Apparatus and Technique for Two-Dimensional Paper Ionophoresis

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While seeking conditions for the paper-ionophoretic separation of amino acids from sugars in hydrolysates of bacteriophage inhibitors before identification of the sugars by the method of Consden & Stanier (1952), it was noticed that, at pH 2.5, a mixture of aspartic and glutamic acids, alanine, valine, leucine, lysine and glucose was separated into six ninhydrin-reactive spots. The sugar formed a separate spot detectable with aniline phthalate (Partridge, 1948). Similar observations have been made by many previous workers, for example Wieland & Fischer (1948), Biserte (1950) and Durrum (1950). Nevertheless, it seemed worth attempting to find conditions for the complete separation of the commoner amino acids by paper ionophoresis because the method should be faster than chromatography, and, depending as it does on different molecular properties, might supplement chromatographic procedures.

It seemed improbable that all the amino acids could be separated at any one pH, but two-dimensional ionophoresis at two different pH values appeared more promising. Two-dimensional paper electrophoresis at one pH was used by Kunkel & Tiselius (1951). Durrum (1951) used two-dimensional ionophoresis at two pH values and separated nearly all the commoner amino acids in a few hours. Durrum also used a combination of chromatography in one dimension with ionophoresis in the other.

Using the apparatus of Flynn & de Mayo (1951) I had tried (with minor modifications) both of Durrum's methods before becoming aware of his paper. The results were encouraging but the spots lacked the symmetry and compactness so characteristic of one-dimensional paper ionophoresis. Using ionophoresis (at pH 2.5 or pH 9.5) followed by ascending chromatography excellent separations were obtained but the method was not pursued as we required a procedure that could be completed in 1 day.

To avoid the distortion of the spots which occurred during earlier attempts we changed the pH of the electrolyte in the paper (after the run in the first direction) by exposing it to the vapour of a volatile acid or base. In the method now described ionophoresis in the first direction is carried out in an acid (phthalate) buffer (*A*) to which may be added a weak acid (boric acid) capable of forming with

ammonia a buffer effective at the pH desired for ionophoresis in the second direction. At the end of the first run the wet paper is exposed to the vapour from a large volume of a buffer (*B*) made by adjusting the pH of *A* with concentrated ammonia solution to the value desired for the second run. If the dilution caused by the addition of ammonia is neglected and the buffer in the paper has not changed in concentration or composition it will absorb ammonia from buffer *B* until both have the same pH. If the volume of *B* is large compared with the volume of buffer in the paper, this pH will remain close to the desired value.

Published designs of apparatus for paper ionophoresis fall into one or other of two main groups, those (e.g. Durrum, 1950, 1951; Flynn & de Mayo, 1951) in which water can evaporate freely from the surface of the paper and those (e.g. Biserte, 1950; Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951; Foster, 1952) in which evaporation is hindered or prevented by immersion of the paper in a solvent not immiscible with water or by sealing it between cooled glass plates. Evaporation, if permitted, causes a flow of electrolyte which is superimposed on that due to electroendosmosis. This flow has an important and, as Durrum (1951) has shown, in extreme cases a predominant effect on the course of ionophoretic separation. For the technique now described the paper cannot be sealed between sheets of glass but is efficiently cooled to minimize evaporation.

MATERIALS AND METHODS

Amino acids. Commercial specimens obtained from the British Drug Houses Ltd. or Hoffman-La Roche were not further purified. Synthetic DL-amino acids, where available, were used in preference to the natural isomers.

Buffer A, 0.02M phthalate (pH 2.5). Phthalic acid (86.4 g.) was dissolved in distilled water (20 l.) by vigorous mechanical stirring and the pH of the solution adjusted to 2.5 with about 20 ml. of 2N-NaOH. The buffer was stored in a 20 l. Pyrex flask fitted with a siphon. CHCl_3 , in which phthalic acid is practically insoluble, was added as preservative.

Buffer B. Ammonia solution (A.R. sp.gr. 0.895-0.90; 85 ml.) was added to buffer *A* (1800 ml.) to give a pH between 10.6 and 10.7.

Dilute ammonia solution (pH approx. 11.6). Water (1 l.) was treated with ammonia solution as above (45 ml.).

