resonance damping. Steric strain should become important only with unusually bulky substituents.

These conclusions are supported by data on the base strengths of phosphines, reported by Davies and Addis.[8b]

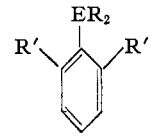
The introduction of a single *ortho* substituent into dimethylphenylphosphine leads to a relatively minor increase in base strength. However, two *ortho* substituents lead to a sharp increase in strength (Fig. 5), in marked contrast to the effect of a second *ortho* substituent in the dimethylaniline series (Fig. 2). In terms of the steric strain hypothesis, steric inhibition of resonance has become appreciable only with the second *ortho* methyl group; steric strain has not yet become significant.

Conclusion

A rigorous quantitative test of the importance of steric strains on the ionization constants of *ortho* substituted aromatic bases will require considerable additional data on compounds in which the number and bulk of alkyl groups attached to the donor atoms and to the *ortho* positions are varied in a systematic manner. It is hoped that such data will be forthcoming. However, even the relatively meager data now available are in good agreement with the steric strain interpretation. This suggests that steric strain should be considered as an important factor in the strength of aromatic bases. Steric strain in conjunction with current ideas concerning the factors affecting the strength of aromatic bases, permits a reasonable interpretation of all available data on the strength of such bases.

Summary

Currently the relative strengths of aromatic bases of the type



where R and R′ are hydrogen or alkyl groups and E is either nitrogen or phosphorus, are attributed to the operation of three major factors: (1) the inductive and hyperconjugative effects of the groups R and R′, (2) resonance involving the atom E and the ring, and (3) steric inhibition of resonance. It has, however, not been possible to account for the behavior of a number of *ortho* substituted amines and phosphines in terms of these factors. Consideration of steric strains resulting from the change in the steric requirements of the $-ER_2$ group accompanying its conversion into the onium ion, $-ER_2H^+$, permits a simple interpretation of outstanding discrepancies. It is suggested that steric strain be added as a fourth important factor in the strengths of *ortho* substituted aromatic bases.

LAFAYETTE, IND. RECEIVED OCTOBER 22, 1949

[CONTRIBUTION FROM MEDICAL DEPARTMENT FIELD RESEARCH LABORATORY, U. S. ARMY]

A Microelectrophoretic and Microionophoretic Technique[1]

BY E. L. DURRUM

In performing electrophoretic and ionophoretic separations, several investigators have utilized an electrical potential applied across various packing materials intended to stabilize migrating boundaries by preventing convection currents in the electrolytes employed. Strain[1a] combined ionophoresis with chromatographic adsorption in the conventional Tswett adsorption column and mentioned utilizing columns filled with cotton for this purpose. Coolidge[2] was able to separate protein constituents in a column packed with ground glass wool across which a potential was applied. Consden, Gordon and Martin[3] described an ionophoretic technique suitable for the separation of certain amino acids which was carried out in silica jelly slabs made up with various buffers. These investigators utilized paper pulp to reinforce the mechanical strength of the silica jelly slabs employed. They also reported an experiment in which their trough was filled with "paper powder saturated with liquids to be analyzed" but abandoned this variation of their method because current densities optimum for their purpose could not be employed. Butler and Stephen[4] have utilized asbestos fiber packed in a segmented polystyrene plastic tube and reported separating glycine from glycylglycine at *p*H 9.3 in this apparatus. None of the above processes was adapted to the separation of small quantities of material.

Recently, Haugaard and Kroner[5] applied electrical potentials across paper partition chromatographs during their development with phenol. They wove thin, flat, metallic electrodes into the edges of the paper which had first been treated with phosphate buffer solution and then dried prior to development with phenol. They reported that the degree of separation of basic and acidic amino acids attainable by paper partition

(1) Presented before the American Chemical Society, Division of Biological Chemistry, March 29, 1949, in San Francisco, California.

(1a) Strain, THIS JOURNAL, **61**, 1292 (1939).

