Journal of Chromatography
Chromatographic Reviews
Elsevier Publishing Company, Amsterdam
Printed in The Netherlands

CHROMATOGRAPHIC METHODS IN THE ANALYSIS OF PROTEIN STRUCTURE

THE METHODS FOR IDENTIFICATION OF N-TERMINAL AMINO ACIDS IN PEPTIDES AND PROTEINS. PART B*

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(Received March 15th, 1972)

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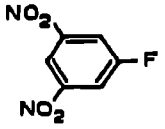
* For Part A, see J. ROSMUS AND Z. DEYL, *Chromatogr. Rev.*, 13 (1971) 163.

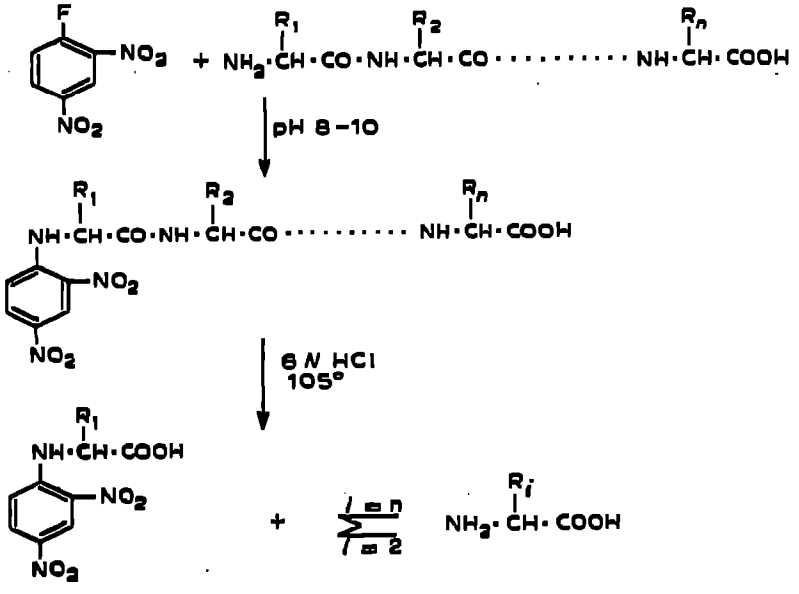
A. REACTION WITH 2,4-DINITROFLUOROBENZENE (Chapter 3.1.3 of the whole review)

Reagent:



2,4-Dinitrofluorobenzene

Reaction scheme:



1. *Introduction*

The merits of dinitrophenylation as the first useful method for the determination of N-terminal amino acids has been mentioned in the INTRODUCTION to Part A of this review. This method was developed by SANGER[108,170] in 1945; the dinitrophenylation reaction was, however, known earlier and was first used for amino acids and peptides by ABDERHALDEN AND BLUMBERG[1] in 1910. For SANGER's success, two factors were decisive: firstly, he used the more reactive 2,4-dinitrofluorobenzene instead of 2,4-dinitrochlorobenzene, which was used by ABDERHALDEN AND BLUMBERG[1], which enabled him to use mild conditions suitable for peptides and even for native proteins. Secondly, the principles of adsorption and partition chromatography had already been developed at the time of SANGER's work and it was therefore possible to use chromatography for the identification of dinitrophenylamino acids instead of the tedious and ineffective separation methods used in classical organic chemistry that were available at the time of ABERHALDEN AND BLUMBERG's work.

Since SANGER's original work, more than 1000 papers have been published on the dinitrophenylation of amino acids, peptides and proteins, on the separation of DNP-amino acids and on various aspects of their identification and characterization. This chapter therefore gives by no means an exhaustive treatment of the dinitrophenylation reaction, as we attempted to do for some of the reactions discussed in Part A. In discussing older work, we relied to a great extent on previous reviews (BISERTE *et al.*[10], BAILEY[8], BRENNER *et al.*[29], FRAENKEL-CONRAT *et al.*[65], JUTISZ[96], MELOUN[122], HOLEYŠOVSKÝ[80], HOLEYŠOVSKÝ AND HOLEYŠOVSKÁ[81], PATAKI[141] and others).

There is another point that must be made: for the separation of DNP-amino acids, almost any method deserving the name chromatography has been applied. It would be possible to write a textbook on chromatographic methods in which the separation of DNP-amino acids could be used as the sole example for all the diverse types of chromatographic techniques. We are not attempting to write such a textbook, and this is a further reason why the topics covered in this Part differ from those covered in Part A of this review.

2. *Dinitrophenylation of amino acids, peptides and proteins*

In spite of the extensive application of dinitrophenylation in biochemical research, the mechanism and kinetics of reactions of amino acids and peptides with 2,4-dinitrofluorobenzene have not received much attention. The rates of reactions of a few amino acids and peptides with 2,4-dinitrofluorobenzene in aqueous solution were determined by BURCHFIELD AND STORRS[35].

The same reaction was studied under heterogenous conditions (2,4-dinitrofluorobenzene was present as a separate liquid phase) by BROUWER *et al.*[30]. In both studies, some attention was paid to variations in pH (covering a range of one pH unit), but the influence of pH has otherwise been little investigated.

In view of the fact that reactions of 2,4-dinitrofluorobenzene with some amines in protic media are catalysed by bases[9,34], while reactions with other amines are not base-catalysed[31,32], BUNNETT AND HERMANN[33] studied the response of the reaction of 2,4-dinitrofluorobenzene with amino acids to catalysis by bases.

The observations of these authors showed that the reactions of 2,4-dinitrofluorobenzene with anions derived from amino acids are not detectably catalysed by sodium hydroxide at concentrations as high as 0.1 *N*. It appears that the only effect of changing the base concentration on the rate of reaction with 2,4-dinitrofluorobenzene is related to equilibrium of the amino acid between its neutral (mainly Zwitterionic) and anionic forms.

For biochemists, the most useful observation of BUNNETT AND HERMANN[83] is the great acceleration of the reaction of 2,4-dinitrofluorobenzene with amino acids which occurs when the solvent is changed from water or aqueous ethanol to dimethyl sulphoxide–water. The reaction rate is more than 100 times greater in 80% dimethyl sulphoxide than in water, aqueous ethanol or acetonitrile. However, no practical use has been made of this important finding up to now.

(a) Preparation of DNP-amino acids

The preparation of the DNP-amino acids has been described in the papers of SANGER[168], PORTER AND SANGER[152], LEVY AND CHUNG[110], FRAENKEL-CONRAT *et al.*[65], RAO AND SOBER[156] and BISERTE *et al.*[10].

Some useful methods of synthesis are presented in detail, covering both general and special methods of preparation.

(b) General methods for the preparation of DNP-amino acids

(i) Method of LEVY AND CHUNG[110]. The reaction is carried out in an aqueous medium at 40°. The respective amino acid (10 mmoles) and Na_2CO_3 (2 g) are dissolved in 40 ml of water at 40°. 2,4-Dinitrofluorobenzene (10 mmoles) is added and the mixture agitated vigorously, the temperature being maintained at 40°. The disappearance of the small drops of 2,4-dinitrofluorobenzene after about half an hour indicates the end of the reaction. The DNP-amino acids are precipitated by adding 3 ml of concentrated HCl. Sometimes it is necessary to use scratching to induce the crystallization of DNP-amino acids. For the crystallization of individual DNP-amino acids, special solvents are used; a survey of useful solvents for this purpose is presented in Table 1.

The bis-dinitrophenyl derivatives of Cys, Tyr, Lys and His require 2 moles of 2,4-dinitrofluorobenzene for each mole of the amino acid.

It is necessary to increase the amount of Na_2CO_3 used to 4 g when cystine and lysine are used and to 3 g when tyrosine, histidine, aspartic acid and glutamic acid are used.

(ii) Method of RAO AND SOBER[156]. The reaction is carried out by shaking the amino acid with 2,4-dinitrofluorobenzene in the presence of a slight excess of $NaHCO_3$ for 2–5 h in 50% aqueous ethanol at room temperature. Ethanol is evaporated off at room temperature and the excess of the reagent is removed by shaking the solution three times with diethyl ether. The aqueous solution is acidified with 6 *N* HCl and the precipitate or oil that separates is washed several times with small volumes of ice-water. For those DNP-amino acids that precipitate as oils, the following method can be recommended.

The substance is dissolved in a large volume of acetone and the solution is dried over anhydrous sodium sulphate and, after filtration, the solution is concentrated to a small volume. An equal volume of benzene is added to the acetone solution and the DNP-amino acid is precipitated with an excess of light petroleum (boiling range 30–

TABLE I

MELTING POINTS AND SOLVENTS FOR CRYSTALLIZATION OF DNP-AMINO ACIDS

DNP-derivative	*Melting point (°C)*	*Solvent*
α-N-DNP-L-arginine	252 (d)	water
α-N-DNP-L-arginine hydrochloride	168	conc. hydrochloric acid
DNP-DL-alanine	172–173	acetone–ligroin
DNP-L-alanine	178	aqueous ethanol
DNP-DL-aspartic acid	196	water
Di-DNP-L-cystine	118–121 (d) 161	aqueous acetic acid
Di-DNP-L-cysteine	155–169 (d)	aqueous acetic acid
DNP-DL-glutamic acid	155–162	(crude product)
DNP-L-glutamic acid	134–136	ethyl acetate–chloroform, acetone–ligroin
DNP-glycine	192–193 (d)	acetone–ligroin, water
Di-DNP-histidine (colourless)		acetic acid
DNP-DL-isoleucine	168–172	acetone–ligroin
DNP-DL-leucine	131.5–132.5	aceton–ligroin
DNP-L-leucine	101	aqueous acetic acid, aqueous methanol
Di-DNP-L-lysine	173–174.5	formic acid
ε-DNP-L-lysine hydrochloride (H_2O)	186	dilute hydrochloric acid
δ-DNP-L-lysine	260 (d)	water
DNP-DL-methionine	107–112, 117	ether–ligroin, ether
DNP-DL-methionine sulphoxide	184.5 (d)	ethanol
DNP-DL-methionine sulphone	184.5 (d)	ethanol
α-DNP-L-ornithine hydrochloride (H_2O)	223 (d)	N-hydrochloric acid
α-DNP-DL-ornithine	227 (d)	water
DNP-DL-phenylalanine	204–206 (d)	acetone–ligroin
DNP-L-phenylalanine	186	aqueous methanol
DNP-L-proline	137–138.5	ether–ligroin
DNP-DL-serine	186–188 (d) 199	acetone–ligroin, methanol
DNP-DL-threonine	177–178	acetone–ligroin, ether–ligroin
DNP-L-tryptophan	196–198 (d)	(crude product)
Di-DNP-L-tyrosine	92–98 (d)	(crude product)
O-DNP-L-tyrosine (H_2O) colourless	202 (d)	water
DNP-DL-valine	182.5–183.5	acetone–ligroin
DNP-L-valine (red)	132	ether–ligroin
DNP-L-valine (yellow tautomer)	131	ether–ligroin

75°). The precipitate is dried in a current of air, dissolved in diethyl ether and again precipitated with light petroleum. The diethyl ether–light petroleum precipitation is repeated several times until the DNP-amino acid crystallizes at a low temperature.

The procedure for those DNP-amino acids that precipitate as solids is much simpler. The precipitate is washed with ice-water and crystallized by using the appropriate solvent (see Table I).

(c) Special methods for the preparation of DNP-amino acids

The synthesis of those DNP-amino acids that require special procedures can be found in the following papers (references for the preparation of some necessary intermediates are also given):

DNP-glutamic acid (RAO AND SOBER[186])

α-Mono-DNP-histidine (RAMACHANDRAN AND MCCONNELL[155]; ZAHN AND PFANNMÜLLER[200])

Di-DNP-histidine (RAMACHANDRAN AND MCCONNELL[155])
Imidazole-DNP-histidine (BERGMANN AND ZERVAS[11]; MARGOLIASH[116])
O-DNP-tyrosine (SANGER[100]; DU VIGNEAUD AND MEYER[46]; FRITZE AND ZAHN[68])
α-Mono-DNP-arginine (PORTER AND SANGER[152])
α-Mono-DNP-ornithine (SANGER[100]; RIVARD[160])
δ-Mono-DNP-L-ornithine (SANGER[160])
α-Mono-DNP-L-lysine (NEUBERGER AND SANGER[135]; BERGMANN *et al.*[12]; RICE[150]; BISERTE *et al.*[10])
ε-Mono-DNP-lysine (PORTER AND SANGER[152])
Mono-DNP-cystine (BETTELHEIM[14])
S-DNP-cysteine (HAUSMANN *et al.*[76])
DNP-cysteic acid (BETTELHEIM[14]).

(d) Dinitrophenylation of a protein hydrolysate

Dinitrophenylation of protein hydrolysates is used mainly in the column chromatography of DNP-amino acids and in the determination of the composition of amino acids by these column chromatographic methods.

(i) Coupling in aqueous medium (LEVY *et al.*[111]).

Reaction procedure: The hydrolysate (3–5 mg in 3 ml) is placed in a synthesis cell (see Fig. 1), 0.1 ml of 3.1 *N* KOH is added and the contents are adjusted to pH 9.0 with about 40 μmoles of 0.2 *N* KOH. The solution is saturated with 2,4-dinitrofluorobenzene at 40° by vigorous agitation with a slight excess (*ca.* 0.1 ml) of the reagent. The pH is maintained at 9 for 80 min by intermittent additions of 0.2 *N* NaOH. This operation can be conveniently carried out by means of an autotitrator[98].

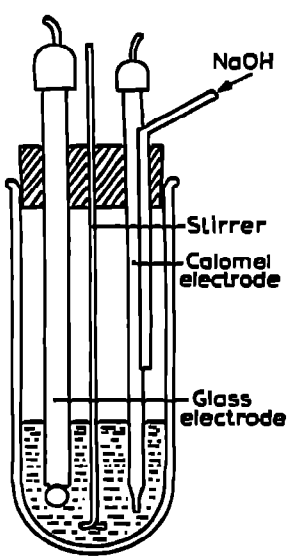


Fig. 1. Synthesis cell for the dinitrophenylation of amino acids.

The kinetics of the reaction can be followed by measuring the consumption of NaOH (for details see LEVY[109] and BUNNETT AND HERMANN[33]). Similar coupling in aqueous medium, using 5% carbonate buffer, was described by FRAENKEL-CONRAT AND SINGER[66].

Extraction of the dinitrophenyl derivatives[10]: After the coupling reaction is completed, the contents of the cell are transferred into a separating funnel and extracted 2–4 times with 5-ml volumes of peroxide-free diethyl ether to remove the excess of the reagent. The reaction mixture is then acidified with 0.5 ml of 5 *N* HCl and the ether-soluble amino acids are extracted 5 times with 5-ml volumes of diethyl ether. The ethereal phase is concentrated carefully to a small volume; the evaporated material, which floats above some droplets of water, is poured into special receptacles for the sublimation of 2,4-dinitrophenol (see below).

The aqueous phase that remains after the extraction of the other soluble DNP-amino acids still contains α-mono-DNP-arginine and α-DNP-histidine (DNP-cysteic acid in those instances when the protein is oxidized with performic acid prior to hydrolysis).

The remaining aqueous phase is extracted several times with a mixture of equal volumes of *sec.*-butanol and ethyl acetate[100].

An alternative procedure for working-up of the aqueous phase was described by LEVY[100].

(ii) Coupling in aqueous alcoholic medium (FRAENKEL-CONRAT AND SINGER[66] *and* BISERTE *et al.*[10]). The hydrolysate produced from 10 mg of protein is dissolved in 5 ml of doubly distilled water at 40°. The pH is adjusted to 9 with *N*/15 NaOH and 0.2 ml of 2,4-dinitrofluorobenzene is added. The solution is stirred for 15 min at 40° while the pH is maintained at 9. Absolute ethanol (10 ml) is then added and stirring is continued for 90 min at 40°, the pH being maintained at 9. After the reaction, the alcohol is removed by evaporation in a current of cold air. The excess of the reagent is extracted 5–10 times with peroxide-free diethyl ether. The medium is acidified with 1 ml of concentrated HCl and again extracted 5 times with peroxide-free diethyl ether and then 3 times with ethyl acetate. The residual aqueous phase is extracted 3 times with a mixture of equal parts of ethyl acetate and *sec.*-butanol. The diethyl ether and ethyl acetate extracts are collected and evaporated to dryness and the 2,4-dinitrophenol present in this phase is removed by sublimation (see below).

The butanol extracts are also evaporated to dryness. After dissolution in a suitable solvent (*e.g.*, acetone for paper chromatography) the evaporated extracts can be used either directly, or after the removal of 2,4-dinitrophenol, for any separation procedure.

(e) Sublimation of 2,4-dinitrophenol (MILLS[124])

This procedure is performed in the sublimation apparatus shown in Fig. 2. This apparatus is designed so that the distance between the heated film and the cooled surface is very small. Sublimation is effected at 70–80° for 30 min.

(f) Dinitrophenylation of a protein

The dinitrophenylation of a protein was first described by SANGER[108]. The following procedures can be used:

(i) Coupling in an ethanol–bicarbonate medium. The protein (0.5 g) and $NaHCO_3$ (0.5 g) are dissolved in 5 ml of water. Then 10 ml of a 5% (v/v) solution of 2,4-dinitrofluorobenzene in ethanol are added and the mixture is agitated mechanically for 2–3 h in the dark at room temperature.

The dinitrophenylation of an insoluble protein requires a prolonged period of

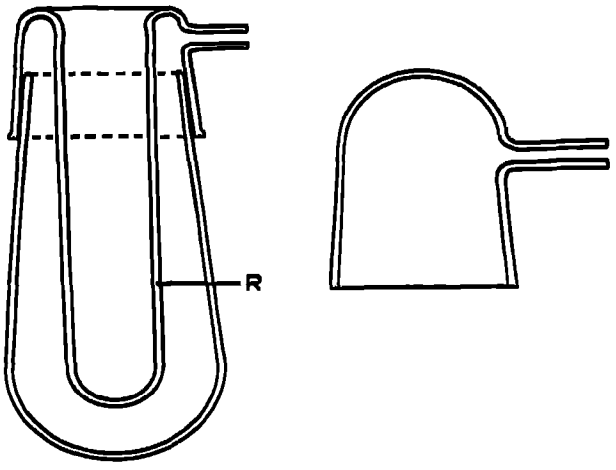


Fig. 2. Apparatus used for the sublimation of dinitrophenol. A standard 29–42 ground-glass joint is used. The cooling finger (R) is filled with acetone + carbon dioxide snow mixture. The glass cap in the right hand part of the figure facilitates the elimination of droplets of water after evaporation of the other solution and before the sublimation is carried out.

agitation (48 h at 40°; 72 h at 20°) and repeated additions of sodium bicarbonate and 2,4-dinitrofluorobenzene (for examples, see FRITZE AND ZAHN[67] and DEYL *et al.*[43]).

After complete dinitrophenylation, the DNP-protein is often insoluble, even in an alkaline medium; on the other hand, after acidification of the medium, most DNP-proteins are precipitated. After centrifugation, the precipitate is washed several times with water to remove mineral salts and with alcohol until a clear liquid is obtained (in order to remove the excess of 2,4-dinitrofluorobenzene and the 2,4-dinitrophenol formed), and is finally washed with diethyl ether.

The washing and isolation procedures vary, however, for individual proteins, depending on the solubilities of the particular DNP-proteins. For example, DNP-albumin and DNP-ovomucoid are soluble in water and can be precipitated with an excess of ethanol; the DNP-derivatives of ovomucoid are soluble in water and ethanol.

The byproducts of the coupling procedure can always be removed by dialysis and the solution of the DNP-protein can be lyophilized.

(ii) Coupling in aqueous medium (LEVY AND LI[112]). The protein (at least 0.2 μmole) is dissolved in 3 ml of 0.1 *M* KCl at 40°. The pH is adjusted to 8 and maintained at this value by additions of 0.05 *N* KOH in an autotitrator. After the addition of 2,4-dinitrofluorobenzene (*ca.* 0.1 ml), the solution is agitated vigorously. The coupling reaction is terminated after agitation for 90–120 min. The graph of KOH consumption against time shows an inflection when the reaction is terminated. The slope of the graph, which then becomes constant, corresponds to the rate of hydrolysis of 2,4-dinitrofluorobenzene.

The reaction medium is then extracted three times with diethyl ether to remove the excess of 2,4-dinitrofluorobenzene and the DNP-protein is isolated as described in the previous method.

(iii) Coupling in a bicarbonate–guanidine hydrochloride medium (PHILLIPS[145]). The protein is dissolved in a solution of 6 *M* guanidine hydrochloride (protein concentration *ca.* 20 mg/ml). Solid $KHCO_3$ is added to a concentration of 10–15 mg/ml and

then 2,4-dinitrofluorobenzene to a concentration of 0.05–0.1 ml/ml. The mixture is agitated at 20° for 6–24 h, then acidified, diluted with three volumes of water and extracted once with diethyl ether.

The precipitate of DNP-protein is washed several times with water, using centrifugation after each washing. The last traces of the reagent and of 2,4-dinitrophenol are removed by three washings with acetone and one washing with diethyl ether.

(g) Dinitrophenylation in acrylamide gel

An important problem in structural protein analysis is the amount of material available. One of the most effective techniques for separating protein is polyacrylamide gel electrophoresis. It is therefore not surprising that attempts were made to apply the sequencing techniques directly to the proteins separated in polyacrylamide gels. Thus CATSIMPOOLAS[37] attempted to stain electrophoretically separated proteins with 2,4-dinitrofluorobenzene. After conventional gel preparation, separation and detection with Amido Black, the extruded gels were placed in the storage tubes, which were filled with a 2.5% (v/v) solution of 2,4-dinitrofluorobenzene in ethanol. The tubes were stoppered and placed in a dark room for 16 h. The reaction was effected by diffusion of the reagent into the gel and proceeded quite smoothly as the inside of the gel was sufficiently alkaline. Shorter incubation times, however, produced incomplete labelling of the protein bands. During the incubation procedure, the gels became opaque and shrank. The gel columns, which at this stage did not exhibit any distinct bands, were washed several times with diethyl ether to remove excess of the reagent and subsequently were transferred into Erlenmeyer flasks containing diethyl ether and 5% of 2 *N* HCl. The whole mixture was shaken until no more yellow colouring was released from the gel columns. During this period, the extractant was replaced several times. At the end of this procedure, which continued for several hours, the gels attained their original length and protein bands appeared as dark yellow zones against a pale yellow background. In comparative experiments with bovine serum albumin, the number of bands was identical to that revealed after staining with Amido Black. The individual yellow bands were then cut away from the gel column and placed in small test-tubes. For adequate results in the next sequencing step, at least 20–30 bands from individual runs have to be combined. The sample of combined gel discs containing DNP-protein was placed in a sealed tube with 6 *N* HCl and hydrolysed in the usual manner. Gel samples containing no yellow protein band were hydrolysed in parallel to prevent artefacts. The hydrolysate was finally filtered and diluted with water and the individual DNP-amino acids were identified by flat-bed techniques.

(h) Hydrolysis of the DNP-proteins

In this particular instance it is not possible to give a general procedure that is useful for the liberation of all types of DNP-derivatives of N-terminal amino acids. The procedures recommended vary with the nature of the N-terminal amino acid.

For a qualitative determination of the N-terminal DNP-amino acid, hydrolysis is usually effected in a sealed tube, which is either evacuated or filled with nitrogen, for 16 h at 105° using redistilled 5.7 *N* HCl.

This type of hydrolysis may cause considerable degradation of DNP-proline and DNP-glycine and also of other DNP-amino acids[151] (see Table 2).

To avoid this degradation as far as possible, it is recommended that, in addition

TABLE 2

APPROXIMATE BREAKDOWN OF DNP-AMINO ACIDS

Amino acid derivative	*Hydrolysis in boiling 5.7 N HCl*		*Hydrolysis for 16 h in 12 N HCl at 105° (minimal figures)*
	Time of hydrolysis (h)	*Amount unchanged (%)*	*Amount unchanged (%)*
DNP-alanine	12	80	75
DNP-arginine	12	90	75
DNP-aspartic acid	24	90	75
Di-DNP-cystine	12	25	0
DNP-glutamic acid	12	75	75
DNP-glycine	8	40	50
DNP-hydroxyproline	4	40	50
DNP-isoleucine	12	80	75
DNP-leucine	12	80	75
Di-DNP-lysine	8	95	75
ε-DNP-lysine	12	95	75
DNP-methionine	12	75	75
DNP-phenylalanine	12	70	50
DNP-proline	2	10	50
DNP-serine	12	90	75
DNP-threonine	24	90	75
DNP-tryptophan	12	90	0
DNP-tyrosine	12	75	50
DNP-valine	12	80	75

to the hydrolysis at 105° for 16 h, two other hydrolyses should be carried out: for DNP-glycine a hydrolysis for 4 h (in this instance a check for DNP-peptides is essential), and for DNP-proline a hydrolysis for 4 h with 11.2 *N* HCl[162].

Other factors that influence the hydrolysis of DNP-proteins are discussed in papers by PHILLIPS[145], SHEPHERD *et al.*[178], HANES *et al.*[74], THOMPSON[180] and DICKMAN AND ASPLUND[44]. The last two papers discuss problems that involve the presence of tryptophan in hydrolysed proteins.

(i) Dinitrophenylation of a peptide

All the methods already described for proteins can also be used for peptides; one has to take into account only the lower molecular weights and different solubilities of peptides compared with those of proteins. In the following paragraphs, the only methods described are those that are designed especially for peptides and that offer some advantages when compared with the techniques used in protein analysis.

For dinitrophenylation in a trimethylamine medium[171], 0.2 μmole of a peptide is dissolved in 0.1 ml of 1% aqueous trimethylamine and an aqueous solution containing 10 μl of 2,4-dinitrofluorobenzene is added. After 2 h, a few drops of water and of the trimethylamine solution are added and the excess of 2,4-dinitrofluorobenzene is removed by extracting the mixture three times with diethyl ether. After evaporating the reaction mixture to dryness, the residue can be used for hydrolysis directly.

In order to reduce the formation of dinitrophenol, LOCKHART AND ABRAHAM[113] replaced trimethylamine with trimethylamine carbonate. Further modification of the trimethylamine carbonate method was described by WALEY[198].

3. *Photolysis of DNP-amino acids, DNP-peptides and DNP-proteins*

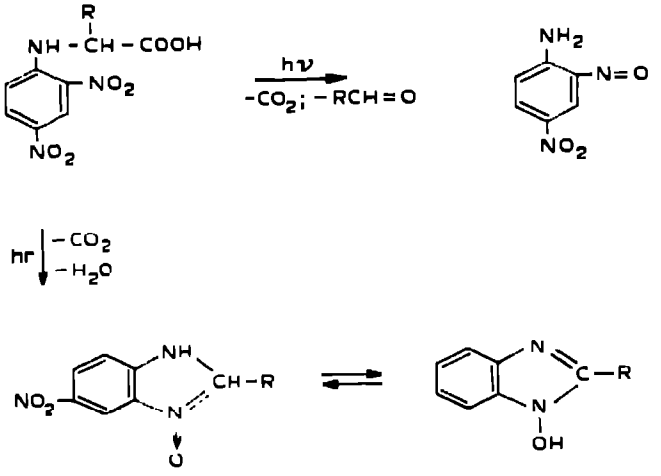
It is well known that the DNP-derivatives discussed in this chapter are decomposed by light.

The pioneering study of AKABORI *et al.*[4] showed that several DNP-amino acids in aqueous solution were decomposed by light at about the same rate, but these authors did not identify the products. They also found that ε-DNP-lysine is stable to light. POLLARA AND VON KOREFF[149] observed that many of these compounds in the solid state are decomposed by light to give the corresponding DNP-alkylamine.

RUSSELL[100,107] found that DNP-leucine, after exposure to sunlight, was decomposed to products whose spectra were different from that of DNP-leucine but also from that of the product, DNP-isopentylamine, which would be expected to result from the photolysis of solid DNP-leucine.

RUSSELL[100,107] identified 4-nitro-2-nitrosoaniline, 3-methylbutyraldehyde and carbon dioxide as being the products of the photolysis of DNP-leucine at pH 8. He also compared the photochemical behaviour of other α-DNP-amino acids at pH 8 and found results similar to those obtained with DNP-leucine. The only exception found was DNP-tryptophan, which was stable under these conditions. β-, δ- and ε-DNP-amino acids were stable; DNP-peptides were decomposed much more slowly than α-DNP-amino acids.

NEADLE AND POLLITT[131] and POLLITT[150] studied the photolysis of DNP-amino acid in acidic media and found that 2-alkyl-4-nitrobenzimidazole-1-oxides are formed in addition to the 4-nitro-2-nitrosoaniline found by RUSSELL[100,107].



The formation of the benzimidazole derivatives is pH-dependent (see Fig. 3) with two optimal pH values at pH 4 and below pH 0; it appears that nobody has attempted to use these photolytic products for the identification of the N-terminal amino acids.

It should be equally attractive to study the products of the photolysis of DNP-peptides under acidic conditions in order to cleave off the benzimidazole derivative corresponding to the N-terminal amino acid.

The photodecomposition of DNP-peptides was studied by AKABORI *et al.*[5].

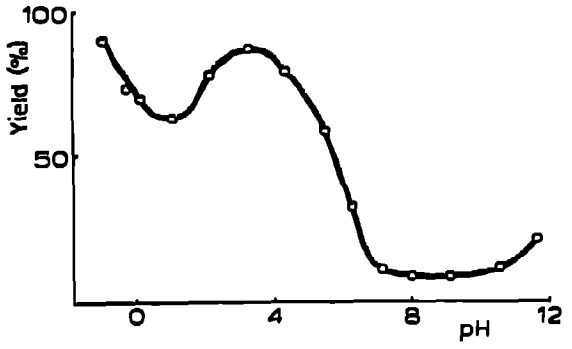


Fig. 3. Yield of 2-methyl-6-nitrobenzimidazole-1-oxide from the photolysis of DNP-alanine in aqueous solution at various pH values.

4. Chemical reactions of DNP-amino acids

This chapter deals generally with the use of DNP-derivatives of amino acids in the identification of the N-terminal amino acids in peptides and proteins. Sometimes it is more advantageous to use the dinitrophenylation reaction only for the labelling and isolation parts of the work and to use some other transformation products of DNP-amino acids for identification and quantitation purposes.

A reaction of this type has already been described in chapter 3.2.2. of this review (Part A, p. 215). In this section, we deal with those reactions of DNP-amino acids that lead to the regeneration of the parent amino acid; the identification or quantitation is then carried out by using an amino acid analyzer or any other method for the identification of free amino acids.

The first worker who applied this principle successfully was MILLS[123], who regenerated the parent amino acid from the DNP-amino acid by heating it with 2 *N* H_2SO_4 containing a trace amount of H_2O_2. He also achieved the cleavage with a saturated solution of barium hydroxide in water.

The ammonolysis of DNP-amino acids was described by LOWTHER[114], who liberated the parent amino acid from its DNP-derivative by treatment with 0.88 *N* NH_3 at 100° for 2 h.

In our opinion, the most useful method for the regeneration of an amino acid from the DNP-derivative is hydrogenation using a platinum catalyst, as described by FASOLD *et al.*[57].

5. Column chromatography of DNP-amino acids

(a) Separation on silica gel columns (PORTER AND SANGER[152])

In all procedures for the column chromatographic separation of DNP-amino acids, the method by which the sorbent is prepared and hydrated is of decisive importance. Therefore, in all procedures of this type, adequate attention must be paid to the preparation of the sorbent.

Sorbent preparation (GORDON *et al.*[49]). Equal volumes of commercial water-glass and ice are stirred well and 12 *N* HCl is added to the mixture stepwise with vigorous stirring until a change in colour of thymol blue indicator is observed. During this operation, the reaction mixture becomes very opaque, but it clears again when the correct pH value is reached. Then the reaction mixture is allowed to stand for 3 h and

HCl is added, if necessary, to maintain the correct pH. The silica gel precipitate is filtered off and washed well with 2 l of water per 250 g of gel. The precipitate is resuspended in 0.5 *N* HCl and allowed to stand for 2 days, filtered and washed until free from chloride. Finally, the precipitate is dried at 100°. As some variations may be obtained with different batches, it is recommended that large batches (5 kg) are prepared to ensure good reproducibility for a reasonable period of time. The variability of different batches of gel may be reduced if they are allowed to stand in an HCl solution of a known concentration before washing and drying them.

Pouring of columns. The wet gel preparation differs slightly according to the solvent system to be used. Most frequently water or the appropriate buffer, equivalent to one half of the weight of the dry silica gel, is stirred into the dry gel. If the prepared silica gel is expected to be used for the separation of basic amino acids, 1 *N* HCl is used instead of water as DNP-lysine is more stable in acidic media.

If organic solvents (water-miscible) are to be used, an amount of the aqueous phase equivalent to two thirds of the weight of the gel is mixed with the dry gel. For the column preparation, 6–10 g of gel are mixed well with the wet organic solvent until the mixture becomes a freely running suspension. In general, columns 33 × 1 cm in size are adequate to ensure good quality of separation. The column is rotated as it settles, to remove air bubbles, and the supernatant solvent is allowed to drain through. Care is taken that the bottom of the column is continuously immersed in the solvent-collecting beaker as otherwise the sorbent column cracks and the column has to be refilled. The same effect occurs if the moisture content of the gel is too high. The use of a small excess pressure during the filling of the column results in sharper bands and improved quality of separation. There are some precautions that should be taken when using organic solvent systems. All the solvents should be kept with a small amount of the aqueous phase and sufficient time for equilibration should be allowed. The only exception to this rule is the ethylene glycol–benzene mixture in which ethylene glycol replaces water and therefore the gel is prepared by mixing the dry gel with an equal weight of ethylene glycol. Chloroform should be washed well to free it from ethanol, which, added to the extent of 1% as a stabilizer, influences the separation maskedly.

Sample application and operational procedures. The sample of a DNP-amino acid mixture obtained by extraction with diethyl ether is dissolved in 1–2 ml of the appropriate solvent and loaded into the column. A bent-tipped pipette is recommended for this application to avoid disturbing the gel surface. The top of the column is washed several times to ensure quantitation. With certain amino acids, previous evaporation to dryness with 12 *N* HCl may help in dissolving the sample.

The amount loaded into the column is important in column chromatographic separations on silica gel columns. The best results are obtained when the amount loaded can just be seen, *i.e.*, 0.1–0.2 μmole. Larger amounts (up to 2 μmoles) cause tailing in most instances. Generally, the mechanism of the silica gel chromatography of DNP-amino acids is that of partition chromatography, but it has been mentioned in the literature that adsorption also plays an important role.

The over-all scheme for the separation of DNP-amino acids on a silica gel column is rather complex. It has been described by PORTER AND SANGER[158] and is carried out as follows.

In the first step, a mixture of ether-soluble DNP-amino acids is subjected to

elution with chloroform and a butanol–chloroform mixture. The original mixture is resolved into five bands, which, except for the third one, are subjected to further fractionation. The third band is the DNP-derivative of hydroxyproline and can therefore be subjected to direct quantitation.

The first band in the chloroform and butanol–chloroform fractionation is eluted in three subfractions with an ethanol–ligroin mixture containing an increasing proportion of ligroin. By washing the column with chloroform–ethanol/ligroin(1:1), the mixture of DNP-Leu and DNP-Ile is eluted. A chloroform–acetone/cyclohexane–ethanol/ligroin(1:2:2) mixture elutes a complex band, which can be separated further with acetone/cyclohexane. If this complex band is treated with chloroform–ethanol/ligroin–acetone/cyclohexane (1:2:1), the DNP-valine band is eluted, while the same solvent system in a ratio of 1:2:2 elutes the DNP-derivative of phenylalanine. The last fraction obtained from the chloroform–ethanol/ligroin fractionation is eluted with this mixture in a ratio of 1:3. The band eluted in this step is again complex, consisting of DNP-methionine, DNP-proline and DNP-alanine. If to the original chloroform–ethanol/ligroin mixture (1:3) another portion of methanol/carbon tetrachloride solvent is added, making the over-all proportions of individual solvents 1:3:1, the band of DNP-methionine is eluted. If the proportion of methanol/carbon tetrachloride is further increased, making the over-all proportion of chloroform–ethanol/ligroin–methanol/carbon tetrachloride 1:3:2, a slow-moving band consisting of DNP-proline and DNP-alanine is eluted. These latter two DNP-amino acids can be separated by adding benzene to the eluant from the previous step. A ratio of chloroform–ethanol/ligroin–methanol/carbon tetrachloride–ethylene glycol/benzene of 1:3:2:1 elutes DNP-proline, while a further increase in the ethylene glycol/benzene ratio elutes DNP-alanine. In this latter instance, the proportions of the components are chloroform–ethanol/ligroin–methanol/carbon tetrachloride–ethylene glycol/benzene 1:3:2:2.

The second band from the chloroform fractionation consists of four amino acid derivatives: DNP-Trp; DNP-Gly; DNP-Tyr and bis-DNP-Lys. These derivatives are separated by eluting the column with an increasing proportion of 33% diethyl ether/ligroin solvent: if the proportion of chloroform–33% diethyl ether/ligroin is 2:1, the mixture of DNP-Trp and DNP-Gly is brought into solution. The further separation of these two amino acid derivatives is carried out by using a 5% solution of propanol in cyclohexane. A mixture of chloroform–33% diethyl ether/ligroin–5% propanol/cyclohexane in the ratio 2:1:1 elutes DNP-Trp, whereas when the components are present in the ratio 2:1:2 DNP-Gly is eluted. The slower band of the 33% diethyl ether/ligroin fractionation eluted with chloroform–33% diethyl ether/ligroin (2:2) consisting of DNP-Tyr and bis-DNP-Lys is separated by using an increasing proportion of ethylene glycol/benzene in the solute. Hence, chloroform–33% diethyl ether/ligroin–ethylene glycol/benzene (2:2:1) washes out the band of DNP-Tyr, whereas when the components are present in the ratio 2:2:2 the band of di-DNP-Lys is washed out.

The third band of the chloroform fractionation, as already mentioned, is pure DNP-Hypro and does not require any further fractionation.

The fourth band of the chloroform fractionation is a mixture of DNP-Thr and DNP-Ser, which is separated by washing first with chloroform–33% diethyl ether/ligroin (4:1) and then with the same mixture in the ratio 4:2. In the first instance, DNP-Thr is eluted, while in the second step DNP-Ser is eluted.

The fifth band of the chloroform fractionation is washed with chloroform–33% diethyl ether/ligroin (5:1), which moves the complex band of DNP-Asp and DNP-Glu, while the same mixture in the ratio 5:2 elutes the last amino acid to be separated, *viz.*, Di-DNP-Cys. The separation of the corresponding derivatives of aspartic and glutamic acid is achieved by using 5% propanol/cyclohexane as the solvent. The solvent system consisting of chloroform–33% diethyl ether/ligroin–5% propanol/cyclohexane elutes DNP-glutamic acid, while the same solvent system with a slightly different ratio of components (5:1:2) elutes DNP-Asp.

Fig. 4 makes the whole system more easy to understand. Of course, all the separation steps cannot be carried out on only one column, as the whole procedure

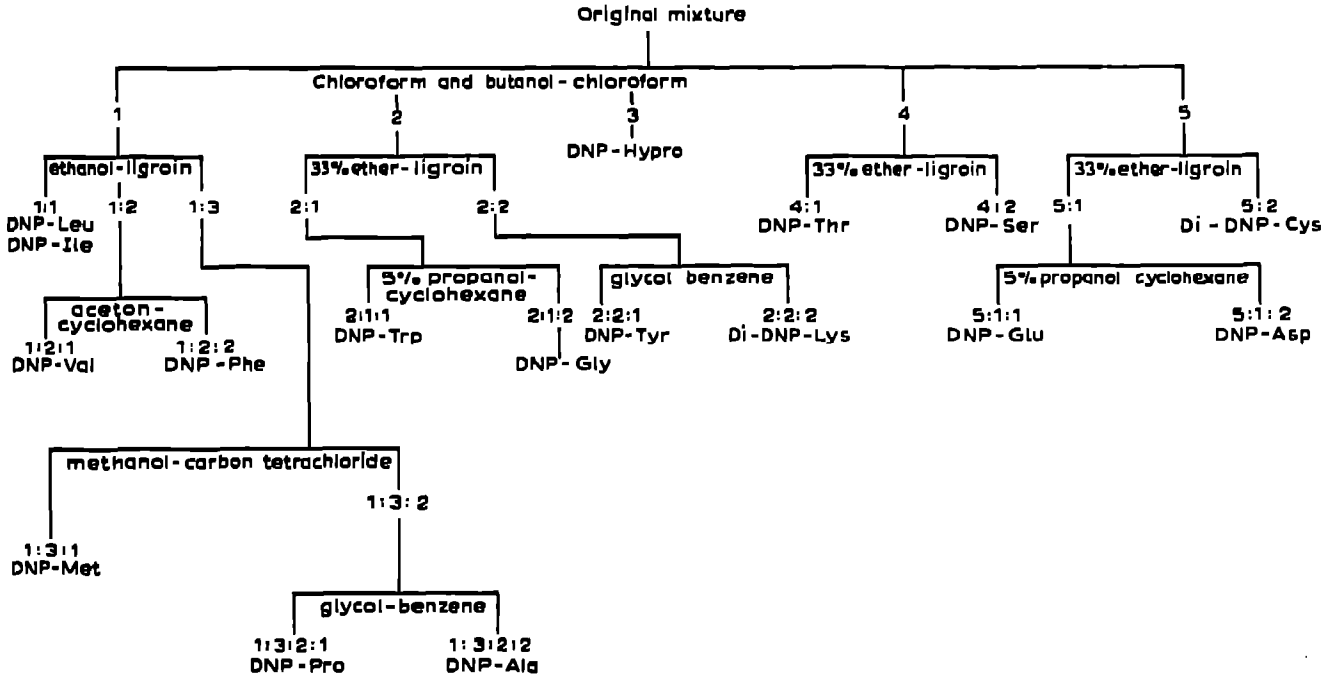


Fig. 4. Scheme for fractionation of ether-soluble DNP-amino acids. (See text for composition of solvents.) (PORTER AND SANGER[158]).

would then be even more tedious and time consuming. PORTER AND SANGER[158] suggested that the following four columns should be used:

(A) Ethanol/ligroin column. One volume of water, 1 volume of ethanol and 10 volumes of ligroin (boiling range 80–100°) are mixed, 1 ml of the lower aqueous phase is added to each 2 ml of silica gel and the upper ligroin phase is used as the mobile phase in the column.

(B) Methanol/carbon tetrachloride column. This column is prepared in a similar manner to column A: 1 volume of water, 1 volume of methanol and 15 volumes of carbon tetrachloride are used.

(C) Acetone/cyclohexane column. This column is prepared by using 1 volume of water, 1 volume of acetone and 10 volumes of cyclohexane.

(D) Ethylene glycol/benzene column. Ethylene glycol and benzene are shaken together, 1 ml of the ethylene glycol layer is added to each 1 g of dry silica and the benzene layer is used as the mobile phase.

These columns when under operation show some artefacts, which should be dealt with in an appropriate way:

(1) A yellow band, which moves faster than any amino acid derivative in the chloroform column.
(2) DNP-Tyr and DNP-Met on hydrolysis result in the formation of coloured bands, which do not move in the chloroform system.
(3) Bis-DNP-Cys yields a band that moves slowly in 3% butanol/chloroform.

In general, certain stable peptides yield corresponding DNP-derivatives that can be easily interchanged with amino acids.

According to GORDON *et al.*[60], the water-soluble DNP-derivatives are eluted from butanol–chloroform or methyl ethyl ketone columns as indicated in Table 3. A 66% solution of methyl ethyl ketone in diethyl ether elutes bis-DNP-His and S-DNP-Cys rapidly, while α-DNP-Arg, ε-DNP-Lys and α-DNP-Lys move with R values of 0.35, 0.20 and 0.11, respectively. These values are, as already mentioned, not absolutely reproducible and are reported here only to give an over-all picture of the separation procedure. Using the butanol/chloroform system (17% butanol), di-DNP-His moves as a fast band, while S-DNP-Cys exhibits an R value of 0.08. Other water-soluble DNP-derivatives are retained on the column under these conditions. In the same system with 30% butanol, S-DNP-Cys moves as a fast band, while DNP-Arg and DNP-Lys move much more slowly, with R values of 0.2 and 0.15, respectively. α-DNP-Lys is retained on the column.

TABLE 3

BAND RATES OF ACID-SOLUBLE DNP-AMINO-ACIDS

Figures are values of R, GORDON *et al.*[60].

DNP-amino acid	*Developing solvent*		
	Methyl ethyl ketone–ether 66%	*Butanol–chloroform 17%*	*30%*
Di-DNP-histidine	Fast	Fast	—
S-DNP-cysteine	Fast	0.08	Fast
α-DNP-arginine	0.35	—	0.2
ε-DNP-lysine	0.20	—	0.15
α-DNP-lysine	0.11	—	—

A further improvement in this procedure could be achieved by adding formaldehyde, which supresses the ionization of the α-amino group of ε-DNP-Lys and thus enables a much clearer separation from DNP-Arg to be achieved. In this particular instance, the column is prepared by mixing 100 g of silica, which contains 9% water, with 40 ml of 1:9 (v/v) formaldehyde–water that has been previously neutralized to pH 7.0 with 0.5 M disodium hydrogen phosphate (BAILEY[6]).

(b) Separation on buffered silica gel columns

BLACKBURN[31] was the first to introduce buffered silica gel columns into the chromatography of DNP-amino acid derivatives. He also was able to show that the differences observed by many workers between different batches of silica gel can be attributed to the variations in the pH of the aqueous phase that is in contact with the gel.

Sorbent preparation. According to the method of BLACKBURN[91], silica gel is treated for 3 h with excess of hydrochloric acid after it has been precipitated from the water-glass solution. The *R* values of amino acid derivatives on buffered columns are summarized in Table 4. The following buffer systems were used:

(A) 0.2 *M* sodium dihydrogen phosphate

(B) 0.5 *M* phosphate, pH 6.61

(C) 0.25 *M* phosphate, pH 5.95.

Acid-soluble DNP-amino acid derivatives may be subjected to fractionation in 0.2 *M* sodium dihydrogen phosphate using Sanger's original fractionation described in the preceding section.

TABLE 4

BAND RATES (*R* VALUES) OF DNP-AMINO ACIDS IN CHLOROFORM AND BUTANOL–CHLOROFORM

Buffer systems: (A) 0.2 M sodium dihydrogen phosphate; (B) 0.5 *M* phosphate, pH 6.61; (C) 0.25 *M* phosphate, pH 5.95.

DNP-amino acid	*R value*			
	$CHCl_3$, buffer A	*$CHCl_3$, buffer B*	*3% BuOH–$CHCl_3$, buffer C*	*17% BuOH–$CHCl_3$, buffer B*
DNP-leucine	Fast	0.7	—	—
DNP-valine	Fast	0.5	—	—
DNP-phenylalanine	Fast	0.7	—	—
DNP-methionine	Fast	0.5	—	—
DNP-proline	Fast	0.3	—	—
DNP-alanine	Fast	0.2	—	0.5
DNP-tryptophan	0.6	0.4	Fast	—
DNP-glycine	0.9	0.03	0.5	0.2
Di-DNP-lysine	0.7	0.06	Fast	Fast
Di-DNP-tyrosine	Fast	0.07	0.9	Fast
DNP-threonine	0.3	Slow	0.18	0.15
DNP-serine	0.11	Slow	0.08	0.07
DNP-glutamic acid	0.17	Slow	0.2	Slow
DNP-aspartic acid	0.06	Slow	Slow	Slow

(c) Automated separation on silica gel columns

The latest achievements in the column chromatography of DNP-amino acids are the automated procedures referred to in the papers of KESNER and co-workers[98,99]. Silica gel is used as the support material and the procedure can easily be repeated in any laboratory that is equipped to carry out standard chromatographic procedures (an over-all scheme of the apparatus used is shown in Fig. 5).

