lactic acid. A series of such estimations can be carried out simultaneously.

3. In blood samples, the lactic acid and glucose

levels can be stabilized by the use of cetyltrimethylammonium bromide, acid citrate buffer and NaF, as previously described for pyruvic acid in blood.

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Ionophoresis in Silica Jelly

A METHOD FOR THE SEPARATION OF AMINO-ACIDS AND PEPTIDES

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As long ago as 1909, Ikeda & Suzuki separated amino-acids into acid, neutral and basic fractions by ionophoresis in a three compartment diaphragm cell, and this method has been followed with various modifications by numerous investigators. However, certain inherent difficulties still remain to be overcome: (a) complete separations have not so far been obtained in one operation, (b) except where a soluble anode is used, decomposition of the acidic and perhaps neutral fractions occurs at the anode, (c) cystine in the cathode compartment will be reduced and reduction of other amino-acids may occur (Gawrilow, Paradashvili, Balabouha-Popzova & Ljapounzowa, 1938), (d) maintenance of the appropriate pH is troublesome. The method has, however, proved suitable for the quantitative separation of the basic from the neutral and acidic acids. The separation of substances of small differences in mobility at a given pH is impracticable in a diaphragm cell. Martin & Synge (1945) have discussed the diaphragm cell in some detail and give a bibliography.

In an attempt to avoid these difficulties we have developed a method in which ionophoretic separations take place in a slab of jelly. The substance to be investigated is inlaid in a gutter cut at right angles to the slab. The function of the jelly is to prevent convection of the electrolyte thus obviating the need for diaphragms and in addition, to prevent contact of the amino-acids with the electrodes. Convection, in the absence of diaphragms, may also be prevented by a density gradient, as in the Tiselius apparatus for the electrophoresis of proteins. Since, in this type of apparatus, the density gradient is provided by the substances under investigation,

complete separations into separate bands cannot be obtained and the analysis is conducted by the observation of fronts. Complete separations would perhaps be possible if the density gradient were provided by an inert substance (e.g. sucrose) to stabilize the column. Attempts have also been made to control convection by conducting the electrophoresis of proteins in a tube packed with glass powder (Coolidge, 1939). When jelly is used, no special precautions need be taken to maintain a constant temperature.

Because there is a swamping concentration of electrolyte throughout the slab the substances being analyzed can move independently and separate into discrete bands. Since the movement of the bands is proportional to the time of running, and their widening by diffusion is only proportional to the square root of the time, substances having relatively small differences in mobility can be separated from one another. In order to minimize the effects of diffusion it is desirable to use the highest possible potential gradient. This is limited by the difficulty of providing adequate cooling. The compromise which we have adopted has been the use of a thin slab of jelly set in a water-cooled glass trough, the breadth of which may be varied according to the amount of material to be analyzed. The apparatus is made of plate glass and its construction requires no special skill. The electrodes consist of a brass cathode and a carbon anode resting in the ends of the trough. Spaces between the electrodes and the slab of jelly are perfused with suitable electrolytes. These solutions are chosen so as to prevent changes arising in the jelly. Throughout the range of pH values which we have used (2-9), we have always

observed that electroendosmosis is towards the cathode. It is important that the ionic strength of the inlay be close to that of the jelly, otherwise differences in the rate of electroendosmotic flow along the length of the slab can cause sweating at the region where there is decreasing rate of flow. Conversely, in a region of increasing rate of flow, a more or less shallow depression is formed owing to concentration of the jelly. Similarly, if the anode perfusion liquor is much different in pH or ionic strength from that of the jelly, depressions or sweating of the surface will occur. These can be almost entirely prevented by using a solution containing the same ions as in the jelly at a slightly higher pH and concentration: where the jelly is neutral and electroendosmosis is slow, the nature of the anion is found to be unimportant as presumably it is not drawn into the jelly under these conditions. The composition of the cathode solution is not so critical, as the electroendosmotic flow is into this compartment. The nature of the cation is unimportant. The concentration and pH of this solution should be lower than that of the jelly. If, on the other hand, as in one of our methods, the jelly is set in contact with a glass lid, it is desirable that the perfusion liquids should be identical with the electrolyte in the jelly, since, in this case, sweating and contraction are equally to be avoided. Both perfusion solutions are run past the electrodes at a rate high enough to prevent more than slight changes in their pH values. In this way, the pH of the jelly remains uniform and practically unchanged throughout the separation.

The progress of the separation may be followed by 'printing off' on to filter paper which is treated with ninhydrin. The prints show bands corresponding in positions to those of the amino-acids in the jelly. These bands, except where method (B) has been used, have always been of uniform width and at right angles to the trough. As soon as prints have shown a clear white space between the bands, it has always been possible to separate the amino-acids without mutual contamination by cutting the jelly.

