ethyl acetate to give a total of 600 μl. The extract is evaporated to dryness, re-dissolved in methanol and treated with an excess of diazomethane in diethyl ether for several minutes. The reaction mixture is then taken to dryness, dissolved in 5 μl of dichloromethane containing the corresponding internal standard and this solution is directly transferred to the gas chromatograph.

If the molecular weight of the peptide or protein is too large, it has been recommended that about 100 nmole of the substance to be analysed be suspended in an acetone–water mixture (1:1) and only then is the N-ethylmorpholine solution added.

(B) Gas chromatography

For the chromatographic separation of N-pivalylamino acids in the form of their corresponding methyl esters, a 35 m × 0.028 cm capillary column, coated with 5% XE-60 and FFAP (0.5%) in dichloromethane was used. Nitrogen served as carrier gas at a flow-rate of 1 ml/min. Temperature programming and separation of individual amino acid derivatives can be seen in Fig. 10. Pivalylphenylalanine ethyl ester served as internal standard.

An identical column was used by Cavadore *et al.*[5] for the separation of benzoylamino acid methyl esters. The column was coated with 1% FFAP in dichloromethane and nitrogen served as carrier gas at a flow-rate of 1 ml/min. The injector heater in both instances was heated to 250°. Temperature programming of the column

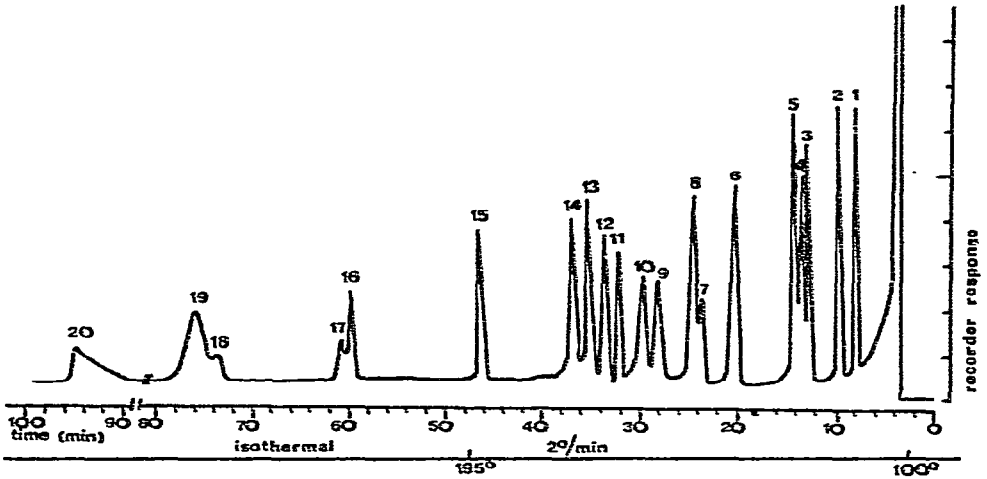


Fig. 10. Gas chromatographic separation of an equimolar mixture of N-pivalylamino acid methy esters (5 nmole) on a glass capillary column (35 m × 0.028 cm I.D.) coated with XE-60 (5%) and FFAP (0.5%) in CH_2Cl_2. Nitrogen carrier gas flow-rate, 1 ml/min.; injector heater at 250°. The internal standard used was pivalylphenylalanine ethyl ester. Peak identification: 1 = Ala; 2 = Val; 3 = Ile; 4 = Gly; 5 = Leu; 6 = Pro; 7 = S-CH_3-Cys; 8 = Asp; 9 = Thr; 10 = Ser; 11 = Met; 12 = Glu; 13 = Phe; 14 = internal standard; 15 = S-CM-Cys; 16 = Tyr; 17 = Asn; 18 = Gln; 19 = Lys; 20 = Trp.

and the quality of separation are shown in Fig. 11; good peaks were obtained after injecting 5 nmole of the derivative into the apparatus.

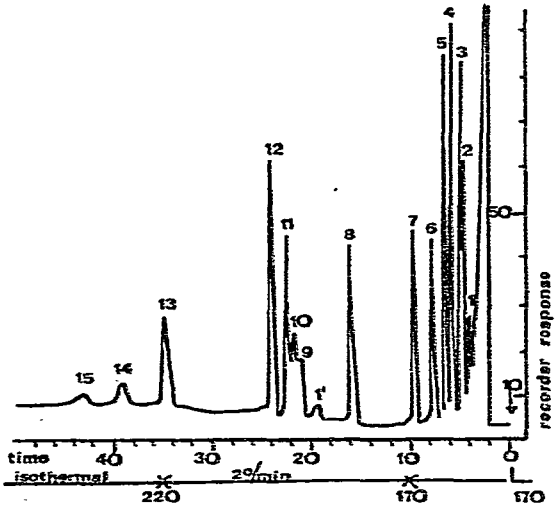


Fig. 11. Gas chromatographic separation of an equimolar mixture of N-benzoylamino acid methyl esters (5 nmole) on a glass capillary column (30 m × 0.028 cm I.D.) coated with FFAP (1%) in CH_2Cl_2. Nitrogen carrier gas flow-rate 1 ml/min; injector heater at 250°. Peak identification: 1, = Ser; 2 = Ala; 3 = Val; 4 = Ile; 5 = Leu; 6 = Gly; 7 = Pro; 8 = Asp; 9 = Thr; 10 = Met; 11 = Glu; 12 = Phe; 13 = Tyr; 14 = Asn; 15 = Gln.

6. PHENYLTHIOHYDANTOINS

(A) Flat-bed separations

For many years thin-layer chromatography of phenylthiohydantoins (PTHs) has been considered a simple and reliable method for the identification of amino acid residues liberated during the individual steps of Edman's degradation procedure[34]. The trends in this field are, like those with the dansylation procedure, directed towards increased sensitivity, which would compensate for stepwise losses and decreased yields recorded during the individual steps of the sequencing process. Recently, chromatography on small-size polyamide sheets has been used for detecting 0.05–0.20 nmole of PTHs. The sheet size used was 5 × 5 cm and detection was carried out by the conventional quenching of a fluorescent indicator added to the layer. Summers *et al.*[35] reported the possibility of detecting as little as 0.3 nmole on 6.3 × 6.3 cm plates using the starch–iodine reaction and Edman[36] himself reported that it was possible to quantitate 0.8 nmole on 20 × 20-cm silica gel plates, and the detection limit in the latter instance was 0.4 nmole.

Generally speaking, small-size plates are currently preferred as the spots, which move a shorter distance, are more compact and smaller amounts can therefore be recovered. On the other hand, Inglis and Nicholls[37], again using 20 × 20 cm plates, succeeded in reliably detecting less than 0.2 nmole and surmised that if smaller plates were used, the sensitivity would accordingly be higher. The method is based on exposing the plate, after careful removal of the mobile phase, to iodine vapour. While Truter[38] does not report this procedure as being particularly suitable for the estimation of PTHs, our own results obtained by the method of Inglis and Nicholls[37] indicated very good applicability of the iodine detection.

It should be stressed that the method of Inglis and Nicholls differs from that involving the iodine–azide reaction, the use of which was not recommended (Feigl[39] and Edman[36]), although it has been used extensively to increase detection limits; the main problem here lies in the fact that the intensity of the spot has a poor correlation with the amount of PTH actually present. This may obviously be a source of serious errors in sequencing. As stressed by Inglis and Nicholls, detection by fluorescence quenching suffers similar problems, while in the iodine reaction these problems are minimized. The reaction with iodine vapour is reversible and non-destructive (Barrett[40]) and therefore offers the possibility of subjecting a particular spot to further investigation.

With regard to other detection methods, that with ninhydrin–collidine mixture in absolute ethanol is still in use (Roseau and Pantel[41]). The chemical reactions involved in this detection were elucidated by Schäfer and Bauer[42]. It has been proved that the red colour obtained after spraying the plate is that of hydrindantin, which is further supported by the results obtained with acetyl-PTHs, as reported by Inglis and Nicholls[43]. These derivatives mostly do not give coloured reaction products, which fact is strongly indicative of the participation of the imino group of the thiohydantoin ring in the detection reaction. The reaction with ninhydrin–collidine in ethanol, although less sensitive by at least by one order of magnitude than the above described procedures, is of considerable diagnostic value as it results in different colours being obtained with different amino acid derivatives, as summarized in Table 4.

TABLE 4

SPECIFIC COLOUR REACTIONS OF PTH-AMINO ACIDS WITH NINHYDRIN–COLLIDINE REAGENT

Amino acid	*Time of heating (min)*	*Colour*
Ser	1	Red-violet
Gly	1.5	Intense orange
Ala	1.5	Red-violet
Methionine sulphone	2	Brown
Cystine	3	Intense pink
Cysteic acid	3	Light pink
Asn	4	Light yellow
Glu	4	Greenish brown
Met	4.5	Brown
Glu	4.5	Dark brown with blue halo
His	4.5	Faint yellow
Asp	4.5	Pink
Arg	4.5	Very faint yellow
Trp	5	Intense yellow
Tyr	5	Light yellow
Thr	5	Light brown
Lys (ε-)	7	Very faint pink
Pro	8	Very faint pink
Phe	9	Very faint yellow
Leu	15	Very faint grey
Ile	15	Very faint grey
Val	—	No colour

With regard to solvent systems used for the TLC separation of PTH-amino acid derivatives, barely any improvements have been made recently and the systems presented earlier[2] may serve to illustrate the older techniques of separation that are still in use and will obviously survive into the future.

