Analytical Separations by High-Voltage Paper Electrophoresis

AMINO ACIDS IN PROTEIN HYDROLYSATES

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The determination of the amino acid composition of peptides and proteins is usually carried out on acid hydrolysates by ion-exchange chromatography, by following the methods developed by Moore & Stein and their collaborators (Moore, Spackman & Stein, 1958). This method is precise but time-consuming, requiring up to 600 individual determinations for a single protein analysis. Automatic scanning of the column effluent (Spackman, Stein & Moore, 1958) obviates this objection, but increases complication and cost.

An alternative group of methods for amino acid analysis are based on paper-chromatographic separation of the individual amino acids, followed by quantitative determination by means of some modification of the ninhydrin reaction. A considerable literature (reviewed by Lederer & Lederer, 1957; Hanes, 1961) describes the many variants of this method, but most are subject to considerable errors due mainly to the limitations of the ninhydrin reaction, and to losses on the paper during the chromatographic separation and the subsequent manipulations. An exhaustive study of these sources of error has been made by Hanes and his associates (Connell, Dixon & Hanes, 1955; Hanes, 1961; Wade, Matheson & Hanes, 1961; Hanes, Harris, Moscarello & Tigane, 1961; Matheson, Tigane & Hanes, 1961; Tigane, Wade, Tze-Fei Wong & Hanes, 1961), who have developed two new paper-chromatographic systems for the separation of 16 of the 18 common amino acids, and also a modified ninhydrin reagent. These improvements constitute a considerable advance over previous methods, but at the cost of a relatively complex technique. Recoveries of individual amino acids were within $\pm 10\%$. No applications to protein hydrolysates were reported.

Levy (1954) has described a method of amino acid analysis based on the paper-chromatographic separation of the dinitrophenyl derivatives, followed by spectrophotometric determination at 360 m μ . This method appears to be markedly inferior to ion-exchange chromatography (see Hedbom, 1961, for a direct comparison). Whitehead (1958) has described an isotope-dilution method based on acetylation of the amino acids with [^{14}C]- and [^3H]-acetic anhydride, and a combined

chromatographic separation of the acetyl compounds. This method does not appear to have been widely used, although it is applicable to microgram amounts of protein.

Recent advances in the technique of high-voltage paper electrophoresis (reviewed comprehensively by Michl, 1958) have provided an analytical method of extremely high resolving power for small molecules. The present paper describes systems for the complete separation of all the amino acids commonly occurring in protein hydrolysates. Combined with the improved cadmium-ninhydrin method of Heilmann, Barollier & Watzke (1957) this provides a new method of amino acid analysis, which reduces the estimations required to one for each amino acid present in the hydrolysate, while the precision is comparable with that obtained with the ion-exchange method; 250 μg . of protein suffices for a complete amino acid analysis. Röwe, Ferber & Fischer (1958) have described an analogous method, which was, however, applicable to only 10 of the 18 commonly occurring amino acids. Preliminary accounts of the present work have already been published (Atfield & Morris, 1960*a, b*).

EXPERIMENTAL

Apparatus

The apparatus used for the electrophoretic separation is based on Gross's (1956, 1961) modification of Michl's (1953) design, in which the paper strip is held horizontally between two cooled surfaces. The apparatus is illustrated in Fig. 1. It consists essentially of two anodized aluminium-copper alloy plates, *AA*, 105 cm. $\times$ 15 cm. $\times$ 2.5 cm., milled to a surface flatness tolerance of 0.001 in. The plates are provided with internal channels, through which aqueous 20% methanol maintained at $2 \pm 1^\circ$ by a thermostated refrigeration unit is circulated at approx. 100 ml./sec. to provide cooling.

The paper electrophoresis strip *C* is insulated electrically from the cooling plates *AA* by the 0.014 in. thick polythene films *BB* (Telegraph Construction and Maintenance Co. Ltd., Farnborough, Kent), which are 5 cm. wider than the cooling plates, to provide adequate insulation under conditions of high humidity. The electrophoresis strip is connected to the electrode vessels by bridges *DD*, which consist of two layers of moist Whatman 3MM filter paper enclosed in cellophan (grade 300, uncoated). The heavy

Fig. 1. Schematic view of high-voltage electrophoresis apparatus. *AA*, Cooling plates; *BB*, polythene insulating films; *C*, paper electrophoresis strip; *DD*, cellophan-covered bridges; *EE*, electrode vessels; *FF*, platinum electrodes; *G*, pneumatic device (see text).

polythene electrode vessels *EE* have a labyrinth arrangement to minimize diffusion of electrolysis products towards the electrophoresis strip. The electrodes *FF* are platinum plates (2 cm. $\times$ 0.5 cm.).