The apparatus

The apparatus consists of a 16 in. square sheet of thin glass having a Perspex electrode vessel along each side and a Perspex cover fitted with trays of large area. The glass is cooled by a water spray beneath. The four electrode vessels (in rotation *N*, *E*, *S*, and *W*) are practically identical and the airtight cover fits over two of them (*E* and *W*) as well as over the glass plate. Vessels *N* and *S* have separate loose covers. A sheet of paper having a small square cut from each corner is laid on the glass and moistened with the acid buffer used for the run in the first dimension. The tongues projecting on each edge of the paper are turned down into the electrode vessels. Vessels *N* and *S* are filled with phthalate buffer (pH 2.5) *E* and *W* are empty. A small volume of solution containing the amino acids to be separated is applied to the paper near *S* and a direct current at 1000 v passed between *N* and *S*, *N* being negative. The amino acids migrate towards *N* in a straight line and are partially separated. After 150 min. the current is turned off, *N* and *S* are emptied and phthalate buffer brought to pH 10.6 with concentrated NH_3 is placed in *E* and *W* and in the trays in the cover. The air in the cover is circulated over the trays and paper by a small fan. Equilibration is complete within 30 min. and a direct current is then passed between *E* and *W* causing separation of amino acids in a direction at right angles to that of the first run. After 150 min. the current is turned off, the cover removed and the paper dried rapidly *in situ* by a fan above and warm water sprayed beneath the glass sheet. The analysis including removal of NH_3 and colouring with ninhydrin occupies less than 9 hr. and all the common amino acids except leucine and isoleucine are separated.

Details of the apparatus are given in Figs. 1–4. Apart from the fan motor which is supported at one side only and the electrolyte-levelling siphons, the apparatus is symmetrical, so allowing different aspects to be presented in the two halves of Figs. 1 and 2. Figs. 1*a* and 2*a* are side and end elevations respectively, Figs. 1*b* and 2*b* being the corresponding median vertical sections. Fig. 3*a* is a plan of the apparatus, Fig. 3*b* a horizontal section at plane *X-X'* and Fig. 3*c* a horizontal section at plane *Y-Y'* (Fig. 1). Fig. 4 is an elevation on a small scale showing the general arrangement. In the drawings Perspex was assumed to be opaque and glass transparent. The thicknesses of the Perspex used were $\frac{1}{8}$ in. and $\frac{1}{4}$ in. indicated by single, thick lines and parallel lines respectively. Numbers in the text refer to the drawings. 1, base of $\frac{7}{8}$ in. laminated board. 2, legs supporting 1 above table. 3, spray tank 48.3 × 48.3 × 11.5 cm. of 19-gauge copper. A thermometer is inserted through the bottom of the tank to indicate cooling-water temperature. 4, 1 in. wide wood strips supporting 3. 5, glass sheet 16 × 16 × $\frac{1}{8}$ in. supported by 3. 6, ring of $\frac{1}{4}$ × $\frac{1}{4}$ in. rubber cemented to glass. 7, apron of thin rubber sheet cemented to 6 preventing leakage of water. 8, gallery of $\frac{7}{8}$ in. laminated board fitting loosely round 3. 9, 4 × levelling screws supporting 8. 10, main cover. Its ends rest on electrode vessels *E* and *W*. 11, 4 × parallel spray tubes pierced with holes (diam. about 1.5 mm.) spaced 1 in. apart in three parallel rows. Holes in outer rows are staggered with respect to corresponding opposite holes in the next tube. 12, water-supply manifold. 13, water inlet connected by glass tubing to 14. 14, no. 12 Stuart pump (600 gal./hr. at 5 ft. head). 15, 2 × 1 in.

copper exit pipes. 16, glass tubes returning water to 17. 17, circulation tank 9 in. diam. × 14.5 in. fitted with loose cover and supported by tripod (neither shown). Connected by 1 in. pipe to 14. The circulation tank is fitted with heaters (200 w) and a thermoregulator. 18, water inlet from tap. 19, overflow (3 in. below top) connected to filter pump. *N*, *E*, *S* and *W* electrode vessels. *N* and *S* are 39.1 cm. long and *E* and *W* 40.8 cm. long. Vessels are divided longitudinally. 20, wicks (6 × $\frac{1}{4}$ in. diam. in each vessel) degreased by boiling with soap and distilled water before installation. 21, electrodes (uncored arc carbons). Terminals and safety covers not shown. 22, levelling siphon and drains. 23, loose covers for vessels *N* and *S* only. 24, rebates in ends of electrode vessels accommodating corners of 5. 25, Perspex sheet 40.7 cm. long fixed inside 10. 26, partitions forming trays at each end of 25. 27, holes 1.7 cm. diam. 28, hole 10.2 cm. diam. for fan 29. 29, four-bladed fan cut from sheet aluminium arranged to blow upwards. 30, thermometer well. 31, transverse struts deflecting draught on to surface of liquid in trays. 32, stainless steel shaft ($\frac{5}{16}$ in. diam.) threaded at lower end to take stainless nuts securing fan. 33, Tufnol bearing. 34, stainless steel collar. 35, 0.01 h.p. motor (3000 rev./min.). 36, plug for electrical connexion to motor.