The recent paper by Walz and Reuterby[44] reports the use of a flat-bed system that is suitable for routine identification of PTH derivatives originating from the automated Edman procedure. In this paper use is made of the solvent systems employed by Inagami and Murakami[45] and by Jeppson and Sjøquist[46], with pre-coated silica gel plates as sorbent. Some additional information about a particular derivative can be obtained by blowing ammonia vapour over the ninhydrin/collidine developed chromatogram and observing the colour change produced (Table 5).

A frequently occurring problem in the chromatographic separation of PTH-amino acids as well as of methylthiohydantoin(MTH)-amino acids lies in the separation of the arginine, cysteic acid and histidine derivatives. Recently, Kulbe[47] published a thin-layer microseparation procedure for these derivatives; 5 × 5 cm polyamide sheets from two different sources gave satisfactory results when developed with ethyl acetate–*n*-butanol–acetic acid (35:10:1) or with ethyl acetate–*tert.*-butanol–acetic acid in the same proportion. The results are summarized in Table 6. Running time for these separations did not exceed 10 min. In order to improve the contrast under UV light it is recommended that some sheets should be pre-run in the developing solvent system.

In the practical procedure recommended by Kulbe[47], the derivatives of polar

TABLE 5

COLOUR PROPERTIES OF PTH-AMINO ACIDS AFTER NINHYDRIN DETECTION AND SUBSEQUENT EXPOSURE TO AMMONIA VAPOUR

GC = Gas chromatography.

Amino acid	*Colour properties*	*Colour change with NH_3 vapour*
Val	Colourless; use GC	
Pro	UV; colourless	Light blue after heating
Ala	Purple	Deeper color
Gly	Orange	
Ser	UV; purple	
Serine breakdown	Faint orange	Weak red
Asn	Yellow	More intense
δ-Carboxymethylcysteine	UV; purple	
Methionine sulphone	Light tan	
Leu	Colourless; use GC	
Met	Faint tan	
Lys	Very faint pink	Weak blue after heating
Tyr	UV; yellow before spraying	Intense yellow
Thr	Colourless	Light tan
Dehydrothreonine	Colourless*	
Gln	Dark green	Dark blue
Ile	Colourless; use GC	
Phe	UV; colourless	Faint yellow
Trp	UV; yellow before spraying	Deep yellow
Asp	UV; pink	Darker
Glu	Grey	Dark blue

* Characteristic scan at 320 nm.

amino acids, *e.g.*, cysteic acid, histidine and arginine, which remain in the aqueous phase during extraction of methyl- or phenylthiohydantoins, are recovered by lyophilization and re-dissolved in methanol. After application of 1 μl of the solution on the polyamide sheet, chromatographic separation is carried out in the solvent systems mentioned above. The results obtained are believed to be better than those

TABLE 6

SEPARATION OF SOME PTH AND MTH DERIVATIVES ON POLYAMIDE SHEETS WITH SPECIAL REFERENCE TO THE MOBILITY OF ARGININE

Mobile phase	*Amino acid*	*Schleicher-Schuell polyamide*		*Cheng Chin polyamide sheets*	
		MTH	*PTH*	*MTH*	*PTH*
Ethyl acetate–*n*-butanol–acetic acid (35:10:1)	Arginine	0.50	0.50	0.44	0.44
	Cysteic acid	0.50	0.50	0.50	0.50
	Histidine	0.85	0.83	0.82	0.82
Ethyl acetate–*tert.*-butanol–acetic acid (35:30:1)	Arginine	0.29	0.28	0.30	0.30
	Cysteic acid	0.50	0.50	0.50	0.50
	Histidine	0.76	0.78	0.80	0.79

achieved by Rabin and Darbre[48]. As reported by Silver and Hood[49], the use of radioactive phenylisothiocyanate in the automated method parmits the N-terminal sequence analysis of 1.5 nmole of protein. Thin-layer chromatography (two dimensional) is used for the determination of individual split-off amino acids. Separation is carried out on polyamide sheets using 45% aqueous formic acid in the first run followed by development with CCl_4–acetic acid (9:2) in the second run. A sketch drawing of the resulting amino acid map can be seen in Fig. 12. The PTH spots were made visible under UV light, cut out and placed in scintillation vials.

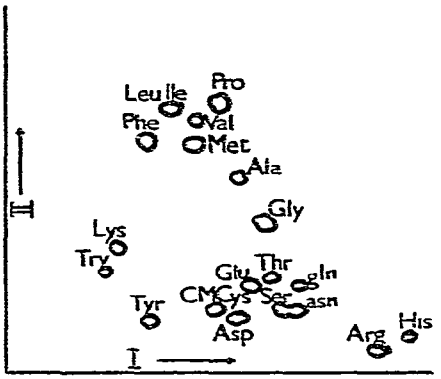


Fig. 12. Separation of PTH-amino acid derivatives by TLC on polyamide sheets. The solvent used in dimension I was 45% formic acid and in dimension II CCl_4–acetic acid (9:2).

(B) Liquid column chromatography and high-speed separations

The application of high-pressure liquid chromatography (HPLC) to PTHs has been introduced only very recently by Frank and Streubert[50] and Matthews *et al.*[51]. Silica columns are used for this purpose and complete separation and quantitation can be achieved in less than 40 min. Good results are also obtained with bonded stationary phases.

In the procedure of Matthews *et al.*[51], a DuPont Model 830 liquid chromatograph was used, with a stainless-steel column (250 × 2.1 mm I.D.) packed with DuPont Zorbax SIL, and operated at 40°. The sample dissolved in methanol (1–10 μl) was injected through a perfluorelastomer septum into the high-pressure line of the column. The system was operated at 1000 p.s.i. which ensured a flow-rate of 0.6 ml/min. Detection was carried out by recording UV absorbance at 254 nm in a 8-μl flow-through cell. Sensitivity of the system ranged between 2 and 5 nmole.

Separations achieved are illustrated in Fig. 13. Elution was carried out with a concave gradient from *n*-hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). The shape of the gradient can be described by $C = Kt^5$, where C is the concentration of the second mobile phase in the first, K is a constant and t is the fraction in time of the completed gradient. As indicated in Fig. 13, the general order of elution of individual amino acid derivatives is that of the increasing polarity of the amino acid side-chain.

The main problem with ethyl acetate extractable PTHs in the above separation is the inability to separate PTH-Gly plus PTH-Thr and PTH-Lys plus PTH-Thr. The

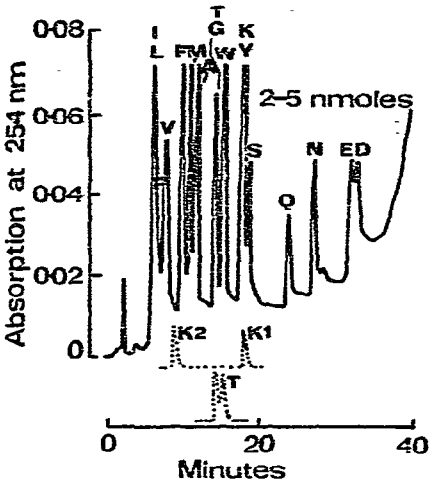


Fig. 13. Separation of PTH-amino acids by HPLC on Zorbax SIL. Elution performed with a concave gradient solvent system from hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). Detection is by UV absorption at 254 nm. The PTH-amino acid peaks are identified by the single letter notation for the corresponding amino acids. The elution positions of the two peaks obtained both from lysine (K1 and K2) and from threonine (T) are shown below the main diagram.

authors, however, indicate that in practice this fact should not cause difficulties, because lysine and threonine give double peaks in actual Edman's degradations, which are probably due to the formation of N^{ε}-phenylthiocarbamoyl-N^{α}-PTH-Lys and dehydro-PTH-Thr, in addition to the expected PTH derivatives.

It should be stressed that the above procedure holds for ethyl acetate extractable PTHs. Therefore, problems arise when PTH-Arg or PTH-His have to be assayed. When adjusting the pH of the aqueous phase (which remains after the ethyl acetate extraction) to about 8.0, PTH-His is susceptible to ethyl acetate extraction and can be chromatographed in a linear gradient from hexane–propanol (95:5) to pure propanol. No system was devised by the above authors for the separation of PTH-Arg.