During operation the cooling plates and insulating films are maintained in close contact with the electrophoresis strip by the pneumatic device *G*, which contains a rubber bag inflated to 0.14 kg./cm.². *G* is attached to the bottom plate by metal straps (not shown), so that a uniform pressure is exerted on the free upper cooling plate. It is essential to maintain the surfaces of the cooling plates in good condition for efficient removal of heat. In practice outputs up to 500 w could be dissipated over 8 hr. periods without evidence of excessive heating.

The potential gradient for electrophoresis is provided by a transformer-selenium rectifier unit giving outputs up to 100 mA at 12 000 v. Variation of the applied potential is obtained by switch selection of multiple secondary tappings, and the output is smoothed by a capacitance-choke-capacitance filter circuit. The entire apparatus is enclosed in a Perspex cover, removal of which interrupts the primary circuit of the power unit.

Materials

Whatman 3MM filter paper has been used as the stabilizing medium throughout this work. Several other grades examined gave inferior resolution.

Preparation and hydrolysis of performic acid-oxidized insulin. The method used is based on that of Hirs (1956). The insulin hydrochloride used was a three times recrystallized specimen (Boots Pure Drug Co. Ltd.). Since Thompson (1954) has shown that performic acid oxidation of tyrosine or tyrosine-containing proteins in the presence of HCl leads to the formation of chlorotyrosine, it was necessary to remove chloride ions before oxidation.

Insulin (15 mg.) was dissolved in conc. NH_3 solution (sp.gr. 0.88; 50 μl .) and water (3 ml.) added. Dowex 2X8 (acetate) ion-exchange resin (50 mg.) was added to the insulin solution, and the mixture shaken for 30 min. The ion-exchange resin was removed by filtration and washed with water, and the combined filtrate and washings were freeze-dried. Performic acid was prepared by adding H_2O_2 (30%, v/v; 0.5 ml.) to formic acid (A.R. grade 98–100%; 9.5 ml.), and keeping the mixture for 2 hr. before use. Freeze-dried chloride-free insulin (14 mg.) was dissolved in formic acid (98–100%; 0.5 ml.) and anhydrous methanol

(0.1 ml.) added with stirring. The insulin solution and the performic acid solution were cooled to 0°, and 1 ml. of the performic acid solution was added to the insulin solution. The reaction was allowed to proceed for 5 hr. at 0° and was terminated by rinsing the contents of the reaction flask into ice-cold water to a total volume of 40 ml. The diluted solution was frozen immediately and the solvents were removed by freeze-drying. The residue was dissolved in water (4 ml.), transferred to a small flask with more water (4 ml.) and again freeze-dried. The product was further dried over P_2O_5 *in vacuo* for analysis.

Hydrolysis was carried out on a 5 mg. sample in a sealed tube with constant-boiling-point HCl (0.1 ml.) for 24 hr. at 110°. The hydrolysate was repeatedly evaporated to dryness with small volumes of water to remove excess of HCl. The final product was dissolved in aqueous 10% (v/v) propan-2-ol (0.1 ml.) for transfer to the electrophoresis strip.

Electrolyte solutions. The compositions given are critical and should be strictly adhered to for best results: (a) Pyridine-acetic acid-water, pH 5.2; pyridine (A.R. grade; redistilled; 20.0 g.), acetic acid (A.R. grade; 99.6%; 9.5 g.) and water to 1 l. (b) Formic acid-acetic acid-water, pH 1.85; acetic acid (A.R. grade; 99.6%; 78.0), formic acid (A.R. grade; 98–100%; 25.0 g.) and water to 1 l. (c) Formic acid-acetic acid-cadmium acetate-water, pH 1.81; acetic acid (A.R. grade; 99.6%; 200.0 g.), formic acid (A.R. grade; 98–100%; 20.0 g.), cadmium acetate (A.R. grade; 1.06 g.) and water to 1 l.

Cadmium-ninhydrin reagent. Cadmium acetate (A.R. grade; 0.050 g.) was dissolved in a mixture of water (5.0 ml.) and acetic acid (1.0 ml.). Acetone (50 ml.) was added and the mixture shaken until any precipitate had redissolved. Ninhydrin (A.R. grade; 0.500 g.) was dissolved in the mixture. The reagent was prepared fresh daily.

Cadmium-isatin reagent. Cadmium acetate (A.R. grade; 0.25 g.) was dissolved in water (1.25 ml.), and acetic acid (A.R. grade; 0.25 ml.) and propan-2-ol (23.75 ml.) were added to the solution. Isatin (0.25 g.) was dissolved in the mixture. The reagent was prepared fresh daily.