(2) Coolidge, *J. Biol. Chem.*, **127**, 551 (1939).

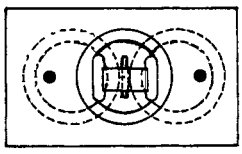
(3) Consden, Gordon and Martin, *Biochem. J.*, **40**, 33 (1946).

(4) Butler and Stephen, *Nature*, **160**, 469 (1947).

(5) Haugaard and Kroner, THIS JOURNAL, **70**, 2135 (1948).

chromatography was enhanced by this expedient. Though their process is applicable to the separation of minute quantities of amino acids, it does not appear to be applicable to protein separations.

This paper is concerned with a microionophoretic or microelectrophoretic technique which has been found useful for the separation of both amino acids and protein constituents in which an electrical potential is applied across the ends of strips of filter paper saturated with buffer or other electrolyte solutions to which are applied, at narrowly circumscribed intermediate areas, mixtures of amino acids, peptides or proteins to be separated. The positions to which components have migrated are determined in the case of amino acids and peptides by spraying the dried strip with ninhydrin (Consden, Gordon and Martin[6]), and in the case of proteins, by "fixing" the protein *in situ* on the paper strips by heat or by coagulation with chemical agents followed by treating the paper strip with a dye selective for the coagulated protein constituents but easily washed from the filter paper in zones free of protein. A third method which has been employed either alone or in combination with the above methods in cases where radioactive constituents are concerned is that of making autoradiographs of the dried or "fixed" strips. The practical applicability of this method appears to be wide enough to make it desirable to report at this time, although its theoretical aspects remain to be investigated more thoroughly.



TOP VIEW

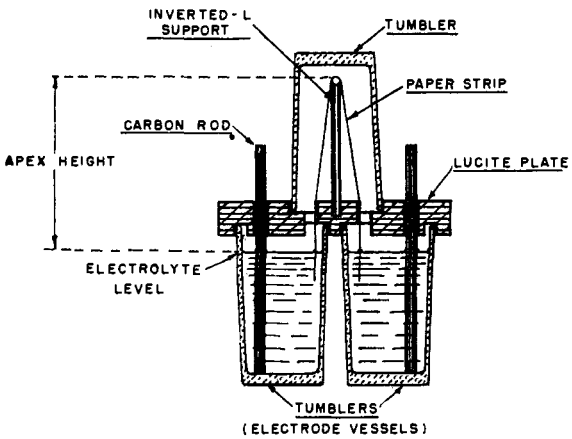


Fig. 1.—Diagram of apparatus.

(6) Consden, Gordon and Martin, *Biochem. J.*, **38**, 224 (1944).

Experimental

In preliminary experiments, narrow (1 cm.) strips of filter paper (about 0.16 mm. thick) were saturated with buffer solutions and the strips draped between two vessels containing the buffer solutions into which were inserted carbon rod electrodes. About the middle of the strips, a drop of serum or amino acid mixture was applied and then a potential of a few hundred volts applied across the carbon rods. These experiments served to show that separations could be practically effected in reasonable periods of time. There were, however, two disadvantages: (1) ill-defined zones of amino acids or proteins were obtained because of the syphoning of the buffer solutions to the low point of the paper with consequent "flooding" in this area; and (2) evaporation from the surface of the paper and temperature could not be controlled readily.

These difficulties were partially avoided by employing a glass bridge arrangement with a filter paper strip placed between somewhat wider plate glass strips resting on the electrode vessels. However, during many of the experiments, puddles of electrolyte were observed to collect irregularly and lateral to the edges of the paper with attendant uncertainties as to uniformity of field strength and as to diffusion of the amino acids into these areas.

This puddling of electrolytes is believed to be due to capillary action between the glass plates in the areas lateral to the paper strip. It was observed that this effect could be minimized by superposing at least three strips of filter paper which then separated the plates at the edges by at least about 0.5 mm. This is a promising method especially for multiple strips, and for single strips where a thicker filter paper is employed. This variation is being investigated further.