The procedure described by Frank and Streubert[50] involves the use of two independent liquid column systems for the separation of PTHs, those which are more and those less polar. It is claimed that the high-resolution liquid column chromatographic separation is superior to both gas and thin-layer chromatography. The less polar amino acid derivatives, *e.g.* the PTH derivatives of Pro, Ile, Val, Phe, Met, Ala, tryptophan (Trp) and Gly, are eluted with the system dichloromethane–*tert.*-butanol–dimethyl sulphoxide (500:4:0.4). The more hydrophilic group consists of the PTH derivatives of Asp, Asn, glutamine (Gln), Glu, Thr and tyrosine (Tyr) and ε-PTH-Lys, for which series of compounds a mixture of dichloromethane–dimethyl sulphoxide–water (80:15:2) was successfully applied. The separations illustrated in Figs. 14 and 15 were carried out with a Siemens-S 200 chromatograph using Merckosorb SI 60 as column packing. Column dimensions were 500 × 3 mm and the column was operated at ambient temperature with a flow-rate of 1.9 ml/min.

Another high-speed procedure that offers excellent results in the separation of

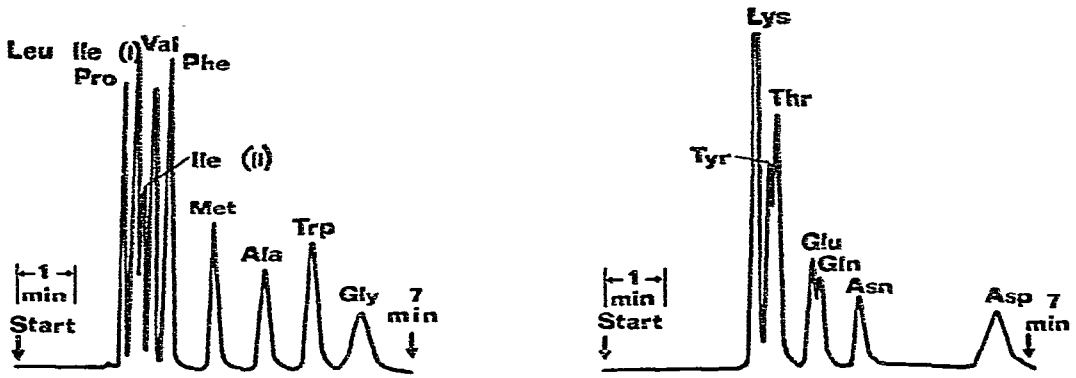


Fig. 14. Separation of PTH-amino acids, "hydrophobic group"; pressure, 250 bar; flow-rate, 1.65 ml/min; mobile phase: CH_2Cl_2–dimethyl sulphoxide–*tert.*-butanol (1000:0.8:8); column: 500-mm tantalum, 3 mm I.D.; pressure, 290 bar; flow-rate, 1.9 ml/min; sensitivity, 1 a.f.s.d.; wavelength, 260 nm; bandwidth, 20 nm; room temperature; sample volume, 10 mm^3; packing: Merckosorb SI 60 (5 μm).

Fig. 15. Separation of PTH-amino acids, "hydrophilic group". Pressure, 250 bar; flow-rate, 0.83 ml/min; mobile phase: organic phase of CH_2Cl_2–dimethyl sulphoxide–H_2O (80:15:2); other conditions as for Fig. 14.

PTH-amino acids is that described by Bollet and Caude[52]: in this instance the separation was carried out with a 25 cm × 2.1 mm column packed with Micropak CN (moderately polar alkyl nitrile phase bonded to silica gel of 10-μm grain size). It is possible to see (Fig. 16) the stepwise elution of individual peaks due to the change in mobile phase composition. Optimum loading capacity in this instance was 3 μl of a sample containing 1–3 mg/ml of the particular amino acid derivative.

Hexane–dichloromethane–isopropanol mixtures of different composition were employed for the preparation of complex gradients used for elution of the individual amino acid derivatives, and the flow-rate varied between 50 and 100 ml/h. The results are summarized in Fig. 17.

(C) Gas chromatography

Gas chromatography has been shown to be a potentially suitable method for the separation of PTHs; however, considerable difficulties are met with when handling some non-volatile derivatives. Trimethylsilyl (TMS) derivatives (Pisano and Bronzert[53] and Harman *et al.*[54]) have proved useful, especially when on-column derivatization has been used. The main disadvantages of the above silylation procedure, as summarized by Inglis *et al.*[55], are as follows: the inability to handle the arginine derivative; widely differing responses in the flame ionization detector; and the limited stability of the derivatives. Recently, Inglis *et al.*[55] and Brian *et al.*[56] reported attempts to overcome these difficulties by acylation. These first indications of the plausibility of the acylation procedure were further developed by Inglis *et al.*[55], who used the acetic anhydride–pyridine (1:1) mixture to acetylate PTHs; usually 100 μl of the reagent were sufficient for a 3-mg sample. The reaction was terminated after a suitable period of time (see Table 7) by shaking the mixture with 1 ml of water.

The actual separation was carried out with a Hewett-Packard Model 7620A

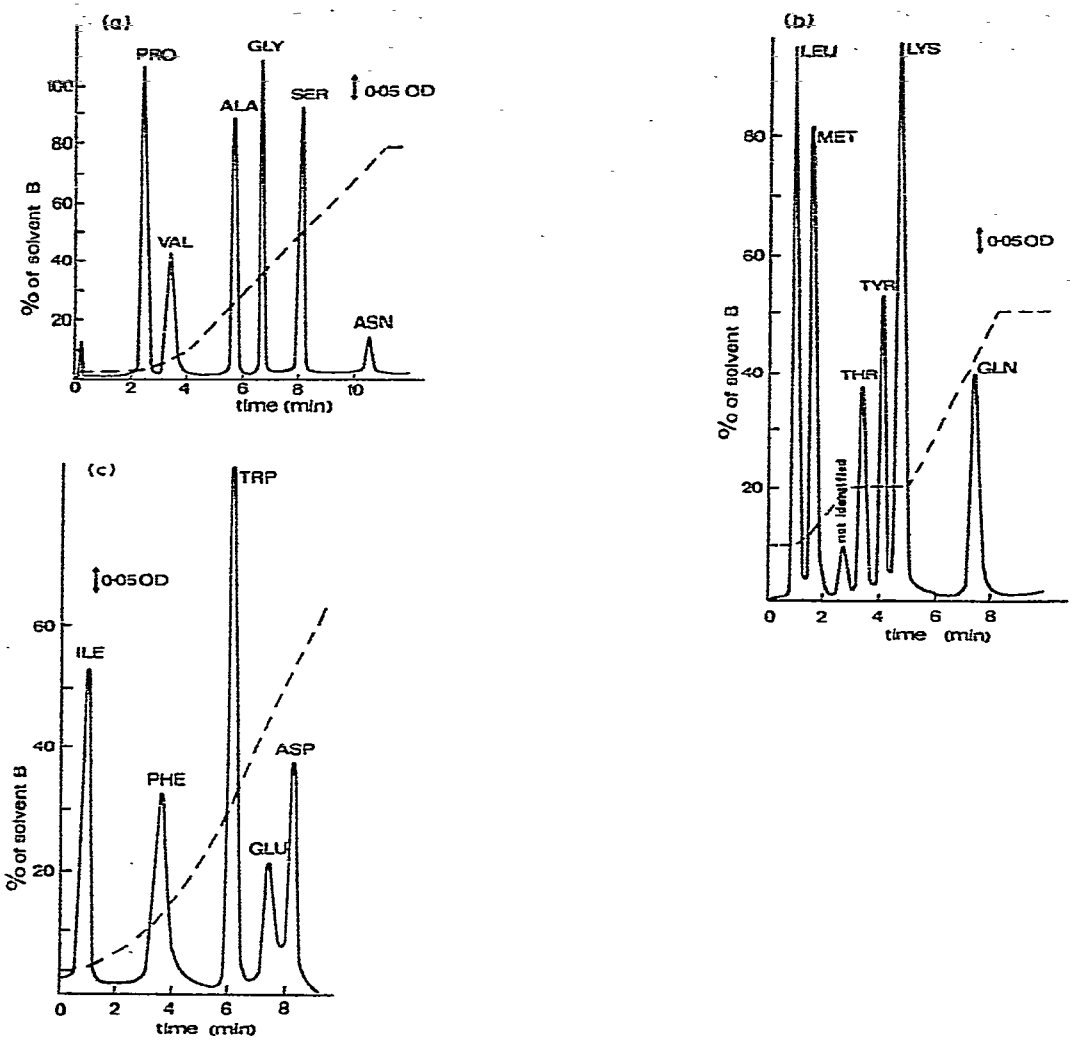


Fig. 16. (a) Separation of six PTH-amino acids (group I): mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (8:20); flow-rate, 100 ml/h; overpressure, 90 bar. (b) Separation of six PTH-amino acids (group II): mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (1:1); flow-rate, 100 ml/h; overpressure, 100 bar. (c) Separation of five PTH-amino acids (group III): mobile phase (A) *n*-hexane and (B) isopropanol; flow-rate, 100 ml/h; maximum overpressure, 300 bar.

gas chromatograph equipped with a dual flame ionization detector. The injection temperature was 270° and the detection temperature was 10° higher. A 1 m × 2 mm glass column containing 5% Dexsil 300 GC on Chromosorb W (acid washed and silanized) was used at 165° for the first 2 min of running time, programmed at 8°/min to 210° and at 10°/min thereafter up to a final temperature of 290° and maintained at this temperature for 4 min. Helium served as carrier gas at a flow-rate of 25 ml/min. Retention times under these conditions are summarized in Table 7.