Sample preparation. For the dinitrophenylation reaction, silicone-coated glassware was recommended (SCHROEDER AND LE GETTE[176]); after the dinitrophenylation reaction is completed, ethanol is evaporated, the dry residue is dissolved in 5 ml of water and the solution is brought to pH 8.0. The solution is then extracted six times with 10-ml volumes of diethyl ether to remove the excess of 2,4-dinitrofluorobenzene and DNP-NH_2. The aqueous layer is finally acidified to pH 1–2 and is then ready for application to the column.

If the mixture to be analyzed contains peptides or dinitrophenylated proteins, the amino acid and peptide derivatives are extracted from the aqueous phase with six 10-ml volumes of water-saturated methyl ethyl ketone. The adjustment of the pH of

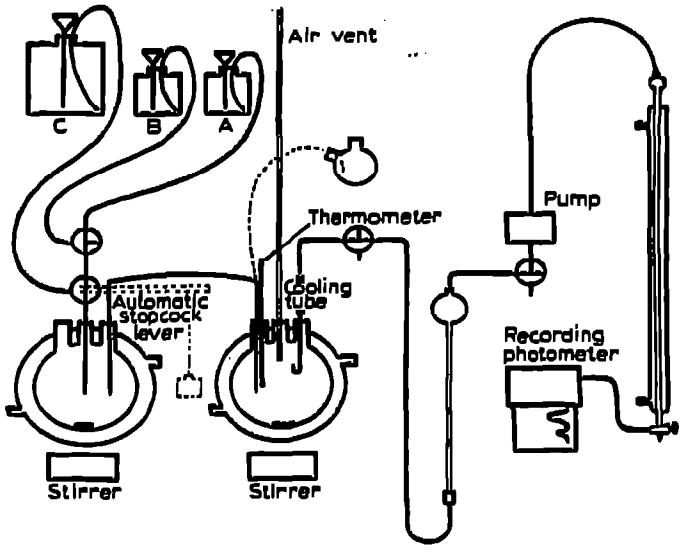


Fig. 5. Two-chamber gradient apparatus using solvent de-aeration, water saturation and continuous spectrophotometric recording.

the reaction mixture before extraction is carried out with concentrated HCl. The extract is evaporated to dryness in a stream of air in a dark hood.

Column preparation and operation. As is usual with silica gel, the final separation is highly sensitive to the moisture content of the sorbent material. It is generally recommended that the sorbent is tested before the column is filled; on the other hand, by changing the activity of the sorbent it is possible to obtain resolutions of DNP-derivatives that in a particular instance had moved together. In conclusion, it should be stressed that constant conditions must be maintained during the automated analysis in order to obtain reproducible results.

Sorbent. 3.5 ml of 0.5 *N* H_2SO_4 are added to 8 g of silica gel. An appropriate amount (about 45 g) is prepared in order to fill a 100 × 1 cm column. Alternatively, the sorbent is prepared by adding 5.2 ml of 0.5 *N* H_2SO_4 per 8 g of silica gel. Before adjusting the humidity of the sorbent, the silica gel is oven-dried and weighed quickly; the appropriate volume of H_2SO_4 is then added and the mixture is stirred well to obtain a free-flowing powder. During this stage the sorbent is transferred to a bottle and mixed in a ball-mill for 15 min.

Column filling. A column of the specified dimensions is filled by using a simple device (see Fig. 6), which, in principle, consists of an infusion bottle clamped to the top of the column and provided with a stirrer blade that extends several inches below the ball joint. The column stopcock is closed and the extra hole is plugged with a short piece of PTFE tubing into which a fine glass rod has been inserted. The whole system is filled with acid-washed heptane until a volume of about 50 ml is present in the infusion bottle. The silica gel sorbent is then transferred into the infusion bottle. Before this transfer is carried out, a 0.3-g amount and a 0.5-g amount of silica gel are retained in two paraffin foil sealed beakers. A further volume of acid-washed heptane is added to the infusion bottle until the mixture reaches the 600-ml mark on the bottle. Then a stopper bearing the stirrer guide is inserted, the stirrer speed is adjusted to maintain a clear heptane layer above the silica gel suspension, the lower stopcock of the column is opened and the silica gel suspension is allowed to sediment under

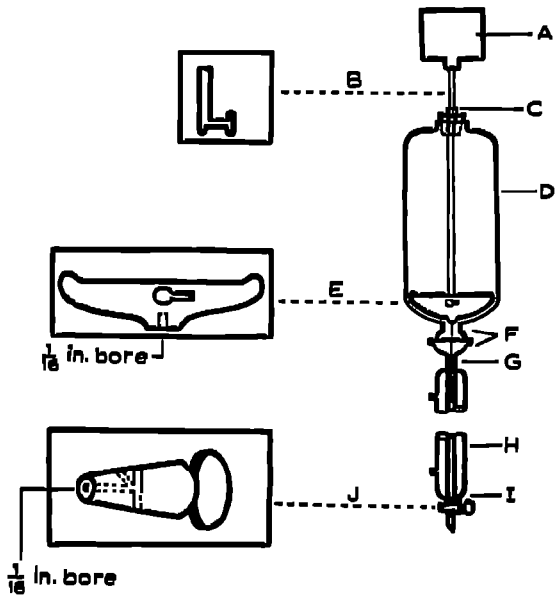


Fig. 6. Column pouring apparatus. A = stirrer with rheostat; B = button-tip glass stirring rod, 6 mm O.D.; C = stirring-rod guide; D = 700-ml infusion bottle, 12 mm outlet bore attached to ball part; E = PTFE stirring blade ($^1/_8$ in. thick); F = ball and socket joint; G = PTFE tubing tail, 0.034 in. I.D., 0.058 in. O.D.; H = jacketed chromatographic column, 1 × 120 cm; I = sintered glass disc; J = PTFE stopcock, bored for connection to flow cell.

gravity. The pouring of the column usually takes 30 min. After this period, the stopcock is closed, the PTFE tubing is attached to a suction flask and the excess of heptane is removed until its level falls to the ball-and-socket joint. The column is now ready to be clamped to the circulating water pump. If, during the pouring of the silica gel suspension, the silica gel particles stick to the column wall in the stirred area, it is recommended that these particles are removed with a narrow glass rod (by moving it back and forth several times) to prevent possible clogging of the column during packing under pressure.

Column packing. The packing is carried out by passing heptane under pressure through the column. Heptane from a reservoir is passed through a stirred three-necked flask in which it is heated to 56°, pumped through the acid-wash columns and delivered to the separation column. The socket area at the top of the separation column is freed from any particles of silica gel by filling it with hot acid-washed heptane and by allowing the particles to fall into the column. Excess of heptane is removed, the socket area is dried with a piece of filter-paper and the column is tightened to the inlet tubing. Care must be taken with the gaskets, which must be prevented from direct contact with heptane which would cause them to swell excessively and cause leaking on the top of the column.

At this stage the thermostat bath is set to 35° and both the column and the mixing chamber (three-necked flask) are heated to this temperature. The column exit is connected to the photometer cell and the hot heptane is allowed to pass through the column at a rate of 180 ml/h. The passage of 45–60 ml of the solvent, which takes 15–20 min, should result in the column packing to *ca.* 120–100 cm in height. Occasionally, the sorbent tends to become clogged during this operation, which can sometimes be prevented by applying a cushioned vibrator to the column walls.

Sample application. To the aliquot (see sample preparation), 0.2 ml of 0.5 *N*

H_2SO_4 is added, together with about 0.5 ml of acetone for washing purposes. The DNP derivatives are dissolved in the solution, by stirring occasionally with a heavy glass rod. Then the appropriate amount of silica gel (0.31 g of sorbent prepared by adding 5.2 ml of 0.5 *N* H_2SO_4 to 8 g of silica gel or 0.36 g of sorbent prepared by adding 3.5 ml of 0.5 *N* H_2SO_4 to 8 g of silica gel) is added to the aliquot and the reaction mixture is stirred and then evaporated to dryness in a stream of air in a dark hood. The dried mixture is transferred quantitatively to a small beaker by using another 0.5 ml of acetone with 0.3 g of silica gel for washing the particles that stick to the wall; the mixture is allowed to dry in a dark hood and the silica gel wash and the original sample are mixed; the heptane layer from the top of the column is siphoned off and the top of the silica gel is levelled with a flat-bottomed glass stirring rod. Some of the acid-washed heptane is added and the sample is transferred into the top of the column, tapping the beaker. Heptane in the socket area is stirred to allow any adhering particles to sediment, the top of the transferred silica gel is levelled with a flat-bottomed glass rod and the top of the sample is covered with 0.5 g of the silica gel sorbent retained at the beginning of the operation. Finally, a circular piece of Whatman filter-paper is used to cover the top of the column. The column is re-filled with acid-washed heptane to a level of about 3 mm above the bottom of the socket, the socket area is dried with filter-paper, the silicone rubber gasket is inserted and the socket joint is connected, observing all the precautions already mentioned. The DNP-derivatives are protected from decomposition by light by wrapping the column with aluminium foil.

Elution. The gradient elution device consists of two heated mixing chambers, each of 500-ml volume. About 150 ml of deaerated 0.1 *N* H_2SO_4 are placed into the acid-wash column (60 × 1.2 cm, with a 150-ml bulb at the top). Then 450 ml of heptane are placed in the first mixing chamber and heated to 56° while the same volume of heptane placed in the other chamber in heated to 35°. Reservoirs, connected to the latter mixing flask contain (1) 3% *tert.*-amyl alcohol in *n*-heptane, (2) 18% *tert.*-amyl alcohol in *n*-heptane, (3) methyl ethyl ketone and (4) *n*-heptane.

The procedure for the separation of peptides is identical with that just described with the exception that the solutions in reservoirs are (1) 18% *tert.*-amyl alcohol in *n*-heptane, (2) 50% methyl ethyl ketone in *n*-heptane, (3) methyl ethyl ketone and (4) *n*-heptane.

The apparatus usually has an additional heptane reservoir, which is used only for column packing.

Operation. The stopcock, which allows the 3% *tert.*-amyl alcohol–*n*-heptane solution to pass down the second mixing chamber, is opened and the pump is set to deliver 180 ml/h of the solvent to the column. After 700 ml of the solvent have passed through the column, which takes almost 4 h (the elution of DNP-phenylalanine is completed by that time), the solvent is changed to 18% *tert.*-amyl alcohol–*n*-heptane. After another 4 h (720 ml), the eluant is replaced with methyl ethyl ketone; about 2200 ml are allowed to pass through the column, by which time all the amino acid derivatives are eluted.

Virtually the same procedure is applicable for DNP-peptides, and the solvents used in each of the three elution steps are those mentioned above.

The separation of DNP-peptides can be improved by altering the methyl ethyl ketone gradient if individual peaks are too close together. In this event, the solvent

system mentioned above is suggested for use in a trial run. Alternatively, the eluate can be further purified by using silica gel of another activity in the same equipment. Some peptides are reported to stick to the column even if it is eluted with pure methyl ethyl ketone; in this event it is recommended that an additional step is introduced; the column is directly eluted with formamide, during which operation the acid-wash column is by-passed and the acid-washed methyl ethyl ketone on the top of the column is first replaced with formamide.

The over-all separation of DNP-amino acids can be seen in Fig. 7.

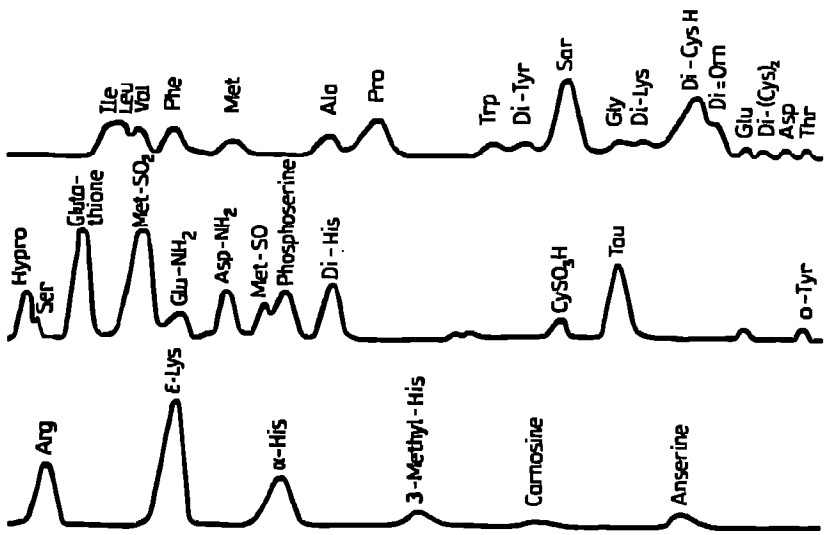


Fig. 7. Separation of a known mixture of DNP-derivatives of amino acids and related substances. Peaks representing unreacted 2,4-dinitrofluorobenzene and dinitrophenol appear before the DNP-amino acids and are not shown. If dinitroaniline is not removed, its peak appears in the alanine–proline area.

A further development of the technique described is based on the use of a standard nine-chamber Technicon Varigrad. A silicone rubber insulated heating tape is wrapped round the bottom third of this chamber and held in place with PTFE adhesive tape. Although the combinations can be adjusted according to the specific requirements for a particular mixture to be separated, the following procedure appears to be generally applicable, according to KESNER and co-workers[98,99]. At a temperature of 45° and a flow-rate of 100–150 ml/h, the following compositions of solvents in the individual chambers of the Varigrad system are used:

Chamber No. 1, 100 ml of 3% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 2, 100 ml of 3% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 3, 100 ml of 3% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 4, 100 ml of 10% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 5, 100 ml of 10% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 6, 100 ml of 3% *tert.*-amyl alcohol in *n*-heptane
Chamber No. 7, 84 ml of methyl ethyl ketone
Chamber No. 8, 84 ml of methyl ethyl ketone
Chamber No. 9, 84 ml of methyl ethyl ketone.

The determination of DNP-amino acids on silica gel can also be carried out at a temperature of 25–28° and at a flow-rate of 250 ml/h. In this instance, chambers 1 to

6 contain 200 ml of 2, 2, 2, 7, 10 and 12% *tert.*-amyl alcohol in *n*-heptane, respectively, while the residual chambers contain 168 ml of methyl ethyl ketone, and sometimes it appears to be necessary to pass additional methyl ethyl ketone through the column in order to elute the slowly moving DNP-amino acid zone.

For mixtures that contain both DNP-amino acids and DNP-peptides, the gradient system should consist of two parts. In the first gradient, which consists of 2, 2, 2, 10, 10 and 15% *tert.*-amyl alcohol in *n*-heptane in chambers 1–6, all DNP-amino acids up to DNP-serine are eluted, the amount poured into each chamber is 150 ml and the column, which is operated at room temperature in this instance, is eluted at a rate of 150 ml/h. The second gradient elutes soluble DNP-derivatives and DNP-peptides; it consists of 15% *tert.*-amyl alcohol in *n*-heptane (200 ml, chambers 1 and 2), 50% methyl ethyl ketone in *n*-heptane (183 ml, chambers 3 and 4) and pure methyl ethyl ketone (168 ml, chambers 5–9).

KESNER *et al.*[98] successfully applied this procedure to the separation of DNP-amino acids and peptides obtained by the action of pronase upon native, oxidized, dinitrophenylated and dinitrophenylated + oxidized ribonuclease. They introduced slight modifications to the above procedure. In the original procedure, 2,4-dinitroaniline is eluted closely to DNP-alanine; any confusion in this respect can be avoided by pre-extracting the mixture with diethyl ether at pH 8, which removes 2,4-dinitroaniline; alternatively, replacing 450 ml of *n*-heptane in the first mixing chamber with 450 ml of *n*-heptane–toluene makes the pre-extraction unnecessary. In the latter instance, the DNP-NH_2 peak is shifted towards the beginning of the chromatogram. In fact, any pre-extraction in this event is unnecessary as DNP-OH, DNP-NH_2 and unreacted 2,4-dinitrofluorobenzene are eluted prior to all amino acids. This buffer change is reported not to change the elution pattern of other DNP-amino acids.

Tris buffers, which are widely used in protein chemistry, are reported to cause a double peak of DNP-glutamine; however, this effect can be avoided by pre-extracting the sample to be analyzed with diethyl ether. It is also recommended that phosphate buffers are used instead of Tris, as they do not exhibit similar effects.

The hydration of the silica gel sorbent appears to be critical for the separation of some DNP-derivatives, such as bis-DNP-cystine from DNP-Glu and DNP-Asp. Failures to separate these DNP-amino acids are ascribed to the day-to-day variations in atmospheric humidity that interfere with the oven-drying procedure. Generally, to avoid such failures, it appears to be of great advantage to prepare large batches of partially hydrated silica gel. A suitable composition comprises 150 ml of deionized water mixed with 1000 g of dried silica gel and stored in air-tight bottles.

During drying, it is possible to use temperatures up to 250°, as silica gel loses water progressively with increasing temperature. However, satisfactory results have been achieved by drying silica gel at 115° for 1 week. This procedure results in a partially hydrated product, the water content of which is used as a basis for calculating the amount of 0.75 *N* sulphuric acid to be added. However, KESNER and co-workers[98,99] reported that even partially hydrated silica gel exhibits some variations with changes in atmospheric humidity. The optimum positioning of the di-DNP-derivatives relative to mono-DNP-derivatives is finally achieved by varying the final hydration of the silica gel. Different degrees of hydration also change the order of elution of amino acids. Thus, for instance, at high hydration (5.7 ml of 0.75 *N* H_2SO_4 per 8 g of silica gel), the order of the eluted peaks runs as follows: Tyr, Gly, Lys, Glu, Lys, Asp, Thr,

Ser, while at low hydration (4.7 ml of 0.75 *N* H_2SO_4 per 8 g of silica gel) the order is Gly, Tyr, Lys, Glu, Asp, Thr, Lys, Ser.

Another system has been described by TENTORI[188] for the automated column chromatography of DNP-derivatives, in which a 0.9 × 120 cm column of silica gel is used. Elution is carried out with a complex gradient system composed of nine chambers. The gradient is formed by different proportions of *tert.*-amyl alcohol, *n*-heptane and methyl ethyl ether as follows:

		tert.-Amyl alcohol	*n*-Heptane	Methyl ethyl ether
Chamber No.	1	5	95	
Chamber No.	2	6	94	
Chamber No.	3	7	93	
Chamber No.	4	10	90	
Chamber No.	5	15	75	8.5
Chamber No.	6	15	60	21
Chamber Nos.	7–9			84

The column was thermostatted at 47° and the running time was about 5.5 h. Before being admitted to the column, the eluant was passed through a saturating chamber containing 0.1 *M* H_2SO_4.

Besides the above mentioned gradient system, it is possible also to use the following system, with which the results obtained are comparable as far as the quality of separation is concerned:

		tert.-Amyl alcohol	*n*-Heptane	Methyl ethyl ether
Chamber No.	1	7.5	142.5	
Chamber No.	2	9	141	
Chamber No.	3	10.5	139.5	
Chamber No.	4	15	135	
Chamber No.	5	22.5	112.5	12.75
Chamber No.	6	10	70	61
Chamber Nos.	7–9			126

The column was maintained for the first 1.5 h at 47° and then at −38°. The separation time was 10 h.

The following method of filling the chambers permitted the separation of DNP-derivatives soluble in water at the operating temperature of 28° in 225 min:

	tert.-Amyl alcohol	*n*-Heptane	Methyl ethyl ether
Chamber Nos. 1–2	15	85	
Chamber Nos. 3–4		46	46
Chamber Nos. 5–9			84

(d) Separation on Kieselguhr columns

It appears that except for the elaborate automated analysis of DNP-amino acid derivatives on silica gel columns (see p. 237), all the other procedures suffer from considerable disadvantages, due mainly to the poor reproducibility of silica gel preparations. For this reason, Kieselguhr columns were introduced by MILLS[124]: acid-washed Kieselguhr is used in this separation. The sorbent is activated before use by shaking it

in a large bottle with a portion of ammonium carbonate enclosed in a cloth bag. The pH of an aqueous extract of this sorbent should lie within the range 6–7. Columns of dimensions 1.5 × 12 cm seem to give an adequate degree of separation. The solvent systems used are different mixtures of chloroform and methyl ethyl ketone (Table 5).

TABLE 5

ORDER OF ELUTION OF DNP-AMINO ACIDS FROM KIESELGUHR

DNP-amino acid	*Solvent*
Di-DNP-cysteine DNP-aspartic acid DNP-glutamic acid	methyl ethyl ketone
DNP-serine Di-DNP-histidine	75% methyl ethyl ketone–chloroform
DNP-methionine sulphone DNP-threonine DNP-glycine DNP-alanine DNP-proline Di-DNP-lysine Di-DNP-cystine DNP-valine DNP-leucine DNP-isoleucine DNP-phenylalanine Dinitrophenol	30% methyl ethyl ketone–chloroform to resolve DNP-threonine and DNP-glycine followed by 45% methyl ethyl ketone–chloroform

The column bed is prepared from 6.5 g of the sorbent compressed in 45% of methyl ethyl ketone–chloroform with a flat-bottomed stainless-steel rod. The optimum load is 2–3 μg of each amino acid band. The order of the eluted bands can be seen in Table 5.

Acid-soluble amino acid derivatives are removed first by extracting the sample into methyl ethyl ketone that has been acidified with 10% of 6 *N* hydrochloric acid. The residue after extraction is evaporated to dryness, dissolved in water-saturated methyl ethyl ketone and loaded into a column prepared with methyl ethyl ketone–chloroform (3:1).

The effect of column buffering is apparent from Table 6.

The buffers used are: 1 *M* sodium dihydrogen phosphate, pH 4.03; 0.25 *M* phosphate, pH 6.5; and 0.4 *M* phosphate, pH 7.0. Each buffer is ground (66% by weight) into dry Kieselguhr and a suspension in the solvent selected is used for preparing a column of dimensions 1 × 12 cm.

Similarly, the procedure can be used for preparative purposes by applying up to 350 μg of a protein to a 3 × 20 cm column[144].

(e) Separation on silicic acid–Celite columns

Silicic acid–Celite chromatography is one of the most rapid classical column procedures used for the separation of DNP-amino acid derivatives. GREEN AND KAY[71] reported the possibility of completing the analysis in less than 2 h. Silicic acid is commercially available, which is another advantage of the technique in addition to the good reproducibility of the results. Most of the commercially available silicic acid prep-

TABLE 6

BAND RATES FOR DNP-AMINO ACIDS ON BUFFERED CELITE 545 COLUMNS
Figures are values of R.

DNP-amino acid	*Ether*			*Chloroform*	
	pH 4	*pH 6.5*	*pH 7*	*pH 4*	*pH 7*
DNP-tryptophan	fast	fast	0.9	fast	—
DNP-isoleucine	fast	fast	0.8	fast	0.2
DNP-leucine	fast	fast	0.8	fast	0.2
DNP-phenylalanine	fast	fast	0.6	fast	0.15
Di-DNP-lysine	fast	fast	0.5	fast	—
DNP-valine	fast	0.7	0.3	fast	0.07
DNP-methionine	fast	0.3	0.2	fast	—
DNP-alanine	fast	0.1	0.07	fast	—
DNP-proline	fast	0.1	0.07	fast	—
Di-DNP-tyrosine	fast	fast	0.1	0.5	—
DNP-glycine	fast	0.05	0.02	0.5	—
DNP-threonine	1.0	slow	slow	0.07	—
DNP-glutamic acid	0.9	slow	slow	0.03	—
DNP-serine	0.6	slow	slow	slow	—
DNP-aspartic acid	0.5	slow	slow	slow	—

arations are suitable without any further pre-treatment. The only problem in the procedure is to ensure an adequate flow-rate, which is difficult to achieve with silicic acid only. The sorbent is therefore mixed with half of its weight of Celite and the fraction that passes a 60-mesh filter is used for column preparation. The column size recommended by GREEN AND KAY[71] is 1–1.4 × 17 cm. The adsorbent can be layered in the column by strong suction and it appears that clogging of the column does not occur. The surface of the column is levelled with a flat-bottomed glass rod and the time required for the preparation of the column takes not more than 30 sec. The top of the column is covered with a fine-mesh disc or a disc filter-paper, which it is usual to use in most of the column separations of DNP-amino acid derivatives to protect the top of the column from disturbances. The performance of the column is distinctly improved by pre-washing the column with redistilled solvents. It has been suggested that pre-washing modifies the water content of the adsorbent. From the experiments of SCHROEDER[174], it appears that the intensity of sorption increases in the following order: alcohol pre-washed adsorbent < non-pre-washed adsorbent < diethyl ether pre-washed adsorbent < acetone–diethyl ether pre-washed adsorbent.

In the procedure described by GREEN AND KAY[71], the columns are pre-washed in several steps. If a volume V equals the volume of the solvent that is just necessary to wet the adsorbent completely, then first a volume 0.2 V of diethyl ether, followed by 1 V of acetone–diethyl ether (1:1), 0.8 V of diethyl ether, 1 V of ligroin and finally 1 V of the developing mobile phase are passed through the column. The columns are operated under a constant under-pressure 70–80 mm. The sample is applied in an acetic acid solution diluted with ligroin. The over-all operating scheme for this procedure is rather complex and it is best understood from Fig. 8.

The original mixture of DNP-amino acid derivatives is eluted with a solvent consisting of 8 ml of acetic acid, 4 ml of acetone and sufficient ligroin to make the volume up to 100 ml. This solvent distributes the mixture into five fractions. The first

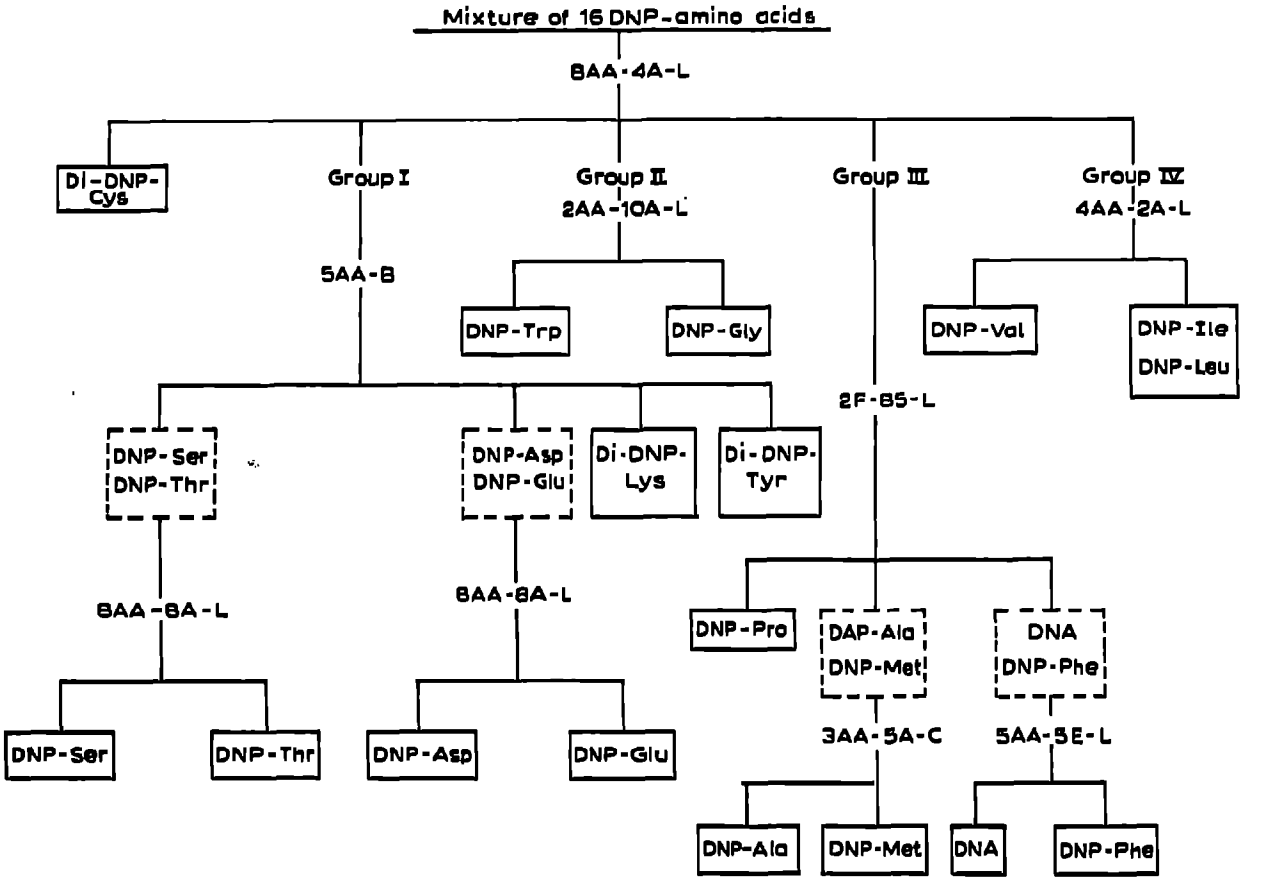


Fig. 8. Chromatographic separation of DNP-amino acids on silicic acid–Celite columns. Development scheme of GREEN AND KAY[71]. AA = acetic acid; A = acetone; L = ligroin; F = formic acid; E = ethyl acetate; B = benzene; C = cyclohexane; DNA = 2,4-dinitroaniline.

band is bis-DNP-Cys and need not be discussed further as it is a pure amino acid derivative. If fractions two and three are absent, the elution with the above solvent may be stopped while the solution is still on the column and it may be changed to further steps of the separation.

Band No. 2 is a very complex mixture, the further separation of which is carried out by using a system containing 5 ml of acetic acid and sufficient benzene to make the volume 100 ml. Kieselguhr–Celite chromatography separates four bands, which consist of:

(A) DNP-Ser and DNP-Thr: these amino acids are separated in a third chromatographic step by using a mixture consisting of 8 ml of acetic acid, 8 ml of acetone and sufficient ligroin to make the volume 100 ml. In this separation, DNP-Ser is the fastest moving band.

(B) The second band is a mixture of DNP-Asp and DNP-Glu. These two amino acids are separated with the same mobile phase as the Ser and Thr derivatives of the first band, *e.g.*, with a mobile phase consisting of 8 ml of acetic acid, 8 ml of acetone and sufficient ligroin to make the volume 100 ml.

(C) This band is bis-DNP-Lys and need not to be subjected to a further separation step.

(D) This band of the acetic acid–benzene step is pure bis-DNP-Tyr and hence it is unnecessary to subject it to any further separation procedure.

Band No. 3, the third zone of the original acetic acid–acetone–ligroin separation, is a mixture of DNP-derivatives of Trp and Gly. These two amino acids are separated on another column by eluting them with a mobile phase consisting of 2 ml of acetic acid, 10 ml of acetone and sufficient ligroin to make the volume 100 ml. The fastest moving band in this separation is that of DNP-Trp.

Band No. 4 is eluted from the original column only after the mixture of 2 ml of formic acid, 8 ml of ethanol and sufficient ligroin to make the volume 100 ml had been introduced. This mixture, again, is very complex, consisting of DNP-derivatives of four amino acids and DNP-OH. The formic acid solvent separates the whole mixture immediately into three zones: DNP-Pro, a mixture of DNP-Ala and DNP-Met, and a mixture of DNP-Ala and DNP-Phe. DNP-Ala is separated from DNP-Met by using another column eluted with a mobile phase consisting of 3 ml of acetic acid, 5 ml of acetone and sufficient ligroin to make the volume 100 ml. Under these conditions, the fastest moving band is that of DNP-Ala. The second complex zone, *i.e.*, the mixture of DNP-NH_2 and DNP-Phe, is separated by using a mixture of 5 ml of acetic acid, 5 ml of ethanol and sufficient ligroin to make the volume 100 ml. Under these conditions, the fastest moving band is that of dinitrophenylaniline while the slower band corresponds to DNP-Phe.

Band No. 5 is a mixture of three DNP-derivatives: DNP-Val, DNP-Leu and DNP-Ile. These amino acids are separated by using a mixture of 4 ml of acetic acid, 2 ml of acetone and sufficient ligroin to make the volume 100 ml. The fastest moving band in this system is that of DNP-Val; DNP-Leu and DNP-Ile move together.

Some DNP-peptides are adsorbed in the region of the second zone (the very complex one appearing in the original procedure immediately after DNP-Cys). Other artefacts have to be avoided in group IV, *i.e.*, in the zone that follows the mixed band of DNP-Pro and DNP-Gly. Besides this, DNP-Pro gives a very pale zone that can be easily overlooked. Also, the zones in the last group (DNP-Val, DNP-Leu and DNP-Ile) are considerably paler than those observed at the beginning of the separation. 2,4-Dinitrophenol, which gives a nearly colourless zone, is less strongly adsorbed in the first separating step and hence can be eluted very easily.

In conclusion, it should be said that the whole system must be studied very carefully if an unknown mixture is to be analysed, as mistakes are easily introduced. Automated analysis, which has been reported in detail earlier (see p. 237), nowadays offers the most reliable procedure for the determination and quantitation of DNP-amino acids, and therefore it is to be recommended. Spare parts of any commercial amino acid analyzer can be adapted for the automated analysis of DNP-derivatives, and therefore virtually every laboratory can apply this procedure for routine sequencing.

(f) Separation on nylon columns

JELLINEK AND DEL CARMEN-VARA[93] used non-stretched nylon 66 and Celite as sorbents for the separation of DNP-amino acid derivatives. The adsorbent was prepared by mixing two volumes of an aqueous solution of non-stretched nylon 66 with one volume of an aqueous suspension of Celite 545. Acetic acid–ammonium acetate and ammonia–ammonium acetate buffers of ionic strength 0.2 (the pH varied from 4 to 10)

were used as eluents, in some instances with the addition of ethanol. The actual separation was carried out at 22° with an elution rate of 1 ml per 8 min. The eluent was evaluated manually by O.D. readings.

(g) Automated separation on nylon columns

Recently, BEYER AND SCHENK[15–17] applied column chromatography on nylon powder columns in DNP-amino acid determinations. The procedure is particularly suitable for water-soluble DNP-derivatives and has been used successfully in the study of structural proteins such as keratin and collagen.

Preparation of nylon powder columns. A 3-l round-bottomed flask provided with a reflux condenser and containing 200 g of unstretched TiO_2-treated nylon fibers covered with 2 l of acetic acid and 100 ml of formic acid was placed on an oil-bath and heated at 180–190° for about 45 min. The turbid solution was then poured into a beaker and allowed to stand overnight. The upper hard layer was removed the next morning and the nylon suspension filtered off. The residue remaining on the filter was allowed to dry for three days and the dry powder was suspended in 1 l of distilled water and homogenized. The slurry was filtered off and the residue remaining on the filter was washed with distilled water until it was neutral. After re-suspending it, the material was screened through a 250-mesh sieve and the fraction that passed through was diluted to 2.5 l and stored at 4° until it was used for column pouring.

Columns of dimensions 50 × 1 cm appeared to be suitable for use in separations. The nylon powder suspensions were boiled before use to avoid the formation of bubbles and then cooled to 30°. At this temperature, the columns were poured with a 3-atm over-pressure to ensure rapid and homogeneous setting. Finally, the column was compressed with air and eluted twice with citrate buffer of pH 3.0. The buffer consisted of 298.5 ml of 1 *N* HCl, 42.33 g of citric acid and 16.12 g of NaOH. This mixture was diluted to 5 l with distilled water.

Operation and sample application. The hydrolysate (2 ml), containing about 200 mg of the sample, was applied to the column, observing the usual precautions. The sample was washed into the column with two 0.5-ml volumes of pH 3.0 buffer and the analysis was carried out using the same buffer at a flow-rate of 30 ml/h. When O-DNP-tyrosine had emerged from the column, the column was ready to be loaded with another sample. However, in practice, after two separations the column had to be regenerated by removing the nylon powder from the column and re-suspending it in 5% ammonia for 15 min. The alkaline suspension was then filtered and the residue washed until it was neutral. The cake was re-suspended in 0.1 *N* HCl, stirred for 15 min, filtered, washed until it was neutral and preserved in 2.5 l of distilled water. After another two runs it was discarded.

For the separation of ether-soluble DNP-amino acids, the chromatographic procedure is only slightly different. The preparation of the carrier material is identical with that described above. However, the columns are filled at 60° and the column is then eluted twice with phosphate buffer of pH 8.0. The buffer used for elution is prepared from 2.7 g of H_3PO_4 and 55 g of $Na_2HPO_4 \cdot 2H_2O$. This mixture was made up to 5 l and used for analysis.

Tracings of the chromatograms obtained with both the ether-soluble and the water-soluble amino acid derivatives on nylon powder columns are presented in Figs. 9 and 10.

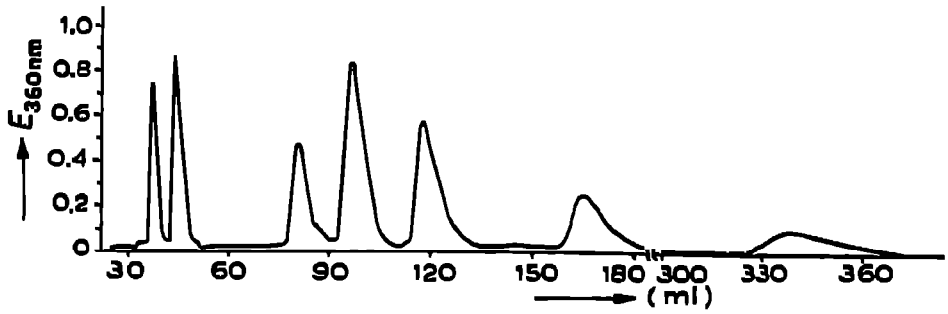


Fig. 9. Nylon powder chromatography of DNP-amino acids in a hydrolysate of DNP-wool. Peak identification, from the left: DNP-Asp, DNP-Glu, DNP-Ser, DNP-Thr, DNP-Gly, DNP-Ala, DNP-Val.

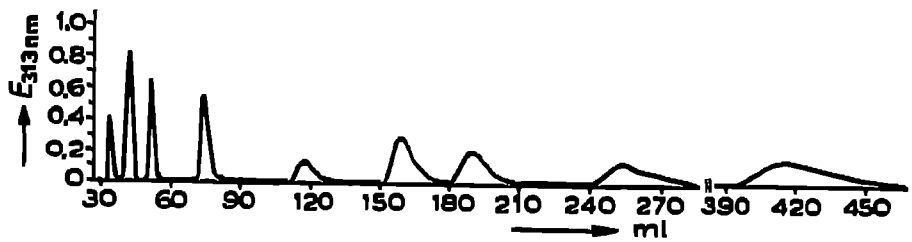


Fig. 10. Nylon powder column chromatography of water-soluble DNP-amino acids. Peak identification, from the left: DNP-His, DNP-Ser, N-DNP-(2-amino-2-carboxyethyl)lysine, DNP-Cys, DNP-Orn, DNP-Lys, DNP-Tyr, DNP-Arg, DNP-$CySO_3H$.

(h) Ion-exchange column chromatography

In column chromatographic techniques, ion exchange can also be used for the separation of water-soluble DNP-amino acids. A suitable procedure was introduced by HEINRICH AND BUGNA[78]. In this particular instance, the cation-exchange resin IRC-50 (Bio Rex 70) in the form of its sodium salt, —400 mesh, was used with considerable success. The column used was thermostatted at 50° and was 15–20 cm in length. The flow-rate applied was *ca.* 0.5 ml/min. The eluting buffer was similar to the "pH 5" buffer specified for the Technicon amino acid analyzer: 14.71 g of trisodium citrate dihydrate are dissolved in 900 ml of water and 25 ml of standardized 2 *N* NaOH are added together with 5.85 g of NaCl and 3 ml of Brij solution (100 g of BRIJ 35 plus 200 ml of water). The buffer is titrated with 6 *N* HCl to pH 5.0, diluted to 1 l and the pH value is adjusted if necessary. Finally, the buffer is de-aerated by heating it to 60°, and cooled. The column effluent is recorded by using a suitable device in the region of 360 nm. The order of amino acid derivatives released from the column (0.5–1 h apart) is α-DNP-Lys, ε-DNP-Lys, DNP-Arg, bis-DNP-His, DNP-Trp and bis-DNP-Lys. The lower limits of this method are 0.01–0.05 μmole provided that standard Technicon equipment has been used.

Excellent results in the ion-exchange chromatography of water-soluble DNP-amino acids were achieved by NISHIKAWA *et al.*[136], using Dowex 50W-X2. The chromatography of DNP-amino acids on Dowex 50-W resins is rather difficult owing to strong non-electrostatic interactions between the dinitrophenyl moiety of the amino acid derivatives and the aromatic constituents of the resin. These interactions result in highly diffused peaks, which are difficult to integrate. A simple approach to avoid these interactions was to introduce aromatic compounds into the eluting buffers;

p-hydroxybenzoic acid gave excellent results in this respect. A Dowex 50-W column of dimensions 0.9 × 29 cm used in the Beckman Spinco amino acid analyzer was applied. Type 15 A resin filled to a height of 11 cm was used and the elution of the column was carried out in stages. Two buffers were used. Buffer I solution was 0.117 *M* in citrate, 0.1 *M* in *p*-hydroxybenzoic acid and 0.411 *M* in final sodium ion concentration at pH 5.28. Buffer II solution was 0.117 *M* in citrate, 0.4 *M* in *p*-hydroxybenzoic acid and 0.69 *M* in sodium ion at pH 6.0.

The column was operated at 54° using a flow-rate of 60 ml/h while the ninhydrin flow-rate was 30 ml/h. The sample was loaded in the usual way and the column was eluted with buffer I for 60 min, and then this was replaced by buffer II. The yellow colour, together with the ninhydrin-positive reactions of amino acids, were recorded. The optimal resolution was obtained if the amount separated was 0.5 μmole per peak. The following order of maxima appeared on the chromatogram: the bulk of DNP-derivatives of neutral and acidic amino acids, Im-DNP-histidine, di-DNP-lysine, DNP-histidine, DNP-NH_2, ε-DNP-lysine, DNP-arginine and O-DNP-tyrosine.

(i) Liquid–liquid partition of DNP-amino acids (Hyflo-Super-Cel chromatography)

DNP-amino acid derivatives are partitioned between the organic and aqueous phases provided that they are undissociated; pH values affect the over-all partition coefficient, as shown by MATHESON[117–119] and MATHESON AND SHELTAWY[120], by changing the equilibrium in the aqueous layer. However, according to MATHESON[117], in principal any of the ether-soluble DNP-amino acids can be partitioned between the aqueous buffers and ethyl acetate in the ionic form. If ionized, the bands of DNP-amino acids are exceptionally narrow and the *R* values are only slightly pH-dependent.

For the liquid–liquid partition chromatography of DNP-amino acids, the following procedure was developed and described by MATHESON AND SHELTAWY[120]. The chromatographic column has dimensions 19 × 1 cm; ethyl acetate and the appropriate buffer (200 and 40 ml, respectively) are shaken together and the resulting phases are separated (the buffer used contains 35 g/l of NaCl). Hyflo-Super-Cel (4 g) is slurried in the upper phase and 2.5 ml of the lower phase are added dropwise. The mixture is shaken vigorously until free from lumps, and the sample is loaded by using a filter-paper disc. All operations involving DNP-derivatives should be carried out in artificial light. The sample is transferred to the top of the column in the minimum volume of water-saturated ethyl acetate. The top of the column is rinsed twice with 1-ml volumes of the water-ethyl acetate solvent and the column is eluted with the following buffers (flow-rate 1 ml/min):

(a) 0.1 *M* Tris–maleic acid–salt buffer, pH 5.4
(b) 0.1 *M* sodium phosphate–salt buffer, pH 7.4
(c) 0.1 *M* Tris–HCl–salt buffer, pH 7.4
(d) 0.1 *M* sodium phosphate buffer, pH 12

Optimum results are obtained in mixtures containing about 1.5 mg of each amino acid present in the form of the mono-DNP-derivative, while the optimum amount for bis-DNP-derivatives is 2–3 mg. A special problem in this type of column chromatography is the separation of DNP-serine and DNP-lysine in complicated mixtures; for this purpose, the Tris–glycine–urea–salt buffer, 6 *N* in urea (the pH of 9.5, however, is not critical) was used by MATHESON AND SHELTAWY[120].

For the column partition of DNP-derivatives of methionine, phenylalanine, ornithine and lysine, buffers consisting of Tris–HCl, 2-amino-2-methylpropane-1,3-diol hydrochloride and alternatively glycine–NaOH were used by MATHESON[117–119]. For water-soluble amino acid derivatives, 0.2 *M* Tris–0.2 *M* glycine buffer of pH 9.1 gives satisfactory results; alternatively, methyl ethyl ketone–ethyl acetate–0.2 *M* Tris + 0.2 *M* glycine buffer (3:2:1 by volume) can be recommended for this purpose.

The sample can also be applied to a column equilibrated with the buffer of low pH, which makes it possible to resolve the slowly moving DNP-derivatives; the fast moving derivatives are eluted and the eluate is applied to another column equilibrated to a higher pH value. It is usually necessary to repeat the process several times until complete separation of all components is achieved. The separation procedure reported by PORTUGAL *et al.*[153] for the separation of glutamic acid and aspartic acid derivatives is, in principal, the same as that reported above.

(j) Reversed-phase chromatography on chlorinated rubber (PARTRIDGE AND SWAIN[130])

The chlorinated rubber (150–200 mesh) was prepared by shaking it with a suspension of *n*-butanol (4 ml per 10 g of chlorinated rubber) in 0.2 *M* citrate–phosphate buffer that had been previously saturated with butanol. The slurry obtained was used for packing the columns by filtering under slightly reduced pressure. The column was eluted with *n*-butanol at three different pH values (3, 4 and 5). The best results were obtained at pH 3, where DNP-Lys, DNP-Asp-NH_2, DNP-Ser, DNP-Asp, DNP-Gly, DNP-Ala, DNP-Pro, DNP-Val and DNP-Leu can be separated (amino acid derivatives are ordered according to the decreasing *R* value). With increasing pH, the fastest moving bands of DNP-amino acids, such as DNP-Lys, DNP-Asp-NH_2 and DNP-Ser, pass through the column virtually with the void volume.

(k) Countercurrent chromatography

The wide applicability of the dinitrophenylation procedure is apparently the reason for the number of chromatographic techniques that are available nowadays for the separation of these derivatives. One of the most recent is the so-called countercurrent chromatography, introduced by ITO AND BOWMAN[89,90]. This technique is essentially liquid–liquid partition without a solid support. The authors describe three different variants of this technique, as follows.

(i) Helix countercurrent chromatography (Figs. 11 and 12). In principle, partitioning occurs in a horizontal helical tube filled with one phase of a two-phase liquid system. The other phase is fed into this coil through one end and passes through the first phase owing to the vertical direction of flow. Segments of the two phases along the helical tube are thus formed. Continued flow may cause displacement of the stationary (first) phase. A liquid–liquid partitioning system is therefore established, although a number of practical problems must be overcome if this procedure is to be of analytical use. The coil must comprise several thousands of turns, the diameter of the tube being less then 1 mm. In order to prevent a situation in which injection of the second phase will simply push the other phase through the helix, it is necessary to increase the gravitational flow by applying a centrifugal force. In the centrifugal field, each phase then moves through each segemt of the other phase and forms alternate segments of phases. Hence the moving phase is capable of moving through trapped

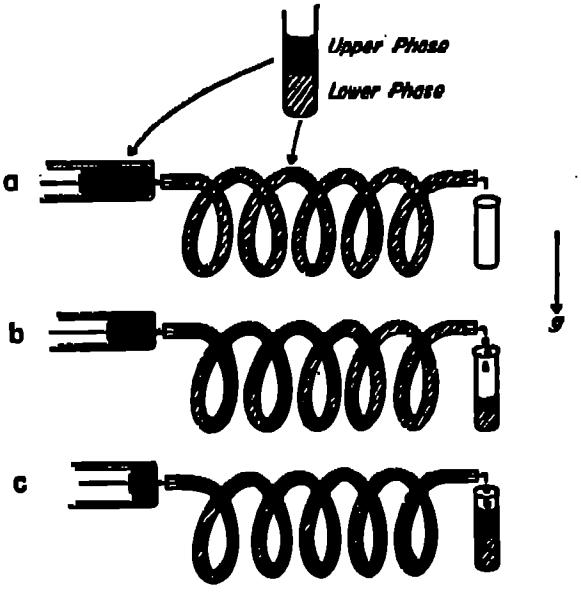


Fig. 11. Model of helix-countercurrent chromatography.