The electrodes are connected to the rectifier through a two-pole two-way switch so that either *N* and *S* or *E* and *W* may be electrified at will. The rectifier is of conventional design and contains a Variac, a step-up transformer, a valve rectifier with smoothing filter and a milliammeter.

The apparatus should be set up with a clear space round it in a well-ventilated room in which the occasional presence of ammonia is of no account.

Two-dimensional separation of amino acids

The surface of the glass plate is cleaned with distilled water and filter paper. Before the first run and occasionally thereafter it is rubbed with cotton wool soaked in a solution of silicone high-vacuum grease (Dow Corning Corporation, Midland, Mich., U.S.A.) in CHCl_3 . Excess grease is removed with fresh CHCl_3 and cotton wool and the surface is polished with a clean cloth (cf. Kunkel & Tiselius, 1951).

A square (side, 46.5 cm.) is cut from a sheet of Whatman no. 20 paper. Margins are drawn in pencil 48 mm. from each edge and also a starting line parallel with and 8 cm. distant from one margin. The axes of the paper are drawn and a mark made for the origin on the starting line 17 cm. from its intersection with one axis. The squares formed by the margins at each corner are cut out.

The paper is placed on the glass with the origin mark near anode vessel *S*. (We have always placed the sheet so that its original long axis lies from *E* to *W*.) The paper is wetted with about 250 ml. of buffer *A*, and wrinkles and excess buffer are squeezed out with a photographic print roller. The paper is rewetted with about 100 ml. of buffer *A* and centred on the glass. The levelling screws 9 (Figs. 2–4) are adjusted to bring the sides of the electrode vessels *E* and *W* about 2 mm. above the plane of the paper. The tongues at each edge of the paper are made to adhere to the inner surfaces of the electrode boxes by capillarity and are pulled down tautly. Excess buffer is again squeezed from the paper with the print roller—care being taken to remove all bubbles of air that may form between the paper and the glass. Some distortion of the ionophoretogram must be caused by siphoning of buffer from the full electrode vessels used for

Fig. 3c

Fig. 4

Fig. 3. Apparatus: *a*, plan; *b*, horizontal section at X-X' in Fig. 1; *c*, horizontal section at Y-Y' in Fig. 1. The scales are marked in 4 cm. divisions. Numbers refer to the text.

Fig. 4. Side elevation of the apparatus showing cooling system. The scales are marked in 10 cm. divisions. Numbers refer to the text.

the first part of the run through the corners of the paper into the neighbouring empty electrode vessels. In practice this effect seems unimportant. Wetting causes distortion of the paper so that the distance between the marginal lines is increased by approximately 6 mm. in the direction of the first run.

The drain to electrode vessels *N* and *S* is closed and buffer *A* (1700 ml.) added—care being taken to displace air bubbles from the levelling siphon. The large Perspex cover is placed in position and screws 9 adjusted so that the cover just clears the paper along each side. Electrode vessels *N* and *S* are covered and the apparatus left for 1 hr. to reach hydraulic equilibrium. During this time the flow of tap water into the water circulation vessel is adjusted so that the filter pump can just deal with the overflow. The circulating pump is then switched on but the thermoregulator and heaters are not used at this stage.

The main cover is removed and the solution to be analysed applied at the origin mark from a 5 μ l. micropipette. Volumes between 0.5 and 3.0 μ l. have been used. For volumes greater than about 1.5 μ l. it may be necessary first to blot the paper round the origin mark. A volume equivalent to about 1 μ l. of a solution 0.02 M with respect to each

amino acid is suitable except for proline of which about twice this concentration is needed.

The covers are replaced, the siphon connecting the filled electrode vessels closed with a screw clip and a potential of about 1000 v applied to the electrodes. The p.d. between the marginal lines should be about 740 v at the beginning and about 700–720 v at the end of the run.

After 140–150 min. the current is turned off, and the thermoregulator and water supply adjusted to bring the temperature of the cooling water to about 0.5° below room temperature. The covers are removed and the edges of the paper cut off along the marginal lines parallel to the filled electrode vessels. The drain and the siphon connecting these vessels are opened, and the exposed margins of the plate dried with filter paper. The drain to electrode vessels *E* and *W* is closed and any liquid in them and in the siphon is removed by suction. Buffer *B* (1800 ml.) is added to vessels *E* and *W* with precautions to remove air from the siphon and to raise the levels in each vessel at about the same rate.