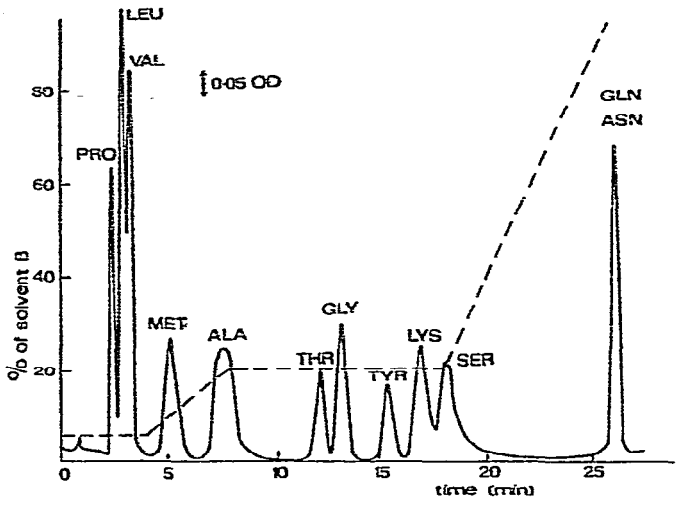


Fig. 17. Separation of PTH-amino acids of all three groups: mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (1:1); flow-rate, 50 ml/h; overpressure, 45–100 bar.

In addition, comparison was made with TLC, using the solvent system previously employed by Inglis and Nicholls[37] and silica gel layers.

Clearly, the above procedure is not yet suitable for routine work. The main problems that give rise to obscure results lie not in the separation technique but in the method of derivatization used. The absence of pyridine in the acetylation mixture, although omitted by previous workers, appears to be detrimental, as the acetyl derivative of valine is not formed. Many acetyl derivatives can be prepared by the simultaneous injection of acetic anhydride and the sample directly into the column. In fact, the acetyl derivatives of PTH-Arg and PTH-His can be prepared exclusively by this technique. Other PTHs, such as those of Thr, Ser and carboxymethylcysteine, gave rise to products that moved with retention times that were identical with that of the corresponding glycine derivative. Another series of problems arises from the fact that it is difficult to elute some of the acetylated PTH derivatives from stainless-steel columns. It has also to be stressed that some disagreement exists between different laboratories on this aspect.

Compared with the unacetylated derivatives, the acetyl derivatives exhibit shorter retention times (by about 6%) and about two- to three-fold better response of the flame ionization detector. It has to be pointed out that the acetylation method is obviously not suitable for the separation of acetyl-PTH-Asp, acetyl-PTH-Asn and acetyl-PTH-Gln, which, although acetylated smoothly as indicated by TLC, do not give any response with gas chromatography. Also acetyl-PTH-Leu and acetyl-PTH-Ile do not separate, though this problem had been overcome earlier by Pisano and Bronzert[53] (non-acetylated derivatives).

(D) Conversion of PTHs into parent amino acids

Considerable attention has been paid recently to the hydrolysis and liberation

TABLE 7

ACYLATION CONDITIONS AND CHROMATOGRAPHIC PROPERTIES FOR ACETYLATED PHENYLTHIOHYDANTOINS (INGLIS AND NICHOLLS[37])

Properties of unacetylated PTHs are given in parentheses.

Amino acid	*Reaction time at 20°*	*Mass-spectral analysis*	*GC retention time (min)*		*TLC R_F value*	
Ala	14 h	Monoacetylated	5.04	(5.28)	0.61	(0.40)
Ser	10–40 min	Mono- + diacetylated	5.60		0.51	(0.11)
					0.68*	
Gly	14 h	Monoacetylated	5.64	(5.96)	0.58	(0.35)
S-CM-Cys**	10 min–6 h	Mono- + diacetylated	5.80	Weak response	0.31	(0.16)
					0.50	
Val	14 h	Monoacetylated	6.08	(6.44)	0.65	(0.46)
Pro	—	Not determined	—	(7.20)		(0.55)
Ile	14 h	Monoacetylated	7.20	(7.52)	0.65	(0.50)
Leu	14 h	Monoacetylated	7.20	(7.52)	0.67	(0.52)
Thr	6 h	Diacetylated	7.00		0.49	(0.18)
			7.36***			
Hyp	14 h	Not determined	9.76		0.54***	
			10.28	(10.00)	0.48	(0.28)
Met	14 h	Monoacetylated	9.88	(10.24)	0.63	(0.44)
Glu	6 h	Monoacetylated	10.24		0.36	(0.20)
Phe	14 h	Monoacetylated	10.40	(11.36)	0.66	(0.45)
Tyr	14 h	Diacetylated	13.12	(13.96)	0.62	(0.25)
Lys	14 h	Diacetylated	13.16		0.63	(0.36)
Trp	6 h	Monoacetylated	14.88***			
			17.28	(17.68)	0.52	(0.41)
Asp	6 h	Not determined			0.32	(0.14)
Asn	14 h	Monoacetylated			0.28	(0.08)
Gln	14 h	Monoacetylated			0.27	(0.09)
His	3 h–6 days	Not determined	13.43***,§			
			13.94			(0)
Arg	3 h–6 days	Not determined	12.50§			(0)

* Spot with $R_F = 0.51$ gradually decreases as second spot increases.
** S-CM-Cys = S-carboxymethylcysteine.
*** Major of two peaks.
§ Must be injected in a fresh aliquot of acetic anhydride for "on-column" conversion.

of the free amino acid from the N-terminal PTH derivative. Mondino *et al.*[57] introduced an open flask system using either a nitrogen or argon atmosphere. In practice, 1 μl of the PTH-amino acid derivative solution containing 0.5 μmole of the derivative is placed in a three-necked flask (25 ml), two lateral necks are stoppered and the contents are evaporated to dryness at 40°. The residue is re-dissolved in 5 ml of 0.1 *N* sodium hydroxide (this solution had argon or nitrogen bubbled through it before use). The flask is then placed on a heating mantle and the reaction mixture refluxed while bubbling inert gas through it at a flow-rate of 0.5 ml/min (the bubbling is begun 1 h before applying heat, the mixture being then heated gently for 16 h). The alkaline solution is then neutralized with 2.5 ml of 0.2 *N* hydrochloric acid, taken to dryness at 40° and re-dissolved in 0.2 *N* hydrochloric acid. A 0.4-ml aliquot of this solution is loaded on to the top of the column of a conventional amino acid analyser.

7. METHYLTHIOHYDANTOINS

At present, all types of chromatographic techniques are available for the separation of methylthiohydantoins as their use for sequencing study has spread considerably during the last few years. Methylthiohydantoin (MTH) amino acids can be used in the manual version of the stepwise degradation in the automated procedure or in solid-phase degradation.

(A) Flat-bed separations

Stepanov and Lapuk[58] reported the possibility of separating MTH-amino acids by TLC. However, not all of the common amino acids could be resolved by their technique. Rabin and Darbre[48] applied polyamide-coated sheets and used toluene–*n*-heptane–acetic acid (60:30:20) and 35% acetic acid as mobile phases. R_F values obtained are presented in Table 8. If these solvents are used consecutively, in a two-dimensional arrangement, they provide a complete separation of the 19 common MTH-amino acids. The spots can be made visible in UV light at 254 nm, in which they exhibit a purple fluorescence.

TABLE 8

$R_F \times 100$ VALUES FOR 19 MTH-AMINO ACID DERIVATIVES IDENTIFIED BY TLC ON POLYAMIDE-COATED PLASTIC PLATES

Amino acid	$R_F \times 100$	
	Toluene–n-heptane–acetic acid (60:30:20)	*35% acetic acid*
Ala	67	70
Arg	2	92
Asn	26	82
Asp	19	70
Cys	45	45
Glu	33	70
Gly	54	74
His	8	93
Ile	89	43
Leu	86	43
Lys	22	60
Met	74	52
Phe	80	35
Pro	90	63
Ser	55	61
Thr	57	45
Trp	42	21
Tyr	18	4–
Val	82	57

Very extensive work on the flat-bed separation of MTHs has been carried out recently by Kulbe[59]. Two generally applicable solvent systems were evolved, which allow the separation of 23 MTH-amino acids. The sensitivity of the method lies within the range 0.05–0.2 nmole, provided that fluorescence detection and double-sided

polyamide sheets were used during the separation procedure. Separation was carried out on small-size polyamide sheets (5 × 5 cm) in order to obtain condensed spots (the spot applied should not exceed 1 mm in diameter). Another interesting feature of this method is that the solvents can be applied consecutively in the same direction of development; a conventional two-dimensional arrangement is also possible. Running time is about 30 min. A review of reported R_F data is summarized in Table 9. The most frequently used solvent systems are toluene–*n*-heptane–acetic acid (100:30:15) and 25% aqueous acetic acid. The latter solvent had previously been used for similar purposes (Kulbe[59] and Kulbe and Nogueira-Hattesohl[60,61]). For some pairs that are difficult to separate in the above two solvent systems, 40% aqueous pyridine–acetic acid (9:1) can be used, the separation being carried out on layers that do not contain the fluorescent indicator. For fluorescence detection the indicator is added instead to the mobile phase or, if two subsequent developments are applied, to the mobile phase that is used for the first run; the mobile phase then contains 250 mg of 2-(4-*tert.*-butylphenyl)-5-(4''-biphenylyl-1,3,4-oxadiazole) (butyl-PBD).