Procedure

A strip of Whatman 3MM filter paper (120 cm. $\times$ 13.5 cm.) was marked with a light transverse pencil line 14.5 cm. from one end and immersed in the appropriate electrolyte solution until saturated. It was then drawn through a twin rubber-roller 'mangle' to remove excess of liquid. The upper roller was spring-loaded with adjustable pressure. It was set to give a moderately dry paper (95–100 g. of electrolyte/100 g. of dry paper). Satisfactory results can also be obtained by blotting with dry filter paper, although reproducibility is then more difficult to achieve. The paper strip was laid on a glass plate with paper strips (14 cm. $\times$ 2 cm.) laid transversely above and below the electrophoresis strip in the vicinity of the starting line. The whole was covered with a 0.014 in.-thick polythene film, and left for 10 min. to equilibrate. The bridge strips were prepared by immersing a strip (30 cm. $\times$ 13.5 cm.) of Whatman 3MM filter paper in electrolyte to saturation, doubling it to form a pad (15 cm. $\times$ 13.5 cm.), roller-pressing to remove excess of liquid, and wrapping the pad in cellophan sheet (grade 300) that had been previously soaked in the electrolyte for at least 12 hr. After 10 min. the paper drying strips were

removed from the electrophoresis strip and the latter was supported on a slotted polythene bridge piece, so that the starting line, although supported, was not in direct contact with a solid surface.

The hydrolysate solution (5 μ l.) was then applied to the paper from a micropipette to give a uniform line approximately 11 cm. long. The application is facilitated by the addition of an anionic dye such as acid fuchsin or neococcin to the test mixture. The regularity of application can then be checked visually, while the dye migrates anodically away from the amino acid mixture on application of the electric potential. A length (2.5 cm.) of the strip at the anodic (starting) end was cut off and transferred to a weighing bottle for moisture determination. The polythene film covering the electrophoresis strip was then inverted and the strip laid on it before transference to the lower cooling plate of the electrophoresis apparatus. A portion (2.5 cm.) at each end of the electrophoresis strip was folded over to form contact pads for the bridge strips, which were placed in a position overlapping the electrophoresis strip by approximately 2 cm., and the whole was covered by another polythene film. The upper cooling plate was lowered and secured, and the pneumatic pressure bag inflated to 0.14 kg./cm.². Good contact between the bridge strips and the electrophoresis strip was ensured by insertion of small rolls of polythene film into the end recesses in the cooling plates. The electrode vessels were filled with electrolyte solution, the cover was put in place, and the potential gradient applied. During operation the electric current tends to increase and it should be checked and if necessary adjusted at 15 min. intervals. A constant current supply would undoubtedly be superior, and the design of a suitable power supply is being currently investigated.

At the end of the separation, the potential gradient was shut off, the air pressure released, the paper strip transferred to a horizontal glass plate, and most of the liquid removed by infrared heating in an air stream for 10 min. The strip was finally dried for 15 min. at 80°. The two-stage drying procedure prevents deterioration of the separated zones by liquid movement within the strip. The dried strip was dipped in the cadmium-ninhydrin reagent, and stored for 20 hr. in a dark, all-glass, air-tight vessel, provided with a dish containing conc. H₂SO₄ to maintain a NH₃-free atmosphere. Quantities (5, 10 and 20 μ g.) of all the amino acids investigated were transferred in 0.1 ml. volumes to strips of Whatman 3MM paper that had been wetted with the appropriate electrolyte solution and roller-pressed free from excess of liquid. These were dried, dipped in ninhydrin reagent and developed together with the electrophoresis strip. Next day the bright-red zones were cut out together with appropriate blank control strips, cut into pieces (approx. 1 cm. $\times$ 0.5 cm.), and transferred to individual glass-stoppered test tubes (12 cm. $\times$ 1.6 cm.). Redistilled absolute methanol (8.00 ml.) was added to each, and the tubes were stoppered and secured and rotated end-for-end in a rotating frame-shaking machine for 30 min. The clear red solutions were freed of paper fibres by pressure filtration through cylindrical funnels provided with sintered-glass filter disks (porosity 2). The extinction was measured at 500 m μ for the amino acids, and at 352 m μ for the yellow reaction product given by proline, by using 1 or 2 cm. cells. The amino acid concentration-extinction graphs showed satisfactory linearity up to an extinction of 0.9 (Atfield & Morris, 1960*b*), and the

individual colour yields remained within $\pm 3.5\%$ over a period of 12 months.