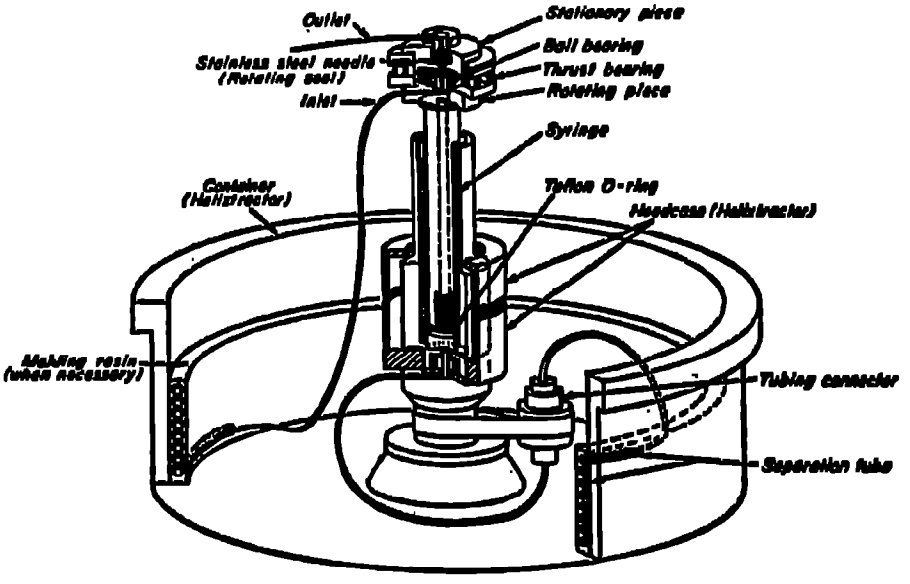


Fig. 12. Diagram of the centrifuge head.

segments of the stationary phase. Of course, it is necessary to maintain the hydrodynamic conditions constant, which implies also that the centrifugal force is constant within very narrow limits to prevent mixing of phase plates. An accurate and constant flow should be provided so as to feed the liquid into a rotating column against the high pressure. When the flow pressure, which increases with increasing g, is established, the number of revolutions must be maintained constant. The equipment used for this purpose consists of a centrifugal head in which the separation tube is supported at its periphery. The separating coil is fed from a coaxially rotating syringe. The usual pressures introduced vary between 16–20 atm. Effluent from the helix is collected from the rotating system through a rotating seal. The tubing used by ITO AND

BOWMAN[80,00] was of 0.2 mm I.D. and two methods of arranging the tubing in the centrifuge head were suggested. In the first method, the tubing is wound tightly on a flexible rod support, which is subsequently coiled in a number of turns around the inside of the centrifuge head. Alternatively, the tubing is folded into two and twisted along its length in a rope-like manner. In this second instance, each individual strand has an appearance of a stretched helix with a small diameter. The coil is wound around a drum support that fits into the centrifuge head.

The phase system used for the separation of DNP-amino acids was chloroform–acetic acid–0.1 *N* HCl (2:2:1) equilibrated at room temperature. DNP-amino acids were dissolved in a portion of the lower phase in a concentration of 1% (or saturated solutions in the lower phase when the solubility did not reach 1%). The separation coil was filled with the stationary phase and 5 μl of the sample to be analysed were introduced into the proximal end of the coil. Then, 2–3 μl of chloroform were aspirated to compensate for evaporation losses. The syringe was filled with the moving phase and centrifugation was carried out at 900–950 r.p.m. Flow rates varied between 125 and 820 μl/h at room temperature. Fractions comprising eight drops were collected. Each fraction was mixed with 3 ml of ethanol and the optical density at 350 nm was measured. Typical chromatograms obtained are shown in Fig. 13a, in which is seen

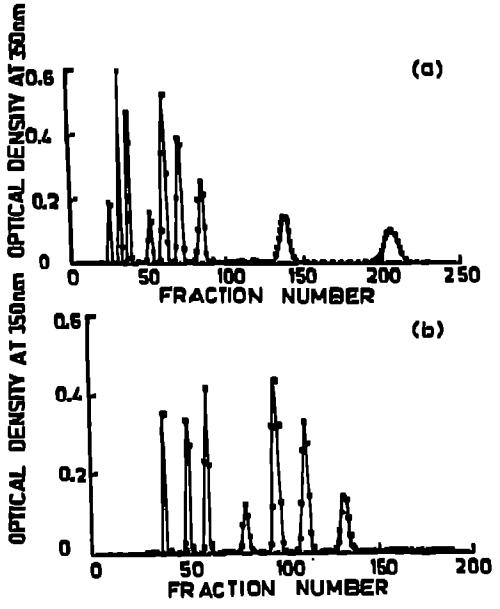


Fig. 13. Result of separation in helix countercurrent chromatography. (a) Coiled column; (b) twisted column. Peak identification, from the left: DNP-Orn, DNP-Asp, DNP-Glu, DNP-CysH, DNP-β-Ala, DNP-Ala, DNP-Pro, DNP-Val, DNP-Leu (valine and leucine derivatives are missing in the (b) version).

the separation of nine amino acid derivatives obtained on a coiled column having 8000 turns with a helical diameter of 0.85 mm and prepared from a 40-m length of PTFE tubing of 0.2 mm I.D. Fig. 13b shows the separation of seven amino acid derivatives on a twisted column having 17,000 turns prepared from an 80-m length of PTFE tubing of 0.2 mm I.D. In both instances the flow-rate was 0.125 μl/h. The pressure used in the first separation was 16 atm overpressure, while it reached 20 atm overpressure in the second.

(ii) Droplet countercurrent chromatography. This technique was introduced by TANIMURA *et al.*[187] and was developed for practical use by ITO AND BOWMAN[80,90]. In principle, the system is based on the observation that a light phase with low surface affinity forms discrete droplets that rise through the more dense phase with distinct interfacial motion. ITO AND BOWMAN[80,90] suggest that under ideal conditions each fluid bubble may be considered to be a plate. The procedure is preferably used for preparative purposes. The system consists of long (20–60 cm) columns of narrow-bore silanized glass tubing with fine capillary tubes to interconnect with wide-bore glass tubes. Discrete droplets at the tips of the finer tube inserted into the bottom of the long glass tube were made to follow one another with a minimal space between and a diameter similar to that of the internal bore of the column. These droplets divide the column into discrete segments that prevent longitudinal diffusion occurring along the length of the columns as they mix locally near equilibrium. The fine PTFE tubing interconnecting the individual columns preserves the integrity of the proces with minimum diffusion and helps to form new droplets at the bottom of the next column.

The equipment used by ITO AND BOWMAN[80,90] consisted of 300 glass tubes 60 cm long and 1.8 mm I.D. The total capacity of the system used was the same as in the previous instance, using chloroform–acetic acid–0.1 *N* HCl (2:2:1). The operational overpressure was 150 p.s.i. at a flow-rate of 16 ml/h. It appears that several hundred milligrams of DNP-amino acids can be separated within 80 h.

(iii) Locular countercurrent chromatography (ITO AND BOWMAN[80,90]). The apparatus used for this particular technique is shown schematically in Fig. 14. Liquid–liquid partition occurs in a column that is inclined at an angle to the horizontal. The column consists of multiple segments of PTFE tubing; longitudinal diffusion of the solute is prevented by centrally perforated partitions spaced across the tube. Circular stirring is ensured by rotating the column. At the beginning of the separation procedure, the column is filled with the lower phase and the upper phase is fed into the column through the first rotating seal connection while the column is set into motion. The upper phase displaces the lower phase in each segment down to the hole that communicates with the next segment. Further feeding of the lower phase results in displacement of the upper phase only, thus leaving the appropriate amount of the lower phase in each segment of the column. Solute fed into the column is therefore subjected

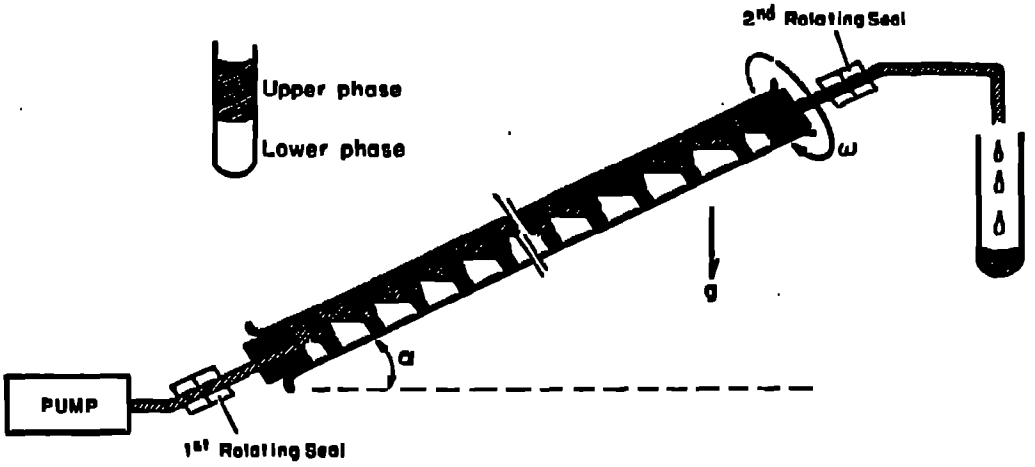


Fig. 14. Mechanism of rotation locular countercurrent chromatography.

to a number of partition steps along the column and is finally collected from the second rotating seal at the top of the device. Essentially, separation occurs by gravity while rotation of the columns ensures adequate mixing.

In the actual apparatus described by ITO AND BOWMAN[80,00], PTFE rings of 2.8 mm O.D., 0.8 mm I.D. and 1 mm thickness, or alternatively 4.8 mm O.D., 1.2 mm I.D. and 1.2 mm thickness, and spaced into PTFE tubing of 5.1 mm O.D. and 4.6 mm I.D., each 47 cm long, at every 3-mm interval to make over 100 locules, were used. In each column, 10–50 such units were mounted side-by-side in an appropriate holder. Connections between the individual column units and between the column units and rotating seals were made of PTFE tubing. The rotation of the column was adjusted to 300 r.p.m. The flow of liquids through the column was controlled by an appropriate pump (Beckman accu-flow pump) or by a syringe driver at a rate of 2–40 ml/h. Finally, the effluent was monitored by a UV monitor. The solvent systems used were the same as those used in the previous arrangements for countercurrent chromatography, namely chloroform–glacial acetic acid–0.1 *N* HCl (2:2:1). DNP-amino acid derivatives were dissolved in a portion of the upper phase so as to make the concentration of each component 0.5–1% (or saturated solutions if the solubility of a particular derivative is lower). Before the operation, the whole system was filled with the stationary phase and 10–40 μl of the sample solution were aspirated into the column. Fractions of 32 drops were collected, diluted with 3 ml of 95% ethanol and the optical density was measured at 350 mm.

The result of a typical separation is shown in Fig. 15. The column consisted of 5000 locules (2.6 mm diameter, 3 mm long) and a total capacity of 100 ml including the dead space of the column, which was estimated to be 5%. The column was rotated at 180 r.p.m. at an angle of 30° to the horizontal. The upper phase was pumped at a flow-rate of 5 ml/h with a Beckman Accu-Flow pump. The maximum pressure during the operation did not exceed 20 p.s.i. The efficiency was of the order of 3000 theoretical plates, which resulted in a 50% efficiency in each locule. The elution time for the first peak was 11 h and for the last peak 68 h.

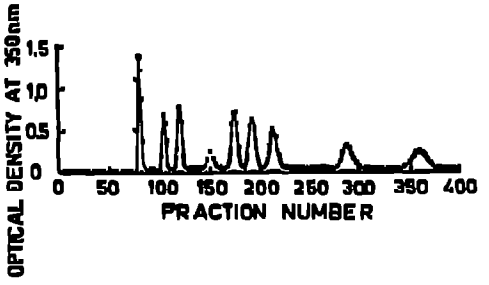


Fig. 15. Result of separation of DNP-amino acid derivatives in locular countercurrent chromatography. Peak identification, from the left: DNP-Arg, DNP-Asp, DNP-Glu, di-DNP-CysH, DNP-β-Ala, DNP-Ala, DNP-Pro, DNP-Val, DNP-Leu. Column efficiency, 3000 theoretical plates; total elution time, 70 h.

According to the experience of ITO AND BOWMAN[80,00], the general efficiency of the procedure increases with an increase in the column length, a decrease in the locular length and a decrease in the flow-rate. For a particular column, the efficiency increases with the angular velocity of rotation up to 180 r.p.m. It has been observed

that a higher speed of rotation fails to improve the separation, probably owing to the cancellation of the gravitational separation by centrifugal force.

The main advantage of this procedure lies in the possibility of using virtually any pair of solvents, without any special demands upon their physical properties, which also means that the procedure is applicable not only to the particular problem of the separation of DNP-amino acids.

ITO AND BOWMAN[80,00] also suggested another variation of this technique, namely gyration locular countercurrent chromatography, and this technique was successfully applied to the separation of DNP-amino acids. Separation occurs in a column constructed in a similar manner as in the previous instance; instead of being rotated, however, the column is gyrated with a fixed excentricity (1.24 cm). The rotation of the holding plate is continuously maintained at 800 r.p.m. As indicated in Fig. 16, the successive positions of one locule as it is gyrated provide a steady change in the direction of the centrifugal force, which prevents the effect of separation and collapse due to increasing centrifugal force. Hence the over-all efficiency of the whole system might be further increased.

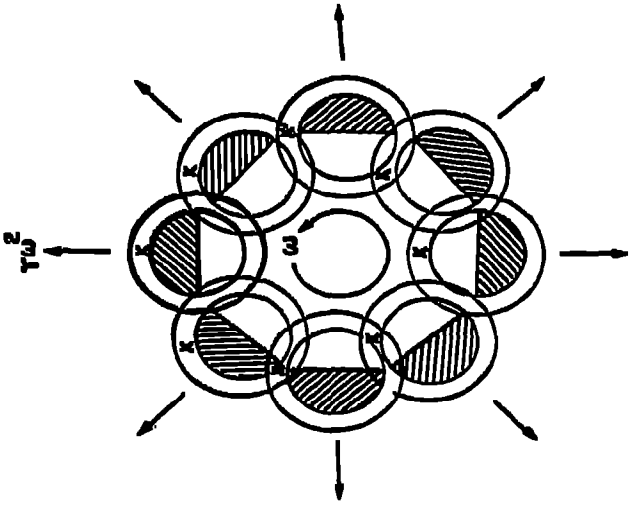


Fig. 16. Mechanism of gyration locular countercurrent chromatography. Gyration effect upon liquid interface in locule on cross-section of successive positions of the same locule.

The column is held in a vertical position and is operated with the same solvent system that was used in the above separations, *i.e.*, chloroform–glacial acetic acid–0.1 *N* HCl (2:2:1). The application of the sample and the operation of the apparatus are, in general, the same as in the above procedure. The column is run at a flow-rate of 20 ml/h with a gyration speed of 500 r.p.m. The efficiency of the system (short-column) was 900 theoretical plates. The efficiency of an individual locule was more than 80%, which is greater than in the previous procedure.

A very useful description of the principle of the countercurrent chromatography of DNP-amino acids is presented in a recent review paper by ITO AND BOWMAN[00].

6. Gas chromatography

During the past few years, a number of papers have appeared that deal with the

possibilities of gas–liquid chromatography in amino acid analysis. Numerous derivatives were prepared, of which N-acyl esters were particularly useful. Acylation considerably increased the volatility of amino acids and the running times for gas chromatographic separations became of the order of 60 min. The other advantage of using gas–liquid chromatography is the extreme sensitivity of detection, which implies that very small amounts of material are needed for the sequential analysis. Thus, for instance, highly sensitive ionizing detectors can detect 10^{-12} mole sec^{-1} of a particular amino acid derivative, which, according to LANDOWNE AND LIPSKY[106], means that 10^{-8}–10^{-10} mole is a sufficient amount for sequencing and analysis. Even higher sensitivity can be achieved by using an electron capture detector. However, the practical application of this method of detection had the disadvantage of the difficulty in finding a votatile amino acid derivative with an electrophoric group that exhibited a high affinity for free electrons. Thus, N-chloroacetylamino acids were suggested for this purpose. On the other hand, it had been noted when working with this particular detection system that a number of chromophoric groups are strongly electrophobic. DNP-amino acid derivatives are an excellent example of this phenomenon.

The first workers to successfully approach the problem of the gas chromatographic separation of DNP-derivatives were PISANO *et al.*[148]. These and other workers used methylsilicone polymer SE-30[107], fluoroalkylsilicone polymer QF-1[106] and phenylsilicone polymer[183] as the stationary phases. Using temperatures in the range 175–200° they observed retention times indicated in Table 7. The DNP derivatives were separated as the methyl esters. Satisfactory results were obtained for derivatives of the simple neutral and acidic amino acids. Serine, threonine, tryptophan, tyrosine and histidine underwent decomposition and did not give satisfactory results. Also, basic amino acids did not chromatograph well.

LANDOWNE AND LIPSKY[106] also used the methyl esters of DNP-amino acids, as the need for increasing the volatility of the derivatives was important. They suggested the following procedure.

Commercially available DNP-amino acids were converted into the corresponding methyl esters by reaction with ethereal diazomethane. If the compound to be esterified was poorly soluble under the reaction conditions, which was indicated by the slow evolution of nitrogen, some methanol was added to the reaction mixture to make the reaction proceed more rapidly. The compounds were diluted in methanol to a known concentration after being evaporated to dryness under nitrogen to remove excess diazomethane and diethyl ether. Samples of the methyl esters were applied to the chromatographic column in the conventional manner.

The DNP-amino acid methyl esters were chromatographed at 220°, the injection temperature being 275–285°. The most convenient stationary phase proved to be neopentenyl adipate or neopentenyl sebacate methyl esters; 3% of the stationary phase was coated on to 90–100 mesh Anakron ABS (Analabs, Hamden, Conn., U.S.A.). The standard 1-cm plate detector was used for identification. Sensivitities were determined at an optimum potential of 14 V applied to the detector, which was maintained at 200°. The flow-rate was 200 ml/min of argon. Significantly, the minimum detectable amounts of the various DNP-amino acids were of the same order of magnitude, namely 3×10^{-16} moles/min. The relative standard deviation was 13%.

In conclusion, one must agree with the three main advantages of this technique as outlined by LANDOWNE AND LIPSKY[106], namely: (1) the extreme sensitivity of the

TABLE 7

RETENTION TIMES OF DNP DERIVATIVES OF AMINO ACIDS

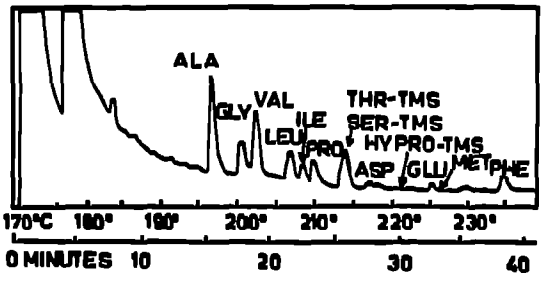
Amino acid	*1.5% SE-30*		*1.0% XE-61*		*1.5% XE-60*
	Isothermal	*Programmed*	*Isothermal*	*Programmed*	*Isothermal*
Alanine	5.4	15.7 (197°)	8.8	13.8 (198°)	8.05
Glycine	6.25	17.9 (201°)	12.6	17.1 (203°)	13.7
Valine	7.0	19.0 (203°)	10.45	14.9 (200°)	8.05
Leucine	8.65	21.8 (207°)	12.6	16.4 (202°)	9.5
Isoleucine	9.2	22.7 (209°)	13.4	17.25 (203°)	9.9
Proline	9.65	23.4 (210°)	22.4	21.5 (211°)	14.9
Threonine-TMS	11.2	25.9 (214°)	15.4	18.75 (206°)	11.3
Serine-TMS	11.2	25.9 (214°)	17.7	19.35 (207°)	13.2
Aspartic acid	12.65	27.8 (217.5°)	28.8	24.0 (215°)	26.2
Hydroxyproline-TMS	15.35	30.1 (221°)	26.0	22.9 (213°)	17.0
Glutamic acid	18.1	32.7 (225°)	43.3	28.0 (222°)	36.7
Methionine	20.1	33.6 (227°)	48.5	29.4 (224°)	36.9
Phenylalanine	28.35	38.5 (230°)	70.5	34.5 (232.5°)	45.0
α-Aminobutyric acid	4.85		6.7		5.7
β-Aminobutyric acid	8.35		15.4		14.25
γ-Aminobutyric acid	12.0		25.5		24.0
p-Methylphenylalanine		41.9 (230°)		37.3 (237°)	
Column	1.75 m × 4 mm	4.0 m × 3 mm	1.8 m × 4 mm	4.0 m × 3 mm	1.0 m × 4 mm
Column temperature	204°	170–230° at 1.7°/min	185°	170–240° at 1.7°/min	210°
N_2 flow-rate (ml/min)	45	25	38	30	20
Inlet pressure (p.s.i.)	30	36	27	40	30

procedure; (2) the uniformity of the response towards all DNP-amino acid derivatives; and (3) the inherent selectivity of the detector that eliminates interference from impurities that would otherwise be a source of considerable problems in a technique of such high sensitivity.

The original procedure described above has been successfully used in practice: ISHII AND WITKOP[87,88] applied this procedure with slight modifications to the identification of the amino acid sequence in gramicidin A. In their procedure, gas chromatographic analyses were carried out on a 60-1A compact chromatograph analysis unit of the Research Specialities Co., equipped with a flame ionization detector. As the stationary phase 1% SE-30 coated on Gas-Chrom P (100–200 mesh) was used. The column used was of stainless-steel, 180 cm long and I.D. 3 mm. During the analysis, the column was maintained at 175°. The flow-rate of the carrier gas (nitrogen) was 10 ml/min.

More recently another alternative, the application of trimethylsilyl (TMS) derivatives of DNP-amino acids, has been described[83], which offers further possibilities in gas chromatographic separations. The separation was carried out on a Shimidazu GC-1C chromatograph equipped with a hydrogen flame ionization detector, a Model TP-2A temperature programmer and a dual column and differential flame system. The column was of the usual U-type and was made of glass. The column parameters are summarized in Table 7. The column packings were prepared according to HORNING *et al.*[92]: 1.5% SE-30 (methylsilicone gum, General Electric Co.), 1.5% XE-60 (nitrile-silicone, General Electric Co), 1.0% XE-61 (phenylmethylsilicone polymer containing 35 mole% of phenyl groups, General Electric Co.), 1% SE-52 (phenyl methyl siloxane, General Electric Co.) and 1.5% QF-1 (fluorinated alkylsilicone, Dow Chemical Co.). Shimalite W (Shimadzu Co.), 80–100 mesh, was used as the support after acid washing and silanization. The retention times of trimethylsilyl DNP-amino acid derivatives are given in Table 7 and Figs. 17–19.

The results can be summarized as follows. While derivatives of alanine, glycine, valine, leucine, isoleucine, proline, aspartic acid, glutamic acid, methionine and phenylalanine can easily be separated by this technique as the methyl esters, the derivatives of threonine, serine and hydroxyproline could be separated only after trimethylsilanization; the methyl esters were prepared in a manner similar to the original procedure of LANDOWNE AND LIPSKY[106], by using diazomethane in methanol. Trimethyl silyl ethers were prepared by the method of SWEELEY *et al.*[186]. It has also been verified that trimethylsilylation gave a virtually quantatitive yield.



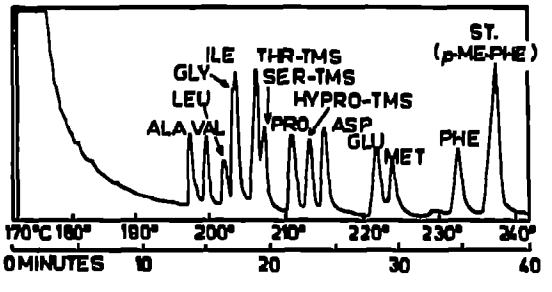


Fig. 17. Analysis of free amino acids in serum as their DNP-derivatives on a 1.5% SE-30 column.

Fig. 18. Gas chromatographic separation of a mixture of 13 DNP-amino acids on a 1% XE-61 column.

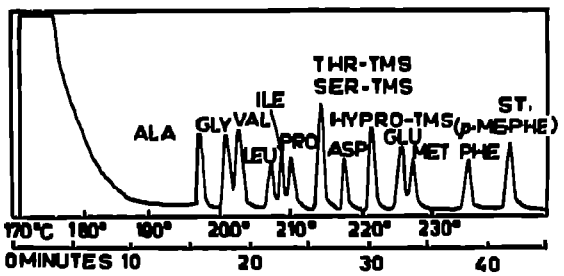


Fig. 19. Gas chromatographic separation of a mixture of 13 DNP-amino acids on a 1.5% SE-30 column.

The corresponding methyl ester was placed in a small test-tube and dissolved in 0.5 ml of dry pyridine, then 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane were added. The test-tube was stoppered tightly and shaken vigorously for 30 sec. Then the tube was allowed to stand for 20 min and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in tetrahydrofuran and a 10-μl aliquot was applied to the column.

A comparison of the stationary phases tested gave the following results. Although all the systems described above are fully applicable to amino acid analysis, the best results obtained were with XE-60 and SE-30. The separation on SE-30 was, however, unsatisfactory for TMS-threonine and TMS-serine. On the other hand, both TMS-threonine and TMS-serine derivatives were separated well on XE-60 and XE-61, but when using XE-61 the separation of glycine and leucine was not satisfactory. The use of temperature programming resulted in a further improvement of the separation, especially with SE-30 and XE-61. Retention times are summarized in Table 8a.

FALES AND PISANO[56] reported the possibility of separating the DNP derivatives of Tyr and Trp on short columns.

TABLE 8a

RELATIVE RETENTION TIMES OF DNP DERIVATIVES OF AMINO ACIDS

Column packing, 1% coating of the liquid phase on 100–140 mesh Gas-Chrom P; 6 ft. glass coils or U-tubes, 3.4–5.0 mm I.D.; 0.1–0.2 atm argon; Lovelock argon ionization detection system. (For experimental details see ref. 183.)

Amino acid	*SE-30*	*QF-1*	*PhSi*
	175°	*194°*	*200°*
Alanine	1.93	2.33	0.44
Glycine	2.24	3.05	0.67
Proline	3.76	4.15	1.16
Valine	2.63	2.67	0.49
Leucine	3.23	3.30	0.56
Isoleucine	3.61	3.37	0.62
Androstane	1.00	—	—
Cholestane	—	1.00	1.00
	202°		
Aspartic acid	5.14	6.95	1.47
Glutamic acid	7.88	10.8	2.01
Methionine	8.80	6.77	2.32
Phenylalanine	12.4	11.6	3.94
Androstane	1.00	—	—
Cholestane	—	1.00	1.00

The peak area per microgram, peak area per 10^{-8} mole and relative molar peak area with *p*-methylphenylalanine as the internal standard for each amino acid analysed are summarized in Table 8b. IKEKAWA *et al.*[83] suggested the alternative use of an *ε*-aminocaproic acid derivative as the internal standard; the peak of the *ε*-aminocaproic acid derivative appears between those of methionine and phenylalanine.

TABLE 8b

DETECTOR RESPONSE OF DNP AMINO ACID METHYL ESTERS BY FLAME IONIZATION[a] AND DETERMINATION OF FREE AMINO ACIDS IN SERUM

Amino acid	*Peak area* ($cm^2/\mu g$)	*Peak area* ($cm^2/10^{-8}$ *mole*)	*R.M.P.A.*[b] (*p-Me-Phe*)	*Amino acids in serum*[c] (*mg/100 ml*)
Alanine	1.73	4.44	1.00	7.1
Glycine	1.17	2.84	0.64	6.2
Valine	2.53	7.23	1.63	3.4
Leucine	2.27	6.79	1.53	1.8
Isoleucine	2.20	6.56	1.48	1.4
Proline	2.27	6.43	1.45	1.5
Threonine-TMS	1.70	4.89	1.10	4.1
Serine-TMS	1.70	4.64	1.04	
Aspartic acid	1.10	3.31	0.74	0.8
Hydroxyproline-TMS	1.40	4.19	0.94	(0.1)
Glutamic acid	1.33	4.18	0.94	1.3
Methionine	1.32	4.17	0.94	0.2
Phenylalanine	1.56	5.20	1.17	2.1
p-Methylphenylalanine	1.28	4.44	1.00	
Cholesterol	3.25	12.52		

[a] A temperature programming system with a 1.5% SE-30 column, 4 m × 3 mm, was used.

[b] Relative molar peak area = $\frac{\text{DNP-amino acid molar response}}{p\text{-Methylphenylalanine molar response}}$.

[c] From a 20-year old normal male.

The relative peak areas could vary to some extent, depending on the over-all peak appearance. According to IKEKAWA *et al.*[83], even when the same packing is used, differences in the temperature rise might result in slight variations in the relative peak areas. Hence considerable attention must be paid to temperature programming.

The above procedure of IKEKAWA *et al.*[83] was originally developed for the routine determination of amino acids in serum, and there is no reason why it could not be used in the determination of N-terminal amino acids. The authors, however, state that they do not have any experience with amino acids other than the thirteen reported, which might raise some problems in routine sequencing and N-terminal analysis. It is, of course, possible to combine gas chromatography with flat-bed techniques, as was done by LANDOWNE AND LIPSKY[106]. In this instance, similarly to the double-checking technique (see Part A of this review), the possibility of introducing mistakes into the sequencing analysis is decreased to the absolute minimum. The advantage of the classical concept of double checking is, of course, precise quantitation, and the disadvantage is the necessity of using rather complex equipment, which is not to be recommended for a single analysis.

7. Paper chromatography

The paper chromatography of DNP-amino acids has been described in numerous classical chromatographic papers, which will be briefly summarized here. Since the pioneering work of BISERTE AND OSTEUX[20] and LEVY[100], DNP-derivatives have been divided according to their solubility into those which are ether-soluble and those which are water-soluble. Both of these groups are dealt with separately in most chromatographic separations.

(a) Ether-soluble DNP-amino acids

In the paper chromatography of ether-soluble DNP-amino acids, toluene and phosphate solvents[20,100] are probably the most frequently used, even now. Both of these solvents are used for two-dimensional identification of the DNP-amino acids in such a way that the first run is carried out in toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonia (30:9:18:18) and this is followed by development of the chromatogram in 1.5 *M* phosphate buffer of pH 6.0. The second run in the original version of the separation was ascending, but there is little difference compared with normal two-dimensional descending separation. This type of two-dimensional chromatography allows the separation of most of the ether-soluble DNP-amino acids, the exceptions being the derivatives of aspartic and glutamic acids; this difficulty can, however, be overcome by using a more concentrated buffer in the second run (2.5 *M* phosphate buffer of the same pH). The development is carried out in this event for 48 h, using a full-size sheet of Whatman No. 1 filter-paper.

The results of the two-dimensional chromatography of DNP-amino acid derivatives on paper are usually not defined in terms of the R_F values, as the R_F values vary to a certain extent, especially in the toluene solvent system, However, the relative positions of individual spots are quite characteristic and the slight variations in R_F values are therefore not a great disadvantage in this method. The reasons why the R_F values in the toluene system vary were discussed in great detail in the classical review by BISERTE *et al.*[10]. It appears that variations in paper size, moisture content in the atmosphere and the ageing phenomenon observed with the toluene solvent influence the final mobilities of individual spots.

Instead of using the toluene solvent in the first dimension, it is also possible to use various systems containing ammonia–*n*-butanol. BRAUNITZER[27] (see also refs. 40 and 100) recommended the use of *n*-butanol saturated with 0.1% ammonia, while others prefer the system propanol–0.2% ammonia (75:25). Both of these systems give quite satisfactory results. Slight variations have also been reported with regard to the development of chromatograms in the second direction. While LEVY[100] and KOCH AND WEIDEL[100] used 1.5 *M* phosphate buffer of pH 6, a more dilute buffer (0.75 *M*) was recommended by DAVIES AND HARRIS[40].

Besides these most frequently used systems, there are, of course, others that can be also used successfully for the separation of ether-soluble DNP-amino acid derivatives. PHILLIPS[145] has described two-dimensional chromatography on Whatman No. 7 filter-paper. 2-Butanol saturated with 0.05 *M* phthalate buffer of pH 6 was used in the first direction while 1.5 *M* phthalate buffer of pH 6 was used in the second run. The paper was pre-impregnated with 0.05 *M* phthalate buffer before the chromato-

alcohol saturated with 0.05 *M* phthalate buffer of pH 6. The alcoholic component can be also replaced by methyl-2-butanol. The second run is carried out with routine development in 1.5 *M* phthalate buffer of pH 6. In this instance also, Whatman No. 7 filter-paper pre-impregnated with 0.05 *M* phthalate buffer of pH 6 before the chromatography was used. Obviously, there are no special demands in this technique with respect to the type of chromatographic paper used, as BISERTE *et al.*[10] reported that Whatman No. 3MM or No. 4 filter-papers are also satisfactory.

To summarize, it can be said that the over-all shape of the individual type of chromatograms is generally the same, whichever solvent system is used in the first dimension. BISERTE *et al.*[10] thus concluded that the separation in the first run is not due only to partition. The mechanism of the separation in the second run is based on salting-out effects. The main differences observed with the above combinations of solvent systems are not those in the positions of individual DNP-amino acid derivatives but mainly those related to the positions of the 2,4-dinitrophenol spots. With the toluene phosphate system, 2,4-dinitrophenol appears close to DNP-Gly and DNP-Ala. With the butanol–ammonia–phthalate and *sec.*-butanol–phosphate systems, the DNP-OH spot moves to the region of DNP-Phe, DNP-Val and DNP-Met. The latter is less convenient as this region of the chromatogram is more populated with other amino acid derivatives. Of course, these problems can be avoided if 2,4-dinitrophenol is removed by sublimation before chromatography. In any event, the original system of toluene–phosphate appears to be generally the most suitable in separating DNP-amino acid mixtures.

As with all chromatographic separations of different compounds, one can meet combinations of derivatives that require special attention in the choice of the solvent system. For these special purposes, a number of solvent systems have been described, and these are briefly described in the following paragraphs.

PARTRIDGE AND DAVIS[138] recommended the system water–benzene–acetic acid in the ratio 1:1:1 for the separation of those derivatives which, in the toluene–phosphate system, exhibit low mobilities. This system gives excellent separation of DNP-aspartic acid, DNP-glutamic acid, DNP-serine and perhaps DNP-cysteic acid. Three alternative systems for a similar purpose were suggested by MELLON *et al.*[121], namely *n*-butanol saturated with water, *n*-butanol–*n*-butyl acetate–1% ammonia (1:2:3), and benzene + 1% of acetic acid.

The system recommended by WILLIAMSON AND PASSMANN[205] is very suitable for the separation of DNP-leucine and DNP-isoleucine, *i.e.*, isooctane–ethylene chlorohydrin–*n*-propanol (20:1:1). For the separation of excess dinitrophenol, the system decalin–acetic acid (1:1) was suggested by BISERTE AND OSTEUX[20]. Dinitrophenylated polymers of amino acids are separated in *n*-propanol (diluted with water to the final specific gravity of 0.813)–acetic acid (containing 1.5% of water)–kerosene (boiling range 100–140°) (20:6:100). In this instance, the paper is impregnated with 0.1 *M* citric acid before use[77].

Other systems do not exhibit any advantages over those already mentioned and are listed here for the sake of completeness. Xylene–acetic acid–0.5 *M* phthalate buffer of pH 6 (10:5:4) was used by LANDMANN *et al.*[105]; the paper was impregnated with the same buffer and then equilibrated with the lower layer 16 h before commencement of the development. *n*-Butanol–ethanol–water (40:10:50) was used by KENT *et al.*[97]; benzyl alcohol containing 10% of ethanol saturated with 0.05 *M* phthalate

buffer of pH 6 and also propanol–cyclohexane or light petroleum (boiling range 100–120°) (30:70) saturated with 0.05 *M* phthalate buffer of pH 6 by BLACKBURN AND LOWTHER[22]; and 1 *M* sodium citrate or 0.7 *M* hydrochloric acid buffer of pH 6.2 by ROVERY AND FABRE[164] and DESNUELLE AND FABRE[41]. The results with the latter buffer are comparable with those obtained with 1.5 *M* phosphate buffer of pH 6 by LEVY[100]. *tert.*-Amyl alcohol containing 10% of 2-propanol saturated with phthalate buffer was used by GREGORY AND YOUNG (unpublished work, quoted by WALEY[198]). SANGER AND THOMPSON[171] used chloroform–1.5 *M* acetic acid–*n*-propanol (10:6:10); this system is recommended for reversed-phase chromatography on silicone-impregnated paper. The paper is saturated with vapours of the organic phase for at least 3 h before chromatography and development is carried out with the aqueous phase. Bis-DNP-Tyr and bis-DNP-Lys do not move from the starting line while other amino acids exhibit rather high mobilities. The variability of the R_F values is high under these conditions and reliable identification is possible only by comparing the results with those for an internal standard.

Other systems used include chloroform–2-propanol–0.05 *M* potassium benzoate (45:49:6) or alternatively cyclohexane–2-propanol–0.05 *M* potassium benzoate (60:36:4)[126], *n*-amyl alcohol saturated with 2 *M* ammonia[25], and *n*-amyl alcohol–methyl ethyl ketone–potassium benzoate (54:40:6)[125].

In the final treatment of a chromatogram one may, especially in sequence analysis, meet the problem of eluting the ether-soluble DNP-amino acid derivatives from the paper. This can easily be carried out by extracting the cut-off spots with 2% sodium bicarbonate solution, preferably in a centrifuge for 15 min. The extractant is preheated to 50–55°. After cooling and acidification with dilute hydrochloric acid, the DNP-amino acids are extracted with peroxide-free diethyl ether. The ethereal solution is extracted with 2–3 ml of 2% sodium bicarbonate to eliminate all traces of the solvent used in the first development. After acidification, the second bicarbonate solution is extracted with diethyl ether. The ethereal solution (2–3 ml) can be evaporated to dryness in the presence of anhydrous sodium sulphate. After re-dissolving them, the samples of DNP-amino acids can be used for further chromatography using the procedure described by BISERTE *et al.*[10].

The problem of incomplete resolution and overlapping of spots has already been discussed to some extent with regard to the individual solvent systems used. However, we shall now approach the same question by enumerating pairs that present some problems from the separational point of view. DNP-aspartic and DNP-glutamic acids comprise the only pair that is not separated by two-dimensional chromatography. BISERTE *et al.*[10] stated that this problem could be solved by a second two-dimensional chromatography in which the second run is carried out with a phosphate buffer with a higher phosphate concentration (2.5 *M*). In this instance, the first separation is carried out using the *n*-butanol–ammonia system. The only reason for this preference is the higher volatility of this solvent system compared with the usual toluene mixture, which enables the whole analysis to proceed more rapidly. Of course, there is no objection to eluting the combined spot of DNP-aspartic and DNP-glutamic acids and running these separately only in the high-concentration phosphate buffer or in the isoamyl alcohol system saturated with 1% acetic acid, as described by BISERTE AND OSTEUX[20]. Another combination of spots that may sometimes interfere is DNP-histidine and DNP-tryptophan. In the classical version of the toluene–phosphate sys-

tem, these two spots appear in the same position. However, it must be realized that during hydrolysis, DNP-tryptophan is destroyed and DNP-histidine does not accompany ether-soluble DNP-derivatives provided that the diethyl ether–ethyl acetate extraction has been carried out. In the toluene–phosphate system, DNP-serine and DNP-methionine sulphone also overlap. Actually, this event occurs only when methionine is oxidized to methionine sulphone, which takes place only in instances when the diethyl ether used for extraction has not been freed from peroxides. If the presence of DNP-methionine is suspected, the zone of DNP-serine and DNP-threonine can be chromatographed again in the system *tert.*-amyl alcohol–phthalate buffer, as described by BLACKBURN AND LOWTHER[22]. Dinitrophenyl derivatives of diamino acids, *e.g.*, bis-DNP-lysine, bis-DNP-ornithine and bis-DNP-diaminobutyric acid, are not separated in the classical version with the toluene–phthalate buffer system. However, the bis-DNP-derivatives of lysine and ornithine are clearly separated in the *n*-butanol–acetic acid–water (4:1:5) system. The identification of these derivatives is always difficult, and it is necessary to regenerate the parent amino acids and to subject them to routine amino acid analysis, as no direct determination of the derivative form is reliable. This is also the situation in the separation of DNP-leucines, which must also be converted into their parent amino acids to obtain a reliable determination.

The following procedure has been recommended by BISERTE *et al.*[10] for the DNP-derivative free amino acid conversion. The sample is heated in a sealed tube at 105° for 1 h with 0.3 *M* $Ba(OH)_2$. $Ba(OH)_2$ is removed by passing carbon dioxide through the sample, evaporating the filtrate to dryness, re-dissolving the residue and subjecting the resulting solution to an appropriate identification procedure (free amino acid chromatography). Proline also presents some problems in DNP-amino acid chromatography. The decomposition of proline upon hydrolysis and formation of α-chloro-δ-DNP-aminovaleric acid and δ-chloro-α-DNP-aminovaleric acid[173] leads to the occurrence of two additional spots on the chromatograms. It is sometimes convenient to identify proline in the N-terminal position by these artefacts[145]. Their chromatographic separation either by the classical toluene–phosphate system or by the *sec.*-butanol–phthalate system is reported to be easy. If the chromatogram is exposed for a long period to UV light, the coloration of the proline spot becomes pink.

Besides DNP-OH, another spot frequently occurs in DNP-amino acid derivative chromatography, namely, that due to 2,4-dinitroaniline. In paper chromatographic separation with the solvent systems already described, the presence of this compound is not a disadvantage, as in all instances it moves far away from other amino acid derivatives. Another artefact that may appear is picric acid, which may interfere with the spot of DNP-phenylalanine as they move very close together. An exhaustive review of other possible artefacts was published by REDFIELD AND ANFINSEN[167]. Some of these artefacts were identified as being decomposition products of lysine, while the others were characterized only by their chromatographic mobilities and spectral parameters and no information is currently available on their chemical nature.

(b) Water-soluble DNP-amino acids

The chromatographic separation of water-soluble DNP-amino acids on paper

may be carried out in various solvent systems, some of which are the same as those recommended for the ether-soluble fraction.

Acid–butanol systems are widely applicable for this purpose. Thus, good separations are achieved in *n*-butanol–water–acetic acid (4:1:5)[178] or in *n*-butanol–acetic acid–water (5:1:1)[86]. Good results are also obtained with *n*-butanol–formic acid–water (75:50:10)[8,116] and with *n*-butanol–acetic acid–water in a different ratio (250:60:250), as described by WOIWOD[307]. In all instances, however, the R_F values are hardly suitable for identification and external standards must be run simultaneously in order to avoid confusing results.

Besides *n*-butanol-based systems, phenolic solvents are equally suitable for separating the water-soluble portion of DNP-amino acid derivatives. Thus, good results were obtained with phenol saturated with water in an atmosphere of 3% ammonia and hydrocyanic acid[10].

In two-dimensional chromatography, a butanolic system is recommended for use in the first direction followed by *m*-cresol–phenol–pH 9.3 buffer (25:25:7)[10] in the second run.

Of systems suitable for the ether-soluble DNP-amino acid derivatives, the *tert.*-amyl alcohol–pH 6 phthalate buffer system or the classical toluene–phosphate system are applicable. With the latter system, the separation of DNP-Arg and DNP Lys (monoderivatives) is incomplete.

Besides a complete separation of water-soluble DNP-amino acid derivatives, it is possible to apply a slightly different approach, which is based on a simplification of the mixture before analysis. For instance, DNP-arginine can be eliminated by chromatography of the whole mixture on a column of talc. The eluate from the talc column, which contains the water-soluble dinitrophenyl derivatives, is treated with fluorodinitrobenzene[165]. Under these condition, ε-mono-DNP-lysine is converted into bis-DNP-lysine and O-DNP-tyrosine into N,O-bis-DNP-tyrosine, and the DNP-derivative of arginine remains unchanged. After the second dinitrophenylation, the extraction of the residue removes bis-DNP-lysine and bis-DNP-tyrosine, while the aqueous phase contains bis-DNP-histidine and α-mono-DNP-arginine. The aqueous phase is subjected to chromatography in *n*-butanol–acetic acid–water (4:1:5) and identification of arginine is completed by the Sakaguchi reaction.

Another procedure has been described by BAILEY[7]. In this procedure, ε-mono-DNP-lysine is converted into ε-mono-DNP-α-methoxycarbonyllysine, which is ether-soluble. The actual procedure used is as follows. After the ether-soluble amino acids have been extracted, the aqueous phase is evaporated to dryness, the residue is dissolved in 0.1 *N* hydrochloric acid and the solution obtained is passed through a talc column. The water-soluble amino acids are eluted with ethanol–1 *N* hydrochloric acid (4:1), and the residue obtained after evaporation is dissolved in 2–3 ml of sodium bicarbonate–sodium carbonate mixture of pH 8.9 (20 ml of 10% sodium bicarbonate and 5 ml of 10% sodium carbonate). The solution is maintained at 20° and four 0.02-ml volumes of methoxycarbonylchloride are added at 10-min intervals with vigorous shaking. The mixture is acidified with hydrochloric acid and extracted four times with diethyl ether to eliminate the corresponding derivative of lysine. The aqueous phase is subjected to chromatographic separation, which may be similar to that described in the previous instance of mixture simplification.

It is also recommended that a special procedure for histidine is applied. For this

purpose, however, it is impossible to use the same technique as for arginine, because during the second dinitrophenylation all the histidine residues, regardless of their positions within a peptide chain, may be converted into bis-DNP-histidine. In order to prevent this problem, it is possible to use continuous ether extraction as described by MILLS[124], or extraction with ethyl acetate. The final identification is carried out with the help of additional colour tests, such as the Pauly or ninhydrin reactions. BISERTE *et al.*[10] state that the identification of histidine may not necessarily always be successful.

As with the chromatography of ether-soluble DNP-derivatives, the occurrence of some artefacts may also cause erroneous results with water-soluble DNP derivatives. Thus, BAILEY[7] observed the presence of a brownish spot that moved in the neighbourhood of arginine, which may be removed by passing the sample over a small column of acidified silica. In this instance, methyl ethyl ketone is recommended as the eluant.

Another spot appears near cysteic acid. The chromatographic behaviour of these artefacts was extensively studied by THOMPSON[100–103].

Chromatographic maps of DNP-amino acids in most of the systems mentioned and in many not mentioned in the text are collected together in the chapter "Pictorial survey of flat-bed techniques used in DNP-amino acid chromatography" (see p. 270).

8. Thin-layer chromatography

The basic approach to the chromatographic separation of DNP-amino acids on thin layers of silica gel is analogous to that in other flat-bed techniques. All possible derivatives of DNP-amino acids are divided into those which are ether-extractable and those which remain in the aqueous phase. A basic study of the TLC separation of DNP-amino acids was published by BRENNER *et al.*[98]. Unlike in paper chromatographic separation, in thin-layer chromatography it is possible to separate most of the derivatives in a single run. Water-soluble amino acid derivatives, namely, α-DNP-Arg, ε-DNP-Lys, α-DNP-His, bis-DNP-His, O-DNP-Tyr, DNP-$(Cys)_2$ and DNP-$CySO_3H$, are separated in the propanol–ammonia (7:3) system[98]. DNP-Arg can be distinguished from ε-DNP-Lys by ninhydrin detection, in which ε-DNP-Lys changes colour from yellow to brownish. During this detection, O-DNP-Tyr, which is normally colourless, turns violet. The components of the pair of DNP-derivatives of Cys and $CySO_3H$, which exhibit identical mobilities in the above system, virtually do not occur side-by-side in natural material.

In a more recent modification by WALZ *et al.*[109], the two-dimensional arrangement was successfully applied. In the first direction the chromatogram is developed three times in pure pyridine followed by butanol saturated with ammonia in the second direction.