Choice of electrolyte. In general, the electrolyte used in the jelly must be a buffer. In the particular case, however, where the substances to be separated are almost entirely charged or uncharged (i.e. acids or bases not within their range of dissociation), non-buffering electrolytes may be employed. If, however, an attempt is made to use a slab of jelly containing a non-buffering electrolyte with material in the inlay which contains a weak acid or base at a pH near its pK, pH changes, which may be very large, occur on either side of the band of the partially dissociated substance. These pH changes result from the movement of the anions away from the undissociated acid. Some of the undissociated molecules left behind ionize, releasing hydrogen ions and some

of the ions which have been moved recombine with hydrogen ions. Thus, a stream of hydrogen ions is produced at the back of the band (cathode side) while the front of the band (anode side) becomes more alkaline. Similarly, the front (cathode side) of a weak base band becomes more acid and the back (anode side) becomes more alkaline. Such changes interfere, of course, with the desired separations and can be limited to small values if the electrolyte consists of a buffer; if the pK of the buffer ion is close to that of the partially dissociated substance, pH changes are negligible.

Where agar is employed as the jelly there is, of course, no difficulty in incorporating any desired buffer. It is, however, very difficult to separate completely the amino-acids from the agar when the ionophoresis is finished. Amino-acids are readily separated from silica jelly, but since we have made the latter from sodium silicate by the addition of acid, the slab, as originally made, always contains sodium. When, therefore, an electrolyte containing no sodium is desired, the slab is washed after setting, in a slow stream of the desired electrolyte, until free from undesired ions. Silica jelly suffers from the further disadvantage that it is mechanically weak and rather brittle, and thus liable to crack after loss of water by evaporation. The addition of paper powder greatly increases its mechanical strength, thereby helping to prevent cracking or mechanical damage.

It is also desirable to choose an electrolyte which can easily be removed from the amino-acids after ionophoresis. This can be achieved by using volatile electrolytes, e.g. ammonium acetate, sparingly soluble electrolytes, e.g. borax, or precipitable electrolytes, e.g. CaCl_2 .

Where the separation does not depend upon a difference in mobility of less than $0.76 \times 10^{-4} \text{ cm.}^2/\text{volt sec.}$ (i.e. not more difficult to separate than alanine and methionine), an open trough is satisfactory. Any syneresis liquid which might otherwise flood the surface of the jelly is evaporated by allowing a rise in temperature. As a result, however, it is impossible to continue the experiment for more than a few hours as the jelly becomes so dry that it is liable to crack. Strengthening the jelly with paper powder will eliminate this cracking except at the joint between the gutter and the main slab, and will delay it here. An attempt to overcome this loss of water by laying on a glass lid after the jelly had set was, indeed, sometimes successful, but often sweating and syneresis caused mixing, and unpredictable movements of the substances took place. Control experiments with dyestuffs showed that to minimize these effects, it was desirable to leave the lid in place for an hour before the potential was applied. This allowed the jelly to be deformed until it was in intimate contact with the glass lid, and reduced un-

wanted flow of liquid. A more satisfactory method for long runs is to set the jelly beneath a lid already in place. The jelly appears to stick to the glass firmly enough to prevent syneresis or sweating, even under rather unfavourable conditions as, for instance, when the salt concentration in the gutter is three-quarters or twice that in the main body of the jelly. The method, unfortunately, prevents the washing of the jelly to change the nature of the electrolyte, e.g. to a volatile salt.

We recommend that the gutter should be filled with jelly of the same ionic concentration as the main slab. Where the ionic concentration of the solution containing the substances to be separated is comparable with that of the electrolyte in the main slab of jelly, it is advisable to reduce the silicate concentration of the gutter jelly so long as a reasonable setting time is retained. It was, however, found possible, when using low current densities on the uncooled trough, to use paper powder, saturated with the liquid to be analyzed. In spite of its greater convenience, we were forced to abandon its use when the optimum current densities were employed in the cooled trough, as its resistance was liable to become excessive.

THEORY OF IONOPHORETIC SEPARATIONS

Consider two acids HA and HB, with dissociation constants K_a and K_b and ion mobilities u_a and u_b . Then the *net mobility* of HA, i.e. the mobility as determined from the movement of a band of the acid in an ionophoretic apparatus will be:

$$U_a = \frac{u_a [A^-]}{[HA] + [A^-]} = \frac{u_a K_a}{[H^+] + K_a}.$$

(*Net mobility* is the mobility of the *ion constituent* as defined by MacInnes (1939). It does not seem to have been named previously.)