The apparatus was further modified to permit the use of single thickness strips of filter paper to which the electrolyte could be confined in a reproducible manner and all the experiments on which the present report is based were carried out in an apparatus of the type illustrated in Fig. 1. This apparatus is comprised of two 150-ml. glass tumblers carrying a lucite plate which seals their tops and supports an inverted L-shaped glass rod. The latter serves to support the apex of the filter paper strips which are draped over it. The ends of the strips pass into the electrolyte solution in the tumblers through slots in the lucite plate. Holes in the plate carry ordinary uncored arc carbon electrodes 8 mm. in diameter. The strips are isolated from the atmosphere by a third inverted 150-ml. tumbler. Annular grooves serve to improve the stability of this arrangement so that no external supports are required.

A larger version of the apparatus having electrode vessels of 500-ml. capacity and wide enough to support seven 1 cm. strips in parallel has proved to be quite convenient and useful when it is desired to compare known and unknown substances simultaneously under identical experimental conditions.

To adapt the apparatus shown in Fig. 1 for experiments of long duration the tumbler electrode vessels were replaced with U-tubes in order to separate the electrode reaction zones from the paper strip ends by a greater distance.

In all of the experiments described, filter paper strips cut from 32-cm. circles of Whatman No. 2 paper were used. Except where otherwise indicated strips 1 cm. wide were employed.

The use of this method is illustrated in the following experiments.

Experiment I.—Separation into five fractions of an equimolar amino acid mixture comprising 19 amino acids: arginine, lysine, histidine, glutamic acid, aspartic acid, glycine, alanine, valine, leucine, isoleucine, serine, threonine, cystine, methionine, tyrosine, tryptophane, phenylalanine, proline and hydroxyproline. Eighty ml. of buffer *p*H 5.9 prepared by mixing 50 ml. of 0.2 *M* potassium acid phthalate and 43 ml. of 0.2 *M* sodium hydroxide and diluting to 400 ml. were placed in each electrode vessel of the apparatus illustrated in Fig. 1. A pencil mark (x) was made across the middle of a 1 × 32 cm. strip of filter

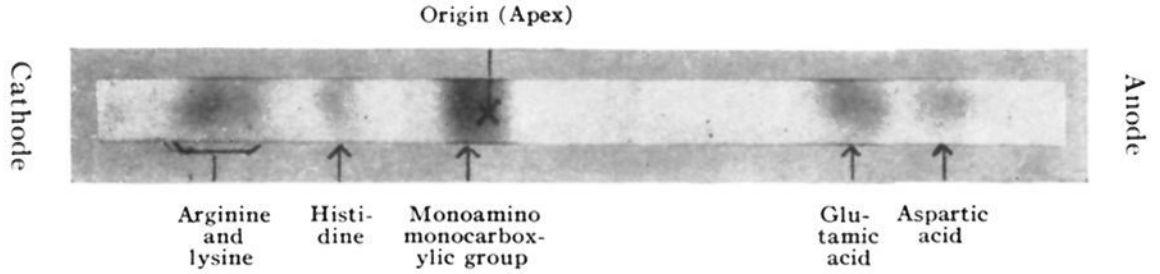


Fig. 2.—Separation of 19 amino acids into 5 groups (Experiment I): electrolyte: buffer (*p*H 5.9—see text) potassium acid phthalate–sodium hydroxide; duration, 120 minutes; current, 1.0 ma./cm. (width), initial 600 volts, final—not recorded; paper, Whatman No. 2; apex height, 14.5 cm.