Ether-soluble DNP-amino acid derivatives are recommended for use in separations by two-dimensional chromatography[98]. A slightly modified toluene system described by BISERTE AND OSTEUX[20] appears to be the best for use in the first direction: chromatoplates are developed in toluene–ethylene chlorohydrin–pyridine–0.8 *N* ammonia (10:6:3:6); it is recommended that 197 mg of octyl alcohol are added to the mixture per 150 ml of toluene. In the second direction, development is continued in any of the solvent systems that contain chloroform or benzene, such as benzene–

pyridine–acetic acid (40:10:1) or chloroform–benzyl alcohol–acetic acid (70:30:3). In general, it can be said that most of the organic solvent systems that are suitable for paper chromatography are also applicable to thin-layer chromatography using silica gel plates. The R_F values vary considerably, which may cause some difficulties in identification. For instance, in the two-dimensional arrangement described above, R_F values depend on the nature of the first run: if the toluene system is applied, the positions of spots, although similar in general appearance, differ considerably from those obtained with other systems applied in the first run. With the system benzene–pyridine–acetic acid (40:10:1) the overflow technique usually has to be used. This holds also for the system chloroform–methanol–acetic acid (95:5:1). In order to obtain reproducible results, it is recommended that a parallel external standard containing about 0.2 mg of each amino acid derivative is used and that a standard procedure for developing the first run in two-dimensional separation and also standard handling and drying procedures are used.

In addition to the separate chromatography of water- and ether-soluble DNP-amino acid derivatives, thin-layer chromatography offers the possibility of almost complete separation of all these derivatives in a two-dimensional separation. For this purpose the modified toluene system described above is recommended for use in the first direction, followed by development in benzene–pyridine–acetic acid (40:10:1). In the second run, which takes about 3 h (overflow), even a mixture such as DNP-Leu, DNP-Ile, DNP-Norleu, DNP-Val and DNP-Norval is separated, which with all other types of amino acid derivatives is rather unusual. The only pair that is not separated under the specified conditions is bis-DNP-Lys and bis-DNP-Tyr. However, this pair is well separated in the system chloroform–methanol–acetic acid (95:5:1). The latter separation is carried out by the overflow technique and the Tyr and Lys derivatives are separated within 3 h. The frequently discussed system benzene–pyridine–acetic acid (40:10:1) gives good separations, although it has one disadvantage, namely that DNP-OH is located in the middle of the chromatogram, in an area that is densely populated with spots of amino acid derivatives, which may be obscured if an excess of DNP-OH is present. Hence the removal of DNP-OH before carrying out thin-layer chromatography in this system is desirable. On the other hand, in chloroform–methanol–acetic acid (95:5:1), 2,4-dinitrophenol moves with the solvent front in a manner similar to that in the chloroform–*tert.*-amyl alcohol–acetic acid (70:30:3) system.

BRENNER *et al.*[28] recommend that the following standard procedure is used in the first run with the toluene system. The chromatographic jar is lined on the inside walls with paper strips that reach to its bottom. A thick grid-like rod is put on the bottom and the aqueous phase of the toluene system is poured over it. The solvent starts to ascend on the paper strips. Two plates without samples are located on the middle of the V-shaped grid, the jar is covered and the system is allowed to equilibrate overnight. It is impossible to load the samples before equilibration, which is the usual practice in paper chromatography, as the spots would become very diffuse. After equilibration, the layer is taken out and covered with a clean glass plate, apart from a 1.5-cm broad edge path on which 1-μl portions of samples are applied. The glass plate cover is then removed and the thin-layer plate is placed in a jar with the organic phase of the toluene system. Provided that the whole operation does not take more than 5 min, the equilibrium disturbances are negligible.

After the chromatogram has been developed, the layer of silica gel is dried in an air stream at room temperature and when it appears to be dry it is heated at 60° for 10 min. Prolonged heating of the layer should be avoided, as otherwise the degradation of methionine may occur, which would result in methionine sulphone, a spot that would coincide with that of histidine.

All the solvents used should be purified before use. An exhaustive survey of the necessary purification procedures has been published[88].

The addition of octyl alcohol to the classical toluene solvent system suggested by MUNIER AND SARRAZIN[120] results in the formation of more compact spots. Of other recommended solvent systems, at least some should be mentioned. MUNIER AND SARRAZIN[120] used ammonium sulphate (saturated solution)–water–sodium dodecyl sulphate (100 ml:700 ml:0.576 g) in the second run in two-dimensional chromatography. Alternatively, BRIJ (0.6 g) can be used instead of sodium dodecyl sulphate. This system, together with the toluene solvent system in the first run, is extremely suitable for ether-soluble DNP-derivatives using cellulose layers. Before chromatography, it is recommended that the cellulose layer is impregnated by spraying it with 0.2 *M* sodium acetate in 60% ethanol followed by rapid drying at 90°. For water-soluble DNP-amino acids, thin-layer electrophoresis is recommended as the second step in a two-dimensional arrangement in which the toluene system has been used in the first separation. For electrophoresis, the silica gel layer is sprayed with 0.033 *M* diethylamine in 0.02 *M* NaCl. Electrophoresis is carried out for 30 min at 385 V and 9.5 mA with a standard 20 × 20 cm sheet.

Glass paper impregnated with silica gel is another material that has been used for the flat-bed separations of DNP-amino acids by DESSAUER *et al.*[42]. The results are reported to be better than those obtained with the traditional version of thin-layer silica gel chromatography. In this particular instance, the whole reaction mixture after dinitrophenylation can be applied, omitting the isolation of the DNP derivatives. The following solvent systems are suitable for use in development: diethyl ether–acetic acid–water in the ratio 100:1:1, 100:3:3 or 100:5:5; and diethyl ether–methanol–7 *M* ammonia in the ratio 100:6:2, 100:8:3 or 100:10:4.

Finally, polyamide is also a suitable material for use in the thin-layer chromatography of DNP-amino acid derivatives. The following systems were recommended for use in development by WANG AND HUANG[200]: methanol–formic acid (9:1), ethanol–water (63:37), ethanol–acetic acid–dimethylformamide–water (4:2:1:6), *n*-butanol–acetic acid (9:1), and chloroform–methyl ethyl ketone–formic acid (7:3:1).

(a) Detection

The application of reagents with DNP-amino acids is not necessary, as the yellow colour of DNP-amino acids is sufficiently intense that 0.1 μg can be made visible in transmitted visible light. The amount is slightly higher in two-dimensional chromatography, being 0.5 μg of each DNP-amino acid. The spots fade within a few hours, however, so that some workers recommended that the chromatogram is copied before it is photographed for documentation purposes in transmitted UV light at 360 nm. In this event, it is recommended that the photographic paper is situated with the sensitive layer adjacent to the chromatographic layer and that the system is exposed to light for a few minutes. DNP-amino acids appear as bleached spots on a dark background. The highest sensitivity is achieved when the background is not allowed to

darken completely. The limits of detection in this technique are about $2 \cdot 10^{-3}$ μmole for two-dimensional separations or about $1 \cdot 10^{-4}$ μmole in one-dimensional chromatography, which represents about 0.02 μg of a separated amino acid derivative.

The layer can be overloaded to a substantial extent: it can easily tolerate up to 100 μg of a DNP-derivative, and it is possible to detect about 0.05% of admixed DNP-amino acid.

(b) Quantitation

Several papers have dealt with the quantitation of DNP-amino acid derivatives in commonly used solvent systems. The layer is scraped off the plate and extracted for 5 min with 1 ml of 0.05 *M* Tris buffer of pH 8.6 at room temperature. Then the slurry is centrifuged off and the clear liquid is evaluated by measuring the optical density at 360 nm, or at 385 nm for DNP-proline. For a blank, a similar extract obtained from the same layer on a clean spot is taken.

A similar procedure to that described above was reported by FIGGE[60], who used a two-dimensional separation in the systems isopropanol–cyclohexane–dimethylformamide–25% ammonia (11:8:5:1) and *n*-butanol saturated with 25% ammonia, with silica gel as sorbent. Both systems are used twice in the same direction.

PATAKI AND WANG[143] recommended the use of direct fluorimetric quantitation (fluorescence quenching) *in situ*. Polyamide or Silica Gel G layers were used as sorbents and chloroform–benzyl alcohol–acetic acid (70:30:3) and *n*-propanol–ammonia (7:3) were used as the developing systems for the Silica Gel G layers. In polyamide layers, the chromatograms were developed with benzene–acetic acid (4:1).

The spots were scanned by using a Camag/Turner Scanner after being dried in a stream of air. The drying period was exactly 30 min and the direction of scanning was perpendicular to the direction of development. The scanning speed was 20 mm/min and the excitation wavelength was 254 nm. During quantitation, the layer was covered, except for a small strip of 12–14 mm that contained the spot under investigation, with a plain silica gel or polyamide sheet. The fluorimeter units were recorded generally at a potential of 10 mV with a recorder speed of 8 cm/min. The technique of fluorescence quenching published previously by PATAKI AND STRASKY[142] is a less elaborate and less precise variation of the above technique.

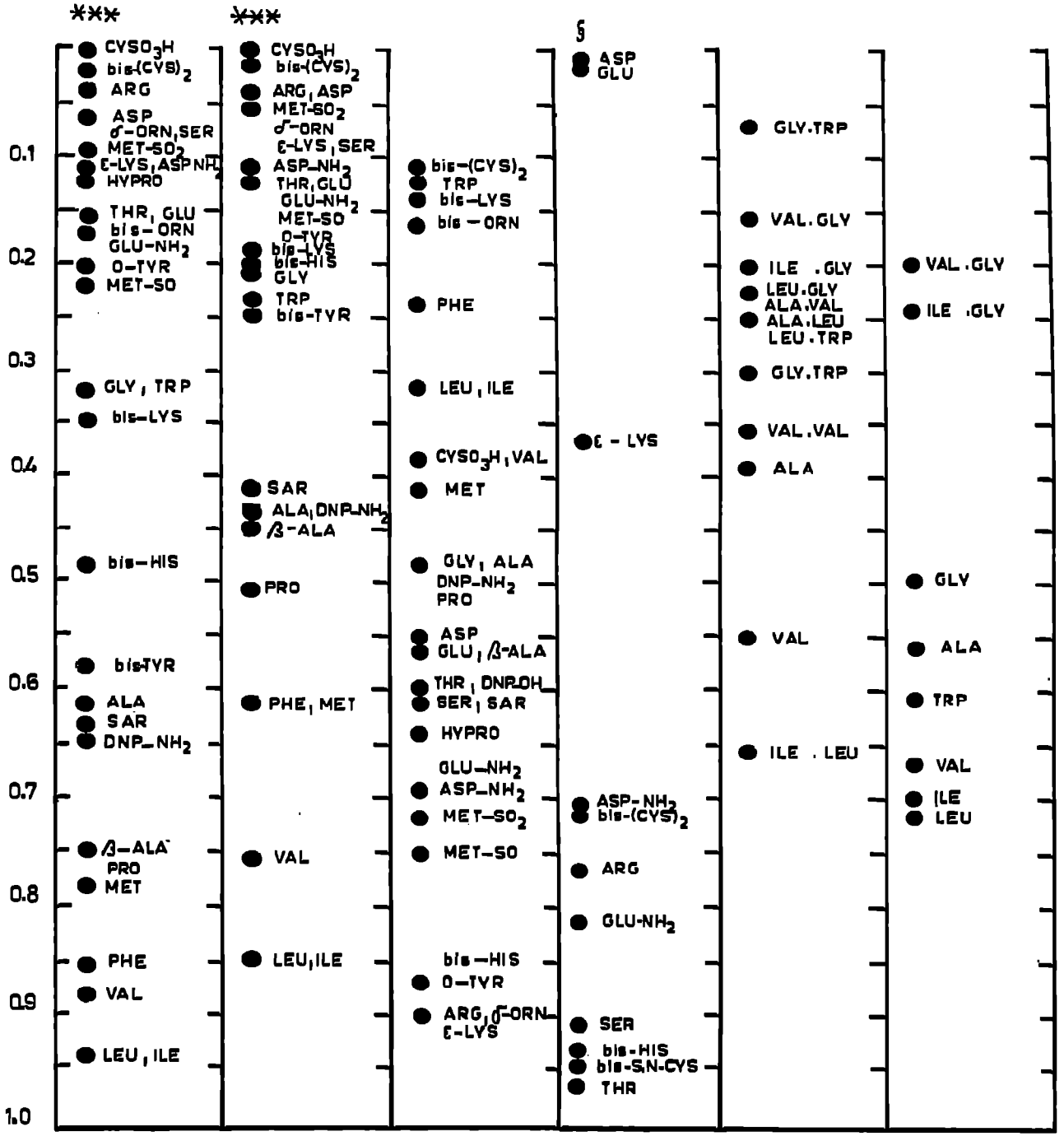
9. Electrophoresis

The electrophoretic separation of DNP-amino acids is always used in combination with chromatography; further details are given in the chapter "Pictorial survey of flat-bed techniques used in DNP-amino acid chromatography" (see below).

10. Pictorial survey of flat-bed techniques used in DNP-amino acid chromatography

This is illustrated in Figs. 20–67. The numbers, used in Figs. 33–67, to identify amino acid derivatives are listed on p. 335.

BENZENE 8 ACETIC ACID 2	CCl_4 8 ACETIC ACID 2	90% FORMIC ACID 1 WATER 1	DIETHYL ETHER 80 METHANOL 5 7M AMMONIA 2	TOLUENE 80 PYRIDINE 10 ACETIC ACID 1 **	BENZENE 3 ACETIC ACID 1 **
WANG AND WANG	WANG AND WANG	WANG AND WANG	DESSAUER et al.	WITKOP	WITKOP
POLYAMIDE	POLYAMIDE	POLYAMIDE	GLASS PAPER *	SILICA GEL.	SILICA GEL



* IMPREGNATED WITH SILICA GEL.

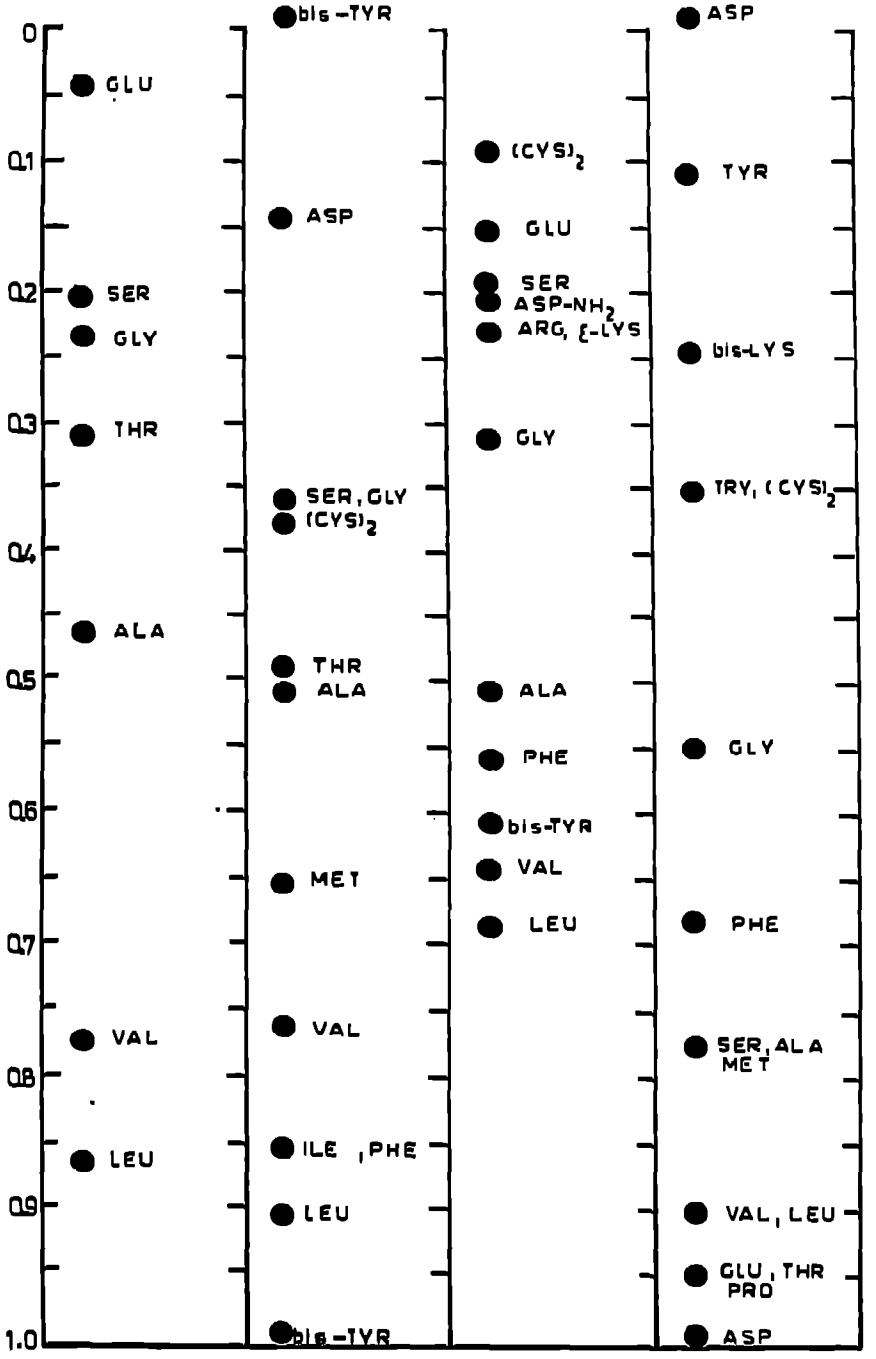
** FOR THE SEPARATION OF BOTH AMINO ACID DERIVATIVES AND PEPTIDE METHYL ESTERS.

*** MOBILITIES RELATIVE TO DNP-OH.

§ MOST OF THE OTHER AMINO ACIDS MOVE WITH THE SOLVENT FRONT.

Fig. 20. See refs. 42, 202 and 203. WITKOP's results are unpublished.

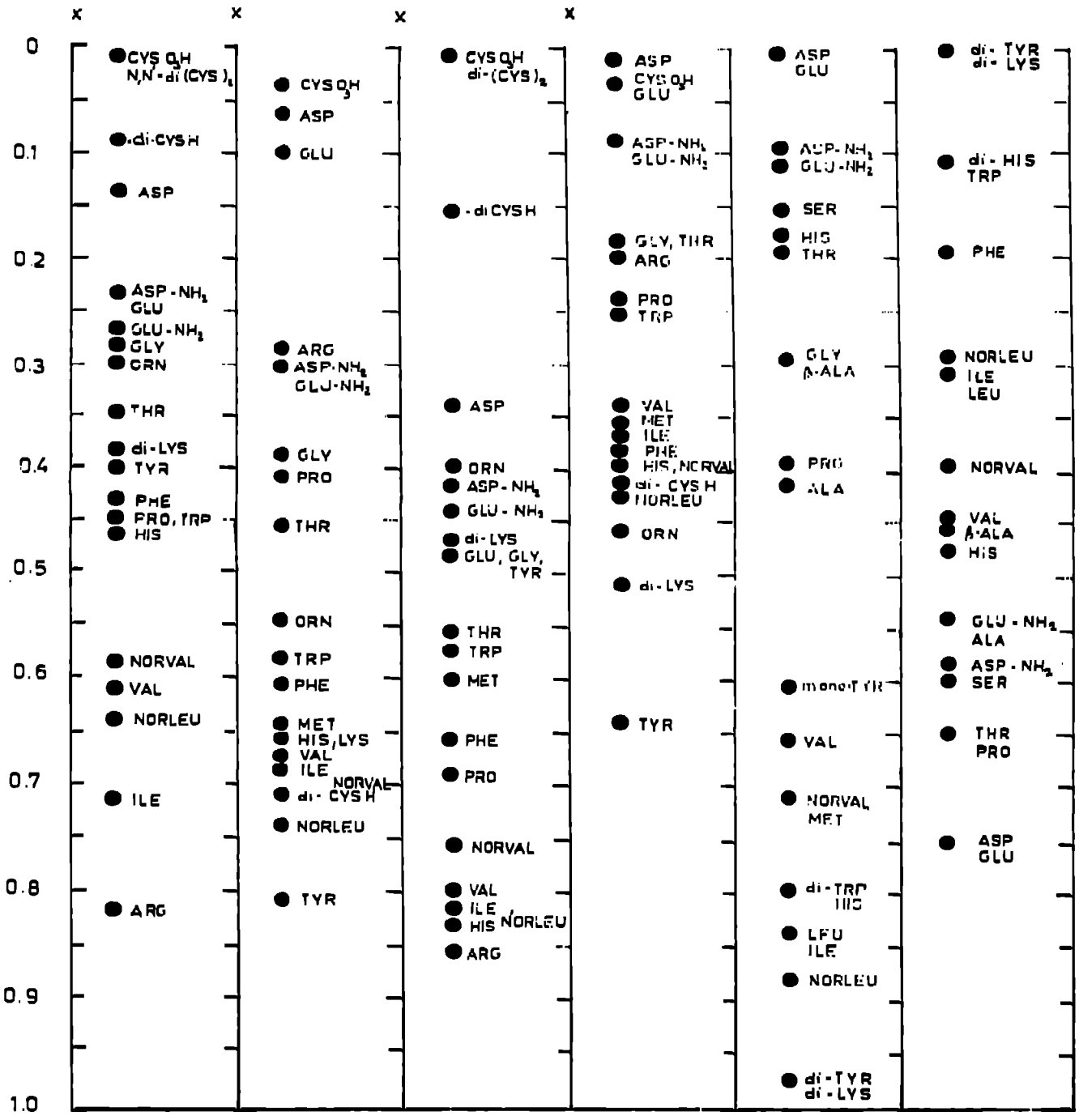
tert.-AMYL ALCOHOL SATURATED WITH 0.5*M* PHOSPHATE BUFFER pH 7	CYCLOHEXANE 15 ISOPROPANOL 9 0.05*M* POTASSIUM BENZOATE 1	*n*-BUTANOL 40 ISOAMYLALCOHOL 40 ETHANOL 13 0.067*M* PHOSPHATE BUFFER pH 6.2 60	1*M* CITRATE-HCl BUFFER pH=6.2
BLACKBURN AND LOWTHER	MONIER AND PENASSE	IWAINSKY	ROVERY AND FABRE
WHATMAN No. 1 *	WHATMAN No. 1	WHATMAN No. 1 *	WHATMAN No. 1 *



* BUFFER IMPREGNATED PAPER.

Fig. 21. See refs. 22, 91, 126 and 164.

n-BUTANOL 30 WATER 20 ACETIC ACID 10	*tert.*-AMYL ALCOHOL 50 0.88*N* AMMONIA 10	*N*-BUTANOL 30 WATER 20 ACETIC ACID 10	TOLUENE 100 PYRIDINE 30 ETHYLENE CHLORO-HYDRIN 60 0.88 AMMONIA 60	TOLUENE 150 ETHYLENE CHLORO-HYDRIN 90 0.8*N* AMMONIA 45 WATER 90	Na_2SO_4 Satd. Soln. 100 WATER 700 SDS ** 0.576g
BRADY AND HOSKINSON	BRADY AND HOSKINSON	BRADY AND HOSKINSON	BRADY AND HOSKINSON	MUNIER AND SARRAZIN	MUNIER AND SARRAZIN
WOOL CORTICAL CELLS	WOOL CORTICAL CELLS	WOOL CORTICAL CELLS, METHYLAT.	WOOL CORTICAL CELLS, METHYLAT.	CELLULOSE LAYER	CELLULOSE LAYER

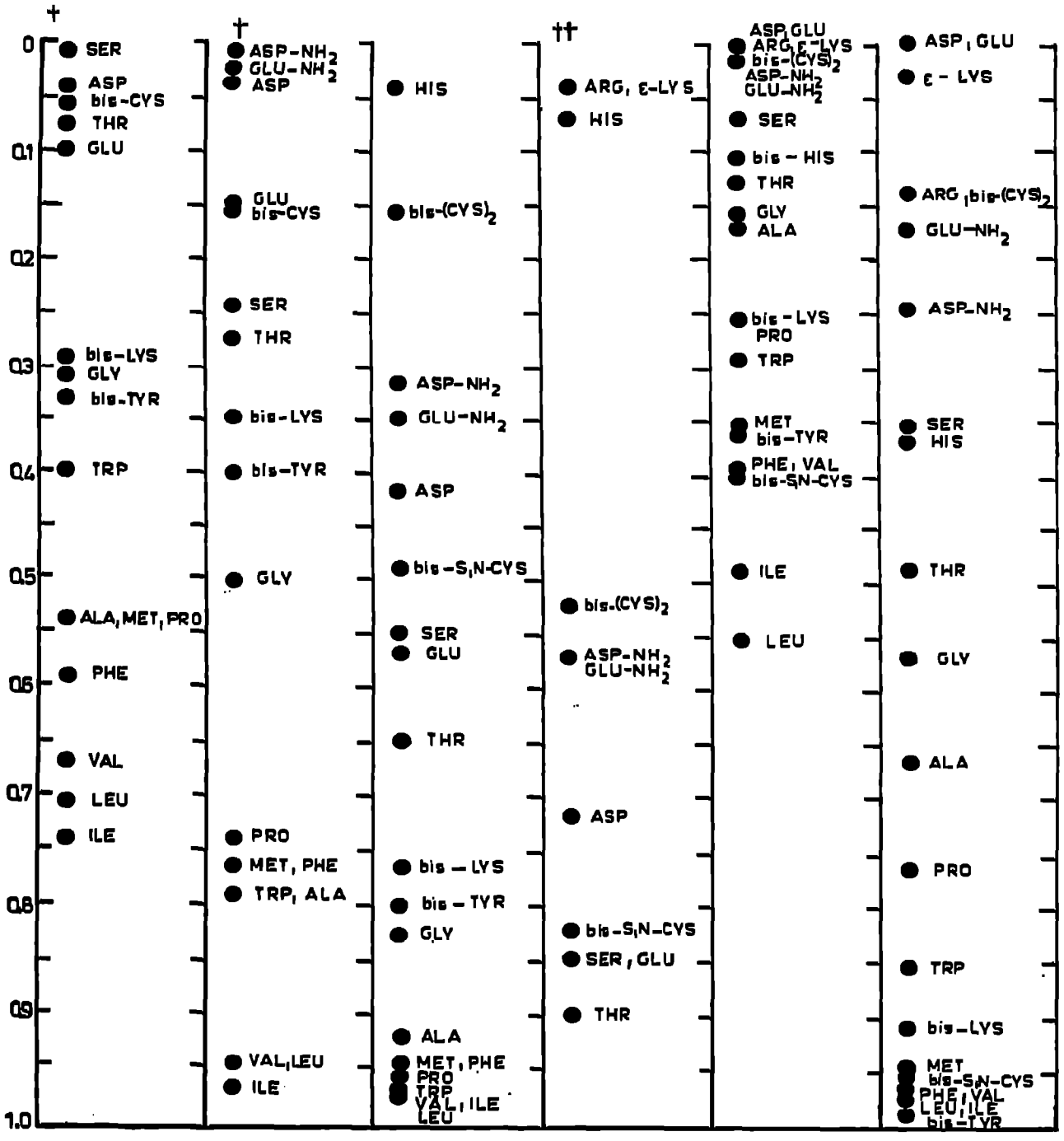


* SULPHUR CONTAINING AMINO ACIDS TEND TO TAIL IN THESE SYSTEMS. R_F OF THOSE WHICH ARE OMITTED HERE CANNOT BE ADEQUATELY DETERMINED.

* * SODIUM DODECYL SULPHATE.

Fig. 22. See refs. 26 and 130.

ISOOCTANE 20 CHLOROFORM 30 ACETIC ACID 1	DIETHYL ETHER 100 ACETIC ACID 1 WATER 1	DIETHYL ETHER 100 ACETIC ACID 3 WATER 3	DIETHYL ETHER 100 ACETIC ACID 5 WATER 5	DIETHYL ETHER 100 METHANOL 6 7M AMMONIA 2	DIETHYL ETHER 100 METHANOL 8 7M AMMONIA 3
DESSAUER et al.	DESSAUER et al.	DESSAUER et al.	DESSAUER et al.	DESSAUER et al.	DESSAUER et al.
GLASS PAPER *	GLASS PAPER *	GLASS PAPER *	GLASS PAPER *	GLASS PAPER *	GLASS PAPER *



* IMPREGNATED WITH SILICA GEL.
† MOST OF THE OTHER AMINO ACIDS AT THE STARTING LINE.
†† MOST OF THE OTHER AMINO ACIDS MOVE WITH THE SOLVENT FRONT.

Fig. 23. See ref. 42.

ISOBUTANOL 100 ACETIC ACID 6 WATER 20	ETHANOL 6 WATER 4	0.5*M* CITRATE 0.1*M* CITRIC ACID pH 5.4	TOLUENE 1 1% ACETIC ACID 1	DIMETHYL - 5 FORMAMIDE ACETIC ACID 10 WATER 30 ETHANOL 20	METHANOL 9 FORMIC ACID 1
MUNIER AND SARRAZIN	MUNIER AND SARRAZIN	KUBOTA	KUBOTA	WANG AND HUANG	WANG AND HUANG
CELLULOSE LAYER	CELLULOSE LAYER	WHATMAN No. 1	WHATMAN No. 1	POLYAMIDE	POLYAMIDE

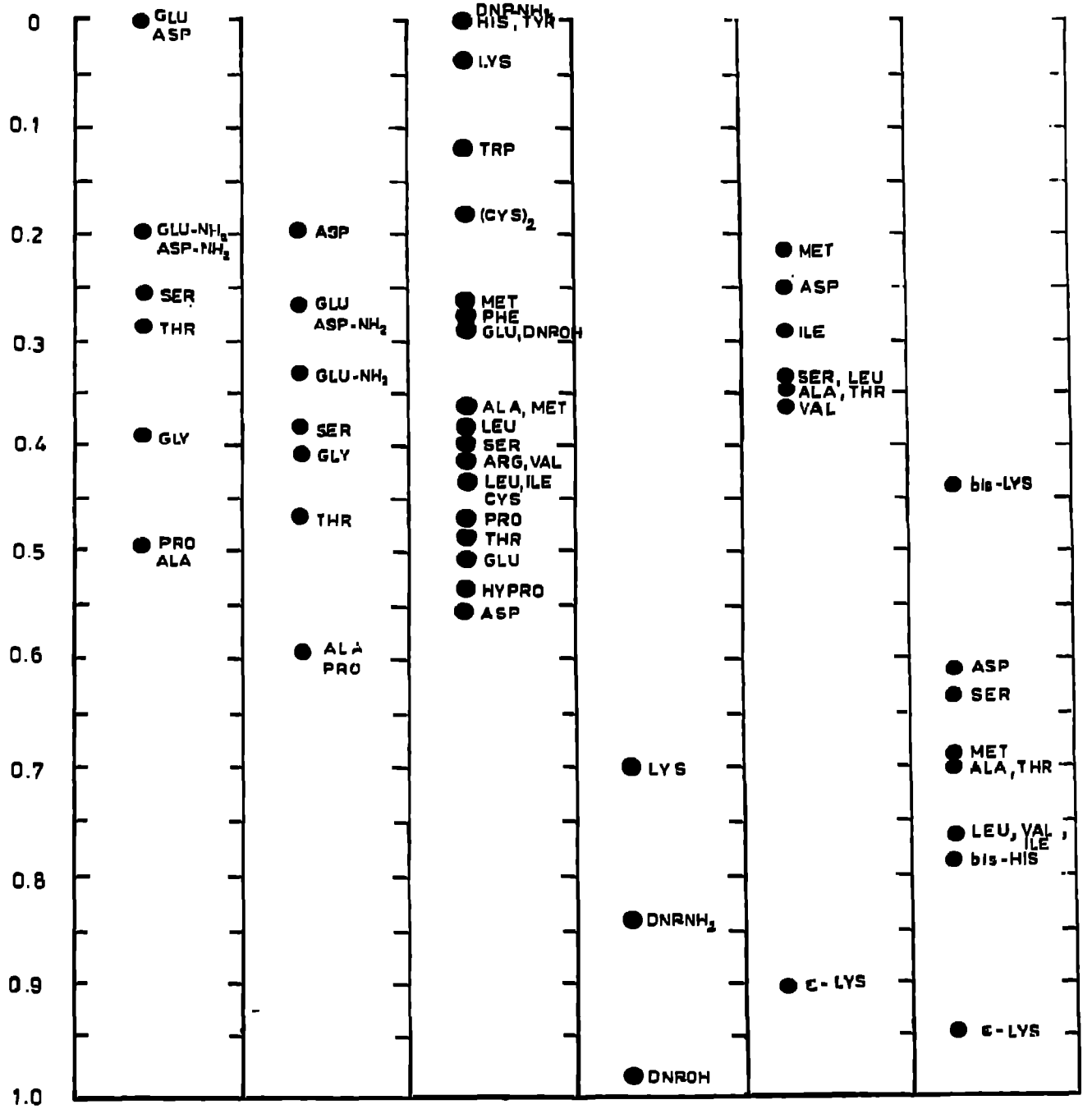
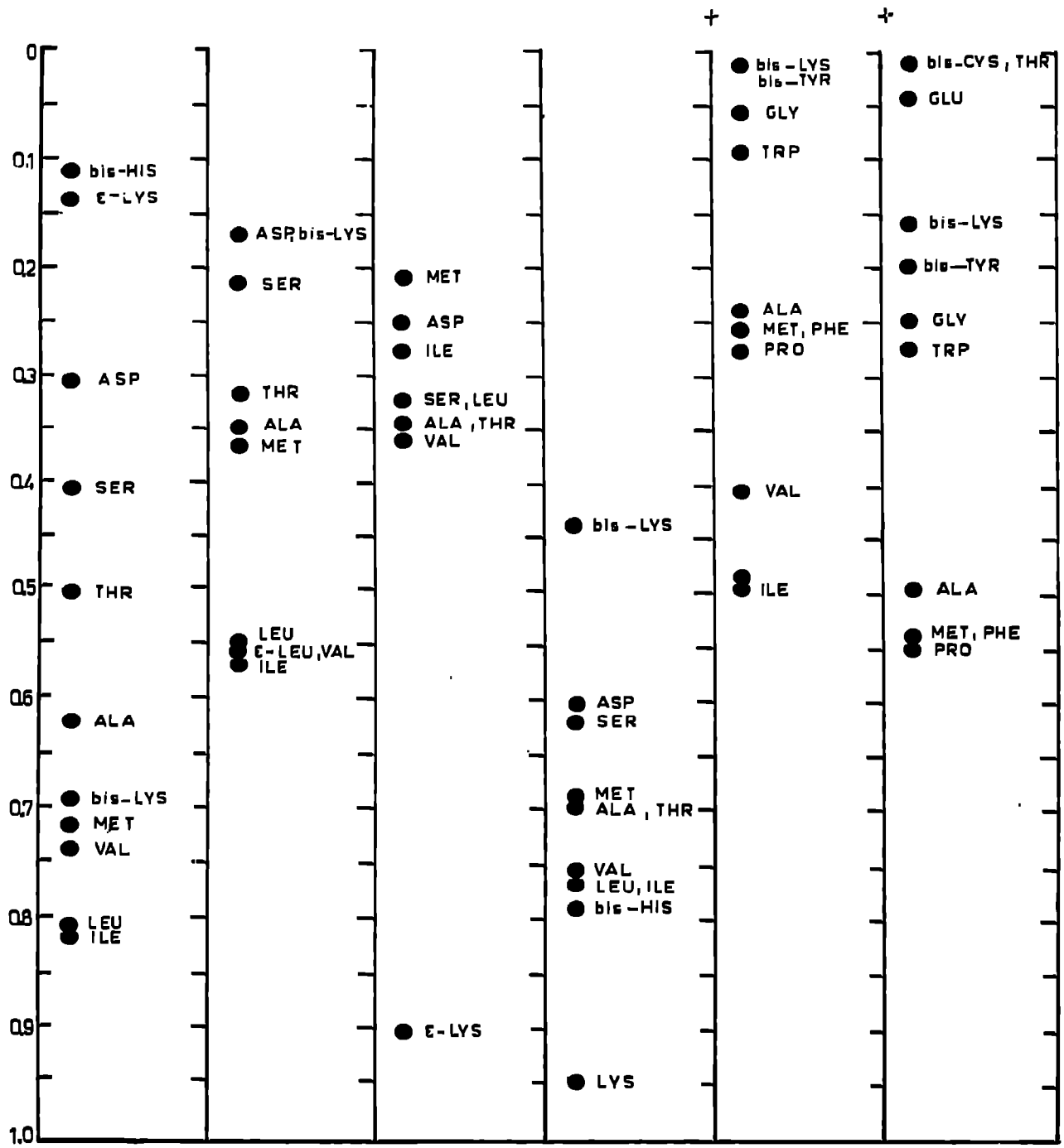


Fig. 24. See refs. 102, 130 and 200.

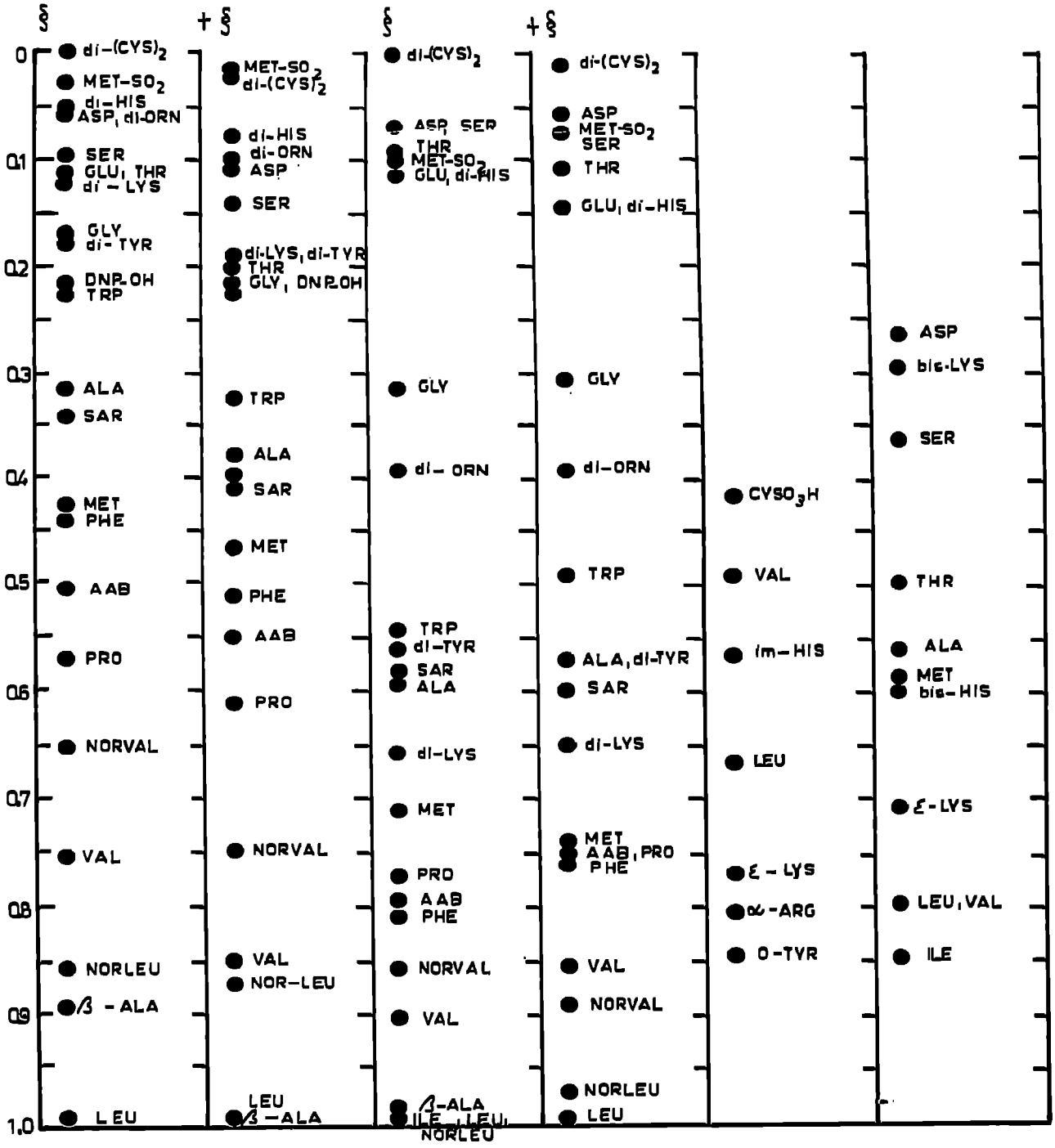
CHLOROFORM 7 METHYL ETHYL KETONE 3 FORMIC ACID 1	ETHANOL 63 WATER 37	DIMETHYL-FORMAMIDE 1 ACETIC ACID 2 WATER 6 ETHANOL 4	METHANOL 9 FORMIC ACID 1	ISOOCTANE 20 CHLOROFORM 10 ACETIC ACID 1	ISOOCTANE 10 CHLOROFORM 10 ACETIC ACID 1
WANG AND HUANG	WANG AND HUANG	WANG AND HUANG	WANG AND HUANG	DESSAUER et al.	DESSAUER et al.
POLYAMIDE LAYER	POLYAMIDE LAYER	POLYAMIDE LAYER	POLYAMIDE LAYER	GLASS PAPER *	GLASS PAPER *



* IMPREGNATED WITH SILICA GEL.
+ MOST OF THE AMINO ACIDS AT THE STARTING LINE.

Fig. 25. See refs. 42 and 200.

BENZENE 40 PYRIDINE 10 ACETIC ACID 1	BENZENE 80 PYRIDINE 20 ACETIC ACID 2 *	CHLOROFORM 95 METHANOL 5 ACETIC ACID 1	CHLOROFORM 95 METHANOL 5 ACETIC ACID 1 *	BUTANOL 4 ACETIC ACID 1 WATER 5	n-BUTANOL 9 ACETIC ACID 1
BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.	SANGER AND TUPPY	WANG AND HUANG
SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL	WHATMAN 4	POLYAMIDE LAYER



* AFTER DEVELOPMENT WITH TOLUENE-PYRIDINE-ETHYLENE CHLOROHYDRIN — 0.8N AMMONIA AND INTERMEDIATE DRYING.
† HORIZONTAL ARRANGEMENT.
§ MOBILITIES RELATIVE TO 2,4-DNP-LEU.

Fig. 26. See refs. 28, 29, 172 and 200. AAB = α-aminobutyric acid.

BENZENE 8 ACETIC ACID 2	CCl_4 8 ACETIC ACID 2	BUTYL ACETATE 9 ACETIC ACID 1	DIETHYL ETHER 9 ACETIC ACID 1	METHYL ETHYL KETONE 1 CHLOROFORM 8 ACETIC ACID 1	ACETIC ACID 1 WATER 1
WANG et al.	WANG et al.	WANG et al.	WANG et al.	WANG et al.	WANG et al.
POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE

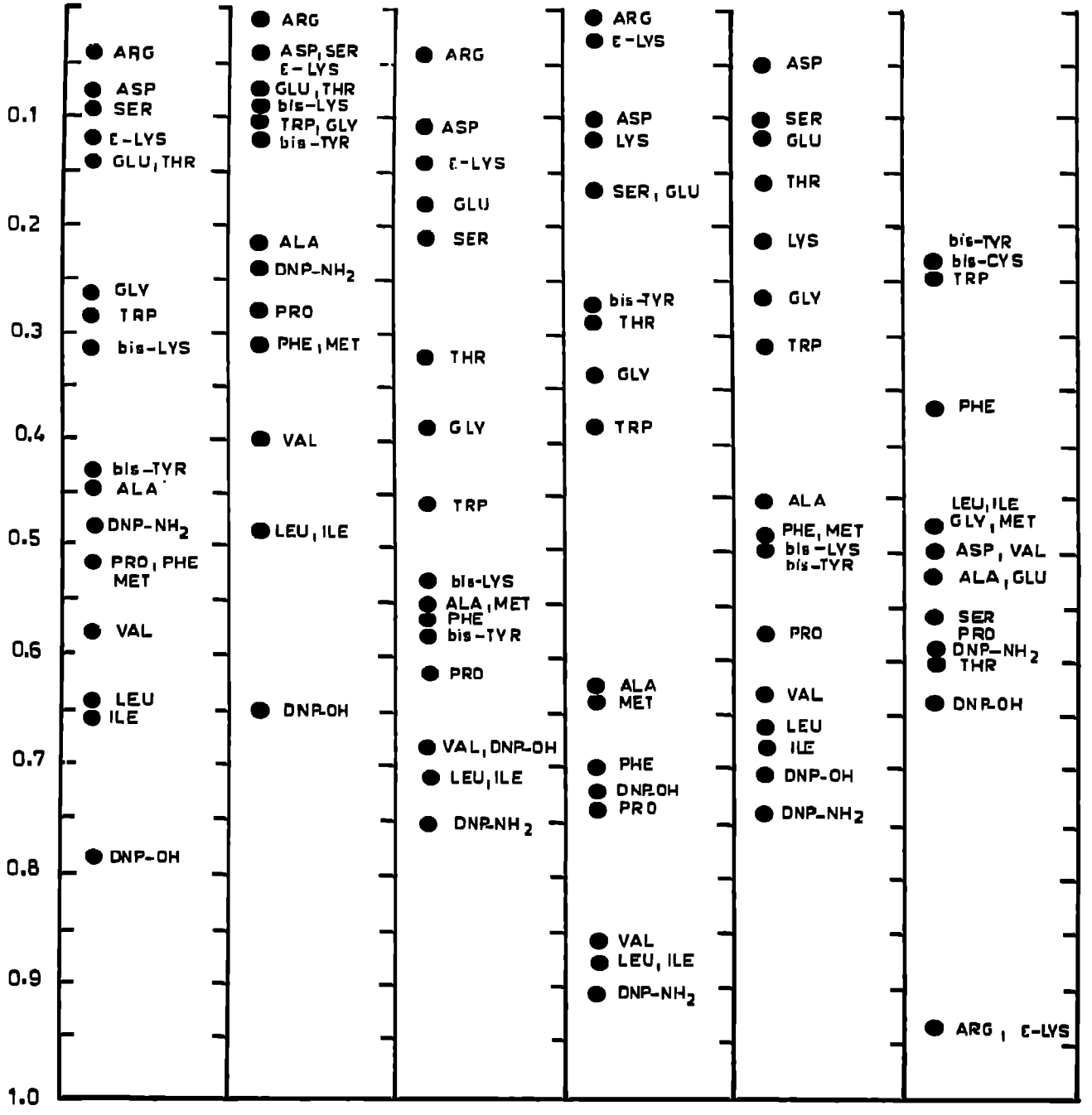
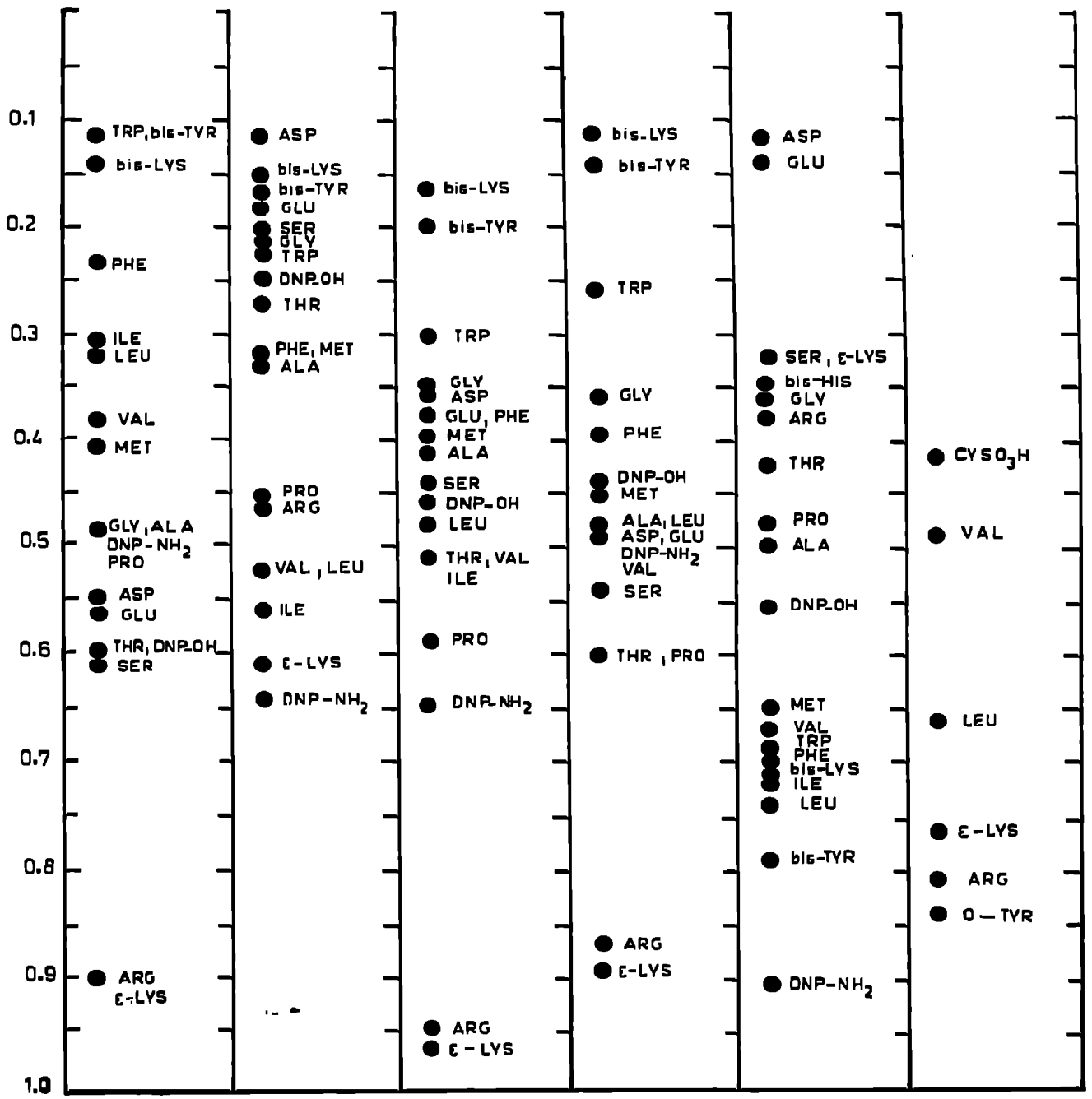


Fig. 27. See ref. 201.