From the point of view of separation we are interested in the difference in net mobility,

$$U_a - U_b = \frac{u_a K_a}{[H^+] + K_a} - \frac{u_b K_b}{[H^+] + K_b}.$$

It can readily be shown that the difference in net mobility is a maximum when:

$$[H^+] = (\sqrt{K_a K_b}) \left(\frac{\sqrt{\frac{u_a}{u_b}} - \sqrt{\frac{K_a}{K_b}}}{1 - \sqrt{\frac{u_a K_a}{u_b K_b}}} \right)$$

or
$$pH = \frac{pK_a + pK_b}{2} - \log \left(\frac{\sqrt{\frac{u_a}{u_b}} - \sqrt{\frac{K_a}{K_b}}}{1 - \sqrt{\frac{u_a K_a}{u_b K_b}}} \right).$$

When $K_a > K_b$ (i.e. $pK_a < pK_b$) and except when K_a/K_b lies between the values of u_a/u_b and u_b/u_a , and if the optimum pH be used, the maximum difference in net mobility may be shown to be

$$(U_a - U_b) = \frac{u_b \left(\sqrt{\frac{u_a K_a}{u_b K_b}} - 1 \right)^2}{K_a/K_b - 1}.$$

Fig. 1 shows $(U_a - U_b)/u_b$ plotted against $pK_b - pK_a$ for various values of u_a/u_b , and the intersecting family of lines shows the pH which must be employed. Equation for pH lines is

$$\frac{U_a - U_b}{u_b} = \frac{K_a/K_b - 1}{(1 + [H^+]/K_b)^2}.$$

It is not important that these pH values should be exactly followed; 0.1 pH unit makes little difference to $U_a - U_b$. Further, in the region where $pH = pK_b + \infty$ is shown, there is little advantage in going beyond $pH = pK_b + 2$. This applies also to the left of this line where the separation depends only on the difference in ion mobility. It is, of course, also possible in the case of substances in which u_a/u_b is low, by working at a high pH, to separate them in the opposite direction to that shown in Fig. 1, using only the ion mobility difference.

The sign convention adopted in Fig. 1 is suitable for the separation of anions, i.e. the acid with the greater pK must be called HB. For the separation of cations, the base with the greater pK must be called A, and to determine the optimum pH, the sign of the addition to pK_b must be reversed. This rule holds also for ampholytes. Thus, in separating glycine and serine in alkaline solution, the ions are anions and pK_b must be greater than pK_a, even though the separation depends on differences in the dissociation constants of the amino groups.

Figures for mobilities of most amino-acids are not available. An estimate may be made, however, by assuming them to be the same as the corresponding hydroxy (or fatty) acid; an extensive table of such values is given in the *International Critical Tables*. A correction must be made for the reduced mobility in the relatively strong solution of electrolyte. We have assumed that

$$u_a = \frac{\Lambda_0 \text{ acid} - \Lambda_0 \text{ H ion}}{96,500} \cdot \frac{\Lambda_c \text{ electrolyte}}{\Lambda_0 \text{ electrolyte}},$$

i.e. the assumption is made that the ratio of the mobilities (of the substances to be separated) at infinite dilution and in the electrolyte actually used, is the same as the ratio of the equivalent conductances of the electrolyte at infinite dilution and at the concentration used.

Agreement of theory and experiment

No experimental verification of the preceding theory has been attempted. Our apparatus was not

suitable for accurate measurements of the mean temperature of the jelly, nor was it easy to obtain a good estimate of the mean potential gradient throughout the course of the experiment, because there was a rather large and unknown drop in potential between the electrodes and the jelly. Further, in the case of the open trough experiments, owing to surface evaporation, an unknown increase

Exp. B1. In the separation of glutamic and aspartic acids the mobilities estimated at infinite dilution and 25° (from *International Critical Tables*) are respectively 2.9 and 3.2×10^{-4} cm.²/volt sec. If we allow a reduction of 15 % to bring the temperature to 18°, and 36 % for working in 0.25M-phosphate, the expected mobilities are 1.86 and 2.05 $\times 10^{-4}$ cm.²/volt sec.

Fig. 1. Chart showing difference in net mobilities of two acids, for a given difference in pK and given ion mobility ratio, when separating at the optimum pH, the value of which is given by the intersecting family of lines.

occurred in the concentration of the electrolyte in the jelly; moreover, the effect of electroendosmosis caused the more concentrated anode solution to run into the jelly. The former makes the mobility difficult to estimate and the latter upsets the potential gradient. The distance that the electrolyte has been moved by electroendosmosis has also to be estimated if a comparison of measured and theoretical mobilities is to be made. We have estimated this by the movement of neutral amino-acids at neutral pH, and by the movement of depressions or lines of sweating at other pH values.