The main cover is replaced and adjusted with screws 9 so that its sides touch the glass and also the top surfaces of the electrode vessels at each end. Vessels *N* and *S* are covered and about 500 ml. of either buffer *B* or dilute ammonia

solution (pH 11.7) added to each tray in the main cover. The fan is started and 30 min. are allowed for equilibration.

The fan is turned off, the siphon connecting vessels *E* and *W* closed and a p.d. of about 870 v applied, *E* being negative. The p.d. between the marginal lines is about 625 v at the beginning and 600–620 v at the end of the run. The current varies between about 48 and 56 ma.

After 145–150 min. the current is turned off and the covers removed. The ends of the paper are trimmed off along the edges of the glass sheet. The siphon is opened and the electrode vessels drained. All electrode vessels and wicks are rinsed thoroughly with distilled water. The water supply to the cooling system is turned off, the thermoregulator and heaters removed and a large burner lighted beneath the circulating-water tank. A fan is arranged to blow across the surface of the paper. All liquid remaining in the electrode vessels is sucked out. During this operation the paper will have become dry or nearly so and is held in place by two large rubber stoppers.

The trays in the cover are emptied and all surfaces washed with a stream of tap water, and the paper is taken for removal of NH_3 and colouring. The apparatus is ready without further attention for another run on the following day.

Method for tracing movements of electrolyte in the paper with a sugar-spot 'grid'

Margins (48 mm. wide) and axes were drawn on a square of Whatman no. 20 paper (46.5 × 46.5 cm.). Lines were drawn on either side of each axis parallel thereto and distant 8 cm. Marks were made at 4 cm. intervals along these lines to give, in all, thirty-two symmetrically arranged origins.

The runs were carried out exactly as described for two-dimensional ionophoresis of amino acids except that (a) 0.5 μl . of a 3% solution of reducing sugars was applied at each origin when the paper had reached hydraulic equilibrium with buffer *A*; (b) in two runs cooled 20% ethanol was circulated from a portable refrigerating machine through about 12 ft. of $\frac{1}{8}$ in. copper tubing coiled in the circulating water tank. In one run the flow of refrigerant was controlled manually, in the other the pump circulating it was controlled through a relay by a thermoregulator placed in the tank; (c) in one run precautions (described below) were taken to reduce liberation of water vapour from the electrode vessels. After each run the dry paper was developed with aniline phthalate in the usual manner and the distances of each spot from the axes of the paper were measured.

Methods for de-ammoniation and colouring of ionophoretograms

With ninhydrin and collidine. (a) The paper is drawn through a trough containing 40 ml. of KOH in isopropanol (1.5%, w/v) first slowly in one direction and then rapidly in the reverse direction; it is then hung from the edge first wetted, fluid collecting at the bottom edge is removed with filter paper and drying is completed by a few minutes exposure to the draught from a fan. (b) The paper is drawn in the same way through 40 ml. of a solution of acetic acid in isopropanol (2%, v/v) and dried with the fan until the odour of acetic acid is barely detectable (about 20 min.). (c) The paper is treated in the same way with 30 ml. of a solution of ninhydrin (0.5%) in anhydrous isopropanol to which com-

mercial 'collidine' (1.5 ml.) has recently been added (Woiwod, 1949). The paper is air-dried for 10–20 min. and heated for about 4 min. in an oven at 80–90° containing a dish of water.

Stainless steel chromatography troughs (Woiwod, 1949) are convenient for dipping the papers. The paper is held beneath the fluid in the trough by a glass tube (diam. 9 mm.) filled with mercury (Polson, 1951). It was found difficult to avoid disturbing the spots, particularly the proline spot, during de-ammoniation by spraying with KOH in methanol as recommended by Boissonas (1950). This difficulty and the necessity for spraying were avoided by the procedure described. The KOH was dissolved in anhydrous isopropanol by boiling under reflux. It was found difficult to obtain uniformly clean ionophoretograms until all details of the procedure had been adopted.

With the ninhydrin reagent of Moore & Stein (1948). Development with ninhydrin and collidine has the advantage of speed, relative freedom from interference by residual traces of ammonia and the diagnostic value of the differences in the colours given by different amino acids. The disadvantage that equivalent amounts of different amino acids do not give the same intensity of colour, can be largely overcome by using Moore & Stein's reagent. This, however, is so sensitive to traces of ammonia that the ionophoretograms must be washed twice with KOH in isopropanol and twice with isopropanol-acetic acid before being sprayed with the reagent (20 ml.) and allowed to develop at room temperature.