90% FORMIC ACID 1 WATER 1	*n*-BUTANOL 9 ACETIC ACID 1	DIMETHYL FORMAMIDE 1 ACETIC ACID 2 45% ETHANOL 4 WATER 4	DIMETHYL FORMAMIDE 1 95% ETHANOL 4 WATER 4	*n*-BUTANOL *satd.* WITH WATER	*n*-BUTANOL 4 ACETIC ACID 1 WATER 5
WANG et al.	WANG et al.	WANG et al.	WANG et al.	MELLON et al.	KENT et al.
POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE *	WHATMAN NO.1	WHATMAN NO. 4



* SOLVENT SYSTEM CONTAINS 0.11g OF SULPHAMIC ACID PER 10ml.

Fig. 28. See refs. 97, 121 and 201.

n-BUTANOL 1 0.1% AMMONIA 1	*n*-BUTANOL 1 BUTYL ACETATE 2 1% AMMONIA 3	ISOBUTANOL 25 METHYL ACETATE 15 0.5*N* HCl 8 ISOPROPANOL 2	BENZENE 1 1% ACETIC ACID 1	DECALINE 1 ACETIC ACID 1	ISO AMYL ALCOHOL SATURATED WITH 1% ACETIC ACID
BRAUNITZER	MELLON et al.	FELIX AND KREKELS	MELLON et al.	BISERTE AND OSTEUX	BISERTE AND OSTEUX
WHATMAN NO.1	WHATMAN NO. 1	WHATMAN NO. 1	WHATMAN NO. 1	WHATMAN NO.1	WHATMAN NO.1

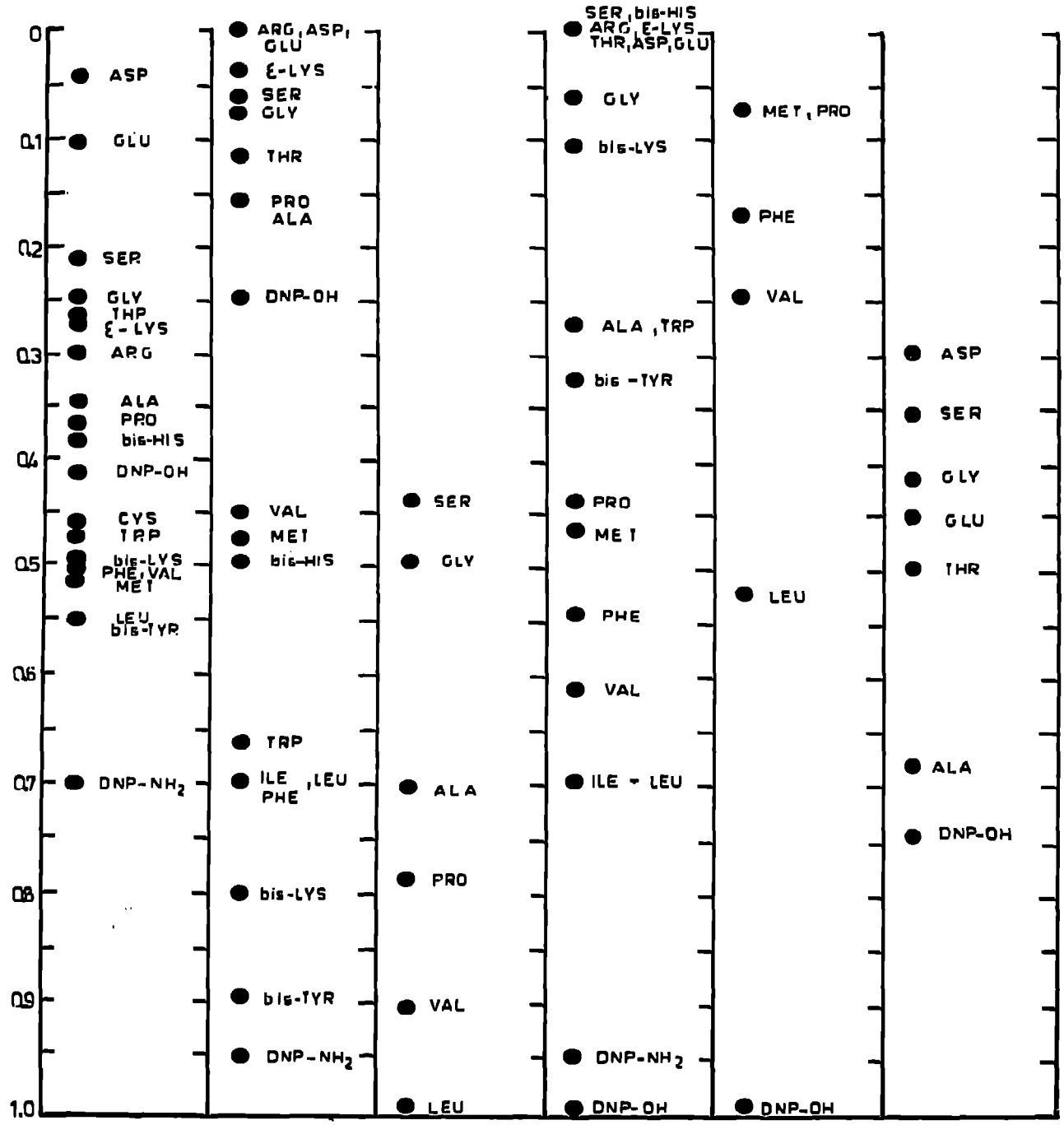
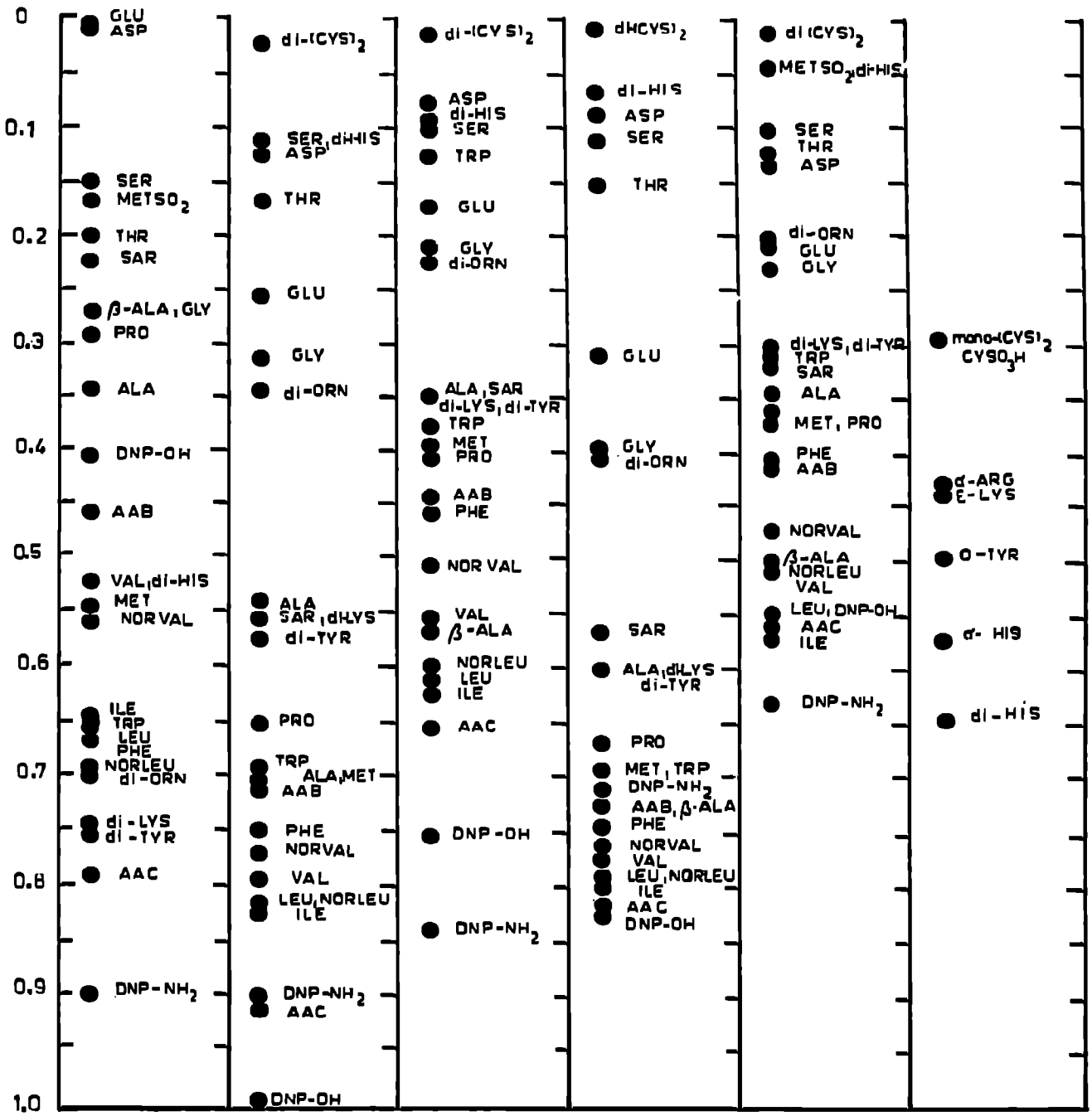


Fig. 29. See refs. 20, 27, 58 and 121.

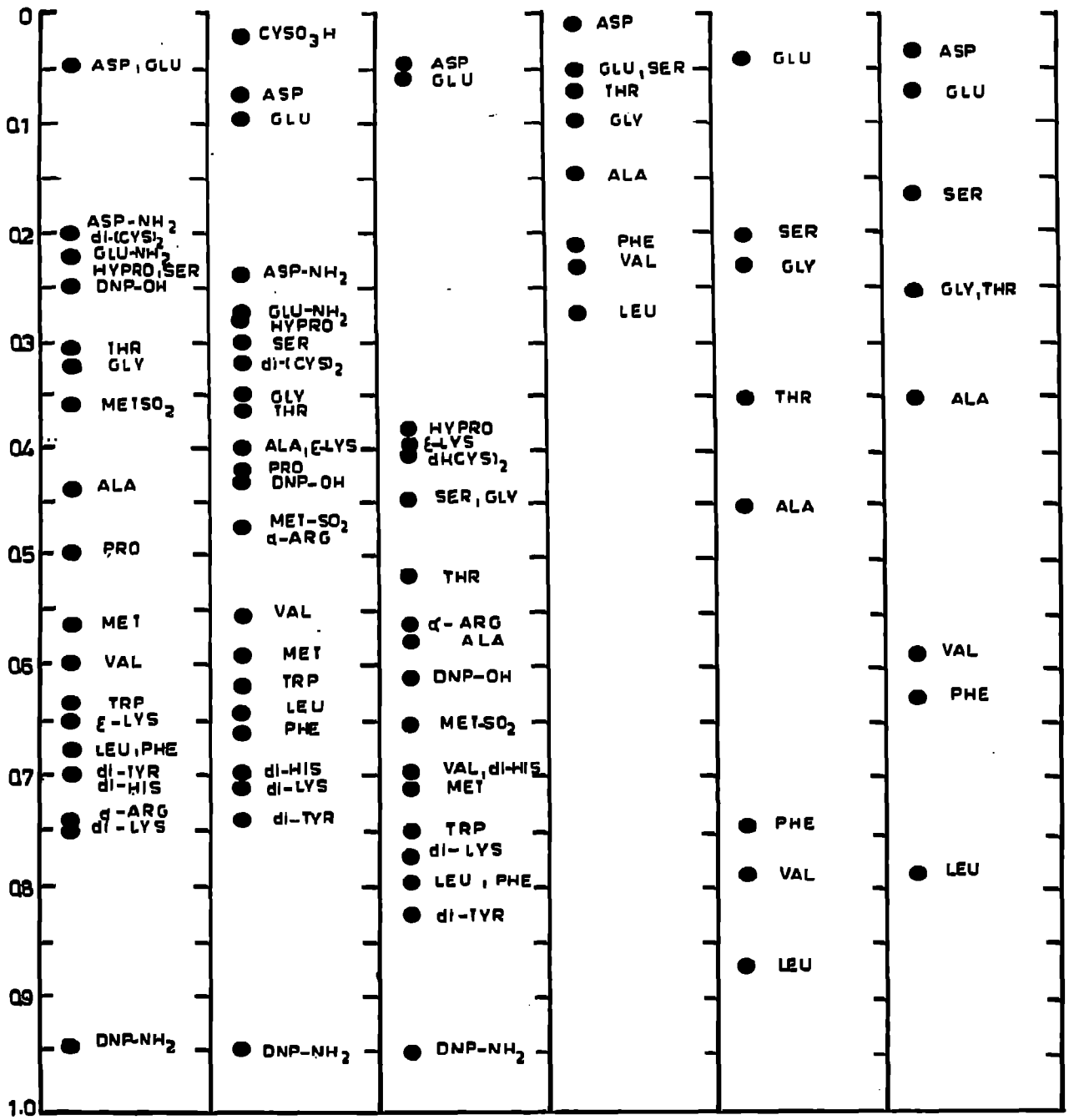
TOLUENE 10 PYRIDINE 3 ETHYLENE 6 CHLOROHYDRIN 6 0.8 *N* AMMONIA 6	CHLOROFORM 70 BENZYL ALCOHOL 30 ACETIC ACID 3	CHLOROFORM 70 BENZYL ALCOHOL 30 ACETIC ACID 3*	CHLOROFORM 70 *tert.*-AMYL ALCOHOL 30 ACETIC ACID 3	CHLOROFORM 70 *tert.*-AMYL ALCOHOL 30 ACETIC ACID 3*	*n*-PROPANOL 7 34% AMMONIA 3
BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.
SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL



* AFTER DEVELOPMENT WITH SOLVENT IN THE FIRST COLUMN (TOLUENE-PYRIDINE--ETHYLENE CHLOROHYDRIN-0.8*N* AMMONIA) AND INTERMEDIATE DRYING.

Fig. 30. See refs. 28 and 29.

PHENOL 1 ISO AMYL ALCOHOL 1 WATER 1	TOLUENE 5 PYRIDINE 1 ETHYLENE CHLOROHYDRIN 3 0.8*N* AMMONIA 3*	PYRIDINE 6 ISO AMYL ALCOHOL 14 1.6*N* AMMONIA 20	30% PROPANOL / CYCLOHEXANE	*tert.*-AMYL ALCOHOL	10% ETHANOL / BENZYL ALCOHOL SATURATED WITH PHTHALATE BUFFER
BISERTE AND OSTEUX	BISERTE AND OSTEUX	BISERTE AND OSTEUX	BLACKBURN AND LOWTHER	BLACKBURN AND LOWTHER	BLACKBURN AND LOWTHER
WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.4	WHATMAN NO.4	WHATMAN NO.4



* UPPER LAYER.

Fig. 31. See refs. 20 and 22.

TOLUENE 1 ETHYL ACETATE 1 ETHANOL 1 1.5*N* NH_4OH 1	TOLUENE 2 ACETIC ACID 1 WATER 1	TOLUENE 1 $CHCl_3$ 1 5% ACETIC ACID 2	CHLOROFORM 4 *n*-BUTANOL 1 1% ACETIC ACID 5	*n*-BUTANOL 9 CYCLOHEXANE 1 0.2% ACETIC ACID 10	*n*-BUTANOL 9 CYCLOHEXANE 1 1.5*N* NH_4OH 10
KUBOTA	KUBOTA	KUBOTA	KUBOTA	KUBOTA	KUBOTA
WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.1	WHATMAN NO.1

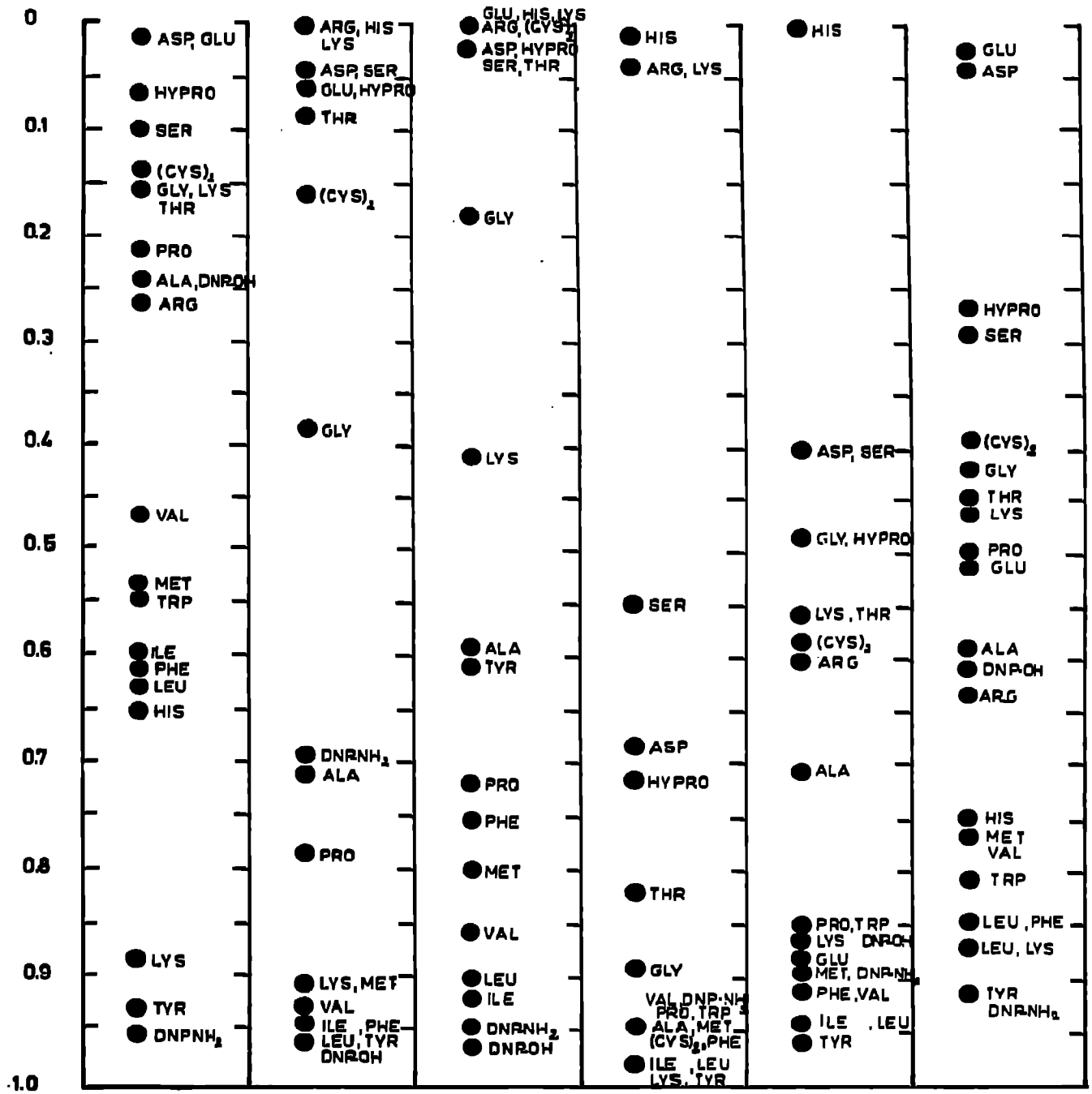


Fig. 32. See ref. 102.

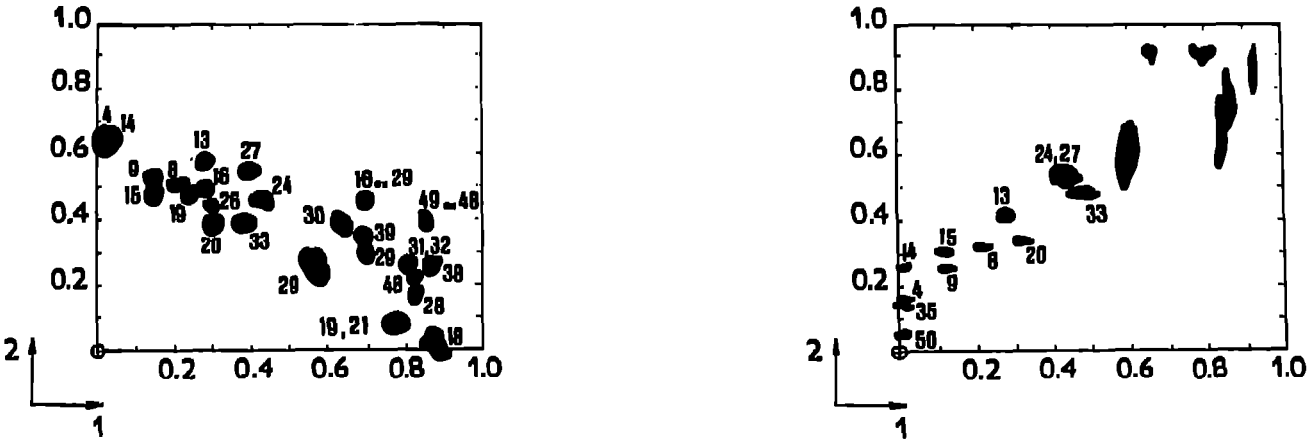


Fig. 33. MUNIER AND SARRAZIN[139]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150:90:45:90); 2nd dimension: water saturated with ammonium sulphate–water–sodium dodecylsulphate (100 ml:700 ml:0.576 g).

Fig. 34. MUNIER AND SARRAZIN[139]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90) + 10 drops of octyl alcohol per 375 ml; 2nd dimension, isobutanol–acetic acid–water (100 : 6 : 20).

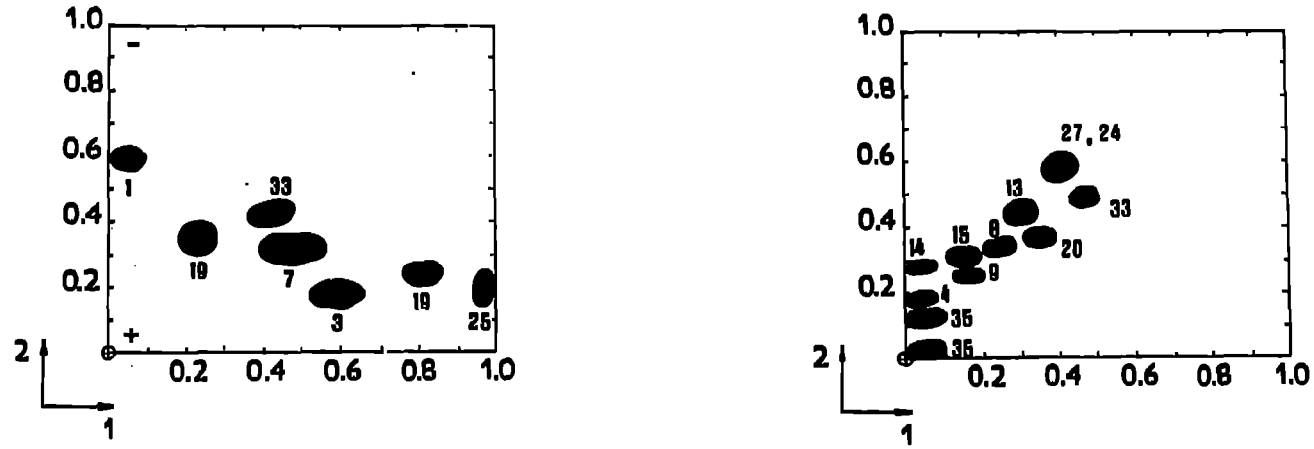


Fig. 35. MUNIER AND SARRAZIN[139]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: 0.1 *M* sodium chloride in water; 0.016 *M* with respect to diethylamine.

Fig. 36. MUNIER AND SARRAZIN[139]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: isobutanol–acetic acid–water (100 : 6 : 20).

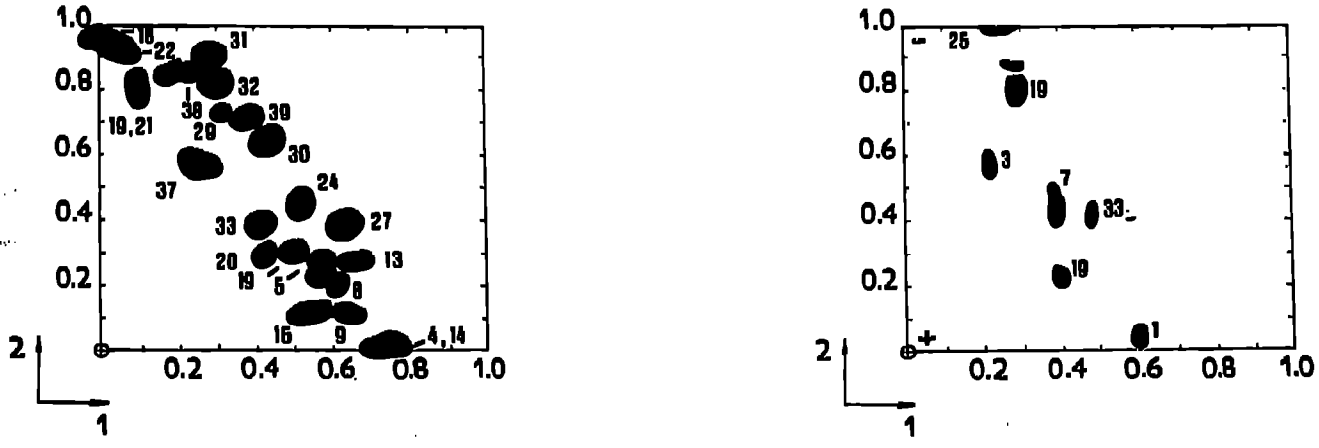


Fig. 37. MUNIER AND SARRAZIN[130]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90) + 10–12 drops of octanol; 2nd dimension: water saturated with ammonium sulphate–water–sodium dodecylsulphate (100 ml : 700 ml : 0.576 g).

Fig. 38. MUNIER AND SARRAZIN[130]. Sorbent, TLC cellulose. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90) + 10–12 drops of octanol; 2nd dimension: electrophoresis in 0.033 *M* diethylamine which is 0.02 *M* with respect to sodium chloride, 25 V/cm.

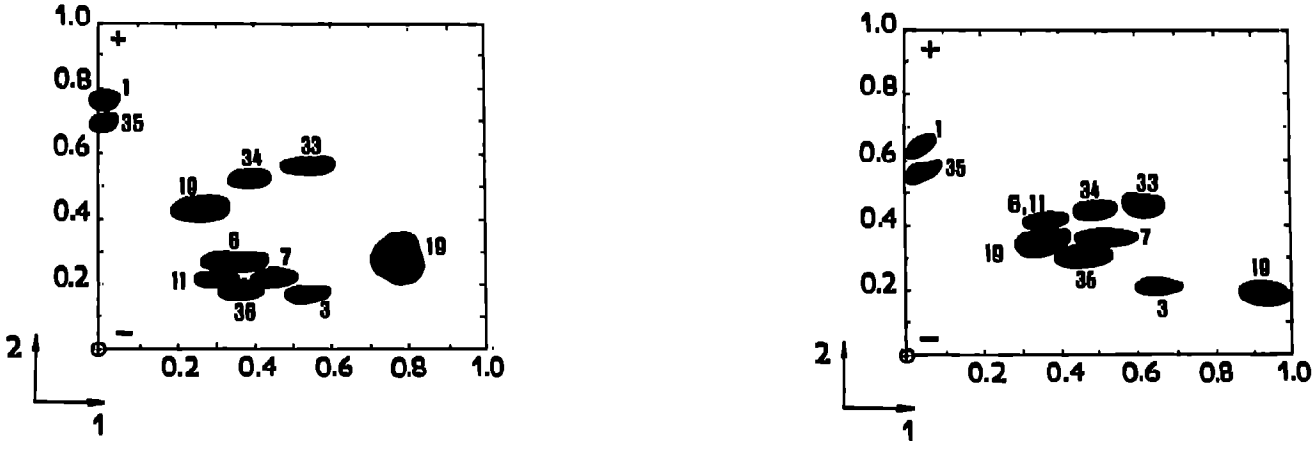


Fig. 39. MUNIER AND SARRAZIN[130]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: electrophoresis in 0.02 *M* borate buffer of pH 8.92; *ca.* 10 V/cm.

Fig. 40. MUNIER AND SARRAZIN[130]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: electrophoresis in 0.25 *M* triethylamine; *ca.* 10 V/cm.

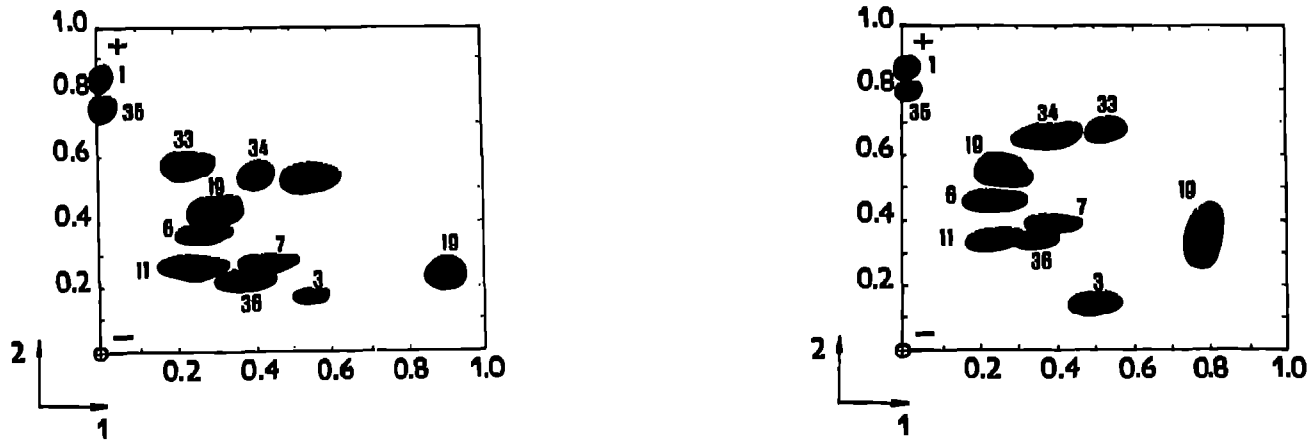


Fig. 41. MUNIER AND SARRAZIN[138]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: electrophoresis in 0.025 *M* diethylamine; *ca.* 10 V/cm.

Fig. 42. MUNIER AND SARRAZIN[138]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: electrophoresis in 0.25 *M* ammonia; *ca.* 10 V/cm.

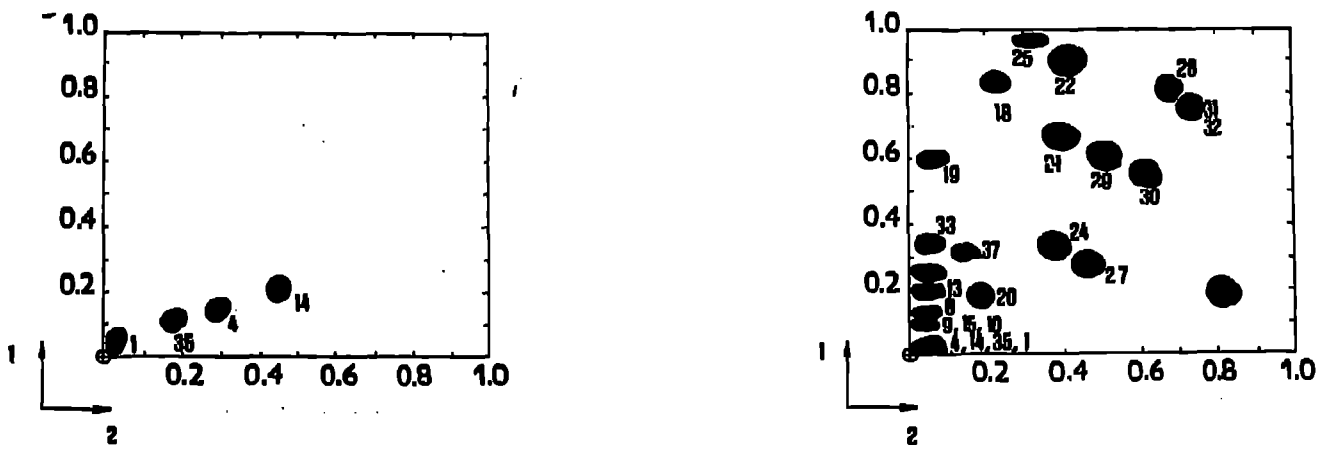


Fig. 43. MUNIER AND THOMMEGAY[181]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: paper impregnated with 0.2 *M* sodium acetate and developed with isobutanol–acetic acid–water (100 : 4.5 : 19).

Fig. 44. MUNIER AND THOMMEGAY[181]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: within 2 h a thin film of formic acid is deposited on cellulose; developed with benzene saturated with formic acid.

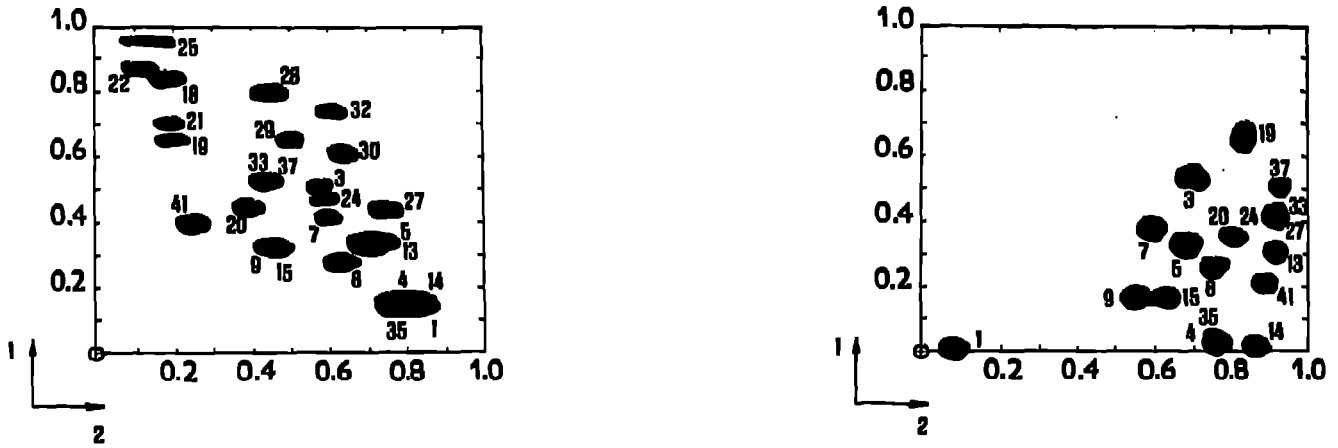
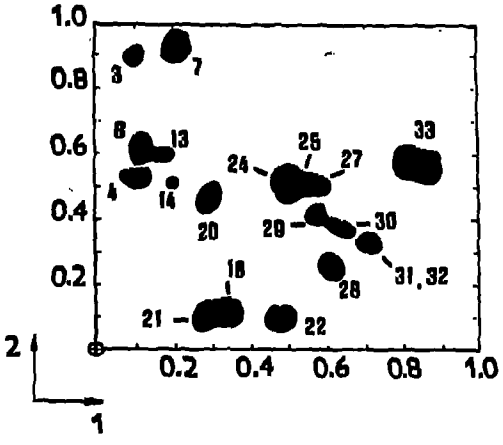


Fig. 45. MUNIER AND THOMMEGAY[101]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: 2.25 *M* sodium acetate.

Fig. 46. MUNIER AND THOMMEGAY[101]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: (impregnated with 0.2 *M* sodium acetate) isobutanol–formic acid–water (100 : 4 : 19).

Fig. 47. MUNIER AND THOMMEGAY[101]. Sorbent, Whatman No. 20 filter-paper. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–0.8 *M* ammonia (150 : 90 : 45 : 90); 2nd dimension: paper impregnated with 1 *M* sodium acetate, then developed with isobutanol–acetic acid–water (100 : 8 : 19)

Fig. 48. WANG *et al.*[201]. Sorbent, polyamide (*ε*-polycaprolactam resin). Development, 1st dimension: *n*-butanol–acetic acid (90 : 10); 2nd dimension: formic acid–water (50 : 50).



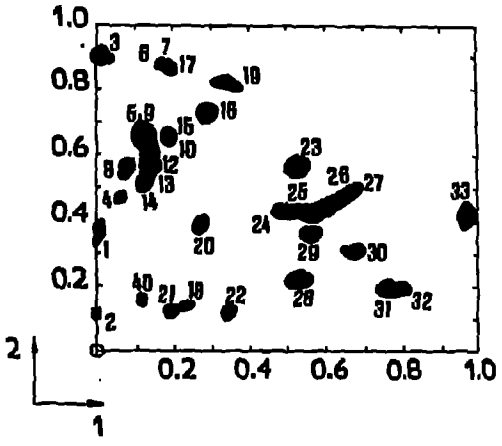
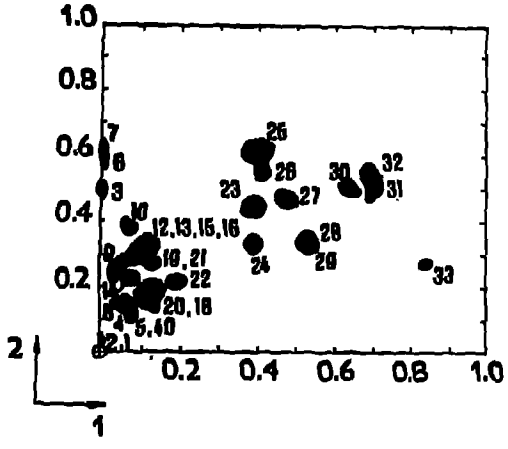


Fig. 49. WANG *et al.*[801]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension; benzene–acetic acid (80 : 20); 2nd dimension: *n*-butanol–acetic acid (90 : 10).

Fig. 50. WANG AND WANG[808]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: benzene–acetic acid (80 : 20); 2nd dimension: 45% formic acid.



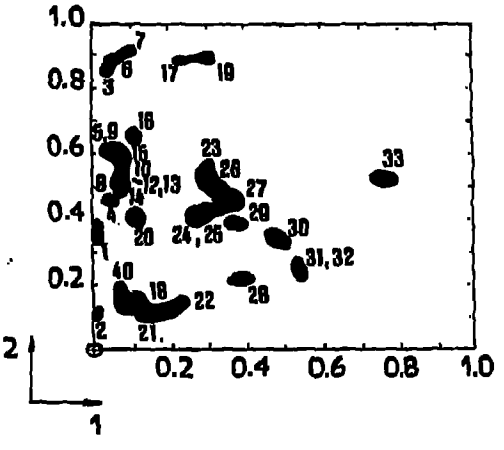


Fig. 51. WANG AND WANG[808]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: carbon tetrachloride–acetic acid (80 : 20); 2nd dimension: *n*-butanol–acetic acid (90 : 10).

Fig. 52. WANG AND WANG[808]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: carbon tetrachloride–acetic acid (80 : 20); 2nd dimension: 45% formic acid.

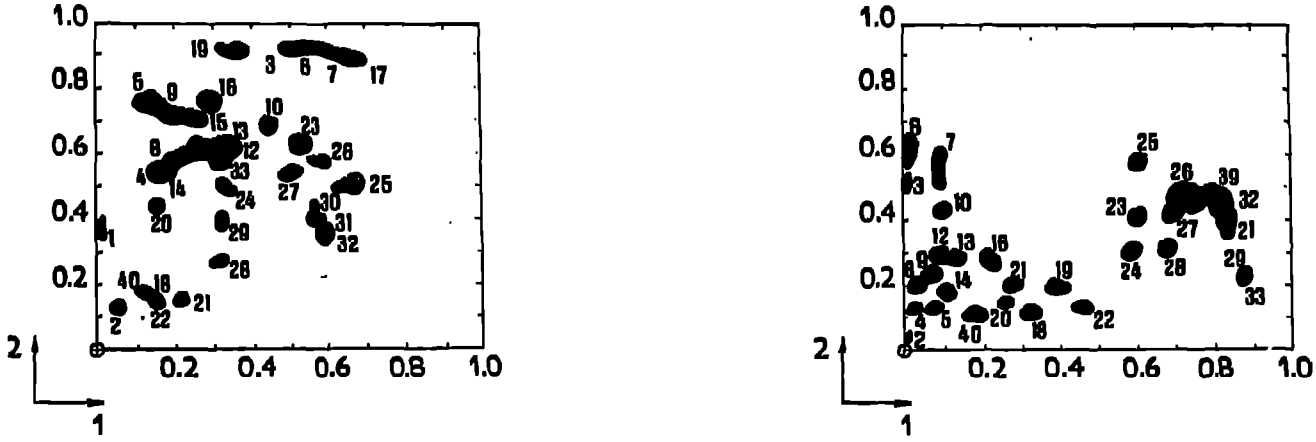


Fig. 53. WANG AND WANG[202]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: *n*-butanol–acetic acid (90 : 10); 2nd dimension: 45% formic acid.

Fig. 54. WANG AND WANG[202]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: benzene–acetic acid (80 : 20); 2nd dimension: *n*-butanol–acetic acid (90 : 10).

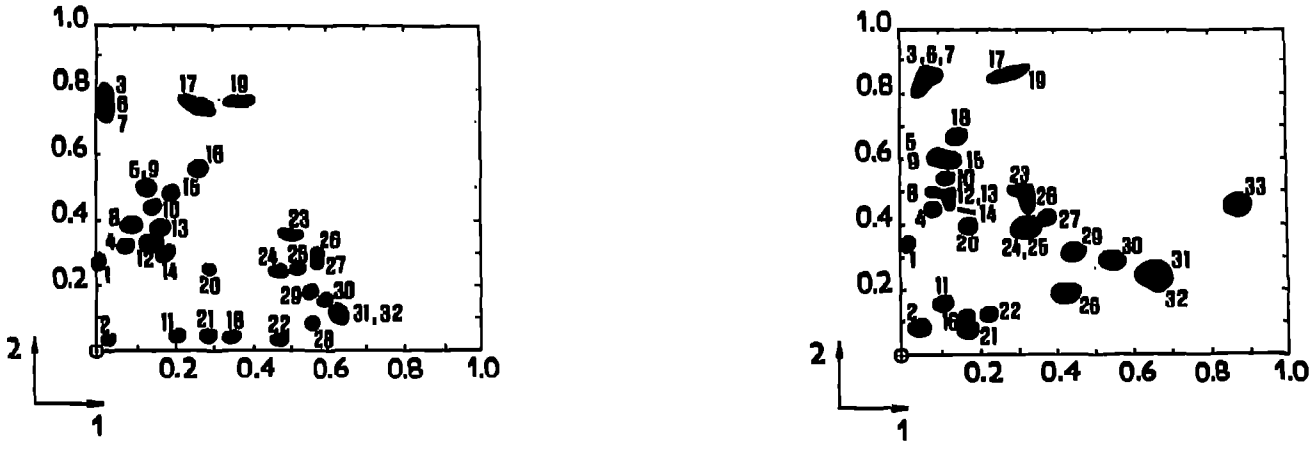


Fig. 55. WANG AND WANG[202]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: benzene–acetic acid (80 : 20) for 1.5 h; 2nd dimension: 45% formic acid for 1 h.

Fig. 56. WANG AND WANG[202]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: carbon tetrachloride–acetic acid (80 : 20) for 3 h; 2nd dimension: 90% formic acid–water (50 : 50) for 1 h.

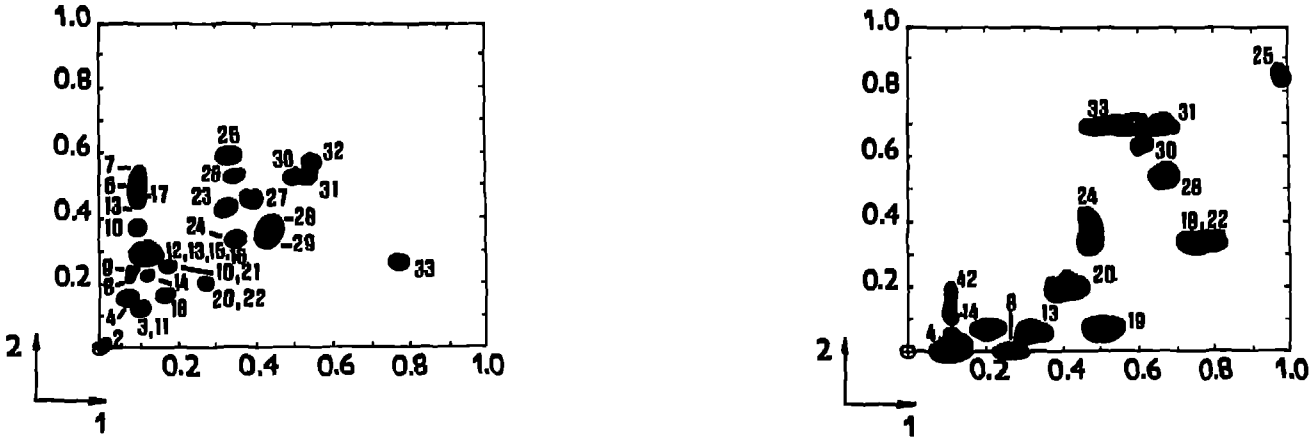


Fig. 57. WANG AND WANG[803]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: carbon tetrachloride–acetic acid (80 : 20) for 2 h; 2nd dimension: *n*-butanol–acetic acid (90 : 10) for 3 h.

Fig. 58. WALZ *et al.*[199]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–25% ammonia (50 : 35 : 15 : 7), developed twice; 2nd dimension: chloroform–benzyl alcohol–acetic acid (70 : 30 : 3).