If the proline content is low (as in insulin), the decreased sensitivity of the ninhydrin reaction may necessitate an alternative procedure based on the colorimetric method of Hrabětová & Tupý (1960). The position of the proline zone was located in the formic acid-acetic acid-cadmium acetate electrophoresis by means of markers on the edges of the strip. The corresponding area of paper was cut out, dried, dipped in the cadmium-isatin reagent and heated for 30 min. at 80°. Proline gives a green zone on a yellow background under these conditions. The strip was washed repeatedly with water at 30° until the background was white or very pale yellow, and the proline zone was deep blue. The paper containing the blue band was cut into small pieces, transferred to a glass-stoppered test tube (12 cm. $\times$ 1.6 cm.) and eluted with water-saturated phenol (5.00 ml.) by end-over-end shaking for 15 min. The blue eluate was filtered through a sintered-glass funnel under positive pressure and the extinction measured at 610 m μ . A control prepared from an adjacent area of the electrophoresis strip, and proline standards (5 and 10 μ g.), were prepared in a similar manner. In agreement with the findings of Heilmann *et al.* (1957) and Hrabětová & Tupý (1960), satisfactory agreement with the Beer-Lambert Law was found for both colorimetric methods up to extinctions of approx. 0.6.

RESULTS

Three electrophoretic separations in different electrolyte solutions are necessary to separate the eighteen amino acids commonly found in protein hydrolysates. These separations are shown in Fig. 2.

The first electrophoresis carried out in pyridine-acetate buffer at pH 5.2 for 200 min. at 75 v/cm. separates the basic amino acids, lysine, histidine and arginine (and also hydroxylysine and ornithine if present), as cations (Fig. 2*a*) and the acidic amino acids, cysteic, aspartic and glutamic acid, as anions (Fig. 2*b*). The neutral amino acids form an unresolved zone that is displaced 1.5–2 cm. towards the cathode by electro-osmotic flow through the paper.

The second electrophoresis is carried out in a 2.5% formic acid–7.8% acetic acid medium at pH 1.85 and gives a good resolution of the majority of the neutral amino acids. The resolved amino acids (in order of mobilities) are glycine, alanine, serine, valine, leucine + isoleucine (partially resolved), threonine, proline (yellow ninhydrin reaction), glutamic acid, phenylalanine + aspartic acid (not resolved), methionine sulphone and tyrosine. Tryptophan, if present, as in an enzymic digest, migrates more slowly than tyrosine. A good separation is obtained in 270 min. at 100 v/cm. (Fig. 2*c*).

A third electrophoresis is necessary to resolve those amino acids that are not resolved in the formic acid-acetic acid medium. This is carried out

Fig. 2. Separation of amino acids by high-voltage paper electrophoresis. (a) Pyridine-acetate buffer, pH 5.2. From the left unresolved neutral amino acids, arginine, histidine and lysine. (b) Pyridine-acetate buffer, pH 5.2. Cysteic acid, aspartic acid and glutamic acid. (c) Formic acid-acetic acid, pH 1.85. Tyrosine, methionine sulphone, phenylalanine + aspartic acid (unresolved), glutamic acid, threonine, leucine + isoleucine (unresolved), valine, serine, alanine and glycine. (d) Formic acid-acetic acid-cadmium acetate, pH 1.81. Tyrosine, methionine sulphone, aspartic acid, phenylalanine, glutamic acid, threonine, *allo*isoleucine, leucine, isoleucine and valine + serine (unresolved).

Table 1. *Mobilities of amino acids relative to alanine*

System A: 2.5% formic acid–7.8% acetic acid, pH 1.85, 100 v/cm. System B: 2.0% formic acid–20% acetic acid–4 mm-cadmium acetate, pH 1.81, 100 v/cm.

A		B	
Amino acid	Relative mobility	Amino acid	Relative mobility
Alanine	1.00	Alanine	1.00
β -Alanine	1.45	Aspartic acid	0.59
α -Aminobutyric acid	0.90	Glutamic acid	0.66
Arginine	1.31	Isoleucine	0.80
Asparagine	0.71	alloIsoleucine	0.76
Aspartic acid	0.61	Leucine	0.78
Cysteine	0.60	Methionine sulphone	0.55
Cystine	0.59	Phenylalanine	0.61
Glutamic acid	0.67	Proline	0.69
Glutamine	0.69	Serine	0.81
Glycine	1.14	Threonine	0.73
Histidine	1.31	Tyrosine	0.53
Hydroxyproline	0.54	Valine	0.81
Isoleucine	0.77		
Leucine	0.77		
Lysine	1.47		
Methionine	0.71		
Methionine sulphone	0.57		
Methionine sulfoxide	0.60		
Ornithine	1.52		
Phenylalanine	0.61		
Proline	0.69		
Serine	0.83		
Taurine	0.03		
Threonine	0.75		
Tryptophan	0.46		
Tyrosine	0.53		
Valine	0.81		

in a 2% formic acid–20% acetic acid solution at pH 1.81, containing 4 mm-cadmium acetate, for 420 min. at 100 v/cm. The separation sequence in descending order of mobilities is valine + serine (unresolved), isoleucine, leucine, *allo*isoleucine, threonine, proline, glutamic acid, phenylalanine, aspartic acid, methionine sulphone and tyrosine (Fig. 2*d*). The basic amino acids, together with glycine and alanine, migrate off the paper during the increased duration of electrophoresis necessary for the separation of the isomeric leucines.