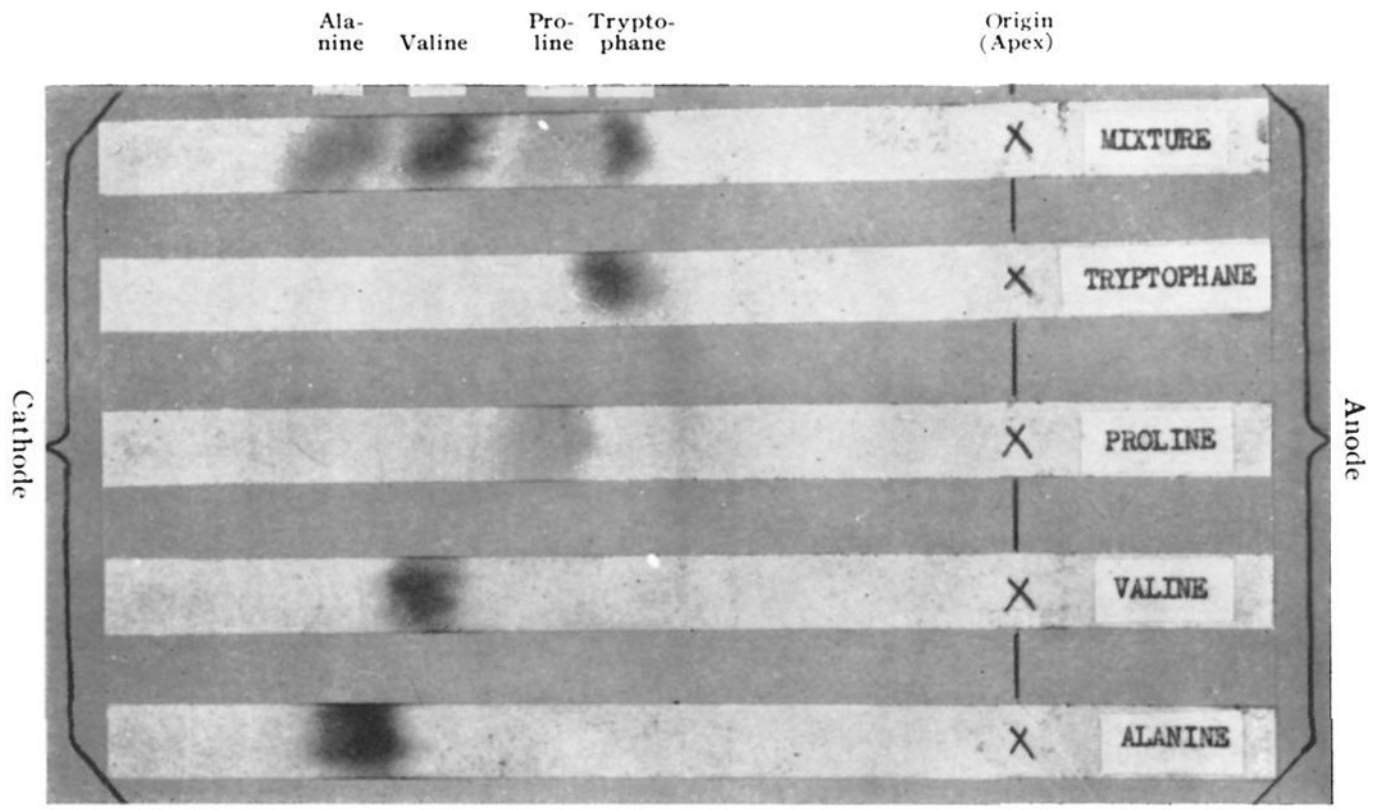


Fig. 3.—Separation of alanine, valine, proline and tryptophane (Experiment III): electrolyte, 5 *N* acetic acid (*p*H 1.7); duration, 120 minutes; current, initial 1.5 ma./5 cm. (width), final 1.7 ma./5 cm. (width); potential, 580 volts; paper, Whatman No. 2 (5 1-cm. strips in parallel); apex height, 11.5 cm.