The practical procedure, as described by Kulbe[59], is as follows. The polyamide sheet is developed first in the system toluene–*n*-heptane–acetic acid (100:30:15), and allowed to run for 8 min. The sheet is then dried in a stream of cool nitrogen and run

TABLE 9

$R_F \times 100$ VALUES OF 23 MTH-AMINO ACIDS CHROMATOGRAPHED ON MICRO-POLYAMIDE LAYERS IN SOLVENTS I AND II (ONE DIMENSIONAL) AND ALSO BY DOUBLE DEVELOPMENT IN I FOLLOWED BY II IN IDENTICAL DIRECTION

Solvent I: toluene–*n*-heptane–acetic acid (100:30:15); solvent II: 25% aqueous acetic acid.

Amino acid	$R_F \times 100$		
	I	*II*	*II after I*
Ala	66	66	86
S-Aminoethylcysteine	12	32	38
Arg	4	93	93
Asn	23	80	86
Asp	15	65	69
S-CM-Cys*	18	44/53	62
Cysteic acid*	0	23	29/65
Glu	28	63	74
Gln	31	79	84
Gly	59	72	88
His	6	96	100
Ile	83	41	90
Leu	82	41	90
Lys	17	45	57
Met	73	51	85
Methionine sulphone	27	82	81
Phe	77	30	82
Pro	93	60	100
Ser	56	52	74
Thr*	68/49	44	80
Trp	44	15	51
Tyr	14	29	43
Val	78	51	88

* More than one spot observed; the first is the main spot.

in the second direction for 10 min. The spots are made visible by fluorescence quenching and identified according to the position of standards chromatographed on the reverse side of the sheet. For simultaneous identification of multiple samples, as required in automated sequence analysis, the same solvents can be applied consecutively in the same direction. Spot distribution on polyamide sheets resembles that of PTH-amino acids.

Some suggestions concerning the quantitation of MTH-amino acids after TLC separation were reported by Amirkhanyan and Stepanov[62], but the procedures involved are not yet ready for routine application. It is also worth mentioning that contrary to the findings of Rabin and Darbre[48], Kulbe was able to prepare the MTH derivative of glutamine, which was clearly separable from the corresponding derivative of glutamic acid.

(B) Liquid column chromatography and high-speed separations

A glass column, 72 cm × 0.9 cm I.D., packed with Dowex 50-X8 (600 mesh) was used for this type of separation (Stepanov *et al.*[63]). Prior to application to the column the resin was washed stepwise with 80% ethanol, water, 2 *N* NaOH, water and 2 *N* HCl. A final wash with water brought it to a neutral condition. After each run the column could be regenerated by washing it with 250 ml of distilled water and five or six analyses run without repacking the column; 0.02–2.5 μmole of each PTH derivative were applied in order to obtain optimum separation. Elution was carried out first with water (for 35 min), then with a linear water–ethanol gradient, which was prepared by using two 300-ml flasks containing the respective solvents. Separation was carried out at a flow-rate of 60 ml/h, the eluate being monitored spectrophotometrically at 235, 265 and 315 nm.

Analogous results can be obtained with Hitachi spherical resins (2612 and 3105), when pre-treated in a way similar to that described for Dowex 50-X8. Examples of these separations are presented in Fig. 18. As one would expect, the separation with spherical resins yield narrower peaks and offer a clearer separation of some MTH-amino acids, namely Gly, Ala and Gln. With these resins it is also possible to separate *allo* and *threo* forms of Thr and the particular derivative of Ile. Hitachi spherical resins, however, require a lower flow-rate (30 ml/h) and a higher overpres-

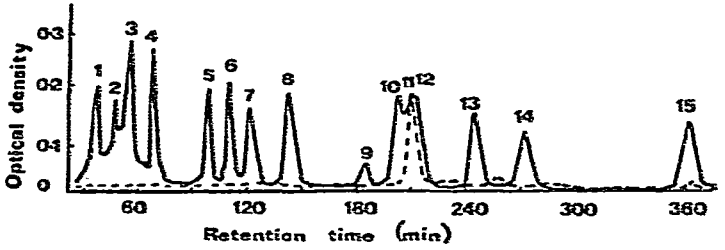


Fig. 18. Separation of MTH-amino acids on spherical analyzing resin. Conditions: resin, Hitachi 2612; elution rate, 60 ml/h; column temperature, 20 ± 0.5°; sample size, 0.2 μmole of each MTH; eluent, water for 0–35 min, then linear gradient water–ethanol (300:300) for 35–340 min. Absorption at 265 nm (———), and at 315 nm (— — —). Peaks: 1 = Asp, S-CM-Cys; 2 = *threo*-Thr; 3 = Glu, Ser, *allo*-Thr; 4 = Asn; 5 = Gly; 6 = Ala; 7 = Gln; 8 = Val; 9 = *allo*-Ile; 10 = Leu, Met, Ile; 11 = Thr (H_2O); 12 = Tyr; 13 = Pro; 14 = Phe; 15 = Trp.

sure on the column; even so, at the end of the analysis the back-pressure did not exceed 12 atp. It should be mentioned that in these separations the order of the eluted amino acid derivatives did not differ from the sequence of parent amino acids chromatographed under identical conditions.

In contrast to flat-bed techniques, this liquid-column chromatographic separation offers an opportunity to quantitate the results provided that the micromolar coefficients are known. The data for the most common amino acid derivatives are summarized in Table 10.

TABLE 10

MICROMOLAR COEFFICIENTS OF MTH-AMINO ACIDS

Amino acid	n^*	C^{**}	$\pm m^{***}$
Asp	5	14.55	0.29
Ser	7	13.27	0.22
allo-Thr	6	15.28	0.35
Asn	9	16.54	0.10
Glu	7	15.56	0.22
Gly	7	13.22	0.18
Ala	9	14.19	0.20
Gln	6	14.70	0.23
Val	7	15.26	0.34
Met	8	10.98	0.39
Leu	6	14.35	0.22
Tyr	6	14.85	0.33
Thr (dehydrated form)	6	17.44	0.35
Pro	5	12.33	0.20
Phe	9	16.03	0.42
Trp	9	16.68	0.30

* Number of parallel experiments.
** Micromolar coefficients.
*** Mean square deviation.

(C) Gas chromatography

While paper and thin-layer chromatography have been used as almost standard techniques for the separation of MTHs, gas chromatography only recently started to invade this area of separation. Several methods are, however, now available for the separation of this category of amino acid derivatives.

Since the work of Waterfield and Haber[64], further improvements in the separation procedure for MTH-amino acids have been reported by Vance and Feingold[65], Pisano *et al.*[66], Eyem and Sjøquist[67] and Lamkin *et al.*[68]. In the procedure described by Vance and Feingold[65] MTH-amino acids are separated in the form of trimethylsilyl derivatives, a 6 ft. × 1/8 in. I.D. glass column packed with 1% OV-17 and another of the same size packed with 1.5% OV-1 being used for the separations. With the OV-17 column the separation is carried out under the following operating conditions: oven temperature was initially set to 160° and, 3 min after the solvent peak appeared, the temperature was programmed to 260° at a 5°/min gradient and maintained at 260° for an additional 12 min. When the corresponding derivative of tryptophan has emerged from the column the analysis is finished. A typical example of a separation

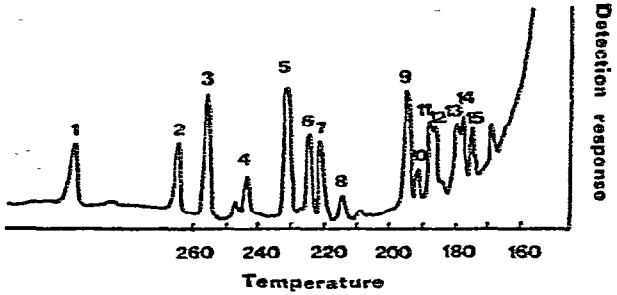


Fig. 19. Gas chromatogram of 18 TMS-MTH-amino acids on an OV-17 column. Oven temperature initially 160°; 3 min after solvent peak appeared, the oven temperature was programmed to 260° for 12 min until TMS-MTH-tryptophan eluted from the column; 2 nmole of each derivative injected, except for the glutamine and lysine derivatives, when 6 nmole was injected. Range setting 10, attenuation 16, resulting in a full-scale deflection of 3×10^{-10} A with a 5-mV recorder. Peaks: 1 = Trp; 2 = Lys; 3 = Tyr; 4 = Gln; 5 = Asn, Phe; 6 = Glu; 7 = Met; 8 = Asp; 9 = Thr, Pro; 10 = Ser; 11 = Leu; 12 = Ile; 13 = S-CM-Cys; 14 = Gly, Val; 15 = Ala.

is presented in Fig. 19. Derivatives of glycine and valine are not resolved under the above conditions and therefore a second run on the OV-1 column has to be carried out. For this purpose, the initial temperature of the oven is 130° and 3 min after the solvent peak has appeared a gradient of 5°/min is set so as to enable the temperature to reach 260° in the final stage of the separation. Amounts ranging between 2 and 6 μmole appear to be the optimum for the analysis. The results of the separation on the OV-1 column is presented in Fig. 20.