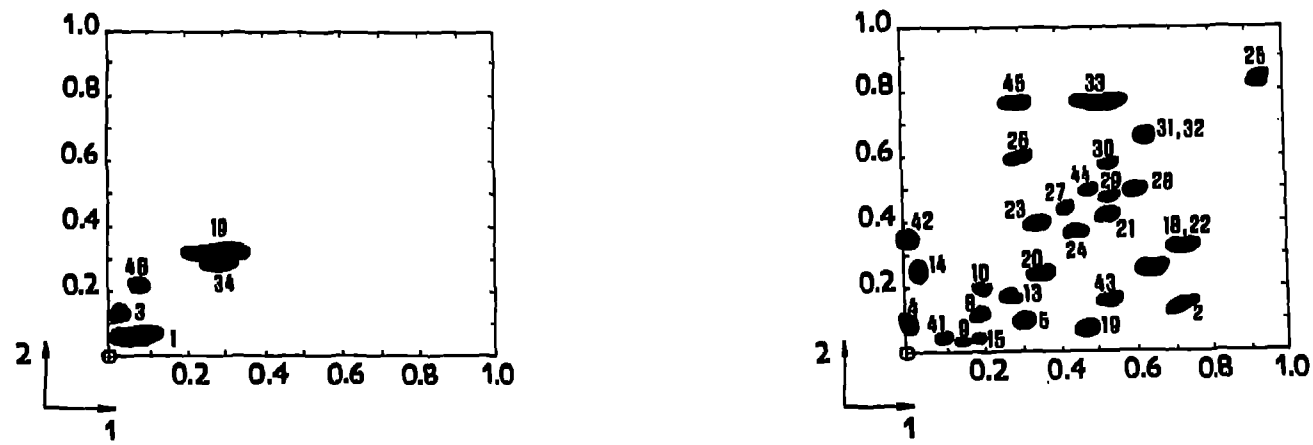


Fig. 59. WALZ *et al.*[199]. Sorbent, Silica Gel G. Development, 1st dimension: pyridine, developed three times; 2nd dimension: *n*-butanol saturated with 25% ammonia.

Fig. 60. WALZ *et al.*[199]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–25% ammonia (50 : 35 : 15 : 17), developed twice; 2nd dimension: chloroform–benzyl alcohol–acetic acid (70 : 30 : 3).

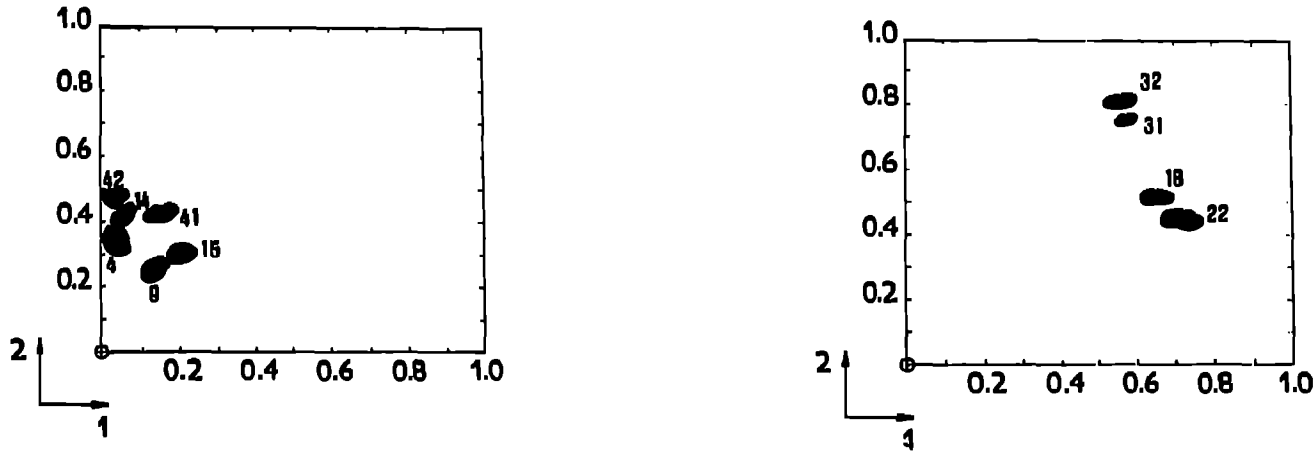


Fig. 61. WALZ *et al.*[100]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–25% ammonia (50 : 35 : 15 : 7), developed three times; 2nd dimension: chloroform–methanol–acetic acid (70 : 30 : 5).

Fig. 62. WALZ *et al.*[100]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–ethylene chlorohydrin–pyridine–25% ammonia (50 : 35 : 15 : 7); 2nd dimension: chloroform–methanol–acetic acid (95 : 5 : 1).

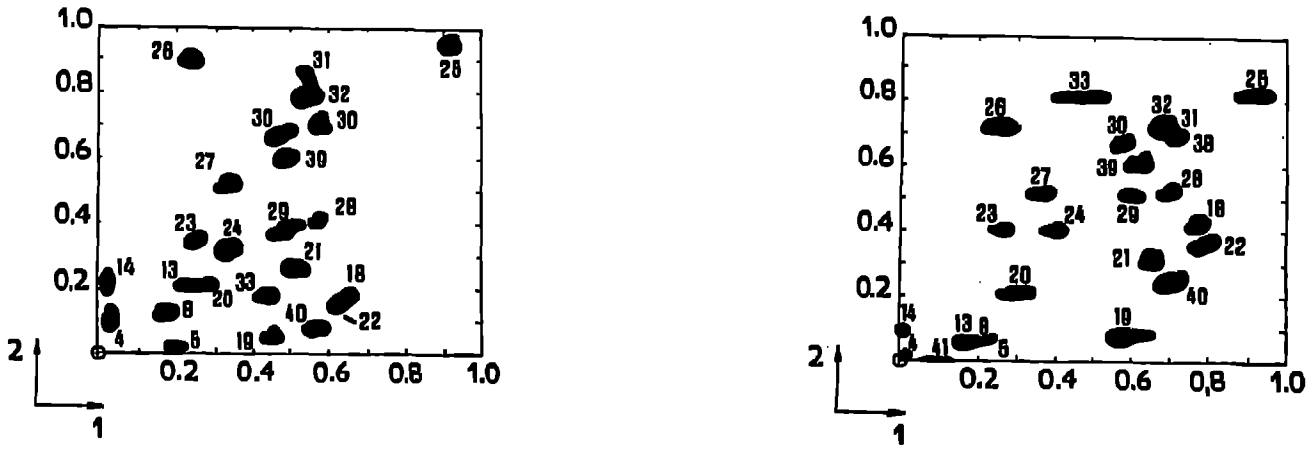
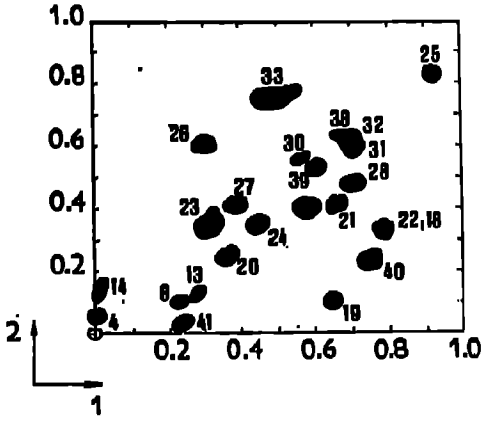


Fig. 63. BRENNER *et al.*[89]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–pyridine–ethylene chlorohydrin–0.8 *M* ammonia (100 : 30 : 60 : 60); 2nd dimension: benzene–pyridine–acetic acid (80 : 20 : 2).

Fig. 64. BRENNER *et al.*[89]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–pyridine–ethylene chlorohydrin–0.8 *M* ammonia (100 : 30 : 60 : 60); 2nd dimension: chloroform–methanol–acetic acid (95 : 5 : 1).



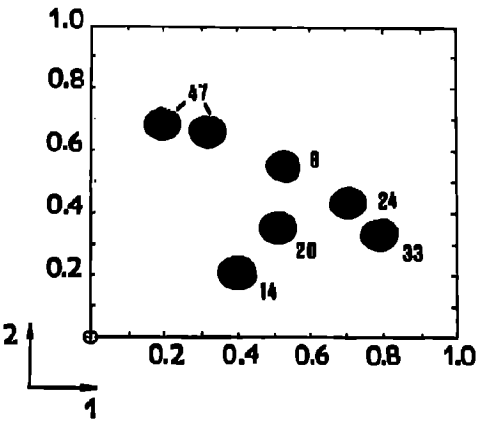


Fig. 65. BRENNER *et al.*[39]. Sorbent, Silica Gel G. Development, 1st dimension: toluene–pyridine–ethylene chlorohydrin–0.8 *M* ammonia (100 : 30 : 60 : 60); 2nd dimension: chloroform–benzyl alcohol–acetic acid (70 : 30 : 3).

Fig. 66. GRANT AND WICKEN[70]. Sorbent, cellulose–silica gel (10 : 4). Development, 1st dimension: 1st development, isopropanol–acetic acid–water (75 : 10 : 15), 2nd development, *n*-butanol–0.15 *M* ammonia (1 : 1) (upper phase); 2nd dimension: 1.5 *M* phosphate buffer of pH 6.0.

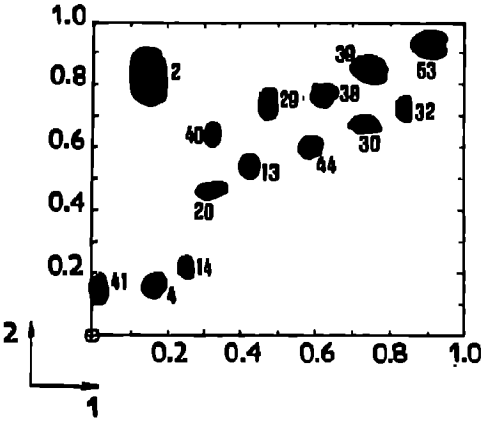
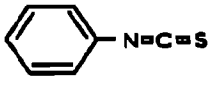


Fig. 67. BRADY AND HOSKINSON[38]. Sorbent, wool cortical cells. Development, 1st dimension: *n*-butanol–water–acetic acid (30 : 20 : 10); 2nd dimension: *tert.*-amyl alcohol–0.88 *M* ammonia (50 : 10).

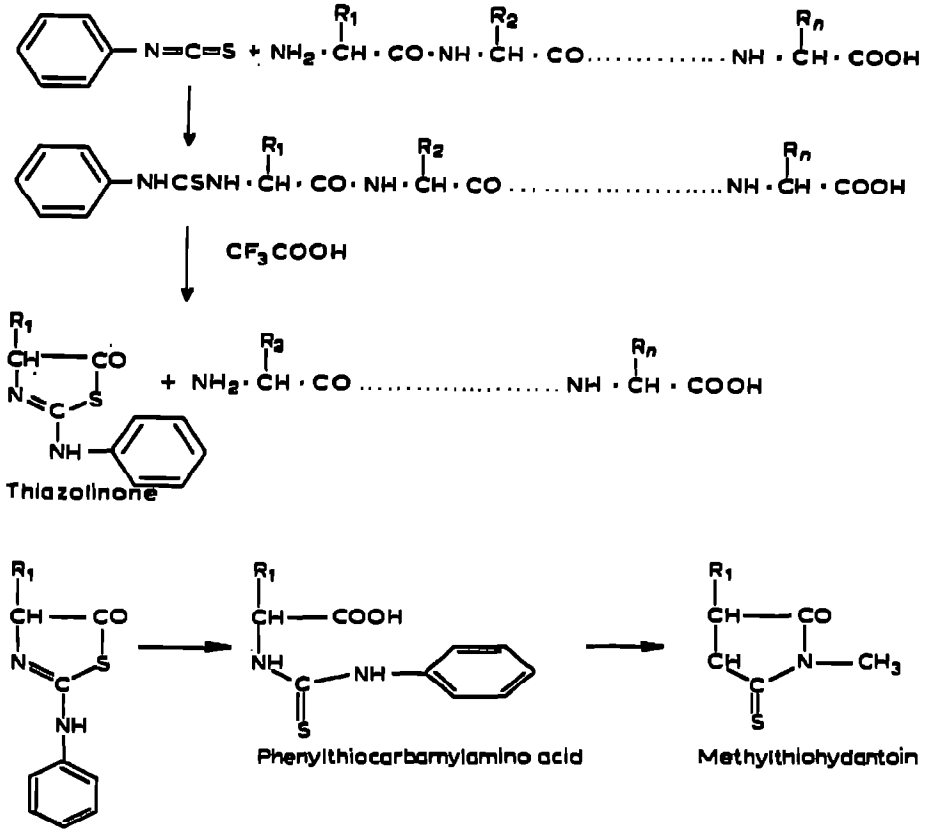
B. REACTION WITH PHENYL ISOTHIOCYANATE AND CYCLISATION TO PHENYLTHIOHYDANTOINS (Chapter 5.2.4 of the whole review)

Reagent:



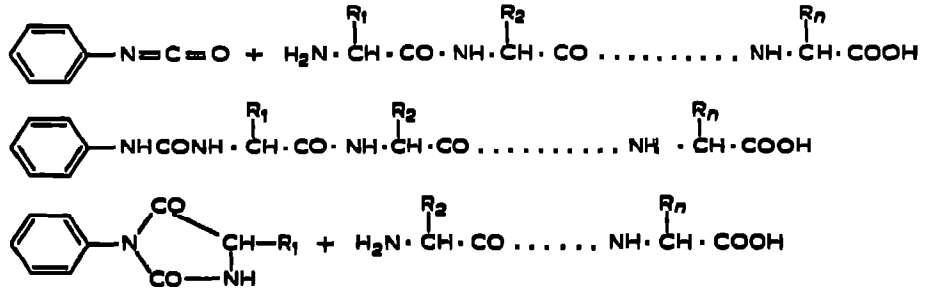
Phenyl isothiocyanate

Reaction scheme:



1. *Introduction*

The principle of the phenyl isothiocyanate method for the identification of N-terminal amino acids was discussed in the introduction to Part A of this review. However, one can trace the origin of this method, now called the Edman degradation, to before Edman's first paper[47] in 1950. BERGMANN *et al.*[10] in 1927 proposed a determination of N-terminal residues of peptides by condensing the latter with phenyl isocyanate. Isocyanates react readily with the amino groups of proteins in an aqueous solution of pH 8.0 at 0°. When the resulting phenylcarbamyl peptide was exposed to cyclizing conditions, *i.e.*, treated with HCl in water or methanol, the N-terminal residue would split off as the highly water-insoluble 3-phenylthiohydantoin:



The procedure was subsequently extended in 1930 by ABDERHALDEN AND

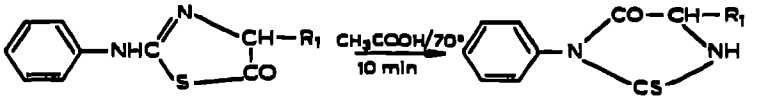
BROCKMANN[2], who suggested that this reaction could be used for a step-by-step method in the degradation of amino acid residues in peptides and proteins starting from the N-terminal group.

Unfortunately, the cleavage conditions used were too drastic for this purpose, and therefore Bergmann's method could not be used in sequential analysis. However, it proved to be a valuable tool in the determination of N-terminal amino acids in the earliest stages of protein chemistry. The first successful N-terminal group analysis of a protein was accomplished by JENSEN AND EVANS in 1935[94], when they condensed insulin with phenyl isocyanate. After hydrolysis in boiling 6 *N* HCl, they isolated the phenylhydantoin of phenylalanine. However, they failed to discover the phenylhydantoin of glycine, probably because of the relative long time required for hydrolysis.

Bergmann's principle, despite many advantages, was abandoned after SANGER[108] discovered the dinitrophenylation method in 1945. The reasons that led to this abandonment are not very clear in view of the fact that the phenyl isocyanate method lacks many of the disadvantages of the dinitrophenylation method. It was Edman, in 1950, who extended Bergman's procedure further by substituting phenyl isothiocyanate for phenyl isocyanate. The resulting 3-phenyl-2-thiohydantoins (PTHs) derived from amino acids have since occupied a central position in protein chemistry.

The first step of Edman's procedure is the formation of a phenylthiocarbamoyl peptide (PTC-peptide). The second is acidic cleavage (using CF_3COOH or CH_3COOH–HCl) of the N-terminal residue in the form of the 2-anilino-5-thiazolinone derivative, simultaneously liberating the intact remainder of the peptide with a free amino group (see reaction scheme above).

The third step is the conversion of the intermediate 2-anilino-5-thiazolinone derivative into 3-phenylthiohydantoin, which can be accomplished by two different methods. In anhydrous media (*e.g.*, CF_3COOH or HCl–CH_3NO_2) and at elevated temperatures, the transformation takes place as an intramolecular rearrangement:



On the other hand, in the presence of aqueous acid, the thiazolinone is rapidly hydrolysed to the corresponding PTC-amino acid, which then slowly undergoes ring closure to give the respective PTH-amino acid derivative (see reaction scheme above).

The kinetics and mechanism of the Edman degradation have been thoroughly studied by Edman[48], EDMAN AND LAUBER[50], BETHELL *et al.*[13] and MADER[115].

2. *Reaction of amino acids, peptides and proteins with phenyl isothiocyanate*

(a) Synthesis of PTC-amino acids (SJÖQUIST[181])

For this synthesis, 0.5 μmole of each amino acid (suitable limits 10–100 μg) is generally used. In order to convert the amino acids to hydrochlorides, thereby simulating an acid protein hydrolysate, they are dissolved in 0.1 *N* hydrochloric acid, an aliquot containing the required amount is transferred to a quartz tube and excess of hydrochloric acid is subsequently removed by gentle evaporation in a desiccator over potassium hydroxide. A few drops of water are added and the evaporation procedure is repeated twice in this way. The dry residue is dissolved in a freshly prepared buffer

(50 μl). Phenyl isothiocyanate (6 μl) is dissolved in freshly distilled acetone (1 ml) and 50 μl of this solution are added to the buffer solution. The phenyl isothiocyanate should be present in at least a two-fold molar excess over the amino acids. The tube is gently shaken to ensure complete mixing, well covered, and placed in a water-bath at 25° for 2.5 h. It is then placed in a desiccator over P_2O_5 and the contents are carefully evaporated for 15 min using a water pump and finally for 2 h in a vacuum of at least 0.01 torr. This operation removes every trace of phenyl isothiocyanate, triethylamine and acetic acid. A blank without amino acid is treated in the same way.

(b) Transformation of PTC-amino acids into PTH-amino acids

To the dry residue resulting from the preparation of the PTC-amino acids, 100 μl of water and 200 μl of acetic acid saturated with HCl are added. The tube is well covered and left in a water-bath at 25° for 6 h. Then the solvent is evaporated in a desiccator over potassium hydroxide in the manner described in the preceding section. The dried sample is dissolved in a suitable volume of ethanol for measurement of the UV absorption.

TABLE 9

MOLAR EXTINCTION COEFFICIENTS OF VARIOUS PTHs

PTH-derivative	$\varepsilon_{345\ nm}$	$\varepsilon_{800\ nm}$	$\varepsilon_{345}/\varepsilon_{800}$
Alanine	6,300	16,000	0.39
Arginine[a]	6,000	15,900	0.38
Aspartic acid[b]	6,500	16,100	0.40
Cystoic acid	5,900	16,600	0.36
Glutamic acid	6,200	15,900	0.39
Glycine	6,300	14,900	0.42
Histidine[a]	6,400	15,50c	0.42
Hydroxyproline	8,900	13,600	0.65
Isoleucine	6,500	17,000	0.38
Leucine	6,000	16,700	0.38
Lysine[c]	21,000	29,000	0.72
Methionine	6,900	17,100	0.40
Phenylalanine	5,700	15,500	0.37
Proline	9,600	14,300	0.67
Serine	5,500	15,500	0.35
Threonine	7,200	15,600	0.46
Tryptophan	8,000	19,700	0.41
Tyrosine	6,600	15,600	0.42
Valine	6,200	16,500	0.38

[a] Monohydrochloride.
[b] Potassium salt.
[c] ε-Phenylthioureido derivative.

The yield is approximately 100% in most instances. Considering that the yield refers to two reaction steps, it seems reasonably satisfactory. The most notable exception is serine, with a deficit of 18%. The cause of this deficit is known to be a side reaction that involves dehydration of the side-chain of serine[47]. The dehydration product is shown by the presence of an extra absorption peak at 320 nm. The tendency of threonine to undergo an analogous reaction is much less and is hardly noticeable un-

TABLE 10

YIELDS OF PTHs IN THE MICRO-SYNTHETIC PROCEDURE

PTH-derivative	*Yield (%)*	$\varepsilon_{245}/\varepsilon_{360}$
Alanine	95.5	0.40
Arginine	101.8	0.39
Aspartic acid	97.5	0.41
Cysteic acid[a]	108.4	0.40
Glutamic acid	97.3	0.41
Glycine	98.2	0.41
Histidine	95.4	0.46
Hydroxyproline	99.8	0.64
Isoleucine	98.6	0.39
Leucine	98.6	0.38
Lysine[b]	96.4	0.72
Methionine	99.0	0.39
Phenylalanine	99.4	0.39
Proline	99.4	0.66
Serine	82.6	0.50
Threonine	98.5	0.47
Tryptophan	98.9	0.44
Tyrosine	99.7	0.43
Valine	98.9	0.39

[a] The free acid.
[b] ε-Phenylthioureido derivative.

der the chosen conditions. It is relevant to the analytical procedure that the deficit in PTH-serine is remarkably constant and hardly affected by the duration of treatment in stage II. The ratio $\varepsilon_{245}/\varepsilon_{360}$ serves as a sensitive criterion of the purity of the products obtained. A comparison of Tables 9 and 10 shows a generally satisfactory agreement of the ratio $\varepsilon_{245}/\varepsilon_{360}$ for the authentic PTHs and for the microsynthetic products with the exception of PTH-serine.

(c) Edman degradation of peptides (FRAENKEL-CONRAT AND HARRIS[64])

The peptide (0.2–0.3 mg) is dissolved in 4 ml of 50% aqueous dioxan, the solution brought to pH 8.7–9.0 with 0.01 *N* sodium hydroxide and the mixture is stirred for 1.5 h at 40° with 0.1 ml of phenyl isothiocyanate while the pH is maintained constant. The reaction mixture is then extracted seven times with benzene and the aqueous solution is evaporated to dryness *in vacuo* over sodium hydroxide.

The sodium salt of the PTC-peptide is re-dissolved in water (2–10 ml) and aliquots corresponding to 0.2–1.0 μmole are made 3 *N* with respect to hydrochloric acid and $0.2–1.0 \cdot 10^{-4}$ *M* with respect to peptide by the addition of the correct amounts of water and 5.7 *N* hydrochloric acid. The rate of release of phenylthiohydantoin can be determined by following the change in the absorption maximum of the solution from 240 nm or lower to 265–270 nm during a period of about 2 h. If the transformation takes place too slowly for a given peptide, the effect of increasing the temperature to 40–45° should be tried. Spectrophotometric observation has been found to be useful for the first two or three stages, but thereafter accumulated artefacts having high UV absorption tend to interfere with the readings.

The PTH-amino acids are extracted into ethyl acetate (with the exception of PTH-arginine and PTH-histidine) and the residual peptide is recovered by evaporation of the aqueous solution as before. The residue is redissolved in 50% aqueous

dioxan and submitted to the same cycle of operations. For applications and modifications of this procedure see refs. 23, 52, 63, 79 and 85.

(d) Edman degradation of proteins (ERIKSSON AND SJÖQUIST[53])

The protein (5–10 mg) is dissolved in 1 ml of saline and mixed with 2 ml of a solution of pyridine–triethylamine–phenyl isothiocyanate (100:3:1). The coupling reaction is carried out at 40° over 1.5 h and the solution is washed five times with 5-ml volumes of benzene–ethylene chloride (3:1) previously saturated with 0.1 *N* sodium hydroxide. The last traces of solvent are removed at 40° in a gentle stream of nitrogen. Cyclization is then brought about by treatment of the solution with 1 ml of water and 2 ml of acetic acid saturated with HCl at 40° for 2 h. The mixture is then freeze-dried almost to dryness and the residue suspended in 2 ml of water previously saturated with ethyl acetate–methyl ethyl ketone (2:1). After extraction with four 2-ml portions of the latter solvent, the PTH-amino acids are dissolved in 0.1 ml of 90% acetic acid and chromatographed by the method described above. It is recommended that the cyclization, freeze-drying and extraction of the PTH-derivatives should be carried out on the same day.

(e) Subtractive Edman degradation (KONIGSBERG[101])

The subtractive modification of the Edman method involves the coupling of the peptide with phenyl isothiocyanate to yield the phenylthiocarbamyl derivative, followed by cyclization of this material with anhydrous acid to give the 2-anilinothiazolinone. The cyclization is a concerted process, in which the sulphur of the thiocarbonyl group attacks the carbonyl carbon of the N-terminal amino acid and causes the fission of the peptide bond, releasing the amino group of the penultimate amino acid residue. The 2-anilinothiazolinone of the N-terminal amino acid can be extracted into an organic solvent and a portion of the remaining peptide taken for hydrolysis and amino acid analysis. If the procedure is successful, the amino acid analysis of the remaining peptide will reveal a decrease in one amino acid residue in comparison with the analysis of the starting material. The amino acid residue that has disappeared is presumed to have been present at the N-terminal end of the peptide undergoing degradation. By successive applications of this method, the amino acid sequence of a peptide can be determined.

In this chapter, the scope and limitations of the subtractive modification of the Edman degradation are discussed.

Coupling of the peptide with phenyl isothiocyanate. The two conditions essential for this reaction are an alkaline pH (it is the unprotonated amino group that attacks the thiocarbonyl group of phenyl isothiocyanate) and a solvent in which both the peptide and the reagent have appreciable solubility. A number of different combinations of solvents have been used, such as (1) 50% aqueous pyridine containing 2% of triethylamine, (2) N-ethylmorpholine (60 ml)–acetic acid (1.5 ml)–95% ethanol (500 ml)–water (438 ml), and (3) 50% aqueous pyridine containing 5% of dimethylallylamine.

Complete reaction can be achieved by using a 50-fold excess of phenyl isothiocyanate at 37° for 2 h. The size of the reaction vessel and the volume of solvent should be kept to a minimum to avoid mechanical losses and to prevent the introduction of impurities that may yield amino acids on hydrolysis.

The importance of excluding oxygen during the coupling process has been stressed by ILSE AND EDMAN[84], as oxygen will replace sulphur in the thiocarbonyl group of the phenylthiocarbamyl peptide. The oxygen of the phenylcarbamyl group will not attack the carbonyl carbon of N-terminal amino acids at an appreciable rate under the conditions used for cyclization. The phenylcarbamyl peptide, if formed but not cyclized, will interfere with the interpretation of the results obtained in subsequent stages. As this material has no free amino group that can react with phenyl isothiocyanate, it cannot be degraded further but will be carried along with the peptide fraction. Upon acidic hydrolysis, this fraction will yield some portion of all the amino acids present in the original peptide (no matter how far the degradation has been carried). If some phenylcarbamyl peptide is formed during each coupling reaction, the gradual accumulation of these products during the course of several stages will contribute increasingly to non-integral loss of the N-terminal amino acid that is being removed. While the presence of phenylcarbamyl peptides will not affect the quantitative interpretation of the results obtained from the direct identification of phenylthiohydantoins, their formation will decrease the yields of hydantoins at each stage.

Removal of reagents. After the phenylthiocarbamyl peptide has been formed, the reaction mixture is usually evaporated almost to dryness and excess phenyl isothiocyanate, phenylthiourea and residual organic solvent can be removed by extraction three times with 1–2-ml volumes of benzene. By avoiding two liquid phases at this stage, the problem of emulsions can be eliminated. The residue left after benzene extraction is evaporated again. The presence of benzene aids the removal of the last traces of water during this operation, as a benzene–water azeotropic mixture is formed, which evaporates at a lower temperature than water. The dry residue is now ready for cyclization.

Cyclization. Edman first emphasized the need for maintaining anhydrous conditions during the cyclization in order to avoid the cleavage of acid-sensitive bonds. Although many variants have been tried, a common procedure has involved the use of acetic acid saturated with HCl gas. This method was used in sequence studies on lysozyme, cytochrome and ribonuclease. With ribonuclease, a serious problem was encountered when using acetic acid–HCl at 100°, as it was discovered that hydroxyl groups of serine and threonine residues are probably esterified in the acetic acid–HCl mixture and losses of these amino acid residues on acidic hydrolysis were excessive. When a serine or threonine residue is situated next to the amino acid residue being removed by cyclization, the esterification of its hydroxyl group places the acetyl group in a position where it can readily undergo an O → N acyl migration when the pH is raised for the next stage of coupling with phenyl isothiocyanate. This results in the prevention of further degradation.

In a search for milder conditions for the cyclization, anhydrous trifluoroacetic acid was used at 25° for 1 h and was found to be effective in promoting complete cyclization of all the potentially degradable material. A second treatment of the residue left after evaporation of the trifluoroacetic acid resulted in no additional decrease of the amount of the N-terminal amino acid (after hydrolysis with 6 *N* HCl), even if the initial decrease was less than one residue.

Trifluoroacetic acid has also been used at 40° for 15 min; these conditions result in complete reaction without fission of additional peptide bonds.

Trifluoroacetic acid used at 25° for 1 h caused no trifluoroacetylation of the

hydroxyl groups of serine when tested with glycyl-L-serine. This peptide, however, undergoes O-acetylation in the HCl–acetic acid mixture used in earlier studies.

Extraction of the N-terminal amino acid derivatives. After the cyclization, the trifluoroacetic acid is evaporated off and the residue, dissolved in 0.2 *M* acetic acid, is heated for 10 min at 40°, which converts the anilinothiazolinones to a mixture of phenylthiocarbamylamino acids and phenylthiohydantoins. The aqueous phase is extracted three times with benzene. This extraction removes the phenylthiocarbamyl-amino acids and phenylthiohydantoins of most of the acidic and neutral amino acids, except for the derivatives of arginine, histidine, aspartic acid, serine, threonine and cysteic acid. These derivatives can be extracted into ethyl acetate, but with this solvent there is some risk of extracting peptide material also, especially if the peptide contains non-polar amino acid residues. The material in the benzene layer can be completely converted to the phenylthiohydantoins by further heating and can then be identified directly by several methods, described in subsequent chapters.

A suitable aliquot of the aqueous solution can be taken at this stage and subjected to hydrolysis followed by amino acid analysis. The remainder of the aqueous solution can be evaporated and the residue then coupled again with phenyl isothiocyanate.

A useful variant of this procedure involved the addition of dichloroethane to the trifluoroacetic acid. The peptide is precipitated and the 2-anilinothiazolinone and trifluoroacetic acid are extracted into the organic solvent. Alternatively, the trifluoroacetic acid can be evaporated and the dry residue extracted with a mixture of benzene and chloroform. The thiazolinones can be recovered from the organic solvent after evaporation, and converted either to the phenylthiohydantoins by heating with aqueous acid or to amino acids in yields of 10–30% by heating with 0.2 *N* NaOH for 2 h at 100°. If the hydrolysis is carried out with a small volume of alkali, the sample can then be neutralized directly without desalting, and used in an amino acid analyzer.

Purification of the remaining peptide. When the subtractive method is used, a problem arises in the interpretation of the results, as was mentioned earlier. If side reactions occur that lead to an accumulation of products that will not react with phenyl isothiocyanate or undergo cyclization, then the amino acid analysis of the remaining peptides will show non-integral decreases of the amino acid residues that are being removed. To overcome this difficulty when it has occurred, the authors of this method separated the remaining peptide, after cyclization, from side-reaction products by a simple ion-exchange procedure. After extraction with benzene, the aqueous layer, which contains the remaining peptide, is adsorbed on a 10 × 2 mm column of Dowex 50-X2 (50–100 mesh) in the acid form. The column is washed with 4 ml of water, followed by 2 ml of an appropriate pyridine acetate buffer (usually 1 *M* in pyridine, pH 5.6) to elute the peptide. The material recovered from the first water wash is ninhydrin-negative, but after acidic hydrolysis all the amino acids present in the parent peptide are found. This indicates that the material eluted in the water wash is a peptide derivative that is devoid of free amino groups. The procedure cannot be applied to acidic peptides that contain cysteic acid, as such peptides are not adsorbed, nor can it be used with peptides that have many basic or aromatic residues, because these peptides are eluted with difficulty from the column, even with volatile buffers at high concentrations.

(f) Automated procedure for Edman degradation

Among various methods for the determination of PTH-amino acid derivatives, special attention must be paid to products that arise from Edman's sequenator. From the point of view of the chromatographer, the sequencing procedure should be considered as a special method of sample preparation and of course all the different techniques, *i.e.*, flat-bed separation, liquid column chromatography or gas chromatographic separation and identification of the cleaved amino acids, can be used. It is unlikely that this review will be used by workers as a practical manual for operating the sequenator. Therefore, the following paragraphs are limited to a general description of the apparatus and special emphasis is placed on specific aspects of the chromatographic separations related to this operation.

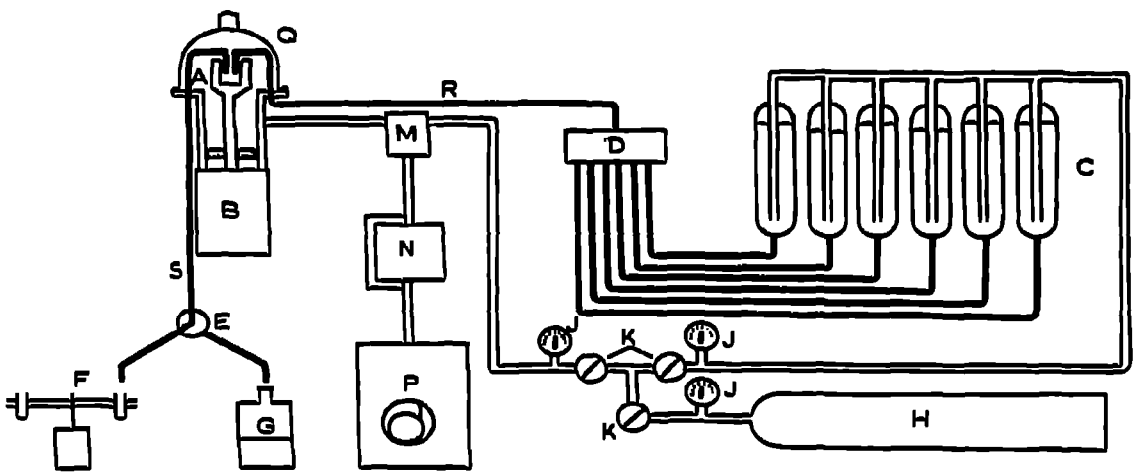


Fig. 68. Schematic representation of the sequenator. A = spinning cup; B = electric motor; C = reagent (solvent) reservoir; D = valve assembly; E = outlet stopcock assembly; F = fraction collector; G = waste container; H = nitrogen cylinder; J = pressure gauges; K = pressure regulators; M = 3-way valve; N = 2-way valve with bypass; P = rotary vacuum pump; Q = bell jar; R = feed line; S = effluent line. Gas lines are doubly contoured; liquid lines are filled.

The general design of the sequenator as described by EDMAN AND BEGG[40] can be seen in Fig. 68. The main part of the apparatus is a spinning cup in which the sample is placed at the beginning of the operation and in which all the reagents and extractants are directed according to a pre-set programme. Reagents and solvents enter through the feed-line to the bottom of the spinning cup and both rise along the walls of the cup in the form of a thin film. Extractants are scooped off and transferred through the collecting line to a fraction collector. The whole system requires the precise addition of reagents and extractants, which is achieved by using overpressure. The reservoirs (six reservoirs are attached to the system) exhibit a slight overpressure compared to the spinning cup space so that the amount of reagent or extractant supplied is regulated by the time period for which the valve assembly is left open. Overpressure in both the reservoir system and the spinning cup area is maintained by nitrogen. The cyclic operation of the sequenator is ensured by the programming unit, which divides the whole preparation of thiazolinone (see p. 294) into thirty steps. The compositions of reagents involved in the individual steps of this procedure are as follows. Reagent No. 1 is a 5% (v/v) solution of phenyl isothiocyanate in heptane.

Phenyl isothiocyanate is unstable in the coupling medium and is therefore kept in a separate system. Reagent No. 2 is 1.0 *M* Quadrol–trifluoroacetic acid buffer (Quadrol = N,N,N,N-tetrakis-2-hydroxypropylethylenediamine) in *n*-propanol–water (3:4), pH = 9.0. Reagent No. 3 is anhydrous *n*-heptafluorobutyric acid. The replacement of the usual solvents in Edman's procedure by heptafluoroacetic acid and Quadrol arises from the decreased volatility requirements. Solvent No. 1 is benzene, solvent No. 2 is ethyl acetate containing 0.1% acetic acid and solvent No. 3 is 1-chlorobutane. As the over-all yield obtained after individual steps in this procedure is decisive for the final result, considerable attention must be paid to solvent purification. This refers especially to traces of aldehydes, which must be completely removed as they tend to react with the terminal amino group and cause a progressive decrease in yield. The purification procedure for solvents has been described in great detail by EDMAN AND BEGG[49].

The applicability of the sequenator, although theoretically unlimited, is in practice restricted by several factors. The first important factor is the over-all recovery per step; when this recovery is 99%, 120 steps are possible; when it falls to 97%, no more than 40 steps can be carried out. Another important factor in the termination of a sequence study is the appearance of increasing background in the chromatography of PTH-derivatives. The appearance of additional spots, which presumably arise from non-specific cleavage of the polypeptide chains, eventually makes the identification of the cleaved amino acid impossible. There is no general agreement about the reason for the background. The problem is that with a high background it might easily happen that the most intense spot is not that of the amino acid cleaved in the particular step but that of the amino acid that occurs most frequently within the structure. This is of decisive importance in the sequencing of proteins or peptides with high internal homogeneity. The problem may lie in the acidolysis of peptide bonds caused by heptafluorobutyric acid, as similar effects were shown to occur with trifluoroacetic acid and there is no reason to suppose that the effects of these two acids would be different.

Another problem is the occurrence of incomplete reactions during the coupling or cleaving steps, which results in overlaps between consecutive steps. Even if this occurred only to a small extent in each cycle, the overlap would become very distinct over many cycles because of cumulative effects. This problem is related mainly to aspartic and glutamic acids, which were shown to undergo incomplete cleavage. EDMAN AND BEGG[40] suggest that this incomplete cleavage is probably due to an equilibrium being reached before complete cleavage has occurred. In the practical version of this procedure suggested by EDMAN AND BEGG[49], the cleavage step is repeated in order to avoid these complications.

In general, there is no problem in identifying any of the common amino acids during the chromatographic separation of the cleaved PTH-amino acids. However, it must be stressed that, due to the occurrence of decomposition reaction during the cleavage step, some of the PTH-amino acids form multiple spots; this occurs mainly to PTH-asparagine, PTH-glutamine and PTH-serine. With PTH-asparagine and PTH-glutamine, the additional spots identified are those of PTH-aspartic acid and PTH-glutamic acid, respectively, which are produced by hydrolysis. The nature of the aditional spots in PTH-serine was not identified.

Another problem that can be expected is the decomposition of some amino

acids within the structure sequenced. This is of particular interest with PTH-tryptophan, but no exact results related to this problem are available.

In addition to the problems discussed above and the limitations of Edman's automated procedure, there are some practical aspects that may place some additional restrictions on the use of the sequenator. These were reviewed recently by VON WILM[206], and a summary of them is given here.

During the prolonged degradation of peptides that are composed mainly of amino acids with long hydrophobic side-chains, solubility problems frequently arise. The stepwise degradation cannot be carried out up to the extreme C-end, as there are only minor differences in the solubilities of short-chain peptides and thiazolinone derivatives of amino acids and therefore quantitative extraction is no longer possible.

(g) Edman degradation in acrylamide gel

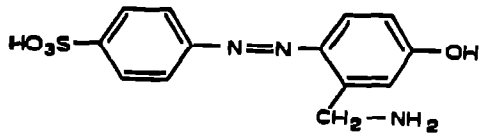
A more advanced variation of the method described by CATSIMPOOLAS[37] for dinitrophenylation (see p. 229) is to carry out the whole of Edman's degradation procedure with electrophoretically separated proteins using polyacrylamide gel as the carrier. SCHYNS[177] was the first to use this idea, with the following procedure. Columns of polyacrylamide gels containing 200–300 μg of the protein to be determined are subjected to an electrophoretic separation. The gels are then extruded and agitated with 0.5 *M* phosphate buffer of pH 8.7 for 15 min. After this period, the gels are removed, pyridine is added and agitation is continued for another 30 min. In the next step, the gels are transferred to a pyridine solution of phenyl isothiocyanate (5%), and agitated for an additional hour. Finally, they are transferred to a 2.5% solution of phenyl isothiocyanate in pyridine and allowed to stand overnight. After the reaction is completed, the expected location of the individual bands is controlled by comparison with a run carried out in parallel and stained by the above dinitrophenylation technique of CATSIMPOOLAS[37] or by the routine Coomassie blue procedure. Gels are dipped in benzene containing 10% of water and are shaken until they are free from colour. The procedure must be carried out for a sufficiently long period of time and the solvent should be replaced at least five times. Hydrolysis of the PTH derivatives is carried out on pooled samples in 1 *N* HCl at 100° for 90 min in a sealed tube. The mixture is then cooled and extracted with ethyl acetate, the organic layer is removed, extracted with a minimum amount of water and the PTH-derivatives are identified in the aqueous extract by a common flat-bed technique (see pp. 311 and 321).

(h) Edman degradation in a paper strip

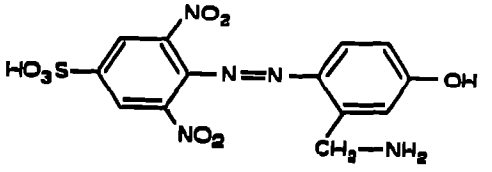
This method is closely related to the paper chromatography of PTH-amino acids and therefore is described in the chapter on paper chromatography of PTH-amino acids (see p. 315).

(i) Edman degradation of peptides fixed on alumina (BIRR *et al.*[18])

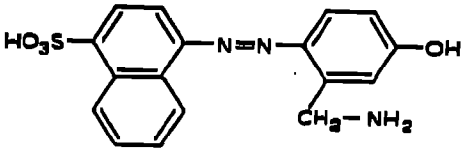
Another solid support that can be used for the binding of peptides for Edman degradation is alumina. The carboxylic groups in peptides are too weakly acidic to be appropriately bound to alumina for a successful Edman degradation. BIRR *et al.*[18] therefore suggested that peptides, after blocking their amino groups with the *tert.*-butyloxycarbonyl (Boc) group, are linked as amides with the aminomethyl group of the arylazosulphonic acids, I–III:



I



II



III

The coloured peptide derivatives are fixed as anions to acidic alumina and subjected in the solid state to stepwise Edman degradation with phenyl isothiocyanate. The actual procedure is as follows. A 0.15 mole amount of Boc-peptide and an equivalent amount of N-methylmorpholine are dissolved in 5 ml of dimethylformamide, then 0.15 mmole of ethyl chloroformate is added at −15° followed by 0.22 mmole of arylazosulphonic acid (I–III) in 5 ml of dimethylformamide and 0.44 mmole of N-methylmorpholine. After 2 h, 12 g of acidic alumina are added and the suspension is filtered.

The Boc-group is split off by re-suspending the peptide–alumina complex in 10 ml of trifluoroacetic acid–tetrahydrofuran (1:1) mixture. The reaction is finished within 10 min and the complex is washed with 10 ml of tetrahydrofuran, 10 ml of a 10% solution of N-methyl morpholine in tetrahydrofuran, and finally with 10 ml of tetrahydrofuran.

The coupling with phenyl isothiocyanate is performed by adding 10 ml of a mixture of phenyl isothiocyanate–N-methylmorpholine–pyridine–tetrahydrofuran (1:1:1:5, v/v) and by shaking at 40° for 5 h. The complex is then washed with tetrahydro-

TABLE II

SEQUENCE OF REACTIONS USED FOR THE EDMAN DEGRADATION OF PEPTIDES BOUND AS AMINOMETHYLAZOBENZENESULPHONIC ACID DERIVATIVES TO ALUMINA BY AN IONIC BOND

(*Ca.* 10 g of alumina per 0.1 mmole of the peptide azo dye.)
NMM = N-methylmorpholine; THF = tetrahydrofuran; Py = pyridine; TFA = trifluoroacetic acid; N_2 = nitrogen atmosphere.

Step No.	*Reagent (10 ml, 40°)*	*Repetitions*	*Total time (min per step)*
1	NMM–THF (1:9)	–	30
2	THF	2	15
3	THF–phenyl isothiocyanate–NMM–Py (5:1:1:1) (N_2)	–	300
4	THF (N_2)	5	30
5	TFA–THF (1:1) (N_2)	–	60
	THF (N_2)	7	40

furan, 10 ml of trifluoroacetic acid is added and shaking at 40° is continued for an additional hour. The complex is filtered, washed with the three solvents as described above and is ready for the next degradation step.

PTH-amino acids are isolated from evaporated washings (5) and (6) (See Table 11) and identified by any of the methods described later in this review.

(j) Concluding remarks

There are many variations and modifications of Edman's degradation, and some recent ones are mentioned below. However, their real value for a protein chemist should be clear after a certain period of time.

SMITHIES *et al.*[185] developed some quantitative procedures for use with the EDMAN AND BEGG[40] sequenator.

VANCE AND FEINGOLD[105] described an additive Edman degradation procedure for the sequencing of small peptides.

ROCHAT *et al.*[161] suggested the alkylation of SH-groups prior to the Edman degradation, in order to overcome problems connected with the degradation of peptides that contain cysteine.

ORLOVSKA *et al.*[137] used $(CF_3CO)_2O$ for the cyclization step of the Edman degradation.

For the identification and quantitation of PTH-amino acids, it is possible to use other methods in addition to those described in this chapter in detail, *e.g.*, IR[132], mass[54,55,108] and NMR[24] spectrometry.

3. Column chromatography

Up to the present time, only one column chromatographic separation of PTH-amino acids has been described, by SJÖQUIST[180,181], who used Celite as the sorbent. The experimental procedure is as follows.

Preparation of the column. Celite (10 g) is mixed with 6 ml of the stationary phase. The mixture is then slurried with 100 ml of the mobile phase and this slurry is immediately added to the column (80 × 0.8 cm). The height of the filled space is *ca.* 50 cm.

Sample application. The sample (about 10 μg of each PTH-amino acid) is dissolved in 0.1 ml of the stationary phase and transferred with a capillary pipette to the top of the column. Two 0.1-ml portions of the stationary phase and three 0.2-ml portions of the mobile phase are used for washing the sample into the column. The space above the column is subsequently filled with the mobile phase.

Development. In the following paragraphs, three different chromatographic operations for the separation of twenty PTH-amino acids are described, which together form the analytical procedure. Two of the operations are carried out on columns, here called Column I and Column II, and these are supplemented by a paper chromatographic separation of PTH-arginine and PTH-histidine.

In column I, the preparation of which is described above, the following solvent systems are used.

(A) Heptane (250 ml) + propionic acid (100 ml) + ethylene chloride (5 ml) + water (30 ml). The lower layer (78 ml) is used as the stationary phase and the upper layer as the mobile phase.

(B) Heptane (85 ml) + propionic acid (5 ml) + ethylene chloride (10 ml). Only one phase is obtained.

The flow-rate is adjusted to 15 ml/h. The elution is started with solvent A, and solvent B is introduced when the peak of PTH-phenylalanine has left the column. The result of a typical separation is shown in Fig. 69.

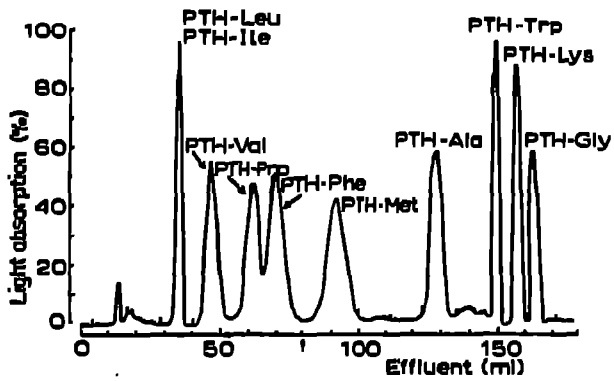


Fig. 69. Chromatographic separation of PTH-amino acids on a Celite column. Optical density recorded at 269 nm. The arrow indicates the change of solvent. Column I.