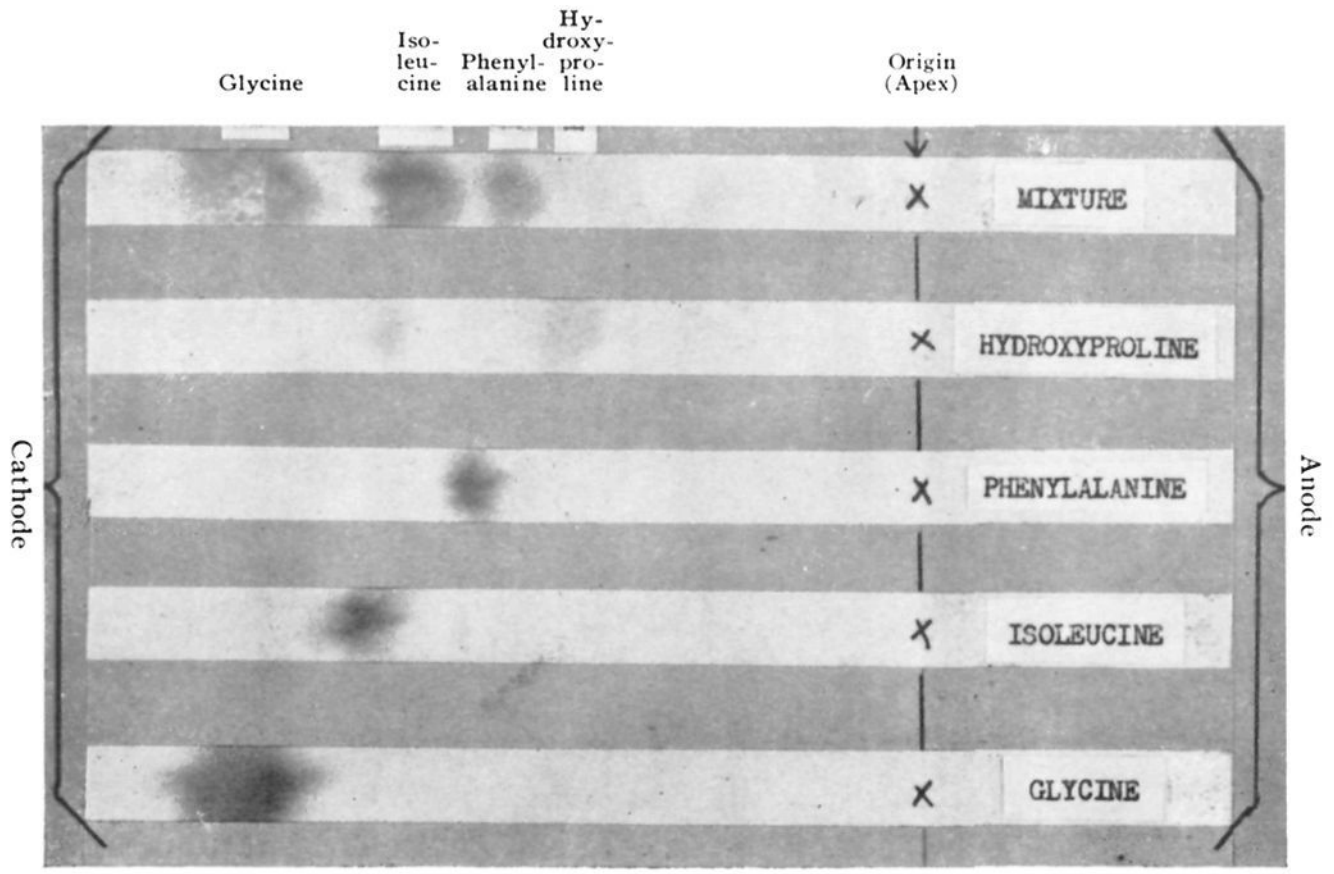


Fig. 4.—Separation of glycine, isoleucine, phenylalanine and hydroxyproline (Experiment IV): electrolyte, 5 *N* acetic acid (*p*H 1.7); duration, 120 minutes; current, initial 1.5 ma./5 cm. (width), final 1.7 ma./5 cm. (width); potential, 580 volts; paper, Whatman No. 2 (5 1-cm. strips in parallel); apex height, 11.5 cm.