The movement of the electrolyte by electroendosmosis was estimated to be 51.5 cm. Therefore the movement of the glutamic acid band was $33 + 51.5 = 84.5$ cm., and of the aspartic acid band $42.5 + 51.5 = 94.0$ cm. Assuming a linear drop of potential between the electrodes, which were 53 cm. apart, the product of potential gradient and time was 4.89×10^5 volt sec./cm.

From these figures the measured mobilities are 1.73 and 1.92×10^{-4} cm.²/volt sec. The ratio of the mobilities, a figure unaffected by errors of estimation of potential gradient or allowance for slowing effect

of the high concentration of phosphate, is calculated 1.10, measured 1.11. The differences in mobility, figures unaffected by errors of estimation of electro-osmosis, are calculated 0.19×10^{-4} and measured 0.19×10^{-4} cm.²/volt sec.

Exp. A 3. In the separation of methionine and alanine, assuming for the former, $u = 2.06 \times 10^{-4}$ and $pK = 9.21$, and for the latter, $u = 2.67 \times 10^{-4}$ and $pK = 9.87$, the net mobilities calculated for a

For a mean value of unity, the r.m.s. value for smooth current is 1.00, for full wave, rectified, sinusoidal current, 1.11, and for half wave, rectified, sinusoidal current, 1.56. Also, except with smooth current, meter readings must be treated with caution, as moving coil instruments give mean values and moving iron or hot wire instruments give r.m.s. values.

The trough. This is constructed of $\frac{1}{4}$ in. plate-glass. The sides and ends consist of strips of such glass, smeared with vaseline and clamped in place. We have experienced no

Fig. 2. Plan and cross-section of apparatus.

temperature of 25° and pH 9.4 are 1.25 and 0.55×10^{-4} cm.²/volt sec.

The movement of the electrolyte by electro-osmosis was 7.1 cm. Therefore the methionine moved $7.1 - 1.0 = 6.1$ cm., and the alanine $7.1 - 4.2 = 2.9$ cm.; giving measured mobilities of 1.05×10^{-4} for methionine and 0.50×10^{-4} cm.²/volt sec. for alanine. The ratio of mobilities was 2.3 (calculated) and 2.1 (measured), and the difference of mobilities was 0.70 (calculated) and 0.55 (measured). These differences between theory and experiment are well within the expected error.

EXPERIMENTAL

Source of electricity. It is undesirable to use unsmoothed, rectified, alternating current. The rate of movement of ions is proportional to the mean potential gradient, and the heat produced, to the square of the r.m.s. potential gradient.

trouble from leakage. The trough rests on a similar but longer trough through which water may be circulated to provide cooling. The plan and cross-section with dimensions are shown in Fig. 2. The glass is bored at *A*, *B* and *C* to provide exit holes for the electrode perfusing solutions and for the cooling water. The tube, which carries away the water at *C*, protrudes $\frac{1}{4}$ in. above the bottom of the cooling trough. This is necessary to maintain the proper water level in this trough. We have found, however, that a sufficient depth of solution is maintained in the anode and cathode compartments without the use of such tubes.

Perfusion solution supply. A suitable rate of drip may be maintained by siphoning the solutions through long capillary tubes connected to Mariotte bottles. Glass bars, resting on small pieces of microscope slides, are placed in front of the electrodes as shown in Fig. 2. These direct the flow of electrolyte away from the jelly, thereby preventing undesirable ions, produced at the electrodes, reaching the jelly. This arrangement, which was found to be an improvement, was used only in the separation of glutamic and

aspartic acids (Exp. B1), which was the last experiment to be conducted.

Preparation of paper powder. Whatman 'Accelerator' paper (170 g.) was broken up and thoroughly stirred with $N/10$ -HCl (1.5 l.). The mixture was allowed to stand for $\frac{1}{2}$ hr. after which the acid was removed and the treatment repeated. After filtration, the paper was thoroughly washed with water until the washings were no longer acid to methyl red. The paper was dried at 90–100°, then powdered by crushing by hand in a canvas bag using rubber gloves, and finally passed through a 30-mesh sieve. The powder thus obtained gave no reaction with ninhydrin.