Owing to the controlled and constant wetness of the stabilizing medium, measured mobilities have shown less than 2% variation over a period of 18 months, and over migration distances from 40 to 80 cm. They provide unequivocal identification of all the amino acids studied. The relative mobilities of a number of naturally occurring amino acids and amines are given in Tables 1 and 2.

Quantitative analyses

Analyses of synthetic mixtures containing approximately 10 and 20 μ g. of 17 amino acids are given in Tables 3 and 4. Glutamic acid can be estimated in all three systems and provides an internal intercomparison standard, and several

Table 2. *Mobilities of amino acids relative to glutamic acid (acidic amino acids) and arginine (basic amino acids)*

System was pyridine–acetic acid, pH 5.2, 70 v/cm.

Amino acid	Mobility relative to	
	Arginine	Glutamic acid
Arginine	1	—
Aspartic acid	—	1.21
Cysteic acid	—	1.30
Glutamic acid	—	1
Histidine	1.06	—
Hydroxylysine	1.04	—
Lysine	1.11	—

other amino acids can be measured in both the formic acid–acetic acid and cadmium–formic acid–acetic acid systems. Isoleucine presents a special problem, as only one preparation of five examined was free of the *allo*-isomer and other ninhydrin-reactive impurities. The low recoveries of this amino acid in Tables 3 and 4 reflect this uncertainty.

Triplicate analyses of the hydrolysates of two samples of performic acid-oxidized insulin prepared as described in the Experimental section are

Table 3. Recoveries from approximately 10 $\mu\text{g.}$ quantities of amino acids

F-A, Cd-F-A and P-A refer to the formic acid-acetic acid, cadmium-formic acid-acetic acid and pyridine-acetic acid media respectively.

Amino acid	Present ($\mu\text{g.}$)	Found ($\mu\text{g.}$)			Error (%)
		F-A	Cd-F-A	P-A	
Alanine	9.1	9.1	—	—	± 0
Arginine	9.4	—	—	9.2	-2.1
Aspartic acid	9.4	—	—	9.1	-3.2
Cysteic acid	14.1	—	—	14.3	+1.1
Glycine	9.5	9.6	—	—	+1.05
Glutamic acid	9.0	9.1	8.7	8.95	-3.2 (mean)
Histidine	9.0	—	—	9.0	± 0
Isoleucine	9.2	—	8.4	—	-8.6
Leucine	9.3	—	9.2	—	-1.1
Lysine	12.1	—	—	12.1	± 0
Methionine sulphone	6.4	6.5	6.2	—	-0.8 (mean)
Phenylalanine	9.8	—	10.1	—	+3.1
Proline	10.3	10.25	—	—	-0.8
Serine	9.2	9.2	—	—	± 0
Threonine	9.9	9.8	—	—	-1.0
Tyrosine	9.7	—	9.9	—	+2.1
Valine	12.0	12.0	—	—	± 0

Table 4. Recoveries from approximately 20 $\mu\text{g.}$ quantities of amino acids

For abbreviations see Table 3.

Amino acid	Present ($\mu\text{g.}$)	Found ($\mu\text{g.}$)			Error (%)
		F-A	Cd-F-A	P-A	
Alanine	20.0	20.05	—	—	+0.25
Arginine	17.15	—	—	17.6	+2.6
Aspartic acid	26.0	—	27.0	25.2	+0.4 (mean)
Cysteic acid	22.0	—	—	21.9	-0.45
Glycine	20.4	20.0	—	—	-1.95
Glutamic acid	21.9	21.7	22.2	21.5	-0.45 (mean)
Histidine	16.3	—	—	16.3	± 0
Isoleucine	18.55	—	17.3	—	-6.7
Leucine	18.0	—	17.7	—	-1.7
Lysine	16.35	—	—	16.1	-1.5
Methionine sulphone	19.8	20.3	20.5	—	+2.0 (mean)
Phenylalanine	19.75	—	18.8	—	-4.8
Proline	23.05	23.2	—	—	+0.65
Serine	19.5	19.7	—	—	+1.0
Threonine	20.7	20.95	19.4	—	-2.4 (mean)
Tyrosine	16.9	16.7	—	—	-1.2
Valine	20.95	20.5	—	—	-2.15

given in Table 5, together with the calculated composition based on the known structure of insulin. The serine and threonine values have been corrected for loss on hydrolysis, and all values are corrected for the moisture and ash content of the oxidized insulin. The proline values were obtained by the isatin method.