The trimethylsilyl derivatives of MTH-amino acids can be prepared by adding N,O-bis(trimethylsilyl)acetamide–acetonitrile mixture (1:3) to the dried MTH derivatives in a small screw-capped vial fitted with a PTFE liner, which is then kept at room temperature for 5 min. The practical procedure used for cleaving the particular N-terminal amino acid from a protein or peptide sample is described by Vance and Feingold[65], and a further description is given by Waterfield and Haber[64].

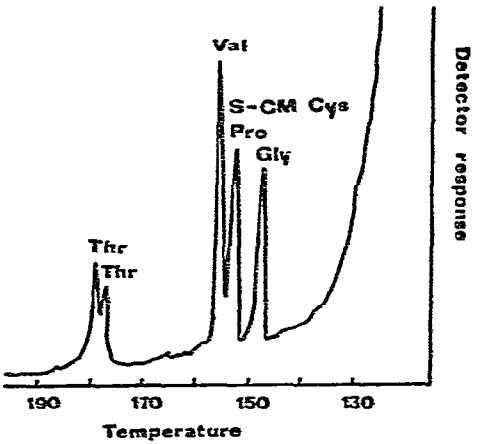


Fig. 20. Gas chromatogram of five TMS-MTH-amino acids on an OV-1 column. Oven temperature initially 130°; 3 min after the solvent peak appeared, the oven temperature was programmed to 260° at 5°/min; 4 nmole of each derivative injected. Range setting 10, attenuation 16.

The application of capillary columns for the separation of silylated MTH-amino acids has been described by Eyem and Sjøquist[67]. In this instance use has been made of a glass column, 4.5 m × 0.13 mm packed with a mixture of OV-101 and OV-225 (95:5). Two different temperature programmes have been developed: in the first, the oven temperature is maintained at 120° for the first 8 min, followed by a temperature gradient of 2.5°/min up to 250°. In the second programme, the initial temperature is 180° for the first 4 min, followed by a 4°/min increase up to the final temperature of 260°. Relative retentions of MTH-amino acids (silylated) are summarized in Table 11. The second programme, outlined above, was designed for the separation of the more labile MTH derivatives, namely the derivatives of Asn, Gln, ornithine (Orn), His and Lys, and for the separation of TMS derivatives of MTH-Glu and Trp.

TABLE 11

RELATIVE RETENTIONS, γ_{ts}, OF TMS-MTH-AMINO ACIDS

Amino acid	*1st Programme*		*2nd Programme*	
	γ_{ts}*	δ	γ_{ts}**	δ
Ala	0.50	0.007		
Gly 1	0.53	0.005		
Val	0.65	0.008		
Pro	0.67	0.005		
Ile 1	0.83	0.007		
Ile 2	0.86	0.001		
Leu	0.88	0.004		
Ser	1.00			
Gly 2	1.11	0.003		
Thr	1.12	0.005		
S-Methylcysteine	1.18	0.007		
Asp	1.39	0.010		
Met	1.43	0.008		
Phe	1.61	0.011		
Glu	1.64	0.011		
Asn	1.71	0.025	0.33	0.005
Gln	1.98	0.020	0.43	0.008
Orn	2.03	0.023	0.46	0.005
His			0.53	0.007
Tyr	2.21	0.023		
Lys	2.27	0.029	0.57	0.003
Trp	2.80	0.031	0.85	0.003

* Relative to phenanthrene.
** Relative to α-cholestane.

The advantage of the procedure described by Eyem and Sjøquist[67] over those reported previously is that by this method it is possible to separate 20 amino acid derivatives in a single run, provided that cysteinyl and arginyl residues have been converted into S-methylcysteinyl and ornithyl residues, respectively. The relative retention in these separations is extremely reliable for identification purposes. Some TMS-MTH derivatives showed a typical pattern (double peaks), which is also of high diagnostic value. Thus isoleucine appears as a double peak, which is ascribed to

the formation of MTH-*allo*-Ile during the silylation procedure. As the mass spectra of the corresponding standard and the newly formed peak were identical, this explanation seems very probable; on the other hand, similar racemization of MTH-Thr was not observed. With the separation of TMS-MTH-Gly, a double peak was also frequently observed, which is reported to be the result of the formation of th bis-TMS derivative.

Precautions have to be taken with the first programme as otherwise the partial decomposition of MTH-Asn, Gln, Orn and Lys and the complete decomposition of His may occur. No problems are reported to arise with the identification of any of the amino acid derivatives except His, because the degree of decomposition is well reproducible. With the second programme higher responses are obtained and also histidine can be identified. Phenanthrene and α-cholestane served as internal standards in the individual programmes devised.

Further investigation of the gas chromatography of MTH-amino acids was devoted to the search for a method that would not require an additional run for the separation of some of the derivatives of commonly occuring amino acids. Although not completely successful, the procedure described by Lamkin and co-workers[68,69] represents at least a further simplification. For this kind of separation two types of column were used: firstly, a column made of borosilicate glass, 165 or 179 cm × 4 mm I.D. in size, packed with 2% OV-17 on 80–100-mesh Gas-Chrom Q was used. For an additional check, a column packed with OV-25 on 80–100-mesh Supelcoport, which served to confirm the presence of asparagine in the mixture, was also used. In the actual separation procedure, the first column was temperature programmed, starting with 145° for the first 15 min, followed by a temperature gradient of 4°/min. Up to 230° and maintained at this temperature for an additional 12 min. For the identification of arginine the temperature was kept constant at 203° (second column). A typical separation is presented in Fig. 21. Retention indices on SE-30 and OV-17, as reported

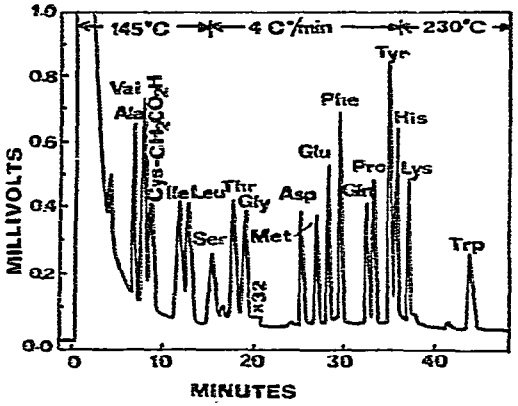


Fig. 21. Gas chromatographic separation of trimethylsilylated methylthiohydantoins (Tracor MT 220). Each peak represents 2.5 nmole of trimethylsilylated methylthiohydantoins, except those for MTH-His and MTH-ε-MTC-Lys, which represent 5 nmole each. Silylation was at 100° for 10.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3). Column: borosilicate glass, 165 cm × 4 mm I.D., containing 2.00% (w/w) OV-17 on 80–100-mesh Gas-Chrom Q. Flow-rate: N_2 at 50 ml/min; H_2 at 50 ml/min; and air at 375 ml/min. Temperature: column programmed as indicated; flash heater, 240°; flame ionization detector, 270°. Attenuation as shown; sensitivity, (attenuation) × 10^{-11} A/mV.

TABLE 12

RETENTION INDICES OF TRIMETHYLSILYLATED METHYLTHIOHYDANTOINS* OF AMINO ACIDS

Amino acid	*Temperature (°C)***	*Retention index*	
		SE-30	*OV-17*
Ala	140	1491	1724
Val	140	1561	1757
S-CM-Cys	140	1546	1773
Ile	140	1649	1837
Leu	140	1658	1857
Δ-Thr	140	1582	1905
Pro***	140	1543	1905
Gly[c]	140	1611, 1743	1764, 1956
Asp	180	1896	2140
Met	180	1912	2203
Glu	180	2016	2246
Phe	180	2005	2307
Asn	180	2016	2307
Gln	180	2154	2447
Tyr	220	2343	2616
His	220	2298	2645
ε-MTC-lys	220	2326	2721
Trp	220	2631	2926

* Silylated at 60° for 5.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3).
** Column temperature at which indices were determined.
*** Not silylated under conditions employed.
[c] Gave two peaks under the silylation conditions employed.

by Lamkin *et al.*[68], are summarized in Table 12. It is interesting to note the dependence of these indices upon the content of the methylphenylpolysiloxane liquid phase employed for the chromatographic separation. This dependence varies for individual amino acid derivatives (as can be seen in Fig. 22), which variation is the cause of changes in the order of elution of individual peaks of amino acids when using different stationary phases.