Titration curves. As a guide to the selection of buffers suitable for runs in the alkaline range, 1 l. amounts of the following were titrated with concentrated NH_3 solution (about 28.9%, w/w) at the glass electrode: (i) 0.02M phthalic acid brought to pH 2.5 with 2N-NaOH; (ii) 0.02M phthalic acid, 0.05M boric acid brought to pH 2.5 with 2N-NaOH; (iii) water. The results appear in Fig. 5.

RESULTS

In the photographs and figures, two-dimensional ionophoretograms are so presented that the direction of the first run is vertical, with the cathode at the top. The axis of the second run is then horizontal, with the cathode at the right-hand side. These conventions apply also to discussion in the text.

Movement of electrolyte in the paper

The movement in different parts of the paper was measured by the sugar-spot grid method described above. A photograph of a completed ionophoretogram indicating the regularity of the electrolyte flow is given in Fig. 6. With the exception of two spots in one experiment, all the spots moved towards the cathode in both acid and alkaline runs. In four experiments, of which brief particulars are given in Table 1, the pH values and durations of the acid and alkaline runs were the same and the potential gradients as nearly the same as possible. Under these circumstances the displacement of a spot varied with its initial position, the pH and the temperature of the cooling water.

In Fig. 7 the displacements of the spots are plotted against their mean positions. The mean position of each spot during migration in one dimension was taken as half-way between its initial and final positions. Each point on the graph (Fig. 7) represents the average of the displacements and positions of two corresponding spots, i.e. spots initially equidistant from the axis from which

measurements were taken (e.g. a and a' , b and b' in Fig. 6). The displacements (in mm.) were measured both in the direction of the first run in each experiment (pH 2.5—broken lines) and in the direction of the second run (pH 10.6–10.7—full lines) displacements towards the cathode being considered positive. The mean positions are plotted as distances (in cm.) from the appropriate centre line of the paper, those in the direction of the cathode being considered positive. All runs at pH 2.5 were made under conditions as nearly identical as possible and the results of only two are plotted. Of the runs at pH 10.6–10.7 one (curve 1) was made under the normal conditions for amino acid separation as described in the experimental section. Curve 2 refers to a run in which the electrode vessels were filled nearly to the top with buffer B (2400 ml.) and three strips of Whatman no. 3 paper ($3\frac{1}{2} \times 14$ in.) were folded over the partition in each vessel to supplement the electrical conductivity of the wicks. These arrangements were intended to reduce the evaporation of water from the uncooled parts of the paper and from the wicks. Curve 3 relates to a run under conditions that were normal except that the temperature of the cooling water was maintained thermostatically at about 4.5° below room temperature. In the fourth run (curve 4) the temperature of the cooling water varied between 4° and about 12° below room temperature. It was in this run that two spots were displaced towards the anode. The significance of the results is discussed below.

Fig. 5. Titration curves of (i) 0.02M phthalic acid brought to pH 2.5 with 2N-NaOH; (ii) 0.02M phthalic acid, 0.05M boric acid brought to pH 2.5 with 2N-NaOH; (iii) water. Initial volumes 1 l. Titrant, ammonia solution about 28.9% (w/w).

Fig. 6. Sugar-spot grid ionophoretogram showing electrolyte displacement. Sugar solution was applied at each of the 32 origins marked with small circles and submitted to two-dimensional ionophoresis under the normal conditions for separation for amino acids. Ionophoretogram developed with aniline phthalate. a , a' ; b , b' examples of spots originally equidistant from the horizontal and vertical axes respectively.

Fig. 7. Electrolyte movement in different parts of the paper as revealed by displacement of sugar spots. Horizontal scale: distances in cm. of mean positions of spots from midline of paper in the directions indicated. Vertical scale: displacement of spots in mm. The point below the abscissa indicates displacement towards anode. Broken lines refer to displacements at pH 2.5, full lines to displacements at pH 10.6–10.7. Curve 1 (○—○), normal conditions; curve 2 (△—△), conditions to diminish condensation on paper; curve 3 (□—□), mild refrigeration; curve 4 (●—●), more vigorous refrigeration. For details see text.