The recoveries from synthetic mixtures were within $\pm 5\%$, with the exception of the isoleucine values, for reasons noted above. The analyses of performic acid-oxidized insulin, although in satisfactory internal agreement (all within $\pm 10\%$, and the majority within $\pm 5\%$), are in less satisfactory agreement with the calculated values, although the majority are within $\pm 10\%$. This may reflect the

purity of the amino acid standards (for example histidine and proline) or the uncertainty in the composition of the actual insulin preparations used. The improved results of the second analysis suggest that an accuracy of $\pm 10\%$ is within the range of the method.

DISCUSSION

The mobility u of an ion of net charge q , and diffusion coefficient D , is given by the expression:

$$u = \frac{qD}{kT}, \quad (1)$$

where T is the absolute temperature, and k is the Boltzmann constant. Equation (1) is independent

Table 5. *Amino acid analyses of two samples of performic acid-oxidized insulin*

Values are given as g. of amino acid/100 g. of oxidized insulin.

Amino acid	Experimental						Calculated
	I			II			
Alanine	4.4	4.4	3.6	3.9	4.0	4.0	4.4
Arginine	2.3	2.3	2.3	2.8	2.8	2.8	2.9
Aspartic acid	6.0	5.9	6.0	6.6	6.6	6.6	6.6
Cysteic acid	14.75	15.35	15.6	15.9	16.5	15.6	16.8
Glutamic acid	16.3	16.4	16.6	16.1	16.1	16.2	17.1
Glycine	5.25	4.75	5.5	5.1	4.95	5.3	5.0
Histidine	4.4	4.55	4.5	4.4	4.65	4.6	5.2
Isoleucine	2.1	2.1	2.1	1.7	1.8	1.8	2.2
Leucine	13.0	13.1	13.1	12.1	12.4	13.8	13.1
Lysine	1.8	1.9	2.1	2.3	2.4	2.1	2.4
Phenylalanine	10.8	10.8	11.2	8.8	9.2	8.7	8.2
Proline	1.6	1.7	1.9	1.6	1.8	1.6	1.9
Serine	4.5	4.5	4.75	3.5	3.6	3.7	5.2
Threonine	1.9	1.9	1.9	1.9	1.9	2.0	2.0
Tyrosine	11.9	11.5	11.9	12.0	11.9	12.0	12.0
Valine	8.0	8.2	9.1	8.8	8.8	9.0	9.7
Total	109.0	109.35	112.15	107.5	109.4	109.8	114.7

Table 6. *Experimental and calculated relative mobilities of amino acid cations in formic acid-acetic acid solution*

Amino acid			<i>u</i> (exp.)	<i>u</i> (calc.)			
pH	...	...		2.1	2.0	1.9	
Histidine			1.31	1.32	1.37	1.42	
Glycine			1.14	1.20	1.19	1.19	
Alanine			1.00	1.00	1.00	1.00	
α -Aminobutyric acid			0.90	0.88	0.87	0.86	
Serine			0.82	0.86	0.88	0.89	
Valine			0.81	0.81	0.81	0.81	
Leucine			0.77	0.79	0.78	0.78	
Threonine			0.75	0.76	0.78	0.79	
Methionine			0.72	0.75	0.76	0.76	
Proline			0.69	0.67	0.70	0.73	
Glutamic acid			0.67	0.68	0.70	0.71	
Aspartic acid			0.61	0.58	0.54	0.52	
Phenylalanine			0.61	0.89	0.87	0.85	
Tyrosine			0.53	0.63	0.64	0.65	
Tryptophan			0.46	0.73	0.72	0.72	

of the shape and size of the ion, but is only valid in media of low ionic strength in which interionic interactions may be neglected. This may be assumed to be approximately true for the formic acid-acetic acid medium used in this work, where the major ionic species will be protons at approximately 0.01 molarity. Absolute mobilities cannot be determined by paper electrophoresis for a variety of reasons (discussed by Svensson, 1956), but mobilities relative to that of a chosen ion can be measured with high precision, provided that the ionic migration is linear both in time and in applied potential gradient. These conditions rarely obtain in conventional paper electrophoresis, owing to the evaporation of solvent from the

electrophoresis strip and the consequent inward flow of buffer from the electrode vessels. This is not the case in the present method, as the use of cellophan-enclosed bridges (Weber, 1951; Michl, 1953) prevents hydrodynamic flows within the paper, and adequate cooling minimizes evaporation. In actual practice the moisture content of the strip changes by less than 5% during operation for 8 hr. at 300 w dissipation. This constancy of paper wetness is reflected in linearity of ionic migration both in time and in applied potential. Measurements of the distance of migration of the alanine cation in the formic acid-acetic acid system as a function of time at constant current (applied potential) and as a function of current for a constant period of migration, show that the linearity of migration (within 1% in both cases) justifies the measurement of relative mobilities up to distances of at least 80 cm.