8. HYDANTOINS DERIVED FROM 2-*p*-ISOTHIOCYANOPHENYLINDONE

These derivatives, the reagent for the preparation of which was synthesized by Ivanov and Mancheva[70], fall into the category of coloured thiohydantoins. For derivatization, the sample (about 1 mg) was dissolved in 0.4 *M* dimethylallylamine buffer (15 ml of pyridine, 10 ml of water and 1.18 ml of dimethylallylamine) and the solution adjusted to pH 9.6 with trifluoroacetic acid. An excess of the reagent (about 4 mg), dissolved in a minimum volume of pyridine, was added and the pH value re-adjusted to 9.6 with trifluoroacetic acid. The coupling reaction was carried out at 40° in an atmosphere of nitrogen, and is usually terminated after 1 h. After completing the reaction, the reaction mixture was extracted four or five times with twice its volume of benzene. Traces of benzene were removed from the mixture with a stream of nitrogen, 0.5 ml of water were added and the aqueous phase taken to dryness by lyophilization. The dry peptide or protein derivative was washed twice with 0.5 ml of

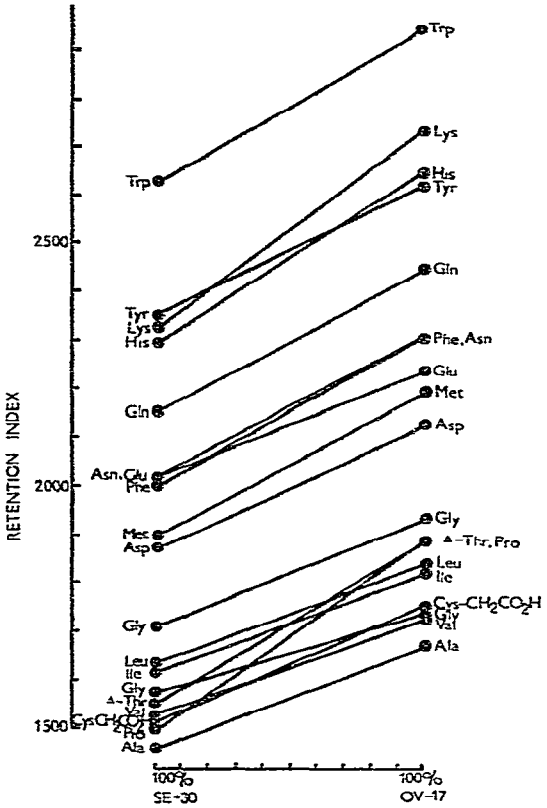


Fig. 22. Retention indices of TMS-MTHs as a function of the phenyl content of the methylphenylpolysiloxane liquid phase employed for chromatographic separation. Silylation was at 60° for 5.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3). SE-30 is 100% methyl substituted and thus has a phenyl content of 0%; OV-17 is 50% methyl and 50% phenyl substituted and corresponds to a phenyl content of 50%.

ethyl acetate, traces of the extractant were removed in a desiccator and 50 μl of trifluoroacetic acid were added. The cleavage occured within 15 min at 40° in a nitrogen atmosphere. The resulting thiazolinone was extracted twice, first with 1 ml and then with 1.5 ml of dichloroethane. The combined extracts were taken to dryness in a stream of nitrogen and 100 μl of a mixture of acetic acid–6 *N* HCl (5:1) were added, the conversion being carried out at 80° for 10 min in a nitrogen atmosphere. The reaction mixture was then diluted with water to bring the volume to 0.5 ml and the resulting amino acid derivatives were extracted three times with 1-ml portions of ethyl acetate. The extract was taken to dryness in an atmosphere of nitrogen and traces of solvent were removed *in vacuo* over potassium hydroxide. The sample was then dissolved in 1,2-dichloroethane, acetone or methanol and spotted on the starting line of a thin-layer plate. The conditions for the reaction have been studied extensively by Ivanov and Mancheva, who, for the separation applied thin-layer chromatography on silica gel G layers[70].

The following mobile phases proved suitable for the separation of these amino

acid derivatives[71]: chloroform–methanol (98:2); chloroform–methanol (90:10); *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20); and chloroform–methanol–acetic acid (80:20:2). R_F values obtained are summarized in Fig. 23. As expected,

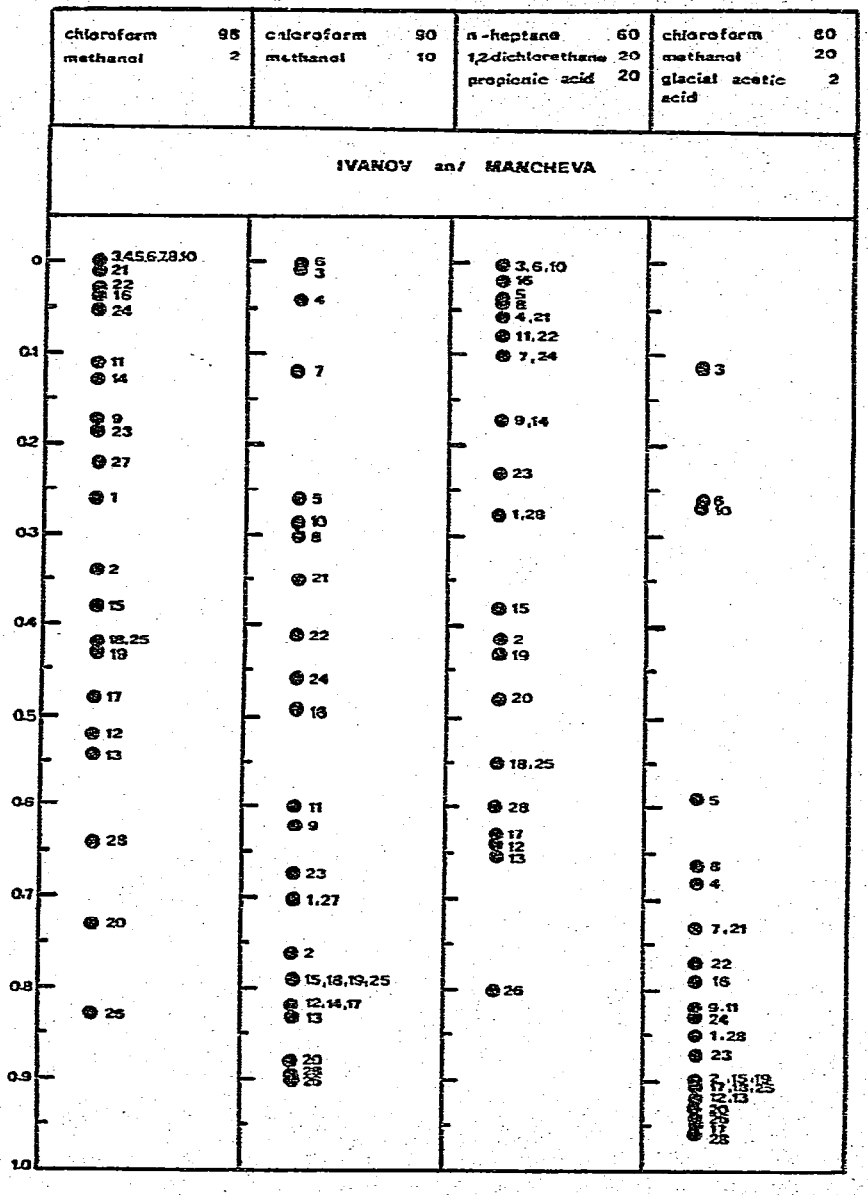


Fig. 23. $R_F \times 100$ values of ITH-amino acids, 2-*p*-isothiocyanophenyl-3-phenylindone and mono- and bisdiphenylindonylthioureas. Peaks: 1 = Ala; 2 = DC-Aminobutyric acid; 3 = Arg; 4 = Asp; 5 = Asn; 6 = $CysO_3H$; 7 = Glu; 8 = Gln; 9 = Gly; 10 = His; 11 = Hyp; 12 = Ile; 13 = Leu; 14 = Lys; 15 = Met; 16 = $MetSO_2$; 17 = NLeu; 18 = NVal; 19 = Phe; 20 = Pro; 21 = Ser; 22 = Thr; 23 = Trp; 24 = Tyr; 25 = Val; 26 = Diphenylindonyl isothiocyanate; 27 = Monodiphenylindonylthiourea; 28 = Bisdiphenylindonylthiourea.