Table 1. *Conditions under which sugar-spot grid runs were made*

	Expt. no.			
	1	2	3	4
Acid run (pH 2.5)				
Potential gradient (v/cm.)	19	19	19	19
Alkaline run (pH 10.6–10.7)				
Potential gradient (v/cm.)	17.8	15.5	17.2	17.7
Heating load (w/cm. ² × 10 ²)	2.2–2.3	2.1–2.2	2.1–2.2	2.1
Air temperature (°)	21	23.3	21	20–19
Water temperature (°)	20–21	22.2–22.8	16.5–16.7	8–16
'Paper temperature' (°)	21–22.5	23.1–24	21–20.2	ca. 16.5

*Two-dimensional ionophoresis of amino acids.
Optimum conditions for separation*

pH. The original experiments on the effects of pH were made in the apparatus of Flynn & de Mayo (1951) in which water evaporates freely from the paper. This evaporation appeared to have no serious effect in the acid range and many experiments merely confirmed that the best results are obtained at the pH (2.5) arbitrarily selected for our original purpose of separating amino acids from sugars. In the alkaline range pH values in the region 9.3–9.6 seemed to give the best results. When, however, pH values in this range were used in the new apparatus the following groups of amino acids were separated poorly or not at all: valine and leucine; serine and threonine; methionine and phenylalanine. One-dimensional ionophoretograms at different pH values in the new apparatus indicated that the results with the older apparatus were misleading and that the optimum pH was at least as high as 10.6. This is about the practical upper limit when ammonia is used as the volatile base.

Buffers. Phthalic acid (0.02M), partly neutralized with sodium hydroxide, was used for the acid runs not only on account of its adequate buffering power but also because it contains no ions which lack buffering value but add to the conductivity and therefore the heating effect of the current. Boric acid (0.05M) was originally added to the phthalate buffer, but as the titration curves in Fig. 5 show, its use is unnecessary in the neighbourhood of pH 10.6 and should be avoided as borates appear to diminish the sensitivity of ninhydrin. For buffer B, which is placed in the electrode vessels for the alkaline run, 0.02M phthalic acid brought to pH 2.5 with sodium hydroxide and then to pH 10.6 with concentrated ammonia was always used. The same mixture may be placed in the trays in the cover. It was later found experimentally that a dilute solution of ammonia (pH 11.6–11.8) is equally satisfactory.

Paper. The following have been tried: Whatman nos. 1, 3, 20, 31, 50 and 542 and Munktell no. 20. The most compact spots were obtained with Whatman

no. 20 which has been used for the experiments reported here.

Temperature. We have not attempted to measure the true temperature of the paper, though 'paper temperatures' recorded by a thermometer in a pocket within a few millimetres of its surface gave useful indications. The gross effects of overheating and overcooling were investigated in two experiments with 17 amino acids in which the conditions during the first half (pH 2.5) were as described in the experimental section for a normal run. In one experiment the heating load during the second half was restricted to 0.014 w/cm.² but the cooling spray was turned off. The 'paper temperature' at the end of the run was 8.5° above room temperature. In the resulting ionophoretogram the amino acid spots were compressed towards the vertical axis of the paper. Aspartic and glutamic acids and cystine formed one zone, serine, threonine, methionine and phenylalanine had coalesced to form another, and alanine, valine and leucine a third. These zones were extended vertically. Only glycine, proline, hydroxyproline and the bases formed separate spots. This behaviour was presumably caused by excessive evaporation with flow of electrolyte from the edges of the paper. In the second experiment the temperature of the cooling water was kept at about 5–11° below room temperature during the second half of the run. The heating load was normal (0.022 w/cm.²) and the 'paper temperature' 1–2° below that of the room. In this ionophoretogram the spots were elongated horizontally and those due to serine, threonine, methionine and phenylalanine had coalesced into a sausage-shaped zone. Arginine migrated off the paper. These results are consistent with the hypothesis that water had condensed on the paper during the run. The earlier experiments were made during the summer when the temperature of the cooling water was only one or two degrees below that of the room. In the winter less satisfactory results were obtained at first, the reason being that the cooling-water temperature was then 3–4° below that of the room, causing excessive condensation on the paper during the

alkaline run. The difficulty was overcome by fitting a thermostat and heaters in the circulating tank. A heating load of about 0.022 w/cm^2 with water about 0.5° below room temperature during the alkaline run appear to give the best results.

*The separation of an artificial mixture of
17 amino acids*

The appearance of an ionophoretogram made under the conditions given in the experimental section is shown in Fig. 8. For this experiment

Fig. 8. Two-dimensional ionophoretogram of 17 amino acids. The small circle at the bottom towards the right is the origin. 1, aspartic acid; 2, glutamic acid; 3, cystine; 4, glycine; 5, serine; 6, tyrosine; 7, threonine; 8, hydroxyproline; 9, methionine; 10, histidine; 11, alanine; 12, phenylalanine; 13, valine; 14, leucine; 15, proline; 16, lysine; 17, arginine. Conditions of ionophoresis as described in experimental section.