Column II is operated in the same way as column I up to the stage when the PTH-Phe has left the column. From that stage, a gradient elution is applied. The gradient consists of 100 ml of solvent A to which is gradually added a mixture of heptane (58 ml) + propionic acid (17 ml) + ethylene chloride (25 ml).

The result of a typical separation is shown in Fig. 70. The only unresolved pair of PTH-amino acids is that of arginine and histidine phenylthiohydantoins. These PTH-derivatives are separated by paper chromatography in heptane–*n*-butanol–75% formic acid (40:30:9). The R_F value for PTH-histidine is 0.37 and for PTH-arginine 0.46. The spots are eluted with 70% ethanol and evaluated spectrophotometrically at 296 nm.

The recoveries both in column and paper separations are better than 95%, with the exception of PTH-serine and PTH-threonine, for which the recoveries are 40% and 70%, respectively.

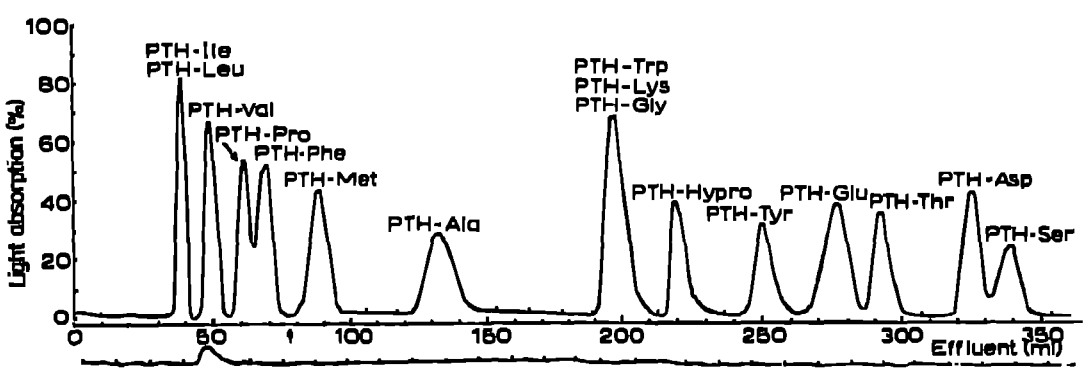


Fig. 70. Separation of PTH-amino acids. Column II. The gradient began at the arrow. The lower curve is a tracing of a blank without PTH-derivatives. Optical density recorded at 269 nm.

4. Gas chromatography

The first attempt to separate phenythiohydantoin derivatives of common amino acids was made by PISANO *et al.*[148]. The gas chromatographic techniques used in this instance were essentially derived from those used for the separation of steroids and other natural products. The column packings used contained relatively thin-film coatings of highly thermostable liquid phases with an argon ionization detection system. The methylsilicone polymer SE-30 is reported by VANDENHEUVEL *et al.*[107] to exhibit the most advantageous properties. Using a column of dimensions 180 × 0.34 cm with 0.75% SE-30 on 100–140-mesh Gas-Chrom P resulted in separation. The argon inlet pressure was 1.5 atm. Most PTH-derivatives were clearly separated (see Table 12). However, derivatives of serine, threonine, asparagine and glutamine, and also of basic amino acids, presented some difficulties. The serine and threonine derivatives underwent dehydration, as demonstrated by the UV spectra of the materials resulting from the chromatographic separation. Asparagine and glutamine derivatives also yielded altered products; in this instance, however, the alteration did not represent a serious problem as a constant pattern was obtained with each substance. As common amino acids have a wide range of molecular weights, one would also expect that their separation would proceed well with the SE-30 polymer. This was found to be virtually true, except for leucine and isoleucine PTH-derivatives, for which PISANO

TABLE 12

RELATIVE RETENTION TIMES OF PTH DERIVATIVES OF AMINO ACIDS

Column packing, 1% of the liquid phase on 100–140 mesh Gas-Chrom P; 6 ft. glass coils or U-tubes, 3.4–5.0 mm I.D.; 0.1–0.2 atm argon; Lovelock argon ionization detection system. Aspartic and glutamic acids on SE-30 and QF-1 chromatographed as methyl esters prepared with BF_3–methanol.

Amino acid	*SE-30*	*QF-1*	*PhSi*
	175°	*175°*	*200°*
Alanine	0.81	0.63	0.35
Glycine	1.03	0.79	0.54
Proline	1.18	1.14	0.59
Valine	1.46	0.80	0.40
Leucine	1.80	1.21	0.53
Isoleucine	1.80	1.10	0.55
Androstane	1.00	—	—
Cholestane	—	1.00	1.00
	200°		
Aspartic acid	2.12	1.89	—
Glutamic acid	3.19	2.31	—
Methionine	3.95	3.04	2.03
Phenylalanine	5.38	3.52	3.10
Androstane	1.00	—	—
Cholestane	—	1.00	1.00
	250°	*255°*	*220°*
Tyrosine	1.12	2.77	9.67
Histidine	1.29	4.07	—
Tryptophan	2.84	5.46	32.3
Cholestane	1.00	—	1.00
Cholesterol	—	1.00	—

et al.[148] used the SF-1 phase, a fluoroalkylsilicone polymer reported previously by VANDENHEUVEL *et al.*[190].

A more recent procedure used for the same purpose is that of HARMAN *et al.*[75]. In this instance, the difficulties arising from the separation of non-derivatized PTH-derivatives were bypassed by using silylation with bistrimethylsilylacetamide. The retention times observed for the trimethylsilylated thiohydantoins of serine and threonine are several times greater than those of the parent dehydrated compounds. This is believed to be due to the presence of a trimethylsilyloxy group, which also seems to suggest that the hydroxyl group has been successfully protected. Trimethylsilylation of the carboxyl group can be substituted for methylation with PTH-aspartic and -glutamic acids, and satisfactory chromatographic results were reported for the trimethylsilylated derivatives of PTH-asparagine and PTH-glutamine, which present some difficulties in the non-derivatized form, as reported by PISANO *et al.*[148]. The explanation for this phenomenon is based on the fact that the polar terminal amide groups have been transformed into less polar derivatives. In addition, trimethylsilylation of other PTH-derivatives that are normally considered to be sufficiently volatile led to considerable alterations in retention behaviour. The actual increase in retention times is evident from Table 13. It has been also reported by HARMAN *et al.*[75] that trimethylsilylation improves the peak shape by increasing the symmetry, which provides more indirect evidence for a considerable decrease in polarity. The above structural considerations concerning trimethylsilylated PTH-derivatives were further

TABLE 13

RELATIVE RETENTION BEHAVIOUR OF PTH DERIVATIVES OF AMINO ACIDS AND THEIR TRIMETHYLSILYLATION PRODUCTS WITH SE-30

A Barber-Colman Model 15 (argon ionization detector) and an F & M Model 402 (flame ionization detector) gas chromatographs were used. The column packing was 1.7% SE-30 (a methylpolysiloxane) coated on acid-washed and silanized Gas-Chrom P.

Amino acid	*Temperature (°C)*	*PTH*	*TMSi-PTH*
Alanine	170	0.79	1.03
Glycine		0.94	1.05
Isoleucine		1.55	2.12
Leucine		1.65	1.98
Proline		1.38	1.38[b]
Serine		0.89[c]	2.24
Threonine		0.97[c]	2.76
Valine		1.12	1.41
Asparagine	200[a]	—	5.53
Aspartic acid		—	3.41
Glutamine		—	8.25
Glutamic acid		—	4.91
Methionine		3.72	3.86
Phenylalanine		5.00	5.23
Tyrosine	225[d]	1.13	1.03
Histidine		1.27	0.98
Tryptophan		2.59	2.13

[a] Relative to androstane.
[b] Does not form a TMSi derivative.
[c] Dehydration product.
[d] Relative to cholestane.

confirmed by combined chromatography–mass spectrometry, which showed quite clearly the presence of one trimethylsilyl group in simple amino acid derivatives such as those of PTH-glycine and two of these groups in more polar amino acid derivatives such as those of PTH-serine, PTH-threonine, PTH-asparagine and PTH-glutamine.

An exception to the generally increased retention times of trimethylsilylated PTH-derivatives are the retention data related to tyrosine, tryptophan and histidine, which are decreased. For tyrosine, this fact is explained by the transformation of the phenolic group into a hydrocarbon-like trimethylsilyl ether, which is an additional reaction to the normal substitution on the hydantoin-ring nitrogen. With the PTH-derivatives of histidine and tryptophan, the decrease in polarity suggests that derivatization of the imidazole and indole rings, respectively, has occurred. This conclusion was shown to be true by mass spectrometry. The great variety in molecular structure resulted, of course, in a wide range of volatilities and hence in considerable differences in the retention times of the particular PTH-derivatives. As a result, three different isothermal data were reported by HARMAN *et al.*[75] and are listed in Table 13. It is quite clear that this is the situation when temperature-programmed separation should be successfully applied. When using a temperature gradient from 165 to 240° at the rate of *ca.* 2°/min, the results for the separation of silylated PTH-amino acids are excellent.

HARMAN's procedure can be further improved by adding sulphur-specific detection to the system as reported by GUERIN AND SHULTS[73].

The most advanced procedure for the gas chromatographic separation of PTH-amino acids, in our opinion, is that described by PISANO AND BRONZERT[147]. The most fundamental contribution of this technique is that it distinguishes three groups of normal PTH-derivatives resulting from Edman's procedure: these three groups differ markedly in their volatilities and compatibilities with different types of liquid phases (see Table 14).

Group I. These derivatives chromatographed well on all the different of phases tested by PISANO AND BRONZERT[147], but separations were better with relatively non-polar phases such as SE-30 and OV-1. The best results were reportedly obtained on DC-560 with 7–10% of coating. The latter phase was capable of separating all members

TABLE 14

GROUPING OF AMINO ACID PTH DERIVATIVES ACCORDING TO GAS CHROMATOGRAPHIC BEHAVIOUR

Group I amino acids are the most volatile and generally give symmetrical peaks. Members of Group II are the least volatile. Histidine, asparagine and glutamine show the greatest tendency to adsorb on the column packing, give tailing peaks and low responses. Group III derivatives include those which must be silylated before analysis (aspartic, glutamic and cystoic acids) and others which, when silylated, have significantly better chromatographic properties. Silylation of Group I and II derivatives is not obligatory but can provide useful confirmatory data.

Group I	*Group II*	*Group III*
Alanine	Asparagine	Aspartic acid
Glycine	Glutamine	S-Carboxymethylcysteine
Valine	Tyrosine	Cystoic acid
Leucine	Histidine	Glutamic acid
Isoleucine	Tryptophan	Lysine
Methionine		Serine
Proline		Threonine
Phenylalanine		

of the group except the leucyl and isoleucyl derivatives. On the contrary, however, the resolution of group I members was poor on columns containing OV-22 or XE-60 as the liquid phase.

As mentioned also with other techniques for the GLC of PTH-derivatives, the separation chromatography of isoleucyl and leucyl phenylthiohydantoins was very difficult. Partial resolution was obtained by PISANO AND BRONZERT[147] on various liquid phases, *e.g.*, XE-60, QF-1, ECNSS-S, HI-EFF-8BP and OV-210. The best results were reported with the OV-210 column.

Group II. These include less volatile PTH-derivatives that are eluted much later than those of group I. Their higher polarity causes considerably more tailing, which it is possible to eliminate by applying a more polar phase, though it has never been completely eliminated. Liquid phases such as XE-60, OV-22 and OV-17 proved to be suitable for separating this group of PTH-derivatives. XE-60 is particularly recommended for this group of compounds.

Group III. These include PTH-derivatives that are much less suitable for direct gas chromatographic analysis. No peaks were obtained for the glutamyl, aspartyl or cysteic acid derivatives. Two peaks were observed with seryl and threonyl compounds, and derivatives of lysine and carboxymethylcysteine gave 15 and 45% yields, respectively. In order to overcome these difficulties, it is suggested that derivatives of this group are subjected to silylation.

The following silylation procedure was recommended by PISANO AND BRONZERT[147]. Amino acid phenylthiohydantoin standards or the appropriate amount of unknown substance were reacted with N,O-bis(trimethylsilyl)acetamide in conical glass reaction tubes of capacity 0.5 ml and an over-all length of less than 5 cm. The tubes were sealed with PTFE-lined rubber septa and the samples were withdrawn with a 10-μl Hamilton syringe by piercing the septa. The syringe needle can reach the tip of the tube, thus enabling total volumes of only a few microlitres to be withdrawn. A typical silylation procedure was carried out as follows. Aliquots of 5–20 μl of a 1 mg/ml solution of the amino acid phenylthiohydantoin standard or of an appropriate amount of an unknown substance dissolved in ethyl acetate were added to the tube with an equal volume of N,O-bis(trimethylsilyl)acetamide and the tubes were immediately stoppered to avoid any contact with atmospheric moisture. The reaction vessels were shaken vigorously for 10–15 min in a water-bath at 50°. Aliquots were injected directly into the gas chromatographic column. For the silylation of the asparagine, glutamine, histidine and cysteic acid derivatives, aliquots of the standard solutions were evaporated to dryness under a stream of nitrogen in the reaction tube. The dry residue was then re-dissolved in a small volume of pyridine or acetonitrile and treated with the silylation reagent as described above.

Grouping the PTH-derivatives according to their volatilities and resulting differences in chromatographic behaviour suggests the use of a dual-column system. This was discussed in great detail by PISANO AND BRONZERT[147]. A column coated with DC-560 could be used primarily for the group I derivatives and, in conjunction with the silylation procedure, also for the indification of the group III derivatives. Derivatives belonging to group II could be resolved much better by using a column coated with XE-60. The over-all procedure for analysing an unknown sample was carried out as follows. The anilinothiazolinone derivatives were converted into phenylthiohydantoins by incubation in 0.2 ml of 1.0 *M* HCl for 10 min at 80° under nitrogen. This

solution was extracted with three 1-ml volumes of ethyl acetate to remove the thiohydantoins of all the amino acids except arginine, histidine and cysteic acid. These derivatives are known to be insoluble in ethyl acetate and therefore they remain in the aqueous layer. The combined ethyl acetate extracts were concentrated under a stream of nitrogen to a volume of less than 0.5 ml. This solution was transferred to the reaction tube as described above and evaporated to dryness. The dry residue was dissolved in a small volume of ethyl acetate, usually at a concentration of 0.1–1.0 $\mu g/\mu l$. Although solubility problems may arise when handling crystalline PTH-derivatives, those arising from individual steps of Edman's procedure did not produce any difficulties in this respect. Re-dissolution of the sample can sometimes be accelerated by gentle heating to 40–50°. The PTFE-lined septa of the tubes prevent evaporation of the ethyl acetate. Aliquots for gas chromatography (1–7 μl) were taken with a 10-μl Hamilton syringe. The first aliquot was injected into the DC-560 column, which permits the identification of most derivatives. The phenylalanine, asparagine, lysine, tyrosine, isoleucine and leucine derivatives were determined with the XE-60 column using another aliquot. A further aliquot of the sample was silylated and injected on to the preferred column when some of the following problems were to be resolved:

(1) no peak is obtained with the first injection and the identification of aspartic or glutamic acid derivatives is required (DC-560);
(2) serine or carboxymethylcysteine derivatives have to be identified (DC-560);
(3) the lysine and tyrosine derivatives have to be identified (XE-60);
(4) a better response is required with S-carboxymethylcysteine, serine, threonine and lysine (DC-560);
(5) confirmatory data are required for any of the remaining amino acid PTH-derivatives.

If all the above possibilities fail to give an appropriate answer, the aqueous phase is examined for the presence of PTH-histidine, PTH-arginine and PTH-cysteic acid. When the hydrochloric acid solution of the histidyl derivative is evaporated to dryness, it cannot be transferred into an organic solvent; consequently, it is treated with 1 *M* Na_2HPO_4, which converts the derivative into the free base and makes it susceptible to the ethyl acetate extraction. The combined extracts (three) are evaporated to dryness under a stream of nitrogen, the residue is dissolved in a small volume of methanol and the solution is applied to an XE-60 column. The arginine derivative cannot be identified by the gas chromatographic method and therefore must be identified by a suitable flat-bed technique, or by the Sakaguchi reaction. The derivative of cysteic acid is suitable to detection as the trimethylsilyl derivative, but this has been carried out only with standards and no data on actual derivatives resulting from Edman's procedure are available.

RODA AND ZAMORANI[162] suggested another procedure for overcoming the volatility problems with common thiohydantoins, involving their conversion into trifluoroacetylated derivatives. The practical procedure is very simple. To about 3 ml of each PTH-amino acid to be determined or to an appropriate amount of an unknown substance, 2 ml of methylene chloride and 0.3 ml of trifluoroacetic anhydride were added. The solution was kept at room temperature for 30 min and then analysed by gas chromatography. The following conditions gave good separations: column 1.5 m × 3 mm; carrier gas, nitrogen at a flow-rate of 20 ml/min; support, Chromosorb W; stationary phase, 5% SE-30; injector temperature, 220°; detector temperature, 250°;

oven temperature, programmed from 130° to 150° at 4°/min and from 150° to the end of the run at 2°/min; and amount of sample injected, 1 μl.

5. *Paper chromatography*

The paper chromatography of PTH-amino acid derivatives is one of the chromatographic procedures that underwent rapid development at first but has since been considerably neglected. About six different solvent systems were reported for the separation of these derivatives by EDMAN AND SJÖQUIST[51], SJÖQUIST[170] and LANDMANN *et al.*[104]. EDMAN AND SJÖQUIST[51] and SJÖQUIST[170] recommended the use of starch-impregnated paper (Whatman No. 1); a 0.5% solution of soluble starch is used and the paper is dried at 40–50°. Samples are applied in the usual manner and the paper is carefully equilibrated in the atmosphere of the system used. The following systems were recommended: *n*-heptane; *n*-heptane–pyridine (7:3); *n*-heptane–*n*-butanol–formic acid (4:2:4); and *n*-heptane–*n*-butanol–formic acid (4:4:2).

The following systems were recommended by LANDMANN *et al.*[104] for separating these derivatives: xylene–acetic acid–0.05 *M* phthalate buffer of pH 6 (3:2:1); and *n*-butanol–0.05 *M* phthalate buffer of pH 6 (7:1).

The use of the iodoazide reagent provides an alternative method for detecting these compounds on paper. Spots of the PTH-amino acids appear as white areas on a dark blue background. The sensitivity of detection is about 0.5 μg. LANDMANN *et al.*[104] used another technique of detection, as follows. After the chromatogram had been developed, the solvents were allowed to evaporate from the paper in a current of air and all traces of acetic acid or butanol, were carefully removed. Then Grote's reagent was applied in a spray form. This detection method provides much better detection than the iodoazide reaction, as different PTH-amino acid derivatives result in different

TABLE 15

SPECIFIC COLOUR REACTIONS OF PTH-AMINO ACIDS WITH GROTE'S REAGENT

PTH-amino acid	*Colour*
Alanine	Blue
Aspartic acid	Blue
Arginine	Violet
Cysteine	Bluish
Glycine	Red
Glutamic acid	Blue
Histidine	Yellow
Isoleucine	Blue
Leucine	Blue
Lysine	Blue–violet
Methionine	Blue
Phenylalanine	Blue, *yellow*[a]
Proline	Blue, *purple*[a]
Serine	Red
Threonine	Blue
Tyrosine	Yellow
Tryptophane	Yellow
Valine	Blue

[a] The definitive spot and its colour are in italics for each compound showing multiple spots in the xylene–acetic acid–pH 6 buffer (3:2:1) system.

coloured spots, as indicated in Table 15. The phenylhydantoins appeared as yellow, red or blue spots after the paper sprayed with Grote's reagent has been held over a boiling water-bath for several minutes. It is necessary to mark the location of each spot while the paper is still damp as the spots fade considerably upon drying. The paper used in this procedure must be buffered with 0.05 *M* phthalate buffer of pH 6 before use. It was also reported by LANDMANN *et al.*[104] that some of the hydantoins give multiple spots. This is particularly true with phenylalanine, leucine and proline. It also has been reported that some amino acids do not form the usual thiohydantoins, namely, serine, threonine and cysteine, and spots detected during the chromatography of phenylthiohydantoins correspond to some intermediates of this rather complex reaction. In the chromatography of amino acid derivatives there are generally some pairs that have identical R_F values and that are hard to differentiate; this applies to PTH-Lys and PTH-Trp, which, however, by using Grote's reagent, can be distinguished by means of the different coloured spots that arise during this detection procedure. PTH valine is impossible to distinguish from PTH phenylalanine and additional hydrolysis and identification of these two amino acids in their free forms is the only reliable method for their identification by paper chromatography. More recently two additional methods of detecting the PTH derivatives of amino acids have been reported. One of these is the direct observation of chromatograms in UV light, as described by PIRKLE[146], who used fluorescent screens coated with activated cadmium borate or with the green phosphor used in colour television tubes to detect UV absorbing spots of PTH amino acid derivatives. The examination of the chromatograms, except for the special method of illumination, was carried out in the usual manner in a viewing cabinet in which the transilluminator consisting of six low-pressure argon–mercury discharge lamps was mounted so that the light was filtered through a filter that transmitted mainly in the region of 254 nm.

In the other recent technique, reported by MORRISON AND JAYASINGHE[127], a spray reagent is used, and this method offers the possibility of a semi-quantitative evaluation of the chromatogram. The spray used is essentially a modification of the iodoazide reagent. A solution of sodium azide (1.5 g per 100 ml) was used to dilute a solution of potassium iodide–iodine solution that contained 2.54 g of iodine and 8 g of potassium iodide per 100 ml. The azide solution is reported to be stable and can be stored for quite a long period of time. The potassium iodide–iodine solution was made up fresh each day. The actual spray reagent was prepared by diluting the potassium iodide–iodine reagent with the sodium azide solution in ratios of 1:128, 1:64, 1:32, 1:16 and 1:4. After development, the chromatograms were sprayed slightly with the

TABLE 16

EFFECT OF CONCENTRATION OF IODINE ON LEVELS OF DETECTION

Dilution of iodine reagent	*Amount of PTH derivative detectable (μmoles)*
1/128	0.001
1/64	0.002
1/32	0.005
1/16	0.01
1/4	0.1

reagent on both sides of the paper. The spots appeared completely in 2 min after spraying. The blue background faded when dilute reagents were used and the chromatogram was slightly heated. The chromatogram was sprayed several times during each detection, starting with the lowest concentration of the detection reagent and proceeding consecutively to higher concentrations, as described by LANDMANN *et al.*[104].

After spraying with the lowest concentration of the spray reagent, in order to detect the lowest possible concentration of the material on the chromatogram, the chromatogram was allowed to dry and immediately sprayed with the next highest concentration of the spray reagent. In this manner, the order of magnitude of the material present on the chromatogram can be detected. According to MORRISON AND JAYASINGHE[187] it is possible to detect 0.001 μmole of a particular PTH-derivative with the lowest concentration of the spray reagent (Table 16).

(a) Quantitation

The following procedure was developed by SJÖQUIST[188].

Separations are carried out by the descending method in four one-dimensional chromatograms during a period of 2.5–3.5 h at 24°. The composition of each solvent is shown in Table 17. The upper phase of systems I, II and III are used; system IV is a single phase. Propionic acid is purified by boiling with chromic acid (5 g per 150 ml) for 3 h. The liquid is poured off and distilled (boiling range 140–141°).

TABLE 17

COMPOSITION BY VOLUME OF THE SOLVENT SYSTEMS

Component	*I*	*II*	*III*	*IV*
Heptane	60	60	30	40
Ethylene chloride	5	30	60	—
Propionic acid	5	—	—	—
n-Butanol	—	—	—	30
75% Formic acid	5	5	5	9

A correct equilibration procedure must be developed for each individual chromatographic unit; the following details apply to glass tanks of dimensions 20 × 30 cm and 60 cm high. The mobile phase is poured into the jars to a depth of about 1 cm. Filter-paper soaked in this solution are attached to the short sides of the jar and wetted before each run for systems I, II and III. A dish containing the stationary phase is placed at the bottom of each jar and a filter-paper 15 cm wide is allowed to hang into the solution. The stationary phase in jars I and II consists of 90% formic acid, in jar III of 75% formic acid and in jar IV of 25% formic acid. Before each run the hanging strips are sprayed with their stationary phase in jars I, II and III. Jar IV is allowed to equilibrate with the moving and stationary phases for 2–3 days before chromatography and the solvents are replenished as they are used up. The solvents in jars I, II and III are changed each week.

Whatman No. 1 filter-papers are dipped in a 0.1% aqueous solution of EDTA, disodium salt, and dried at 100°. The paper may contain as many as seven or nine strips; each spot of test solution is flanked by a spot of the sample solution and a spot of the blank solution.

The test solutions A and B (see Table 18) contain 5 μmoles of each PTH-amino acid dissolved in 2 ml of 90% acetic acid, and these solutions are stable for several months at 5°. Solution A (10 μl) is applied to chromatograms I and II and 10 μl of solution B to chromatograms III and IV.

TABLE 18

COMPOSITION OF TEST SOLUTIONS

No.	*Solution A*	*No.*	*Solution B*
1	PTH-leucine	2	PTH-isoleucine
3	PTH-valine	4	PTH-proline
5	PTH-phenylalanine	9	PTH-glycine
6	PTH-methionine	10	PTH-lysine
7	PTH-alanine	11	Phenylthiourea
8	PTH-tryptophan	12	PTH-tyrosine
9	PTH-glycine	14	PTH-glutamic acid
12	PTH-tyrosine	15	PTH-aspartic acid
13	PTH-threonine	17	PTH-arginine
16	PTH-serine	18	PTH-histidine
		19	PTH-cysteic acid

The papers are allowed to equilibrate for 15 min in jars I, II and III, and for 30 min in jar IV. The solvent systems are freshly prepared before each run and the papers are irrigated to a depth of about 35 cm. Papers I, II and IV are dried at 100° for exactly 30 min and paper III for exactly 1.5 h.

To locate the spots, use is made of their very strong UV absorption when viewed against a fluorescent screen (transparent Perspex sprayed evenly with a suspension of fluorescent zinc silicate in chloroform). If the UV light is filtered to remove all visible light except red and red spectacles are worn by the operator, the PTH-amino acids appear as black spots on a red background.

The excised spots and blanks are extracted in 2 ml of 95% ethanol for 1 h at 40° in glass-stoppered tubes. The extinction values of the PTH-amino acids are determined at 269 nm. The PTH-derivatives of serine and threonine undergo dehydration during the drying of the paper and the eluted decomposition products show an additional absorption peak at 320 nm. PTH-threonine is therefore determined at 320 nm, but for PTH-serine the extinction at 269 nm must be determined and subtracted from the sum of the values for PTH-aspartic acid and PTH-serine at 269 nm. The ratio $\varepsilon_{269}/\varepsilon_{320}$ is 0.43 and this constant is used for the conversion.

PTH-leucine and PTH-isoleucine overlap and they are determined together. PTH-proline travels with PTH-phenylalanine in system I and with PTH-valine in system II. As PTH-valine and PTH-phenylalanine are resolved completely in systems I and II, respectively, a value for PTH-proline can be calculated. PTH-lysine is determined similarly from chromatogram II. The appearance of a phenylthiourea spot indicates that ammonium ions are present as a contaminant or released from amide groups. PTH-hydroxyproline will interfere with the ammonium determination. On black strips, an artefact sometimes appears between PTH-phenylalanine and PTH-valine in system I. It has the same R_F value as that of diphenylthiourea and appears to be connected with the purity of the triethylamine used for the buffer.

TABLE 19

EXTINCTION VALUES OF ELUTED PHENYLTHIOHYDANTOINS AT 269 AND 320 nm

Amino acid	*ε*		*Yield (%)*	*C*
	Found	*Calculated*		
Alanine	0.334	0.320	104.2	0.120
(Ammonium chloride)[a]	0.121			0.330
Arginine	0.284	0.318	89.4	0.141
Aspartic acid	0.306	0.322	95.0	0.131
Cysteic acid	0.305	0.332	92.0	0.131
Glutamic acid	0.304	0.318	95.6	0.132
Glycine	0.290	0.298	97.4	0.138
Histidine	0.250	0.310	80.6	0.160
Hydroxyproline	0.254	0.272	93.4	0.157
Isoleucine	0.308	0.337	91.5	0.130
Leucine	0.308	0.337	91.5	0.130
Lysine[b]	0.455	0.580	78.5	0.088
Methionine	0.345	0.342	100.8	0.116
Phenylalanine	0.294	0.310	94.8	0.136
Proline	0.274	0.286	95.8	0.146
Serine[c]	0.221			0.181
Threonine[c]	0.300			0.133
Tryptophan	0.340	0.394	86.8	0.118
Tyrosine	0.297	0.312	95.2	0.135
Valine	0.300	0.330	90.0	0.133

[a] Determined as phenylthiourea.
[b] Determined from chromatogram II.
[c] Measured at 320 nm.

The extinction values for 0.04 mole of each PTH-amino acid applied at 269 nm are shown in Table 19. Percentage yields are over-all values; the factor C is the ratio of the amount of sample applied to the extinction. The amount A of PTH-amino acid in the sample in μmoles/mg is calculated from the equation

$$A = \frac{C \cdot \varepsilon \cdot a}{b \cdot w}$$

where C is the coefficient in Table 19; ε is the extinction value at 269 or 320 nm; a is the volume of 90% acetic acid in microlitres in which the sample is dissolved before application to the chromatogram; b is the volume of solution in microlitres applied to the chromatogram; and w is the weight of sample in milligrams. A linear relationship exists between the amounts of amino acids applied in the procedure and the extinction values of the PTH-derivatives. Lysine is the only exception and the extinction of PTH-lysine should be compared with a standard curve. The reproducibility in each determination is ±4%.

(b) Flat-bed arrangement of Edman's procedure

As mentioned already, the chromatography of phenylthiohydantoins is intimately related to the EDMAN[47] degradation procedure. Thus the analysis of N-terminal groups is extended to the identification of amino acids during the stepwise degradation. In our opinion, the most advanced procedure in this respect is that described by

SCHROEDER[175], who was able to transfer almost all operations from test-tubes to a paper sheet (Whatman No. 1 paper is used throughout this procedure) so that the whole procedure, including degradation and identification of the split-off amino acid, is carried out in the flat-bed arangement.

A total amount of 0.3–1.0 μmole of the peptide to be determined is applied to a number of paper strips, each of which carries about 0.2 μmole of the peptide to be determined. In the original version of the procedure, it is recommended that four strips are used, regardless of the amount, in order to prevent total loss of the sample by accident. The strips are allowed to dry most conveniently by hanging them on a suitable rack.

In the next step, the reaction with phenyl isothiocyanate is carried out. Each strip is thoroughly saturated with a 20% solution of phenyl isothiocyanate in dioxan. The reagent is applied with a pipette or a small dropper. Alternatively, the paper strip can be dipped in the same solution, but there is always the risk of eluting an unknown proportion of the sample during the latter procedure. In order to prevent the contamination of the sample at this stage, it is necessary to hold the paper strip with tweezers or to use rubber gloves. Reagent is added to the strip slowly and no more than two or three drops are allowed per strip.

Each pair of strips is then put into a screw-capped jar into which about 15 ml of the mixture consisting of dioxan, pyridine and water (1:1:1) have been added. The jars are tightly closed and heated at 40° for 3 h in an oven. The volume of 15 ml of the pyridine–dioxan–water mixture is essential, as if more strips are placed into the same jar the reaction is incomplete.

Next, it is necessary to extract the excess of the reagent. In the original version of this procedure, described by FRAENKEL-CONRAT[62], the strips were extracted first with benzene followed by ethanol–diethyl ether (1:1). However, according to SCHROEDER[175], this procedure is rather inconvenient as a number of peptides are soluble in the alcohol–diethyl ether mixture. Hence it is impossible to avoid undesirable losses of PTH-peptides during this step if ethanol–diethyl ether (1:1) is used. Extraction with benzene alone is quite adequate and does not remove the peptide derivatives from the paper strips. In any event, it is recommended that some tests are carried out to determine the possible losses in the benzene fraction during this operation. After removal from the reaction atmosphere, the somewhat translucent strips are allowed to dry just to the stage when they loose this transparency. The strips are then dipped in test-tubes that contain a volume of benzene sufficient to cover them. Omission of the drying steps results in some loss of the PTC-peptide; on the other hand, if the paper strips are dried too much then benzene does not remove the diphenylthiourea, which is a side product of the reaction; failure to remove the diphenylthiourea results in obscuring of PTH-methionine.

The benzene extraction is repeated three times over a period of 1.5 h each. The benzene washings are discarded. It is also recommended that the last benzene washing is carried out overnight.

In the next step, the degradation of the PTC-peptide is carried out in a desiccator. After the strips have been washed, they are aerated in the atmosphere and placed into a desiccator, into which 15 ml of glacial acetic acid and 15 ml of 6 *N* hydrochloric acid in individual beakers have been placed. The over-all pressure is decreased to 100 torr and the degradation is carried out at ambient temperature for

7 h, which time period appears to be adequate in most instances; however, the degradation period can be extended if necessary.

After degradation, the resulting PTH-amino acid must be extracted from the paper. After being removed from the degradation atmosphere, the strips are aerated in a ventilated chamber or in the laboratory atmosphere overnight. A 2-h extraction with acetone is recommended by SCHROEDER[175] to remove the PTH-amino acid. Also, in a humid atmosphere, the peptide may be only partially extracted. The acetone extract is then evaporated to dryness under reduced pressure and at a temperature not exceeding 40°.

For the identification of PTH-amino acids, the procedure published first by EDMAN AND SJÖQUIST[51] has been recommended. Starched chromatographic paper is used for the separation as it makes the final detection easier. Alternatively, UV light may be used to make the spots visible provided that a fluorescent screen has been used.

The starching procedure recommended by EDMAN AND SJÖQUIST[51] and used also by SCHROEDER[175] is carried out as follows.

A 0.5% solution of soluble starch is made by pouring a thin slurry of 5 g of soluble starch in 100 ml of cold water into 900 ml of boiling water. The mixture is allowed to boil until the starch is completely dissolved. After cooling, the water lost by evaporation is replaced. The starch solution is poured into a tray and Whatman paper sheets are passed through and allowed to dry in the laboratory atmosphere. The starched sheets usually become wrinkled and must be flattened under pressure before use.

In the chromatographic procedure, adequate standards of PTH-amino acids are used side-by-side with the sample being analysed. Standards that contain 2 mg/ml in acetone are usually suitable. These standards are stable over 1 month except for the standard solution of histidine, which is said to decompose within 1 week. The occurrence of multiple spots in chromatograms is the first sign of possible decomposition of the standard solution. According to SCHROEDER[175], it is more convenient to use standard solutions of individual amino acids rather than to use a complete mixture. However, several amino acid derivatives can be placed on the same spot on the chromatogram as no more than 5 ml of the above standard solutions are applied. For the first orientation, a mixture of PTH-proline, PTH-leucine, PTH-valine, PTH-alanine and PTH-glycine appears to be very useful. After evaporation of acetone, the dry sample is dissolved in 0.5 ml of the same solvent and an amount equivalent to 0.1 μmole of the peptide is applied to the chromatographic paper. However, some additional treatment of the paper may either preceed or follow the sample application, as described by SCHROEDER[175].

The following solvent systems are recommended: (1) heptane–pyridine (7:3); (2) *n*-butanol–heptane–90% formic acid (2:2:1); (3) xylene; (4) water-saturated butyl acetate containing 3% of propionic acid; this mixture is saturated with formamide and the starched paper used is dipped into 20% formamide in acetone before chromatography; and (5) benzene–heptane (3:2).

There are some further requirements that must be fulfilled in order to obtain satisfactory separations of the PTH-amino acids in the above systems. With the first system, which is the most widely used, equilibration in an atmosphere of 45% humidity gives the best results.

A closed chamber containing a saturated solution of potassium carbonate provides an atmosphere of the required humidity. If the humidities is too high (above 60%), the solvent forms complexes with poor separation efficiencies. When the humidity is too low, almost all PTH-amino acids move with the solvent front. A period of 3 h in the atmosphere of 45% humidity proved to be satisfactory. An alternative procedure to satisfy the humidity requirement is to dip the paper into a 10% solution of water in acetone. In this instance, samples are applied during the evaporation of acetone. Another method, which requires some experience, is to dry the chromatogram appropriately if the humidity is too high.

With the *n*-butanol–heptane–formic acid system, some difficulties may arise if the temperature falls, as this usually results in the separation of this solvent system into separate phases. In order to prevent erroneous separations resulting from this fact, it is recommended that the lower part of the chromatographic chamber is placed in a water-bath heated to 30° during the separation. In general, this solvent system is used much less frequently than that mentioned above.

With the solvent system based on *n*-butyl acetate, it is also recommended that the starched paper is dipped into 20% formamide in acetone before applying the sample.

The use of the last solvent system, benzene–heptane, makes the identification of some PTH-amino acids difficult, so it is recommended that several PTH-standards are used on each side to make the amino acid derivative identification sufficiently reliable.

The final detection procedure is as follows. After the chromatograms have been removed from the jars, they are put into a ventilated hood and dried for at least 10 min. Chromatograms to which formamide has been applied are then heated at 100° for at least 30 min, but longer heating is of considerable advantage. The sheets are then sprayed with the iodine–azide reagent of SJÖQUIST[170]. For preparing this reagent, 82.5 g of potassium iodide and 2.5 g of iodine are dissolved in 1 l of distilled water. The other solution contains 32.5 g of sodium azide in 1 l of distilled water. Both solutions are mixed in the ratio 1:1 before use. There are some physiological hazards related to the use of this reagent and therefore it is essential that this reagent is applied in a well ventilated location as inhalation causes nasal bleeding. PTH-amino acids appear as white spots on a purple background, and these spots rapidly turn brownish in colour. If the chromatograms are interleaved with clean filter-paper and stored in a freezer they are indefinitely stable, but at room temperature the spots disappear after about a week.

From the standpoint of purely flat-bed identification (without converting the PTH-amino acid into the original amino acid), some amino acids require special care. Thus PTH-valine and PTH-phenylalanine have similar R_F values in most instances and PTH-histidine is a problem in itself. Usually, the identification of an unknown PTH-amino acid involves several steps. In the first step, the amino acid derivative is developed in the heptane–pyridine mixture. After the tentative identification of the particular amino acid derivative, another solvent system is chosen for the final identification. Xylene is recommended for use in the second chromatographic step for amino acid derivatives that move with a velocity equal to/or more rapid than that of PTH-glycine. A solvent system based on butyl acetate is recommended for use with amino acid derivatives which move more slowly than PTH-glycine. In general, in the

first chromatographic run PTH-amino acid derivatives are grouped into rather simple combinations of amino acid derivatives, which are identified definitely in the second run in a different solvent system, as described. Thus, for instance, PTH-proline, PTH-leucine and PTH-valine move with identical mobilities in the first solvent system, while they are easily distinguished in xylene. Similarly, PTH-valine, PTH-methionine and PTH-alanine form a single spot in heptane–pyridine while they are well separated in the system based on *n*-butyl acetate. On the other hand, amino acid derivatives that are well separated in the first run, such as PTH-phenylalanine, and PTH-valine or PTH-serine and PTH-aspartic acid, cannot be distiguished in the other solvent systems.

There are special combinations for which two runs are still inadequate for unequivocal identification of an amino acid derivative, and heptane–butanol–90% formic acid must therefore be used for the identification of PTH-histidine and PTH-arginine. A similar situation occurs with PTH-methionine sulphone and PTH-glutamic acid or with PTH-leucine and PTH-isoleucine. Sometimes the colour of the spot after iodine–azide detection may serve for the further identification of the particular spot. Usually, PTH-tyrosine, PTH-phenylalanine and PTH-tryptophan appear as spots with yellow centres while PTH-glycine, PTH-serine and PTH-threonine give spots with pink centres.

In quantitation, heavy spraying of the chromatograms is recommended so that a relative comparison can be made of the amounts of amino acids originally present in a sample. Light spraying will result in bleaching of the iodine–azide reagent, which usually leads to an erroneous conclusion about the presence of PTH-amino acids observed on the chromatogram.

As a rule, in peptide chromatography the amount of the material available is of decisive importance. It frequently happens that the amount of a valuable material that is available is not sufficient for several subsequent runs to be made to give an unequivocal identification of the N-terminal amino acids. In these situations, two-dimensional separation is useful, and can be carried out in the following manner. The unknown sample is placed at position 1 (see Fig. 71) and appropriate standards at position 2. The whole sheet is developed with xylene (solvent D) in the direction indicated. After the chromatogram has been developed, part E is separated from the wet chromatogram and appropriate standards are applied at position 3. After the xylene has evaporated, the E strip is developed in the solvent system (solvent E) based on *n*-butyl acetate. Before both developments, the whole sheet of paper is dipped into a solution of formamide in acetone. Part D is cut off as indicated and both parts D and E are detected with the iodine–azide reagent.

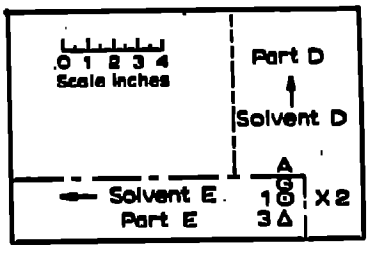


Fig. 71. Chromatographic paper marked for two-dimensional chromatography.

The idea of the separation is as follows. PTH-alanine and those amino acids which move more rapidly than PTH-alanine will move in xylene to position A and will be identified in the first run (with the exception of PTH-valine and PTH-phenylalanine). PTH-glycine will move approximately to position G in xylene and PTH-glycine and the more slowly moving amino acid derivatives will therefore be identified in the perpendicular run in the solvent system based on *n*-butyl acetate. There is only one method of eliminating uncertainities in this situation, *viz.*, by total amino acid analyses carried out in parallel.

Quantitation, besides the general requirement regarding the amount of the detection reagent applied, can be carried out by the dilution technique with an appropriate series of standards.

Peptides that contain histidine and subterminally located arginine require special care. The main problem with these amino acids is based on the fact that they are not extracted by acetone. According to SCHROEDER[175], the PTH-derivatives of these two amino acids can be extracted with a 5% solution of water in acetone, but this procedure is of little use as the residual peptide is also extracted and offers no possibility of further degradation. Usually, it is recommended that a certain portion of the chromatogram is cut off, so that the identification is carried out on this cut-off sheet while the remainder is used for further degradation steps. Tryptic peptides with C-terminal arginine do not pose this particular problem, but if more of these amino acids are present per peptide then the over-all degradation scheme should be altered. The next degradation step should not be started unless the presence or absence of acetone-soluble PTH-amino acid derivatives has been established. In the absence of such a derivative, the identification is carried out with heptane–*n*-butanol–acetic acid (2:2:1) as described above. In the presence of such a derivative, the chromatogram developed with the heptane–*n*-butanol–formic acid system is cut into two halves, one of which is sprayed routinely with the iodine–azide reagent, while the other is subjected to the specific detection of arginine and histidine.

Another method for the identification of PTH-amino acid derivatives in this procedure is electrophoresis. Starched Whatman 3 MM filter-paper is used for this purpose together with pyridine–acetate buffer of pH 6.4; 190 ml of water are mixed with 10 ml of pyridine and 0.4 ml of glacial acetic acid. The running time in this buffer is 2 h at 15–20 V/cm. The final detection is carried out with the iodine–azide reagent in a similar manner to chromatography.

A problem arises with N-terminal glutamine, which, under the conditions of the degradation, is converted into pyrrolidone carboxylic acid, so that when splitting off of the N-terminal group does not occur in a peptide known to contain glutamine it can be tentatively concluded that pyrrolidone carboxylic acid is to be found at the N-terminal position. However, if glutamine is located in any other position (subterminally in a peptide), in most instances no problems arise although there is a similar possibility of cyclization to pyrrolidone carboxylic acid. Similarly threonine, serine and asparagine do not give rise to special problems in the degradation procedure. However, with lysine, it happens that no derivative is found in the lysine position while subsequent steps proceed without complication. The missing lysine must therefore be determined from the over-all amino acid analysis or from other results. With histidine, conflicting results may also be obtained on occasions, arising from the fact that when histidine is or becomes the N-terminal group, the histidyl residue is removed in

the reaction with phenyl isothiocyanate and the succeeding residue reacts also, so that after cyclization both residues are removed.

In general, a sequence of 6–8 residues can be detected in this manner. Sometimes, for reasons that have not yet been explained, the degradation in the first step proceeds quite normally, but the next degradation step fails to release the N-terminal amino acid.

Also, repeating sequences must be considered very carefully because in every step some evidence related to the proceeding step can always be obtained. Therefore, when identical residues occur in two subsequent steps, quantitation is essential with a yield of over 80%.

Several variations of the above procedure have been described. According to SCHROEDER[175] and according to our own experience, prolonged heating of the sample as well as double treatment of the sample with phenyl isothiocyanate, repeating extractions, different times of cyclization or re-application of the reagent are of little use. According to SCHROEDER[175], prolonged cyclization times lead to scission of the residual peptide at positions that are sensitive to acid. Other precautions were mentioned when discussing the individual reaction steps.

6. *Thin-layer chromatography*

The thin-layer chromatography of PTH-amino acid derivatives is now one of the most widespread techniques used for separating these compounds. The simplicity of the technique, the high sensitivity and the absence of special demands upon the equipment make thin-layer chromatography very useful for both these and other derivatives. The sensitivity of the method is about 0.01 μmole of the peptide or protein to be determined.

The first solvent system used for the thin-layer chromatography of PTH-amino acids was that of CHERBULIEZ *et al.*[38]. These workers separated, however, only three PTH-derivatives of glycine, proline and leucine using *n*-heptane–pyridine–acetic acid (5:3:2) as the mobile phase. They specially activated the silica gel layer by heating it at 140° for 2 h and allowing it to cool again in a desiccator. However, it was later proved that this activation procedure is unnecessary and may even be harmful because of cracks appearing in the layer and because of its high sensitivity to atmospheric moisture, which may result in variations in the R_F value. The first extensive study on the separation of PTH-amino acid derivatives was published by BRENNER *et al.*[39], who devised special solvent systems for thin-layer chromatography such as chloroform, chloroform–methanol (9:1), chloroform–formic acid (20:1), chloroform–methanol–formic acid (35:15:1) and *n*-heptane–ethylenechlorohydrin–formic acid–propionic acid (30:10:7:6).

BRENNER *et al.*[39] were also the first to use two-dimensional thin-layer chromatography for the separation of PTH-amino acid derivatives. According to their procedure, samples are loaded in amounts of 0.5 μg of each component to be separated in 0.5 μl of methanol. It is recommended, as in other amino acid determinations, that a known mixture of amino acid derivatives is spotted simultaneously in order to simplify the identification. If a complex mixture of unknown PTH-amino acid derivatives is being studied, the use of two-dimensional separation seems inevitable. In the first run, the mixture is separated in the solvent system chloroform–methanol (9:1)

and in the second run the mixture chloroform–formic acid (20:1) is applied. With this procedure it is possible to determine eleven PTH-amino acids side-by-side; the derivatives of aspartic and glutamic acid remain unresolved together with the so-called leucine group, which contains six additional amino acids. There is the possibility of separating PTH-Asp and PTH-Glu in a subsequent one-dimensional run in chloroform–methanol–formic acid (35:15:1), in which system almost all other amino acids migrate with the solvent front. The members of the leucine group, *i.e.*, the PTH-derivatives of Leu, Ile, Pro, Val, Met and Phe, are separated further by using pure chloroform. After this separation, it is possible to identify PTH-Pro in addition to a double spot of PTH-Leu and PTH-Ile and also a combined spot of PTH-Val, PTH-Met and PTH-Phe.