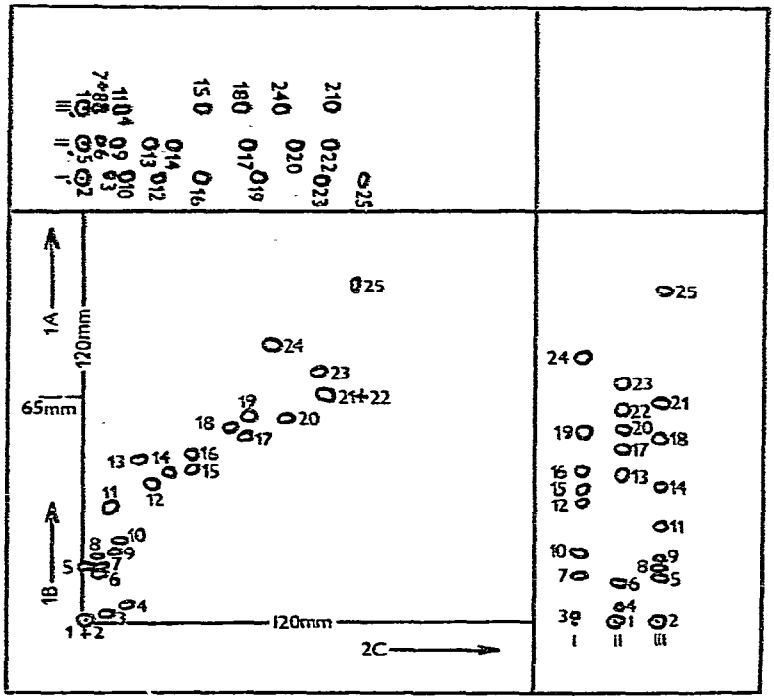


Fig. 24. Two-dimensional separation of ITH-amino acids, diphenylindonyl isothiocyanate and mono- and bisdiphenylindonylthiourea. Sorbent: Kieselgel G; layer thickness, 0.5 mm. First run: solvent system B, chloroform–methanol (90:10), up to 65 mm from the starting line, and in solvent system A, chloroform–methanol (98:2); second run: solvent system C, *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20). Spots: 1 = ITH-cysteic acid; 2 = ITH-Arg; 3 = ITH-Asp; 4 = ITH-Glu; 5 = ITH-His; 6 = ITH-Asn; 7 = ITH-Gln; 8 = ITH-Ser; 9 = ITH-Thr; 10 = ITH-Tyr; 11 = ITH-Hyp; 12 = ITH-Gly; 13 = ITH-Lys; 14 = ITH-Trp; 15 = monodiphenylindonylthiourea; 16 = ITH-Ala; 17 = ITH-α-aminobutyric acid; 18 = ITH-Met; 19 = ITH-Phe; 20 = ITH-Val; 21 = ITH-Leu; 22 = ITH-Ile; 23 = bisdiphenylindonylthiourea; 24 = ITH-Pro; 25 = diphenylindonyl isothiocyanate.

none of the mobile phases specified above is capable of completely separating all of the common amino acid derivatives. Therefore, a special type of two-dimensional chromatography has been applied: the plate is developed in chloroform–methanol (90:10) up to 65 mm from the starting line, and the mobile phase is then changed abruptly to chloroform–methanol (98:2). The development is carried out for an additional 120 mm and the plate is then dried, rotated through 90° and developed in the second dimension with *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20). The result of such a separation is presented in Fig. 24. No detection is needed in this instance because 10^{-9} mole of amino acid derivatives is already visible as a yellow-orange spot.

9. 4-N,N-DIMETHYLAMINOAZOBENZENE-4'-THIOHYDANTOINS (ISOTHIOCYANATE DERIVATIVE OF THE DABSYL REAGENT)

4-N,N-Dimethylaminoazobenzene-4'-isothiocyanate has been synthetised by

TABLE 13

BASIC DATA RELATED TO THE DIFFERENT IDENTIFICATION PROCEDURES IN PROTEIN SEQUENCING

GC = Gas chromatography; LC = liquid chromatography; TLC = thin-layer chromatography.

Type of derivative	*Separation technique used*	*Sensitivity range (method used for quantitation)*	*Unseparated double peaks or amino acid derivatives causing problems in identification*	*Notes*
Dns	TLC (polyamide)	5–20 pmole (photocopying densitometry)	Complete separation with three runs except Dns-Cys	
^{14}C-Dns	TLC (polyamide)	100–500 pmole (radioactivity counting)	Complete separation with three runs except Dns-Cys	
Dns	LC, high speed (silica)	Nanogram (UV recording)	Two runs with differently polar mobile phases necessary	
Dns	LC, classical (polyamide)	Nanogram (luminescence measurement)		
Bansyl	TLC (polyamide)	0.1 nmole (luminescence measurement)	Some double peaks may cause problems (not yet clear)	
Dabsyl	TLC (polyamide)	10–20 pmole (coloured and fluorescent spots)	Complete separations require more than one two-dimensional separation	Sensitivity can be increased by exposing the plate to HCl vapour
Pivalyl derivatives (as methyl esters)	GC, 5 % XE and 0.5 % FFAP, temperature programming	≈5 nmole	Some double peaks may cause problems (not yet clear)	
Benzoyl derivatives (as methyl esters)	GC, 1 % FFAP, temperature programming	~5 nmole		
PTH	TLC (polyamide)	0.05–0.2 nmole (5 × 5-cm sheets) 0.8 nmole (20 × 20 cm sheets) (fluorescence quenching)		
PTH	TLC (polyamide)	≈10 nmole (ninhydrin–collidine)		Different colours of different amino acids

PTH	LC, high speed (Zorbax SIL, Merckosorb SI®, Micropak CN)	2–5 μmole	Depends on the type of column packing and mobile phase used. Hydrophilic derivatives frequently cause problems in separation	
Acetyl-PTH	GC, 5% Dexsil on 300 GC Chromosorb W	Data not available	S-CM-Cys, Ser, Thr and Gly, and Leu and Ile, give a single peak	
PTH, after conversion into parent amino acids	Conventional amino acid analysis	>0.5 μmole		
MTH	TLC (polyamide)	0.05–0.2 nmole (5 × 5-cm sheets, fluorescence quenching)	Leu and Ile are difficult to separate. The derivative of glutamine is not easy to prepare	Spot applied should not exceed 1 mm in diameter
MTH	LC, classical (Dowex 50-X8) LC, high resolution (Hitachi spherical resin)	0.02–2.5 μmole (spectrophotometric detection at 235, 265 and 315 nm)		Order of elution of MTH derivatives is the same as that of the free amino acids
MTH (trimethylsilylated)	GC, 1–2% OV-17, then 1.5% OV-1; alternatively, OV-101, OV-225 or OV-25 on Supelcoport. Temperature programming	Data not available		Some trimethylsilylated derivatives of MTH-amino acids give double peaks; 2% OV-17 on Gas-Chrom Q allows the complete separation in a single run
Hydantoins derived from 2-*p*-isothiocyanophenyl-indone	TLC (silica gel G)	10^{-9} mole (orange-yellow spots)		
Isothiocyanate derivative of the dabsyl reagent	TLC (polyamide)	Picomole level		Leu and Ile do not react with the reagent. The rate of the colour change from purple to blue is of diagnostic value

Creaser[33] in order to improve the sensitivity and ease of determination of amino acids liberated during sequence analysis. Derivatives of all amino acids are readily prepared with this reagent (except Leu and Ile, which do not react) and are susceptible to complete two-dimensional separation on thin-layer polyamide sheets. The sensitive azo group permits the detection of the dabsyl isothiocyanate derivatives down to the picomole level as red spots directly on the sheet. Two to three subsequent amino acids at the N-end can be identified by this reagent. The colour change of the spots from purple to blue and red after being exposed to HCl vapour, which corresponds to the conversion of isothiocyanates into the corresponding thiocarbamyl-amino acids, is an additional feature of these derivatives that is of high diagnostic value.

10. CONCLUSIONS

In our opinion the separation techniques presented above characterise the new trends that have been applied for the separation and identification of N-terminal amino acids during sequence analysis. So far, no basically new concepts have appeared in the available literature, the procedures presented performing, in principle, only more precise variations of techniques that have been known for at least 5 years (for a summary, see Table 13). However, interest is such that publication of new procedures for sequence analysis of proteins and peptides surely cannot long be delayed. During a recent discussion at the Meeting on Protein Structure and Evolution, S. W. Fox suggested the possibility of making use of the different rates of splitting of peptide bonds in which different amino acids participate, and subjecting these data to computer analysis, which would result in information being obtained on the most probable sequence of the protein studied. Currently, the techniques are limited to rather short sequences. Another idea concerning sequence analysis relates to mass spectrometry. If the first of these trends materializes, the chromatographic techniques will shift towards more precise quantitation of complex peptide mixtures, with high preference for automated systems. In the second instance, the basic problems will require the use of gas chromatography for their solution. It may be of interest to review such techniques sometime in the future, but at present they lie outside the scope of the this review.

11. NOTE

During the preparation of this manuscript we became aware of the fact that a new technique had been developed for the flat-bed separation of mansyl (N-methyl-2-aniline-6-naphthalenesulphonyl) derivatives of amino acids[72].

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13. SUMMARY

New trends in the chromatographic analysis of N-terminal amino acids have been reviewed. It appears that while attempts have been made to achieve more precise separations of PTH and Dns derivatives, separations that could be subjected to quantitative evaluation are preferred. Other trends favour the application of different fluorescent derivatives, such as the dabsyl or bansyl derivatives, which could be used for sequencing of very small amounts of proteins. Miniaturization of scale can be discerned as the second major trend in this area of chromatographic techniques.

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