$1.3\ \mu\text{l.}$ of a solution 0.02 M with respect to 16 amino acids and 0.04 M with respect to proline was applied to the paper.

A rough idea of the reproducibility attainable is given by the results in Table 2 taken from two runs made with a month's interval between but under conditions as nearly as possible the same. The ionophoretograms were selected before the measurements were made. In this table the movements of each amino acid in the first and second dimensions are considered separately. The largest discrepancies are between the positions of threonine and phenylalanine in the acid runs and those of threonine, alanine and arginine in the alkaline runs. Irregularities in the paper or slight displacements during washing and development may have been partly responsible. The agreement between the two runs appears reasonable and the reproducibility of the map adequate for the immediate detection of any of the 17 amino acids listed.

DISCUSSION

*The movement of electrolyte in the paper and
its effect on the efficiency of separation*

Movement of fluid in the paper during electrophoresis may be due to inadequate hydraulic equilibration of the paper with the liquid in the electrode vessels, to electroendosmosis, to loss or gain of water by evaporation or condensation and possibly to other undefined causes. If the flow varies from one part of the paper to another it may cause distortion of the spots and inefficient separation. The effects of electroendosmosis (which is usually cathodic and constant over the whole paper) and evaporation (which causes irregular movement

Table 2. Distances travelled by amino acid spots on two-dimensional ionophoretograms made under the conditions described in the experimental section

Amino acid	Acid runs (pH 2.5) Distances from origin (mm.)			Alkaline runs (pH 10.6-10.7) Distances from starting line* (mm.)		
	19. xi. 53	18. xii. 53	Average	19. xi. 53	18. xii. 53	Average
Aspartic acid	102	99	100.5	299	293	296
Glutamic acid	121	121	121	277	272	274.5
Cystine	108	107	107.5	244	245	244.5
Glycine	206	208	207	201	208	204.5
Serine	144	140	142	187	184	185.5
Tyrosine	116	109	112.5	178	180	179
Threonine	135	125	130	168	160	164
Hydroxyproline	100	92	96	152	150	151
Methionine	129	123	126	148	142	145
Histidine	324	327	325.5	136	138	137
Alanine	183	180	181.5	142	128	135
Phenylalanine	122	112	117	129	123	126
Valine	150	146	148	127	121	124
Leucine	151	145	148	109	104	106.5
Proline	112	108	110	27	24	25.5
Lysine	342	340	341	-11†	-19†	-15†
Arginine	311	312	311.5	-61†	-71†	-66†

* A line through the origin in the direction of the acid run.

† Migration towards cathode.

Table 3. *The effect of electrolyte movement on the position of amino acid spots at the conclusion of a normal two-dimensional run*

Amino acid	pH 2.5			pH 10.6-10.7		
	Measured movement towards cathode (mm.)	Correction for electrolyte flow (mm.)*	Corrected migration towards cathode (mm.)	Measured movement towards anode (mm.)	Correction for electrolyte flow (mm.)*	Corrected migration towards anode (mm.)
Aspartic acid	100.5	53	47.5	296	65	361
Glycine	207	45	162	204.5	72	276.5
Valine	148	49	99	124	80	204
Lysine	341	36	305	-15†	88	73‡

* Estimated from data in Fig. 7.

† Movement towards cathode.

‡ Corrected movement towards anode.

of fluid) have been discussed by previous authors, but not measured by observing the displacement of spots of non-electrolyte applied at several points along the paper in the direction of the current flow except by Macheboeuf and his collaborators, who have shown that an uneven flow of electrolyte may under some conditions give improved separations (Macheboeuf, Rebeyrotte, Dubert & Brunerie, 1953). In the present work undistorted spots and effective separation were not achieved until the movement of fluid over the whole paper had been studied by the sugar-spot grid method and irregularities reduced by adjustment of the cooling.

Durrum (1950, 1951) has pointed out that evaporation causes movement of buffer towards the centre of the paper, the flow being greatest near the ends. When there is electroendosmosis towards the cathode as well as evaporation the displacement will be greatest near the anode and least near the cathode. The broken curves in Fig. 7 showing the displacements at different areas in the paper during the acid run follow this pattern and indicate that some evaporation occurs. The rate of evaporation depends on the difference between the temperature of the paper and that of the film of moisture on the cover of the apparatus and is diminished by cooling the paper (rather than the whole apparatus) as efficiently as possible. During the alkaline run, however, the paper is exposed to water vapour from the wicks (which have a relatively high resistance) and the uncooled electrolyte in the electrode vessels. Water therefore tends to condense on the paper and if the temperature of the cooling water is more than about 1° below that of the room this causes distortion of the spots and poor separation. The effects of condensation at different cooling-water temperatures are shown by the full-line curves in Fig. 7. The approximate effects of electrolyte flow on the migration of four amino acids are shown in Table 3.