The separation of PTH-Met from PTH-Val and PTH-Phe presents a considerable problem, which can be solved by oxidizing the methionine derivative with hydrogen peroxide. As the result depends on the relative proportions of the methionine derivative and the hydrogen peroxide used, the procedure is rather elaborate. A clean plate is spotted with four spots of the material that is suspected to contain a methionine derivative and to each of these spots an additional spot of hydrogen peroxide is added after the original sample has been dried, which takes usually a few minutes. Individual sample spots are covered with hydrogen peroxide spots containing different concentrations of the reagent: 1, 2, 4 and 8%, respectively. The reaction is allowed to proceed for 2 min. The chromatogram is then developed in chloroform and the run in which a minimum number of additional spots appear is used for identification. A spot that remains on the start proves the presence of PTH-Met while a spot with the same mobility as that of the original sample proves the presence of PTH-Val or PTH-Phe, or possibly of both. This method offers no opportunity of distinguishing between the PTH-derivatives of leucine and isoleucine or between the PTH-derivatives of valine and phenylalanine. It is recommended that these derivatives are hydrolyzed after elution (1 μg of the particular derivative is necessary) in 6 *N* HCl in a sealed tube for 12 h at 120° and that the unknown substance is identified as the free amino acid by an appropriate flat-bed technique or by using an automated amino acid analyzer. It must be stressed that the precautions usually taken in the chromatography of free amino acids have to be respected, especially the complete removal of hydrochloric acid. For this purpose, the hydrolysate is evaporated to dryness, the residue is re-dissolved in distilled water and the solution is evaporated to dryness again. The whole procedure is repeated at least twice. If there are too many different PTH-amino acid derivatives present it is recommended that the identification is carried out in the following manner. Run a two-dimensional chromatogram in the solvent systems specified above, scrape off the combined spot of aspartic and glutamic acids near the start and the fast-moving leucine group spot, disperse these separately in 1 ml of methanol, heat the mixture for 2 min at 40–50°, remove the adsorbent by filtration, wash the residue twice with hot methanol and evaporate the combined filtrates to dryness. Individual amino acids supposed to be present in these two spots are identified as described above.

BRENNER *et al.*[20] described an alternative procedure that in some respects may be more useful. At the beginning, two two-dimensional and one one-dimensional chromatograms are prepared from the same sample according to the following scheme. The first two-dimensional chromatogram is run in the system chloroform–methanol

(9:1) and chloroform–formic acid (20:1). The second is developed by chloroform in the first direction followed by development in *n*-heptane–ethylenechlorohydrin–formic acid–propionic acid (30:10:7:6) in the subsequent step. The one-dimensional run is carried out in chloroform–methanol–formic acid (35:15:1). The reasons for carrying out these chromatographic runs are as follows. Most of the expected PTH-amino acid derivatives are identified in the first two-dimensional run. In this run, it is also desirable to distinguish between monomethylthiourea and the PTH-derivative of glycine, which is achieved by exposing the chromatogram to ammonia vapour: a reddish spot that does not fade appears in the presence of PTH-Gly. In the second two-dimensional chromatogram, the amino acid derivatives identified are PTH-Pro, PTH-Met, PTH-Phe, PTH-Val, PTH-Leu, PTH-Ile and diphenylthiourea. The one-dimensional run is carried out to achieve the separation of PTH-Asp and PTH-Glu.

Since the pioneering work of BRENNER *et al.*[20], various other solvent systems have been used for the thin-layer separations of PTH-derivatives. First, systems that contained different alcohol–chloroform ratios were applied with great success: chloroform–methanol (5:2 and 9:1) by PATAKI[140] and FITTKAU *et al.*[60], and chloroform–ethanol (49:1); the latter system was recommended for use with alumina layers. For alumina layers, it is also possible to use pure chloroform or chloroform–isopropanol–formic acid (35:4:1), as described by CHERBULIEZ *et al.*[30]. Other systems were developed for silica gel layers, as follows: chloroform–isopropanol–water (28:1:1), chloroform–ethyl acetate (19:1) and chloroform–ethyl acetate–water (6:3:1) by CHERBULIEZ *et al.*[30]; chloroform–methanol–light petroleum (100:40:7) and chloroform–acetic acid–light petroleum (25:4:1) by FITTKAU *et al.*[60]; and ethyl acetate–pyridine–water (7:2:1) by CHERBULIEZ *et al.*[30].

The above systems and their properties were reviewed by HOLEYŠOVSKÝ[80] and HOLEYŠOVSKÝ AND HOLEYŠOVSKÁ[81].

More recently, some additional systems have been used for the thin-layer chromatography of PTH-derivatives on silica gel layers. JEPPSON AND SJÖQUIST[95] and DUS *et al.*[45] recommended the following: heptane–propionic acid–ethylene chloride (58:17:25)[95], heptane–*n*-butanol–90% formic acid (40:40:20)[45] and heptane–*n*-butanol–75% formic acid (50:30:9)[95].

GRÜNER[72] and DUS *et al.*[45] were successful in separating PTH-amino acids in the following systems: acetic acid–ethylene chloride (4:30), butyl acetate–water–propionic acid–formamide (60:1:1.8:8) (with 20% of formamide in acetone as the stationary phase), *o*-xylene (with 20% of formamide in acetone as the stationary phase) and *o*-xylene–acetone (5:1) (with a saturated solution of glycerine in acetone as the stationary phase).

The recent developments in separations on polyamide layers, most of which were made by WANG *et al.*[204], can also be applied to the separation of PTH-derivatives. The following solvent systems were used by these workers: 90% formic acid–water (1:1), *n*-heptane–butanol–acetic acid (40:30:9), carbon tetrachloride–acetic acid (9:1), benzene–acetic acid (9:1) and 2-butanone–*n*-hexane (1:3).

The above systems were used also for the two-dimensional separation of PTH-derivatives on polyamide layers. In this instance, either *n*-heptane–*n*-butanol–acetic acid (40:30:9) or carbon tetrachloride–acetic acid (9:1) are recommended for use in the first run, followed by formic acid–water (1:1).

The most recent paper on the thin-layer chromatography of PTH-amino acids

on polyamide layers was published by KULBE[103]; however, it was not available at the the time when this review was being prepared and therefore it is not discussed here.

In order to make the survey of different sorbents for flat-bed arrangements complete, separations carried out on glass-paper sheets must be mentioned. The following systems were used for this purpose by RADHAKRISHNAMURTHY AND ROSENBERG[154]: *n*-heptane–chloroform (1:1), *n*-heptane–chloroform–acetic acid (10:9:1) and *n*-heptane–chloroform–pyridine (10:9:1).

(a) Detection

The simplest method of detecting PTH-amino acid derivatives involves the use of a fluorescent indicator-containing layer in which the spots of PTH-derivatives appear as dark areas on a pink fluorescent background. However, there are other detection techniques which make the spots of PTH-amino acid derivatives visible without using fluorescent light. Thus, it is possible to use the iodoazide reaction, as used for the detection of these derivatives in paper chromatograms. In thin-layer separations, this method of detection, however, is not sensitive enough. Therefore, BRENNER *et al.*[29] devised another method based on the chlorination reaction of REINDEL AND HOPPE[158]. The fluorescent detection can, of course, be used in its original version, *i.e.*, a plain layer of silica gel can be sprayed with a diluted solution of fluorescein and the spots made visible as dark areas against a yellow background under UV light.

ROSEAU AND PANTEL[163] devised the ninhydrin detection of PTH-derivatives of amino acids, which is of considerable diagnostic value as the colours with different amino acid derivatives are also different.

The reagent is used as follows. Ninhydrin (100 mg) is dissolved with 5 ml of

TABLE 20

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

PTH-amino acid	*Time of heating (min)*	*Colour*
Serine	1	Red–violet
Glycine	1.5	Intense orange
Alanine	1.5	Red–violet
Methionine sulphone	2	Brown
Cystine	3	Intense pink
Cysteic acid	3	Light pink
Asparagine	4	Light yellow
Glutamine	4	Greenish brown
Methionine	4.5	Brown
Glutamic acid	4.5	Dark brown with blue halo
Histidine	4.5	Faint yellow
Aspartic acid	4.5	Pink
Arginine	4.5	Very faint yellow
Tryptophan	5	Intense yellow
Tyrosine	5	Light yellow
Threonine	5	Light brown
Lysine (ε-)	7	Very faint pink
Proline	8	Very faint pink
Phenylalanine	9	Very faint yellow
Leucine	15	Very faint grey
Isoleucine	15	Very faint grey
Valine	—	No colour

collidine in 100 ml of absolute ethanol. The layers are dried at 110° for 15 min and sprayed with the ninhydrin–collidine reagent. The layers are returned to the drying oven and left there for an additional 15 min. Individual PTH-derivatives then give different colours (Table 20).

(b) Quantitation

The quantitation of PTH-amino acid derivatives involves both methods carried out *in situ* and methods carried out after elution. The *in situ* techniques were introduced by PATAKI AND WANG[143] and PATAKI AND STRASKY[142]: *in situ*, the fluorescence quenching areas of PTH-derivatives are usually measured relative to the fluorescent background. According to PATAKI AND WANG[143], the spots are scanned (after drying the chromatoplates in a stream of cold air for exactly 30 min) with a Turner Fluorometer 111 fitted with a door for scanning chromatoplates. It appears that the good results obtained by the above workers are the property of the PTH-derivatives rather than the property of the sophisticated equipment used in this particular instance. The excitation wavelength recommended for the fluorimetric determination of PTH-amino acids is 254 nm. It should also be pointed out that *in situ* quantitations on polyamide layers gave much better results than with silica gel layers, presumably owing to the greater uniformity of the former plates. Areas of fluorescence quenching peaks for PTH-amino acid derivatives on both silica gel and polyamide layers are summarized in Table 21. Although these areas may vary according to the experimental conditions used in different laboratories, their relative values may certainly serve as a valuable guide for other workers who may decide to use this technique.

In order to obtain a reasonable degree of reproducibility, certain rules must be obeyed that were discussed in detail in papers by PATAKI and co-workers[142,143]. Briefly, the important influencing factors are the position of the scanner, the standardization of time between scanning and the end of chromatography, the loading volume, the developing distance and the layer thickness. In order to avoid interference from neighbouring spots, it is advisable to cover the plate with a Polygram Sil-sheet, for example, except for a small strip that contains the spot under investigation.

The other group of quantitation techniques is that which involves the use of the UV absorbances of PTH-derivatives after they have been eluted from the layer. A very precise variation of this technique has been reported by SMITH AND MURRAY[184]. The spots were removed from the plate by placing the mouth of a clean test-tube against it from below, inverting the arrangement and slowly twisting the tube while applying pressure upwards towards the surface. Each compound is removed in this manner, appropriate volumes of methanol are added and the corked test-tubes are allowed to stand overnight at room temperature. Blank areas are cut out at random from empty lanes of the plate, and these blanks are treated in a similar manner to the samples. One blank is sufficient if the plate has been treated with a chloroform wash after chromatographic separation, while two or three blanks are necessary if this step was omitted. After overnight extraction, the scraped-off layer is centrifuged for 30 min at 300 r.p.m. and the spectra of portions of the resulting liquid are recorded in the range from 320 nm to about 230 nm. There is one important precaution that must be observed: the layers before separation must be washed with methanol, as otherwise no reproducible UV absorbances are obtained. The procedure involving washing the plates, firstly with methanol before chromatography and secondly with chloroform

TABLE 21

AREAS OF FLUORESCENCE QUENCHING PEAKS OF PTH-AMINO ACIDS (2 μg) ON POLYAMIDE AND SILICA GEL–ZINC SILICATE LAYERS

PTH-amino acid	*Polyamide*		*Silica gel*	
	Peak area (mm^2)	*t^a (min)*	*Peak area (mm^2)*	*t^a (min)*
Δ-Serine	560	1	440	7
α-Aminobutyric acid	1710	1.5	2310	1.5
Cystine	750	2	750	8
Alanine	2230	3	2300	2
Histidine			740	3
Glutamic acid	2060	9	1380	16
Homocitrulline	1520	11.5	1130	16.5
Leucine	1950	12.5	2540	16
Glycine	2120	16	1820	20
Valine	2670	22.5	2250	19
Phenylalanine	2490	27.5	1270	30.5
Methionine	2630	36.5	1800	34
Proline	2220	40	2660	40.5
Citrulline	1180	40	1150	44
Glutamine	2180	49	2160	52
Tryptophan	970	60.5	1570	60.5
Hydroxyproline	1590	65.5	1390	62
α-Aminoisobutyric acid	1320	84.5	1310	84
Methionine sulphone	2260	91.5	1240	78
Cysteic acid	1020	112.5	1210	105.5
Arginine	3140	124.5	1190	123
Asparagine[b]	1430	137.5	850	140
Aspartic acid	1710	142	1780	132
Lysine	1620	156	1560	147
Tyrosine	1340	171	1490	158
Isoleucine	1730	180	1300	168
Δ-Threonine	820	191	1250	187.5
Threonine	1270	197	1780	181.5

[a] t is the time period elapsing between drying the plate and scanning.
[b] Impure, gives a secondary spot.

after the separation has been carried out, provides suitable conditions for the quantiprotation of PTH-derivatives after elution.

TKOCZKO AND SZWEDA[104] used another technique for the quantitation of PTH-derivatives in the eluate, involving the use of the iodine reaction for the determination. The developed plates were exposed in a chamber to iodine vapour for 1–2 min, the brownish spots of PTH-amino acid derivatives were scraped off, eluted with 5 ml of 96% ethanol and the optical densities of aliquots at 269 nm were estimated; appropriate blank determinations were carried out in parallel.

The technique for the quantitation of PTH-derivatives described by WRONSKI[208] is based on desulphurization with *o*-hydroxymercuribenzoic acid and quenching of the fluorescence of tetrakis(acetoxymercurio)fluorescein. To detect hydrolyzable sulphur, it is recommended that $1–5 \cdot 10^{-8}$ mmoles of PTH-derivatives in 1 ml of 5% ethanol, 1 ml of 96% ethanol and 2 ml of $5 \cdot 10^{-5}$ *N* tetrakis(acetoxymercurio)fluorescein is heated in 0.5 *M* NaOH for 20 min on a boiling water-bath. The mixture is then cooled and 0.6 ml of a solution consisting of 0.75 *M* H_3PO_4 + 0.25 *M* ethanolamine + 0.23

M H_3BO_3 and 2 ml of a solution of dithiofluorescein (4 mg dissolved in 2 ml of a solution containing 20 g of EDTA and 20 g of triethylamine in 1 litre), are added. When the blue colour develops, the absorbance is measured at 587 nm. The reaction gives almost a 100% yield with most of the PTH-derivatives of common amino acids, with the exceptions of proline and hydroxyproline, which exhibit a low recovery in the range 26–59%, while methionine gives an apparent recovery of over 140%, indicating that splitting of the thioether bond has occurred. This technique, of course, necessitates adquate elution of the separated spots from the layer, which can be carried out in a manner described previously.

It is possible to classify the isotope dilution technique described by CALLEWAERT AND VERNON[36] and by LAURSEN[107] among the methods which deal with quantitation after elution. In a typical example of the stepwise Edman procedure, the individual steps of LAURSEN's procedure were as follows*. A sample (0.35 μmole of a hexapeptide) was attached to aminoethylaminomethylpolystyrene and was degraded through eight cycles using the automated version of the Edman degradation. During each 2-h cycle, the peptide was treated for 20 min with about 100 μl of [^{3}H]phenyl isothiocyanate and for 50 min with trifluoroacetic acid–dichloroethane (1:1) with washing steps in between. Thiazolinones were collected after each cycle in a fraction collector in tubes each containing 0.1–1.0 μmole of the phenylthiohydantoin derived from glycine, and also other amino acids expected to occur in the analysed sequence, and 0.1 ml of water in order to promote isomerization *in situ*. The tubes were kept at room temperature and were not protected from air. At the end of the degradation (16 h), each tube contained about 2 ml of trifluoroacetic acid and 4 ml of dichloroethane in addition to the phenylthiohydantoins.

The contents of each tube were evaporated to dryness and the residue was heated in 0.2 ml of 20% trifluoroacetic acid at 70° for 10 min to ensure that isomerization occurred. The resulting mixture was diluted with 0.5 ml of methanol and the solution was passed through a column (5 × 20 mm) of Dowex 50 (H^+-form) to remove basic salts. The column was washed with 4 ml of methanol, the effluent was evaporated to dryness and the residue was dissolved in 40 μl of dichloroethane. Alternatively, the extraction procedure of EDMAN AND BEGG[49] was used.

A 4 μl-aliquot containing about 0.035 μmole of the radioactive phenylthiohydantoin was applied to a silica gel sheet containing the fluorescent indicator. The plate was developed for 13 cm in chloroform–ethanol (98:2). After locating the spots under UV light, absorbing areas were scraped off and the silica gel was transferred to scintillation vials. Ethanol (0.2 ml) and 15 moles of toluene scintillator solution were added.

An alternative procedure using the same principle is that described by CALLEWAERT AND VERNON[36]. While the above procedure is applicable to short-chain peptides, the procedure described below is particularly suitable for use with high molecular weight proteins. The protein sample is dissolved in propanol–water (3:4). The amount of sample taken is *ca.* 0.25 μmole and the volume of the solvent is 1 ml. Before being added to the protein, the solvent is made 0.4 *M* with respect to N-dimethylallylamine and sufficient trifluoroacetic acid to make the apparent pH equal 9.5 is added. ^{35}S-labelled phenyl isothiocyanate (20 μl; specific activity 0.5 mCi/mmole) is added

* See also Chaptor 5.2.9 (Part A, p. 294).

and coupling to the protein is achieved by maintaining the mixture at 40° for 1.5 h. Excess of phenyl isothiocyanate is then extracted by repeated washing with benzene, and the remaining liquid is removed by freeze-drying with the use of a rotating evaporator. The phenylthiohydantoin derivative is now formed under the conditions described by ERIKSSON AND SJÖQUIST[53]: 1 ml of water and 1 ml of acetic acid saturated with HCl are added and the mixture is stirred at 40° for 2 h. Isotope dilution is then carried out by adding a known proportion of unlabelled phenylthiohydantoin (*ca.* 2.5 μmole) dissolved in 1 ml of ethanol. The next step in quantitation is the isolation of pure phenylthiohydantoin and the determination of its specific activity, which is carried out by the described flat-bed techniques, and the estimation of the specific activity, which is based on the ratio of activity to optical density.

7. Electrophoresis

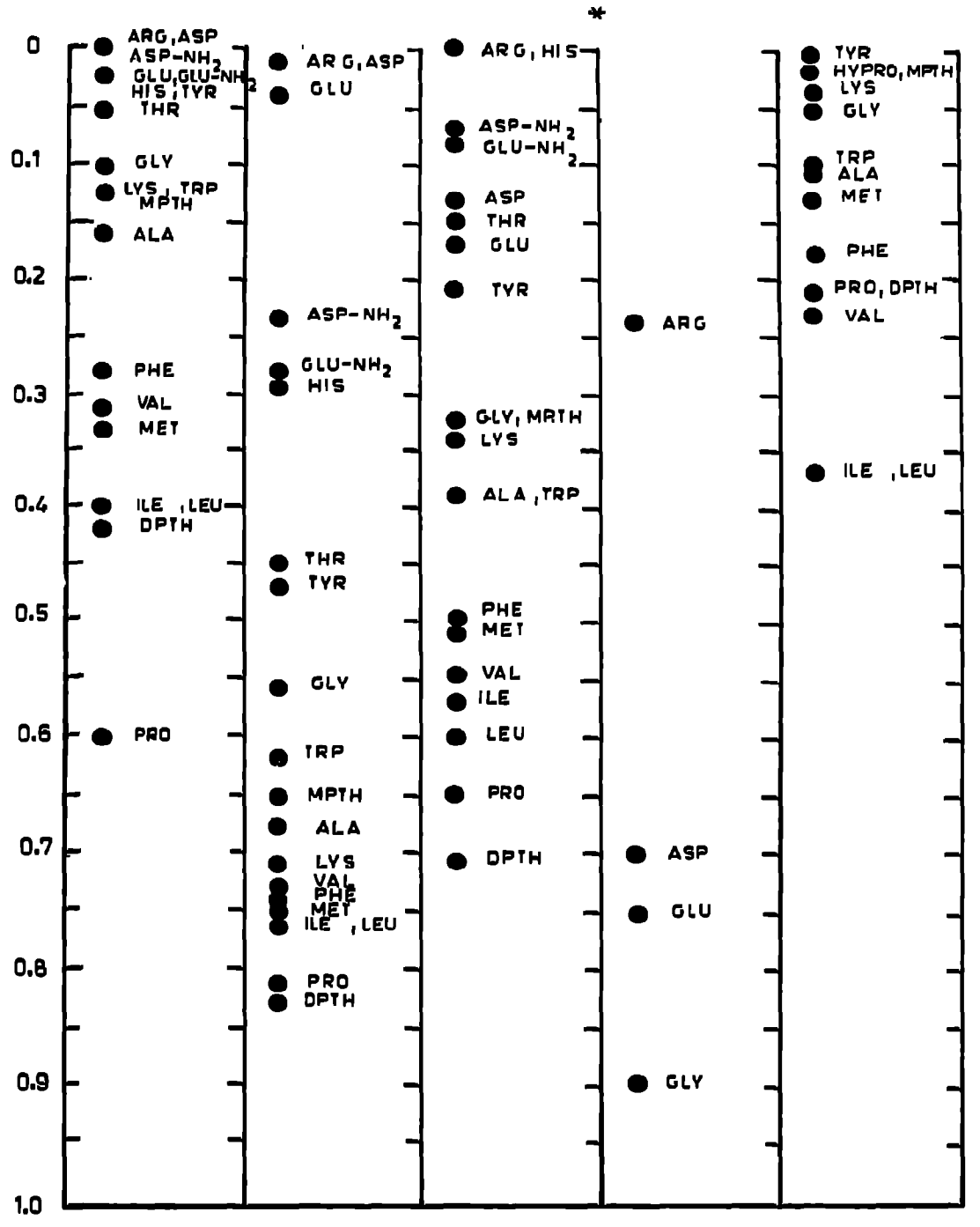
Electrophoretic separation of PTH-derivatives is a rather rare technique for identifying these compounds. As reported by NAKAJIMA *et al.*[133], electrophoresis can be carried out on paper using a high potential (2000–3000 V/cm) with a running time of 2–3 h. The final appearance of the chromatograms is strongly dependent on the pH: while PTH-Leu, PTH-Pro, PTH-Val and PTH-Lys are well separated at pH 1.0, at pH 2.0 it is possible to identify PTH-Ala, PTH-Ser, PTH-Gly, PTH-Thr, PTH-Leu, PTH-Ile and PTH-Met side-by-side. With a further increase in the pH it is possible to obtain a satisfactory separation of PTH-Asp, PTH-Glu, PTH-Trp, PTH-Tyr, PTH-Gly and PTH-Thr. Finally, at pH 6, the zones of PTH-Ser, PTH-Asp, PTH-Glu, PTH-Arg, PTH-His and PTH-Cys are seen.

Another electrophoretic separation of PTH-amino acid was described by SCHROEDER[175]; this method is used in combination with the Edman degradation in a paper strip combined with paper chromatography, and is described on p. 315 of this review.

8. Pictorial survey of flat-bed techniques used in PTH-amino acid chromatography

This is illustrated in Figs. 72–77. For key of numbers used to identify amino acid derivatives see p. 335.

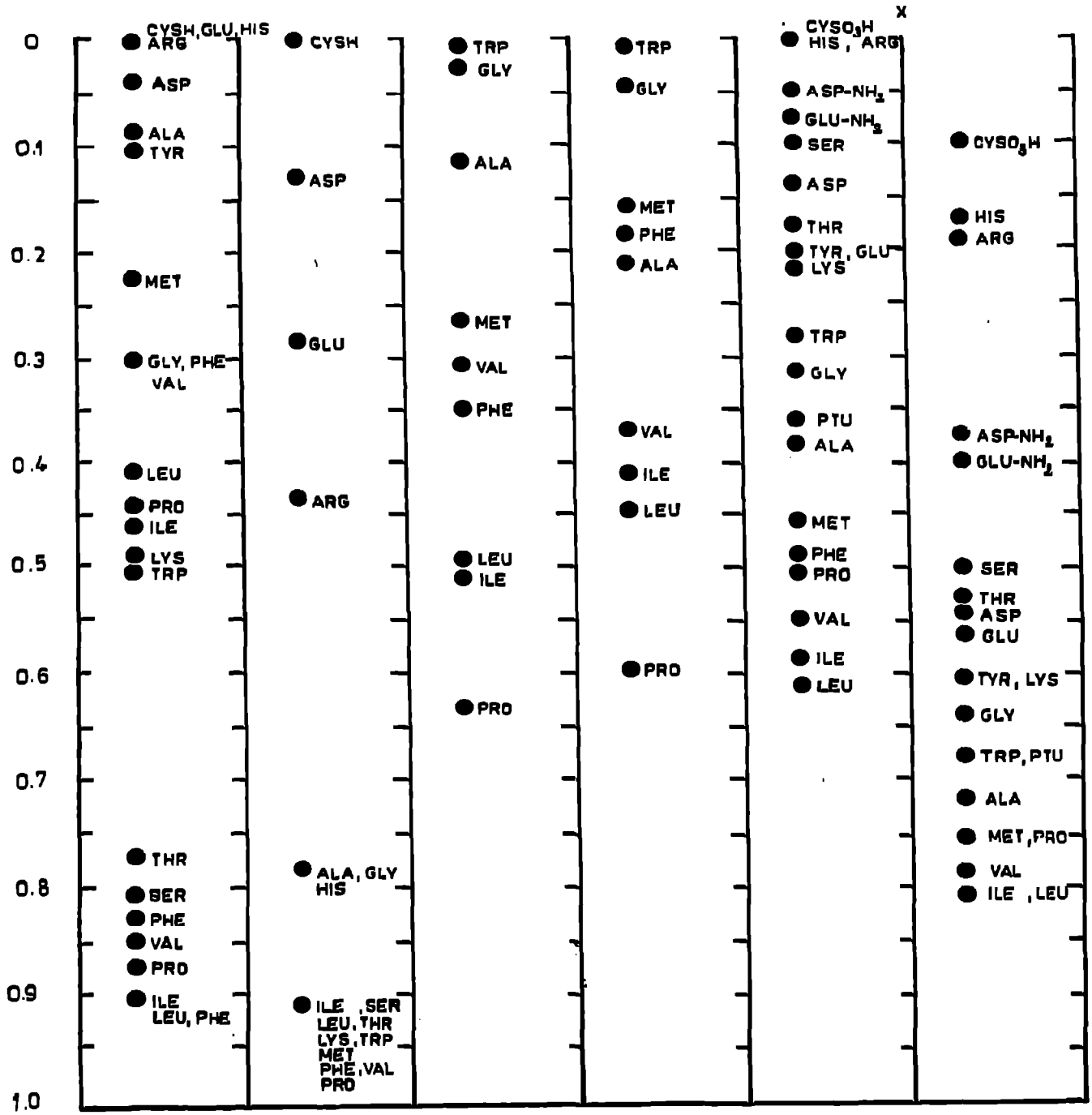
CHLOROFORM	CHLOROFORM 9 METHANOL 1	CHLOROFORM20 FORMIC ACID 1	CHLOROFORM35 FORMIC ACID 1 METHANOL 15	HEPTANE 30 ETHYLENE CHLORIDE 10 FORMIC ACID 7 PROPIONIC ACID 6
BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.	BRENNER et al.
SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL



* MOST OTHER AMINO ACIDS RUN WITH SOLVENT FRONT.

Fig. 72. See ref. 29.

XYLENE 3 ACETIC ACID 2 PHTHALATE 1 BUFFER pH 6	BUTANOL 7 PHTHALATE 1 BUFFER pH 6	o-XYLENE FORMAMIDE	XYLENE 5 ACETONE 1	HEPTANE 58 PROPIONIC ACID 17 ETHYLENE CHLORIDE 25	HEPTANE 50 n-BUTANOL 30
LANDMANN et al.	LANDMANN et al.	GRÜNER	GRÜNER	JEPPSON & SJOQUIST	JEPPSON & SJOQUIST
WHATMAN NO.1	WHATMAN NO.1	SILICA GEL	SILICA GEL	SILICA GEL	SILICA GEL



x THE SAMPLE WAS PRE-RUN IN HEPTANE–PROPIONIC ACID–ETHYLENE CHLOROHYDRIN (58:17:25) (THE PRECEDING SYSTEM OF JEPPSON & SJÖQUIST).

Fig. 73. See refs. 72, 95 and 104.

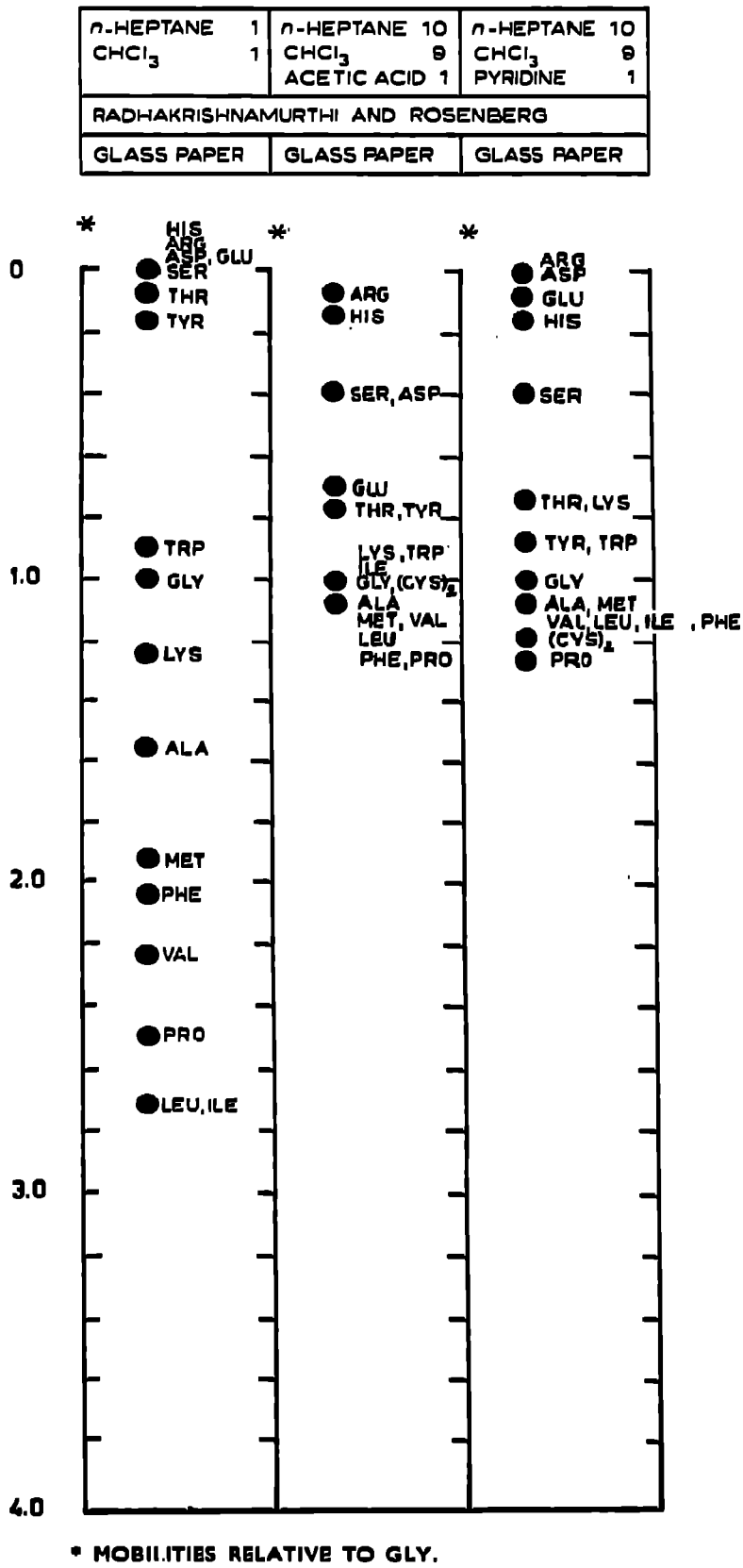


Fig. 74. See ref. 154.

90% FORMIC ACID 1 WATER	*n*-HEPTANE 40 *n*-BUTANOL 30 ACETIC ACID 9	CCl_4 9 ACETIC ACID 1	BENZENE 9 ACETIC ACID 1	2-BUTANONE 1 *n*-HEXANE 3
WANG et al.	WANG et al.	WANG et al.	WANG et al.	WANG et al.
POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE	POLYAMIDE

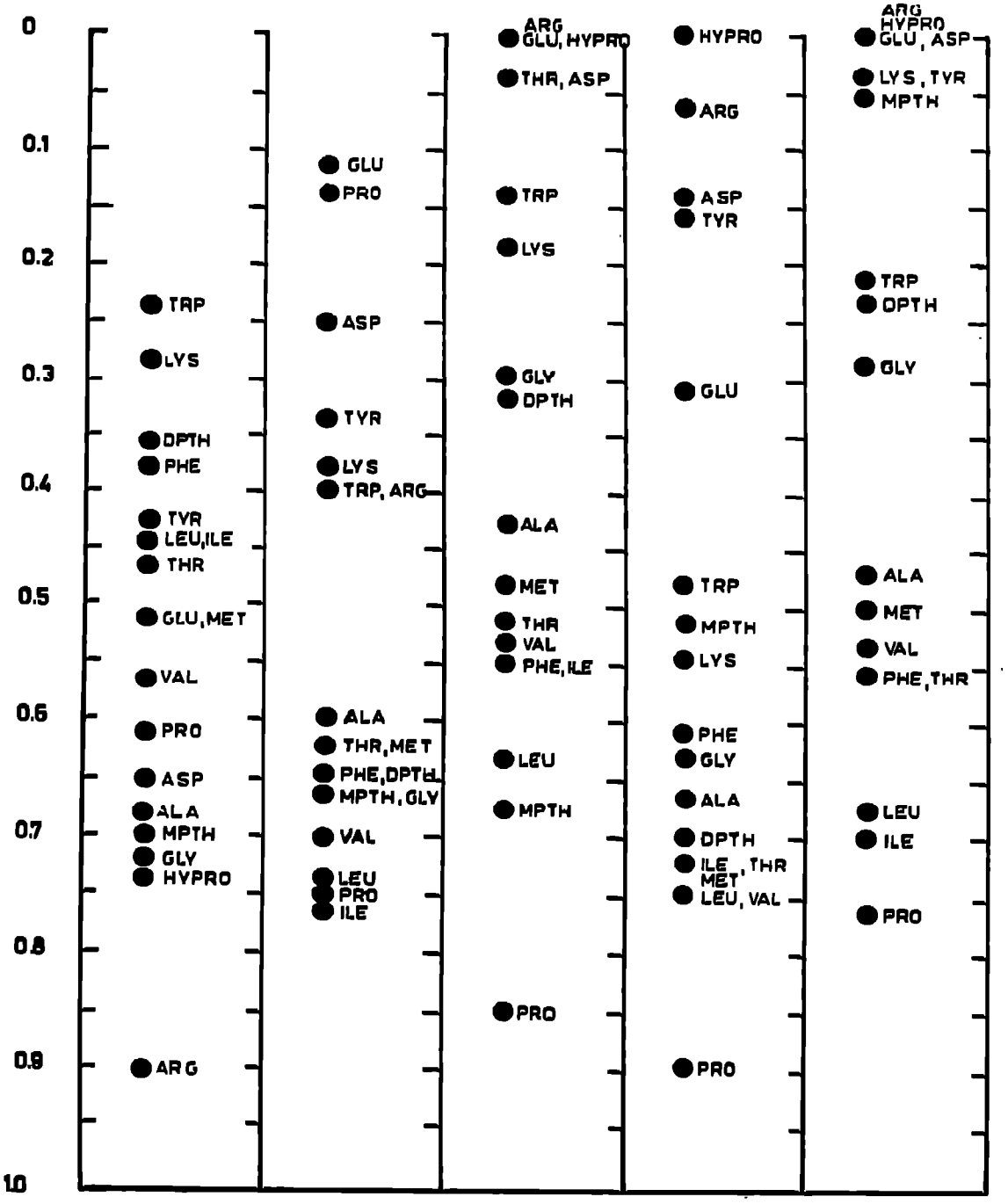
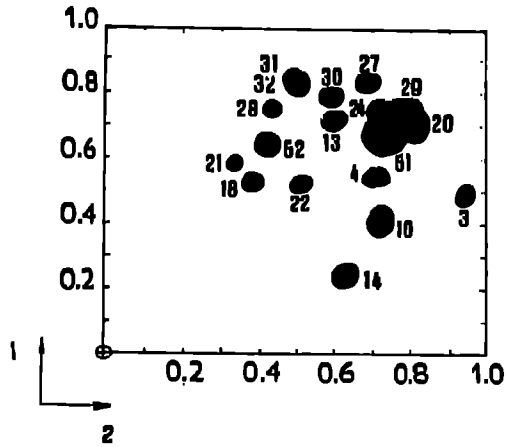


Fig. 75. See ref. 204.



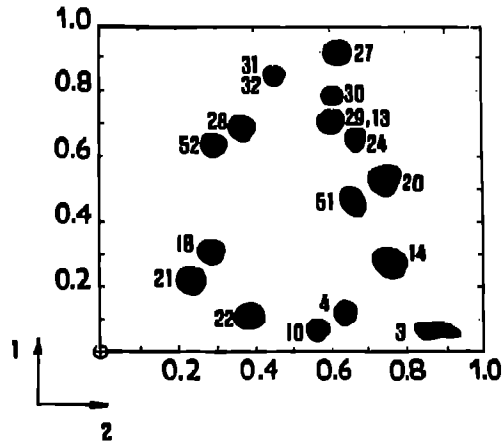


Fig. 76. WANG *et al.*[804]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: *n*-heptane–*n*-butanol–acetic acid (40 : 30 : 9); 2nd dimension: 45% formic acid.

Fig. 77. WANG *et al.*[804]. Sorbent, polyamide (ε-polycaprolactam resin). Development, 1st dimension: carbon tetrachloride–acetic acid (9 : 1); 2nd dimension: 45% formic acid.

CONCLUSION

If one compares the present state of knowledge surveyed in this review with the first review, devoted solely to the problems of identification of N-terminal residues in proteins, written by SIDNEY W. FOX[61] in 1945 and actually covering the pre-war period, the results are amazing.

FOX described in his review about six or seven methods, but he indicated only three of them as being adequate for proteins and useful for future development. The development of protein chemistry confirmed his positive as well as his negative judgment. The three methods he recommended are as follows:

(a) reaction with arylsulphonyl chlorides
(b) reaction with nitrophenylchlorides
(c) reaction with arylisocyanates

Apart from the experimental details, already described in great detail in individual paragraphs of this review, there is nothing to be added to the ingenious foresight of S. W. FOX expressed nearly thirty years ago.

From the viewpoint of recent practice and the status of the chromatographic techniques the following methods deserve to be stressed for use in the identification of N-terminal amino acids.

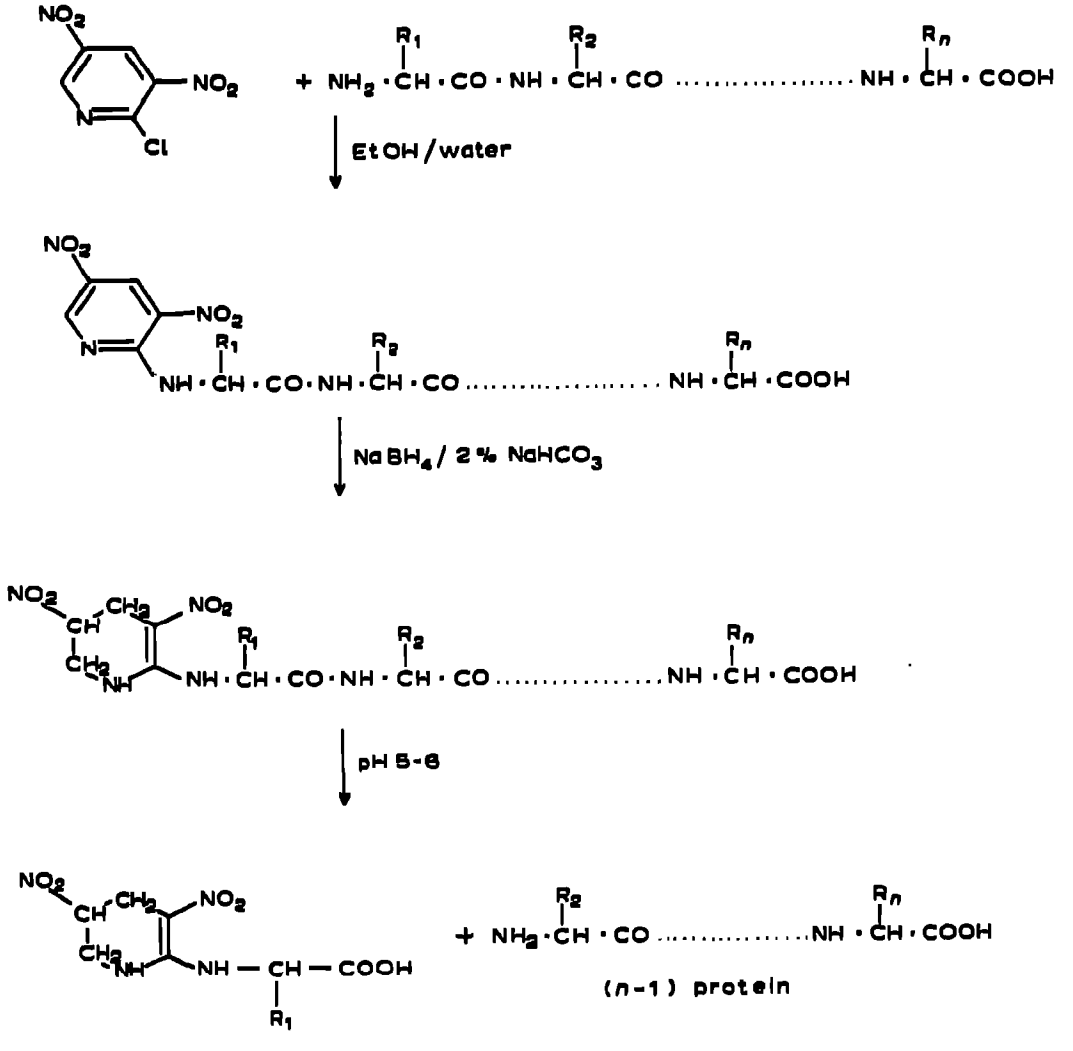
(1) The dansylation method (see Chapter 4.1.4), because of its usefulness mainly in the determination of the low-molecular-weight peptide structures and in being capable of rapidly supplying information on protein N-terminal groups.
(2) The dinitrophenylation method (see Chapter 3.1.3), being useful for the determination of N-terminal groups in proteins that contain a limited amount of impurities; its lower sensitivity allows an unequivocal determination of the N-terminal amino acid in the above instance when dansylation fails to give a reliable result.

(3) The alkyl isothiocyanate method in any of the three modifications described in Chapters 5.2.3, 5.2.4 and 5.2.9, because of its applicability in the automated version of protein sequencing (stepwise degradation from the N-end of the molecule). The method using simple isocyanate (Chapter 5.2.2) also offers some advantages, but the possibility of automation is by no means clear here and therefore the phenylthiohydantoin and methylthiohydantoin methods (see Chapters 5.2.3 and 5.2.4) are preferred in laboratories in which primary structures are determined all the year round.

We would like to try to follow S. W. Fox's foresight and to suggest at least one method in addition, which, in our opinion, should be of some value mainly in smaller laboratories where there is no possibility of acquiring the expensive automatic sequenator.

Such a method is the reaction with 3,5-dinitro-2-chloropyridine (Chapter 3.2.6), which combines the advantages of two general principles pointed out in the introduction to Part A of this review.

In this method, an alkylation reaction similar to that used by SANGER is used for labelling, which results in the formation of a bond between the N-terminal amino group and the reagent that is much more stable to hydrolysis than the peptide bond.



For cleavage of the labelled N-terminal amino acid, the labilizing influence of the neighbouring pyridyl species was used, with the basicity enhanced by selective reduction of the aromatic nucleus by $NaBH_4$. This enhanced basicity displaces the catalytic effect to the range pH 5–6, where all structural features of proteins usually remain unaltered.

This method enables the whole of the N-terminal amino acid determination to be carried out in the pH range 5–8, which makes it possible to isolate the $(n - 1)$ protein in the "native" conformation and to perform further studies even of a biological nature with such proteins.

It is unfortunate for chromatographers (including the present authors) that there is no chromatographic information about the derivatives that result from this method.

Finally, we would like to pay tribute to those who have written earlier reviews on similar topics. It would have been impossible to evaluate critically all the data presented in this paper without using the experience and insight of our predecessors. An incomplete list of the authors of these reviews is as follows: S. W. FOX[61], F. SANGER[170], B. MELOUN[122], V. HOLEYŠOVSKÝ[80], V. HOLEYŠOVSKÝ AND H. HOLEYŠOVSKÁ[81], G. PATAKI[141], N. SEILER (ref. 123 in Part A), G. R. STARK (refs. 142 and 143 in Part A), H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY[65], G. BISERTE, J. W. HOLLEMAN-DEHOVE AND P. SAUTIÈRE[10], M. JUTISZ[90] and M. BRENNER, A. NIEDERWIESER AND G. PATAKI[20].

NUMBERS USED TO IDENTIFY AMINO ACID DERIVATIVES IN TWO-DIMENSIONAL CHROMATOGRAMS

The numbers below are valid for both DNP- and PTH-amino acid chromatograms unless otherwise stated.

1 = Cysteic acid (mono); 2 = cysteine (mono); 3 = arginine (mono); 4 = aspartic acid (mono); 5 = methionine sulphone (mono); 6 = ornithine (δ-mono); 7 = lysine (ε-mono); 8 = serine (mono); 9 = asparagine (mono); 10 = hydroxyproline (mono); 11 = ornithine (α-mono); 12 = allothreonine (mono); 13 = threonine (mono); 14 = glutamic acid; 15 = glutamine; 16 = methionine sulphoxide (mono); 17 = tyrosine (O-mono); 18 = lysine (bis); 19 = histidine (bis); 20 = glycine (mono); 21 = tryptophan (mono); 22 = tyrosine (bis); 23 = sarcosine (mono); 24 = alanine (mono); 25 = 2,4-dinitroaniline (for DNP-amino acid chromatograms only); 26 = β-alanine (mono); 27 = proline (mono); 28 = phenylalanine (mono); 29 = methionine (mono); 30 = valine (mono); 31 = leucine (mono); 32 = isoleucine (mono); 33 = 2,4-dinitroaniline (for DNP-amino acid chromatograms only); 34 = taurin (mono); 35 = carboxymethylcysteine (mono); 36 = lysine (α-mono); 37 = tyrosine (N-mono); 38 = norleucine (mono); 39 = norvaline (mono); 40 = ornithine (bis); 41 = cystine (N-bis); 42 = α-aminoadipic acid (mono); 43 = hydroxylysine (all derivatives); 44 = α-aminobutyric acid (mono); 45 = γ-aminobutyric acid (mono); 46 = citrulline; 47 = diaminopimelic acid (all derivatives); 48 = ethionine (mono); 49 = ethionine sulphoxide (mono); 50 = carboxymethylcysteine sulphoxide (mono); 51 = monophenylthiourea (for PTH-amino acid chromatograms only); 52 = diphenylthiourea (for PTH-amino acid chromatograms only); 53 = α-aminocaprylic acid.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the following collaborators for their valuable help in preparing the manuscript of this review: Mrs. M. CIPROVÁ, Miss R. KESSLEROVÁ, Mrs. H. MÁLKOVÁ, Mr. J. MARC, Mrs. M. PETŘÍKOVÁ, Miss J. SVOBODOVÁ, Mrs. H. ŠTOLCOVÁ and Mrs. E. TOMÁŠKOVÁ. To the late Dr. V. HOLEŠOVSKÝ we are obliged for the opportunity of using his unpublished materials on the topics dealt with in this review.

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