For two ionic species A and B, equation (1) may be written in the form:

$$\frac{u_A}{u_B} = \frac{q_A D_A}{q_B D_B}, \quad (2)$$

where the subscripts identify the species. Table 6 shows the mobilities of several amino acid cations measured relative to the mobility of alanine in the formic acid-acetic acid system at pH 1.85 and 4°. The Table also shows the relative mobilities calculated from equation (2) from values of the net charges obtained at three pH values from the Henderson-Hasselbalch equation, without correction for thermodynamic activities, and values of the diffusion coefficients at 1° obtained either from Longworth's (1952) measurements, or calculated

from the molecular weights by means of his empirical equation. Several points emerge from this comparison:

(1) The agreement is in general good enough (within 5%) to justify application of equation (2) to the prediction of relative mobilities in this system.

(2) The aromatic amino acids phenylalanine, tyrosine and tryptophan migrate more slowly than predicted. This is undoubtedly due to reversible adsorption on the paper. This phenomenon is already well established for these substances, and tryptophan can in fact be separated from other amino acids by paper chromatography in aqueous solutions (e.g. Dalglish, 1956).

(3) The calculated values for pH 2.1 agree better with the experimental values than those calculated for pH 1.9, nearest to the actual pH of the electrophoresis medium. This is particularly evident for threonine, which should migrate faster than leucine at pH 1.85, whereas in fact it migrates more slowly in agreement with the predicted behaviour at pH 2.1. This anomaly can be explained by the suggestion of Davies (1949) that the H^+ ion potential within a porous solid phase will differ from that in free solution by the potential difference (mainly electrokinetic) through which a proton must pass in order to reach the interior of the porous solid. Thus if pH_i is the pH inside the porous phase and pH_o is the pH in free solution in equilibrium with the solid phase:

$$pH_i - pH_o = \frac{V}{0.059}, \quad (3)$$

where V is the electrokinetic potential. The value of V as estimated from electro-osmotic flow in the formic acid-acetic acid system is small and positive, so that the direction of the observed anomaly is predicted correctly by equation (3), and its magnitude, 0.25 pH unit, would require the reasonable value of 15 mv for V . The neglect of activity coefficients in the calculation of q may also affect the values of u although this may be expected to balance out in the relative values.

The present method appears to be capable of separating completely ions differing in mobility by about 1%. This degree of resolution is a result of the linearity of migration in time, and there appears to be no reason why the migration path should not be extended to provide further resolving power. A 2.5 m. machine in construction in this Laboratory may enable the majority of the amino acids to be separated in a single electrophoresis.

The use of Cd^{2+} ions in the third electrophoresis medium presents some interesting features as this and certain other heavy-metal ions produce a small but definite specific retardation of the dicarboxylic and hydroxy amino acids relative to the aliphatic

or aromatic amino acids (Table 1). This effect has been exploited in the present work to separate aspartic acid from phenylalanine, and it also accounts for the decreased resolution of serine and valine in the cadmium-containing medium. Although only a small proportion of the amino acid would be expected to form complexes with the metal at pH 1.81 (Gurd & Wilcox, 1956), this may nevertheless be sufficient to account for the retardation, and it suggests that paper electrophoresis under carefully controlled conditions may be a useful method for the investigation of complex formation.

The analytical results on performic acid-oxidized insulin provide evidence that the method is a practical one for the amino acid analysis of proteins on the milligram scale. Several modifications of the ninhydrin method for the colorimetric determination of the amino acids were examined in the course of this work, but the cadmium-ninhydrin procedure of Heilmann *et al.* (1957) was clearly superior as regards low blanks, linearity of colour response over a wide concentration range and reproducibility.

SUMMARY

1. An apparatus and experimental details for high-voltage paper electrophoresis under conditions of constant temperature and paper wetness are described.

2. The application of the method to the separation of the amino acids commonly occurring in protein hydrolysates is described. Eighteen amino acids can be separated completely, by three electrophoretic experiments at pH 5.2, 1.85 and 1.81, at potential gradients of 70–100 v/cm.

3. The mobilities of several amino acid cations relative to alanine are tabulated, and a method for calculation of these mobilities from the net charge and diffusion constant is described and compared with experiment.