Suggested modifications to the apparatus

To avoid condensation on the paper, water vapour from the electrode vessels should be ex-

cluded from the main cover which would then fit over the glass plate only, all the electrode vessels having separate lids. The trays would have to be narrowed to allow air to circulate between them and the ends of the cover, but must present sufficient area of dilute ammonia solution for equilibration of the paper to pH 10.6 in a reasonable time. If the possibility of condensation has been excluded, it should be easy to avoid excessive evaporation by matching heating load with the difference between the temperature of the room and that of the cooling water.

As experiments have shown that separation (e.g. of valine from leucine) improves with increased migration, the whole apparatus could with advantage be made much larger especially in the direction of the alkaline run, during which amino acids move against the electrolyte flow.

Scope of the two-dimensional ionophoretic method

The method now presented permits a qualitative estimate of the amino acid composition of protein hydrolysates to be obtained within 9 hr. This may be an advantage in clinical or other work in which daily samples are taken and the course of the experiment must be guided by the analytical results. The usefulness of the method for revealing ninhydrin-reactive substances other than the common amino acids is as yet uncertain but simultaneous ionophoresis and chromatography would seem preferable to reliance on either process alone. The disadvantages of the process are that only one specimen can be examined at a time, that it is at present a qualitative method only, and that leucine and isoleucine are not separated.

There seems to be no reason why any of the methods for quantitative estimation of amino acids that have been applied to chromatography should not be applied to ionophoresis provided that all ammonia is removed from the paper and that the spots are adequately separated—a condition which should be more readily satisfied in larger apparatus.

The method has been applied to hydrolysates of human γ -globulin and of a protein from the surface antigen of *Pseudomonas aeruginosa* L2 (Mead & van den Ende, 1953) and also to concentrated normal urine. The globulin hydrolysate gave spots corresponding to aspartic and glutamic acids, cystine, serine, threonine, tyrosine, glycine, alanine, valine, leucine (isoleucine), phenylalanine, proline, histidine, lysine and arginine. Spots at the positions taken by 13 of the common amino acids were detectable on the ionophoretogram given by the antigen-derived protein. The urine concentrate served to indicate that the method may be applied directly to solutions rich in dissolved solids. Taurine, glycine, serine, asparagine, methionine, alanine and histidine were tentatively identified.

Two-dimensional ionophoresis should give better separation than one-dimensional in all cases where change of pH affects the relative as well as the absolute rate of migration of the substances to be analysed. In practice, however, difficulties in finding sufficiently volatile buffer components may be a limiting factor. Mixtures of proteins are an obvious field for experiment also lipoprotein-polysaccharide complexes such as some bacterial antigens for at least one of which (that of *P. aeruginosa* strain L2) the excellent method of detection on paper due to Rydon & Smith (1952) may be used.

SUMMARY

1. In a new apparatus for two-dimensional paper ionophoresis, the pH of the buffer solution in the paper is changed, after ionophoresis in one dimension, by exposure to the vapour from a solution exerting a suitable and substantially constant partial pressure of ammonia.

2. A method for determining the flow of electrolyte at different points on the paper is proposed. The results of applying it in the new apparatus are discussed.

3. The following amino acids are separated qualitatively by the new apparatus: glutamic and aspartic acids, cystine, glycine, alanine, valine, leucine, phenylalanine, serine, threonine, tyrosine, hydroxyproline, proline, histidine, lysine and arginine. Leucine and isoleucine are not separated. The developed ionophoretogram is ready within nine hours of starting an experiment.

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Separation of Neutral Proteins on Ion-Exchange Resins

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In recent years, chromatographic procedures using ion-exchange resins have been applied to the separation of a large number of substances, but their successful application to the separation of proteins has been confined mainly to basic proteins of low molecular weight, such as cytochrome c (Paleus &

Nielands, 1950) ribonuclease (Hirs, Moore & Stein, 1953) lysozyme (Tallan & Stein, 1953) and chymotrypsinogen α (Hirs, 1953). The ion-exchange resin used in the above studies was the carboxylic cation exchanger, Amberlite IRC-50. Columns of this resin have also been used to purify the active substances from a preparation of adrenotropic hormone (Dixon, Moore, Stack-Dunne & Young, 1951).

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