Method A. Jelly uncovered. Suitable for short runs

Exp. A1. Separation of a wool hydrolysate into acid, basic and neutral fractions. Commercial water glass (cf. Gordon, Martin & Syngé, 1943) was diluted approximately twice with water and filtered to give a 4.3 N- solution with respect to alkali. 28 ml. of this solution were diluted to 350 ml. with water and 60 ml. of approximately 1.6 M- H_3PO_4 solution added until the pH was just above 7 (blue-green to bromthymol blue). This solution was poured into the trough and allowed to set. The ends of the jelly were cut away to provide compartments for the electrodes (see Fig. 2). A gutter, 1 cm. wide, was now cut out, 23 cm. from the anode and on the anode side of the middle to compensate for electroendosmosis. The hydrolysate was a solution containing 5 mg. N/ml., obtained by refluxing wool with 6 N-HCl for 24 hr., removing excess HCl *in vacuo* and diluting with water. The hydrolysate (1 ml.) was made to 5 ml. with sufficient of the above silicate and phosphoric acid solutions to give the same Na^+ concentration and pH as that of the main jelly. The gutter was filled to the brim with this solution which then set in about 5 min. After the electrodes had been put in place, the perfusion was started. A solution of Na_3PO_4 and Na_2HPO_4 , which was grey-blue to thymol blue and $N/3$ with respect to Na^+ , was used for the anode compartment. The cathode perfusion liquor was $N/8$ NaH_2PO_4 brought to pH 6.5 (green to bromthymol blue) with NaOH. Next, a potential of 68 volts was applied which produced a current of 90 ma. After $\frac{1}{2}$ hr. the voltage was raised to 190, giving a current of 430 ma. At this point the cooling water was turned on. After a further 250 min. the current was stopped and a print was taken by laying a strip of dry filter paper along the length of the jelly, allowing it to become saturated, removing and drying. The strip was sprayed with ninhydrin solution (0.1% in *n*-butanol) and again dried for 5 min. at 100°. Three purple bands appeared, one 3.5 cm. wide had moved 3 cm. towards the anode. (The movement of all bands is measured from the centres of the bands.) The second band was 2.7 cm. wide and had moved 4 cm. towards the cathode. The trailing edge of this band appeared yellow, perhaps due to proline, which always gives a yellow colour with ninhydrin. The third band was 1.8 cm. wide and had moved 7.8 cm. towards the cathode. A second print was now taken as above, but after the first drying, it was sprayed with 0.14% solution of α -naphthol in 0.5 N-NaOH. This was partially dried off and then the paper was resprayed with a concentrated solution of NaOCl. A single red band appeared, identical in position with the third ninhydrin band. This was presumably due to arginine (Sakaguchi, 1925).

By reference to the prints, the jelly was cut into three sections which were each thoroughly mixed with 10 g. of paper powder in beakers. After standing overnight *in vacuo*, the dry powders were tightly packed into 1.5 cm. diameter glass tubes and extracted chromatographically with about 30 ml. each of water. These solutions were reduced to small volume *in vacuo*, cooled in ice water and filtered from most of the sodium phosphate. The fractions were then tested by partition chromatography on Whatman no. 4 paper (Consdén, Gordon & Martin, 1944), using *s*-collidine for the cathode band and phenol-coal gas- NH_3 for the anode. In this and all subsequent tests, where partition chromatography on paper was used, except where the two-dimensional technique was employed, the appropriate amino-acids were run parallel as controls. The cathode band was found to contain lysine and arginine only and the anode band aspartic and glutamic acids only. A two-dimensional chromatogram was performed on the material from the middle band, using *s*-collidine in one direction, followed by phenol-coal gas- NH_3 in the other. No spots corresponding to basic or dicarboxylic acids were found, whilst those for the neutral amino-acids of wool were all present.

Exp. A2. Separation of glycine and serine. The jelly for this separation was made up as follows: 4.3 N-sodium silicate (25 ml.) was made to 300 ml. with water and hot boric acid solution (about 90 ml. of 12% (w/v)) was added until the pH was 9.2 (blue-grey to thymol blue and faint blue to thymolphthalein). The jelly was allowed to stand overnight before cutting out the compartments. No syneresis was observed. A solution was made up containing 1 mg. each of glycine and serine per ml., with the same concentration of sodium silicate and boric acid and the same pH as that of the rest of the jelly. The central gutter, 1 cm. wide, was filled with 7 ml. of this solution and allowed to set. The anode was perfused with saturated borax solution and the cathode with $N/8$ borax solution. A potential of 180 volts was applied causing the passage of a current of 300 ma. Slight sweating was observed on the cathode side of the inlay, but after about $\frac{1}{2}$ hr., this had dried off, so the cooling water was started. After 6 hr. a print was made, using the same ninhydrin solution as above but with the addition of 0.02 ml. concentrated HCl per ml. This revealed two bands 4 cm. apart and each about 2 cm. wide. Both had moved towards the cathode, one had moved 4.7 cm. whilst the other had moved only 0.5 cm. A second print was made and developed by spraying with a solution made by adding Nessler's reagent to solid periodic acid until the precipitate first formed just redissolved. (This method was suggested to us by Dr R. L. M. Syngé.) A dark brown band slowly appeared, corresponding exactly in position with that of the slower band of the ninhydrin print. As this test is given by serine and not by glycine, serine was present in the slower band and absent from the faster. The jelly corresponding to each band was cut out and extracted as described above, and the concentrated extracts were tested by partition chromatography on paper using phenol-cupron as solvent. This revealed that the slower band contained serine only and the faster band glycine only.