4. A method of quantitative determination of the resolved amino acid zones by use of the cadmium-ninhydrin reaction is described, and analyses of synthetic amino acid mixtures and replicate analyses of hydrolysates of performic acid-oxidized insulin are given to illustrate the utility of the method.

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REFERENCES

- Atfield, G. N. & Morris, C. J. O. R. (1960a). *Biochem. J.* **74**, 37f.
- Atfield, G. N. & Morris, C. J. O. R. (1960b). *Proc. 8th Collq., Protides of the Biological Fluids*, p. 82.

- Connell, G. E., Dixon, G. H. & Hanes, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 416.
- Dalgleish, C. E. (1956). *Biochem. J.* **64**, 481.
- Davies, C. W. (1949). *Biochem. J.* **45**, 38.
- Gross, D. (1956). *Nature, Lond.*, **178**, 29.
- Gross, D. (1961). *J. Chromat.* **5**, 194.
- Gurd, F. R. N. & Wilcox, P. E. (1956). *Advanc. Protein Chem.* **11**, 311.
- Hanes, C. S., Harris, C. K., Moscarello, M. A. & Tigane, E. (1961). *Canad. J. Biochem. Physiol.* **39**, 163.
- Hanes, C. S. (1961). *Canad. J. Biochem. Physiol.* **39**, 119.
- Hedbom, A. (1961). *Biochem. J.* **79**, 469.
- Heilmann, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
- Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
- Hraběťová, E. & Tupy, J. (1960). *J. Chromat.* **3**, 199.
- Lederer, E. & Lederer, M. (1957). *Chromatography*, 2nd ed. Amsterdam: Elsevier Publishing Co.
- Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
- Longworth, L. G. (1952). *J. Amer. chem. Soc.* **74**, 4155.
- Matheson, A. T., Tigane, E. & Hanes, C. S. (1961). *Canad. J. Biochem. Physiol.* **39**, 417.
- Michl, H. (1953). *Mh. Chem.* **83**, 737.
- Michl, H. (1958). *J. Chromat.* **1**, 93.
- Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
- Röwe, K., Ferber, E. & Fischer, H. (1958). *Hoppe-Seyl. Z.* **313**, 174.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Svensson, H. (1956). *Ciba Foundation Symp., Paper Electrophoresis*, p. 86.
- Thompson, E. O. P. (1954). *Biochim. biophys. Acta*, **15**, 440.
- Tigane, E., Wade, E. H. M., Tze-Fei Wong, J. & Hanes, C. S. (1961). *Canad. J. Biochem. Physiol.* **39**, 427.
- Wade, E. H. M., Matheson, A. T. & Hanes, C. S. (1961). *Canad. J. Biochem. Physiol.* **39**, 141.
- Weber, R. (1951). *Helv. chim. acta*, **34**, 2031.
- Whitehead, J. K. (1958). *Biochem. J.* **68**, 653, 662.

Biochem. J. (1961) **81**, 614

An Apparatus for the Automatic Measurement of Oxygen Uptake by Electrolytic Replacement of the Oxygen Consumed

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In studies of the respiration of plant tissue, particularly in relation to terminal oxidases, measurement of the rate of uptake of oxygen from an atmosphere of controlled oxygen pressure is necessary. For short periods of time and small amounts of tissue a Warburg type of apparatus is suitable but in studies of intact tissue, where whole organs must be used (0.5–200 g.) and where the duration of an experiment is prolonged, an apparatus that maintains the partial pressure of oxygen constant while giving a continuous record of uptake is needed. The apparatus to be described replaces the oxygen consumed by the tissue by oxygen generated electrolytically, and automatically records the volume added. This principle of electrolytic replacement of oxygen has been used before (Fernandes, 1923; Wetzel, 1927; Bennet-Clark, 1932; Ulrich, 1940), but the accuracy of the estimations has been greatly increased by the use of a new type of electrolytic oxygen generator based on the use of palladium and by a new method of measuring the oxygen added. The apparatus was designed to measure the oxygen uptake by 5–10 g. of peas and has been extensively used in conjunction with an automatic method for measuring the carbon dioxide produced (Wager, 1957).

Recently it has been used to determine the oxygen uptake by 100–150 g. of potatoes from atmospheres of different oxygen content (Mapson & Burton, 1962).

METHODS

Principle of the method (Fig. 1)

The tissue is enclosed in an airtight respiration chamber above a solution of sodium hydroxide, which serves to absorb the carbon dioxide given out. This chamber is connected to a compensating volume through a narrow U-tube manometer and also to an electrolytic oxygen generator (Figs. 1 and 3). A platinum electrode is fixed

Fig. 1. Block diagram of the apparatus.