Exp. A3. Separation of methionine and alanine. The jelly for this separation was made up similarly to that described in Exp. A2 except that the pH was slightly higher (9.4, glass electrode). The solution for the gutter which was the same as the body of the jelly as regards pH, silicate content and ionic concentration, contained 6 mg. each of alanine

and methionine. The electrode perfusion solutions were similar to those in Exp. A2 above. A potential of 180 volts was applied which produced a current of 200 ma. Some sweating occurred on the inlay and this line of droplets travelled towards the cathode. Some flooding occurred at the surface of the jelly near the anode which was not prevented by adding solid borax to the anode compartment. This flooding was controlled by mopping off occasionally with strips of filter paper. After about 1 hr., the cooling water was started and after $4\frac{1}{2}$ hr. the current was stopped. A print developed with acidified ninhydrin solution showed the presence of two bands, 2 cm. wide, both on the cathode side of the gutter, one having moved 4.2 cm. the other 1.0 cm. The distance between the centre of the bands was 3.2 cm. Another print was taken and developed with sodium iodoplatinate solution, freshly prepared from equal parts of 0.17% PtCl_4 and 0.83% NaI . (This reaction seems to have possibilities for the analysis of the S-containing amino-acids.) A yellow band appeared immediately corresponding in position to the slower band of the ninhydrin print, confirming the presence of methionine. A second yellow band, corresponding with the faster band of the ninhydrin print appeared more slowly and consistent with the presence of alanine. From the movement of a depression it was estimated that electroendosmosis had moved the electrolyte 7.1 cm. towards the cathode.

The jelly was then cut up into two sections corresponding with these bands, extracted as in Exp. A1 and the concentrated filtrates tested by partition chromatography on paper using both *n*-butanol and phenol-cupron as solvents. The slower band was shown to be methionine only, whilst the faster band was alanine only.

Method B. Suitable for difficult separations. Jelly completely enclosed

Exp. B1. Separation of glutamic and aspartic acids, making use of their difference in ion mobilities. The jelly for this separation was made up similarly to that used for Exp. A1 except that before setting, two pieces of glass 3.0 and 42.5 cm. long respectively were lowered into the trough and allowed to rest on a line of microscope slides placed end to end along the edges of the trough. This arrangement gives a slab of jelly, 1.4 mm. thick and 10.2 cm. wide. Bubbles were readily avoided by placing one end of each lid in the liquid and gently lowering the other. A space, 1 cm. wide, was left between the lids and after setting, the jelly was cut out from here. A solution containing 2 mg. each of aspartic and glutamic acids and similar in all other respects to the rest of the jelly was run in. To prevent the solution wetting the edges of the lids, these were previously slightly greased with vaseline. When the inlay solution had set, it was covered with a 1 cm. wide glass bar and the joints were sealed with vaseline. Both anode and cathode compartments were perfused with sodium phosphate solution of the same normality and pH as in the body of the jelly. The cooling water was started and a potential of 180 volts was applied for 1 hr. The voltage was then raised to 270 for 26 hr. producing a current of 150 ma. The lid was slid off transversely after first removing one side of the trough, and a print was taken and developed with acidified ninhydrin solution. Two bands were revealed, each 5 cm. wide, which had moved 33 and 42.5 cm. respectively towards the anode. The jelly was cut into two sections corresponding

with these bands and extracted as described above. The filtrates were tested by partition chromatography on paper, using phenol-coal gas- NH_3 . The faster band consisted of aspartic only, the slower, of glutamic acid only.

Exp. B2. Separation of lysine and histidine. The jelly for this separation was made up similarly to that used in Exp. A1 except that *N*-HCl was used instead of H_3PO_4 and the pH was brought to 6.6 (yellow-green to bromthymol blue). The lids were inserted as in Exp. B1 except that they were reversed to give a gutter near the anode. A solution containing 2 mg. each of the monohydrochlorides of lysine and histidine and similar in all other respects to the rest of the jelly, was run in. After setting, this was covered and sealed as in Exp. B1. The anode compartment was perfused with 0.5*N*- Na_2CO_3 and the cathode with 0.5*N*-HCl. An uncooled trough, 56 cm. long, was used for this separation. A potential of 100 volts was applied for 7 hr. giving a current of 200 ma. Next, the lids were removed and a print was taken. On development with ninhydrin, two bands appeared, one immediately and the other, nearer the middle, after an hour's delay. This delay is consistent with the behaviour of histidine which does not give such a satisfactory reaction with ninhydrin as most of the amino-acids. Both had moved towards the cathode, one having travelled 7.8 cm. and the other 23 cm. A second print was taken and sprayed with diazotized sulphanilic acid (0.1-0.2% in *N*- Na_2CO_3 solution) (Pauly, 1904). An immediate red colour appeared corresponding in position to the slower band. The jelly was cut into two sections corresponding with the bands, dried and extracted. The concentrated solutions were tested by partition chromatography on paper using *s*-collidine as solvent. Lysine was found only in the faster band. No spot corresponding to histidine appeared with ninhydrin, perhaps owing to the presence of salt. However, on spraying with the diazo reagent, the strip from the slower band gave a red spot in the expected position whereas that from the faster band gave no colour. The remainders of both solutions were also tested with the diazo reagent, that from the slower band gave a strong red colour and that from the faster remained unchanged, confirming the absence of histidine in this fraction.

Method C. Experiments using a volatile electrolyte. Glass lid laid on jelly after setting

Exp. C1. Separation of glycine and glycyglycine. The jelly for this separation was made up similarly to that used in Exp. B2 above, but it was set in an uncooled trough 120 cm. long and 15 cm. wide. A solution of $(\text{NH}_4)_2\text{CO}_3$ (*N*/2) was allowed to run slowly over its surface for 17 hr. A glass cover resting on the edges of the trough but not in contact with the surface of the liquid, prevented evaporation. This washing sufficed to reduce the Cl^- concentration of the jelly to negligible proportions (AgNO_3 test on excised portions) and to bring its pH to about 9.2. Next, the trough was slightly tilted to allow draining. When this was complete, a gutter 8 mm. wide was cut out and filled with 1.8 ml. of a solution containing 35 mg. of glycine and 65 mg. of glycyglycine and brought to pH 9.2 with NH_3 . Sufficient paper powder was now added to the gutter to pack it level with the surface of the jelly. A rectangular piece of plate glass, the same length as, but 2 mm. narrower than the jelly, was then carefully laid on the surface and pressed firmly home. Both anode and cathode compartments were

perfused with $N/2$ $(NH_4)_2CO_3$ solution. Next, a potential of 170 volts was applied for 41 hr. producing a current of 120 ma. After removal of the lid, a print was taken which on development with ninhydrin revealed one orange-yellow band, 6 cm. wide, which had moved 22 cm. towards the anode and two purple bands 7 and 6 cm. wide which had moved 5.5 and 12.0 cm. respectively towards the cathode. The jelly was cut into three sections corresponding to these bands, mixed with paper powder and dried *in vacuo* over H_2SO_4 and soda lime to remove $(NH_4)_2CO_3$. The extracts were tested by partition chromatography on paper using *s*-collidine as solvent. The presence of glycylglycine and absence of glycine in the material from the anode band was shown both by the characteristic colour (orange-yellow, later turning to purple), given by glycylglycine in the ninhydrin reaction and the position of the spots. That the other two bands contained nothing but glycine was similarly shown by mixed chromatograms of these materials with glycine. The existence of two glycine bands is attributable to the movement of liquid between the surface of the jelly and the glass lid.

Exp. C2. Separation of cysteic from aspartic and glutamic acids. For this separation an uncooled trough, 56 cm. long and 15 cm. wide, was used. The jelly was made up similarly to that used in Exp. B2 but it was strengthened by the incorporation of paper powder. 60 g. were placed in the trough which was then filled with silicate solution and vibrated horizontally to ensure even distribution of the paper throughout. After setting, the jelly was treated as in Exp. C1, except that $N/50$ HCl was used as the wash liquor. The gutter, 8 mm. wide was filled with a solution (2 ml.) containing 10 mg. each of cysteic, aspartic and glutamic acids dissolved in $N/50$ HCl. Paper powder was added and the glass lid was laid on as in Exp. C1. Both anode and cathode compartments were perfused with $N/50$ HCl. A potential of 100 volts was applied for 17 hr. producing a current of 50 ma. After removal of the lid, a print was taken which on development with ninhydrin revealed two bands, one 10 cm., and the other 4.5 cm. wide. The broader band had moved 10 cm. towards the cathode, while the narrower band had moved 3 cm. towards the anode. The jelly, corresponding to these bands, was cut out, dried and extracted and the eluates were tested by partition chromatography on paper, using phenol-coal gas- NH_3 as solvent. The material from the anode band was found to be cysteic acid only; that from the cathode band was aspartic and glutamic acids only.

DISCUSSION

The ionophoretic method described above is suitable for the separation of substances by reason of differences either in their ion or in their net mobilities. It is not primarily concerned with the presence or absence of a positive or negative charge on the molecule, but with its mobility. In this it differs essentially from ionophoretic methods used heretofore. In illustration, it may be pointed out that the isoelectric point does not occupy any special or favoured place. In fact, the 'neutral' amino-acids with considerable differences of isoelectric point, e.g. tyrosine pI 5.63, proline pI 6.3, could not in any apparatus be made to separate at a pH near

their isoelectric points because the net mobility of these acids at this pH is negligible. On the other hand, at the optimum pH the separation would be extremely easy. Williams (1935) has maintained a pH gradient between the electrodes, and suggests that substances will move towards the region corresponding to their isoelectric points. The small mobility of many substances near their isoelectric point would account in part for the lack of clear-cut separations by this method.

Since either ion or net mobility is the basis of separation, there is an almost unexplored field of separations based on ion mobilities (cf. Kendall, 1942). In addition, ancillary means are available to alter mobilities differentially, as, for example, by treatment with formaldehyde or sulphurous acid (producing S-cysteine sulphonc acid). Many other possible compounds or complexes can no doubt be profitably used in this way.

With increasing size of molecule the silica must inevitably prevent movement of the molecule or ion, but it is probable that a molecular weight of several thousand may be used successfully. It is to be noticed that since the mobility is equally involved in diffusion and movement in an electric field, the small mobility of large molecules does not hinder the separation appreciably, since the bands stay correspondingly narrower owing to reduced diffusion.

Adsorption of the substances on to the silica would, of course, be a complicating factor if it occurred and could increase or decrease the separations obtained. Sufficient data have not been collected to decide as yet how important it is when it is likely to occur. We have had some evidence suggesting that positively charged ions are adsorbed preferentially (cf. Synge, 1945*a*). As in the case of partition chromatography on silica gel (Martin & Synge, 1941) it may be possible to work in the presence of eluting agents.

The separations effected by ionophoresis, in so far as they depend on differences in net mobility, run parallel in many respects to the separations which may be effected by the use of ion exchange materials (cf. Martin & Synge, 1945). The latter have a complication in that Van der Waals forces, in addition to electrostatic forces, play a part in determining the exchange and hence the behaviour is less predictable and less easy to control than ionophoresis. Ionophoretic separations depending on ion mobility differences do not correspond or go parallel with those obtainable by other means.

The method has already been used preparatively by Synge (1945 *a, b*) in the isolation of ethanolamine from gramicidin and of ornithine from gramicidin-S. It is of interest that the behaviour of ethanolamine was sufficiently characteristic to provide useful evidence of its identity.

In the particular field of separation of the products of partial hydrolysis of proteins, the use of ionophoresis several times successively at different pH values on the same fractions promises to effect separation into a number of classes; and though it is improbable that its use alone will serve for the isolation of any single substance, its use as part of the separation will probably prove essential.

With the apparatus we have constructed, not more than one or two millimoles electrolyte can be used in the sample to be analyzed. These quantities are too small for convenience for many purposes, but could be increased by increasing the area of the apparatus. The thickness of the jelly cannot profitably be increased very far. The use of thinner glass and water cooling of the lid would increase the permissible thickness by a factor of about three. Thus, there seems no reason why several grams of materials could not be treated in an apparatus of laboratory size.

Further work would be required to make this method fully quantitative. So far we have obtained recoveries ranging from 80 to 100 %. It is also inherently probable that by running the bands sufficiently far apart, mutual contamination can be reduced to any desired degree. In the experiments described above, the method of test employed would have revealed a contamination of more than 1 or 2 %, but we believe that the actual figure was much lower than this.

SUMMARY

1. A method for the separation of substances of different mobility by ionophoresis in a slab of silica jelly has been developed.

2. A theory of the movement of charged substances in jelly containing a swamping concentration of electrolyte is presented, and a chart showing the relationship of net mobility, ion mobility and dissociation constant is given.

3. A wool hydrolysate has been separated into basic, neutral and acidic fractions at neutral pH.

4. The following pairs of substances have been separated by taking advantage of their different net mobilities at the optimum pH for separation. Glycine and serine; alanine and methionine; lysine and histidine; glycine and glycyglycine; glutamic, aspartic, and cysteic acids.

5. Glutamic and aspartic acids have been separated at neutrality by taking advantage of their differing ion mobilities.

6. Colour reactions, applicable to paper prints have been developed which reveal the presence and position of bands of arginine, histidine; methionine and serine.

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The Determination of Nitrogen and of Protein in Pooled Samples of Human Plasma

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The determination of plasma proteins has assumed importance in recent years on account of the value of the result in dietary surveys and in clinical medicine. The main methods available are: (i) the determination of protein-N (total N minus non-

protein-N) and subsequent conversion of N into protein by use of the factor 6.25 assuming that the total proteins contain 16 % N; (ii) gravimetric estimation. Here the acetone-treated, heat-coagulated proteins are washed, dried and weighed. A good