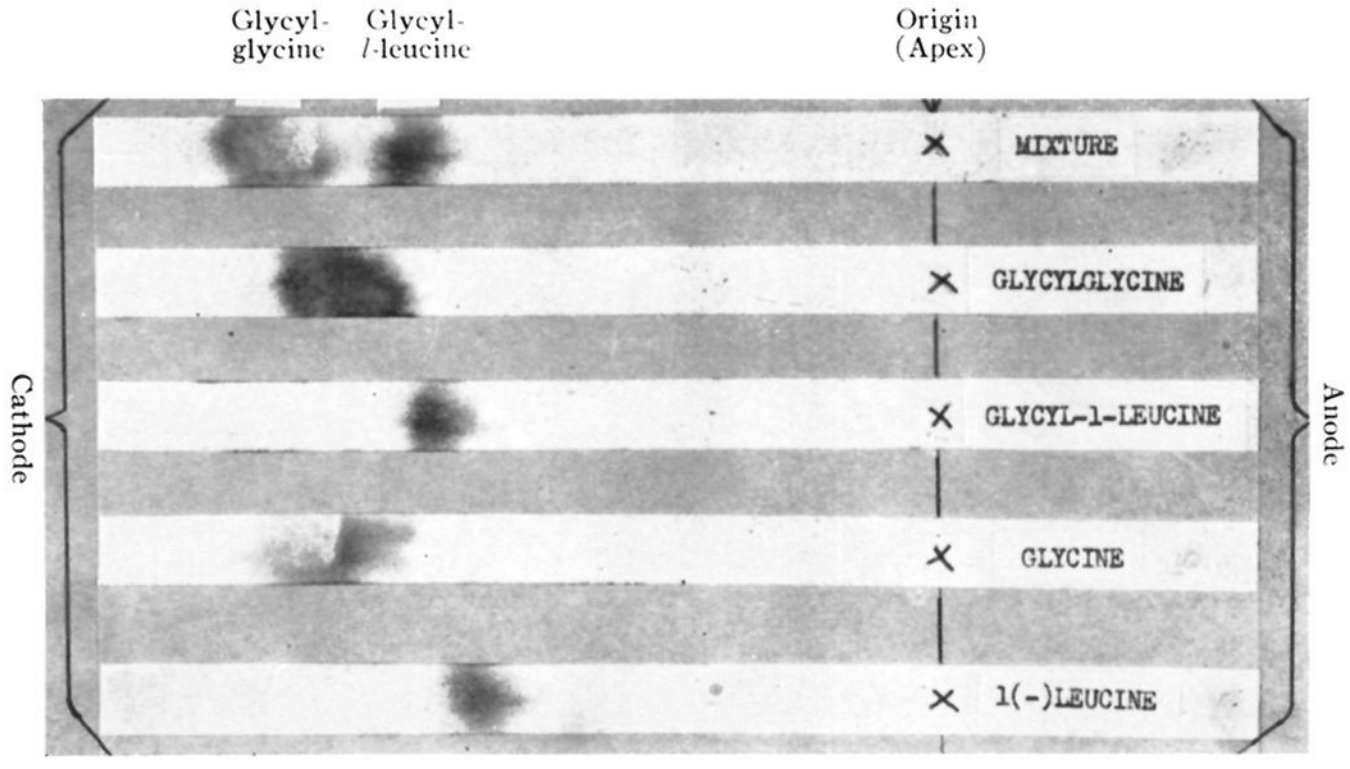


Fig. 5.—Separation of glycylglycine and glycyl-*l*-leucine (Experiment V): electrolyte, 5 *N* acetic acid (*p*H 1.7); duration, 120 minutes; current, initial 1.5 ma./5 cm. (width), final 1.7 ma./5 cm. (width); potential, 580 volts; paper, Whatman No. 2 (5 1-cm. strips in parallel); apex height, 11.5 cm.

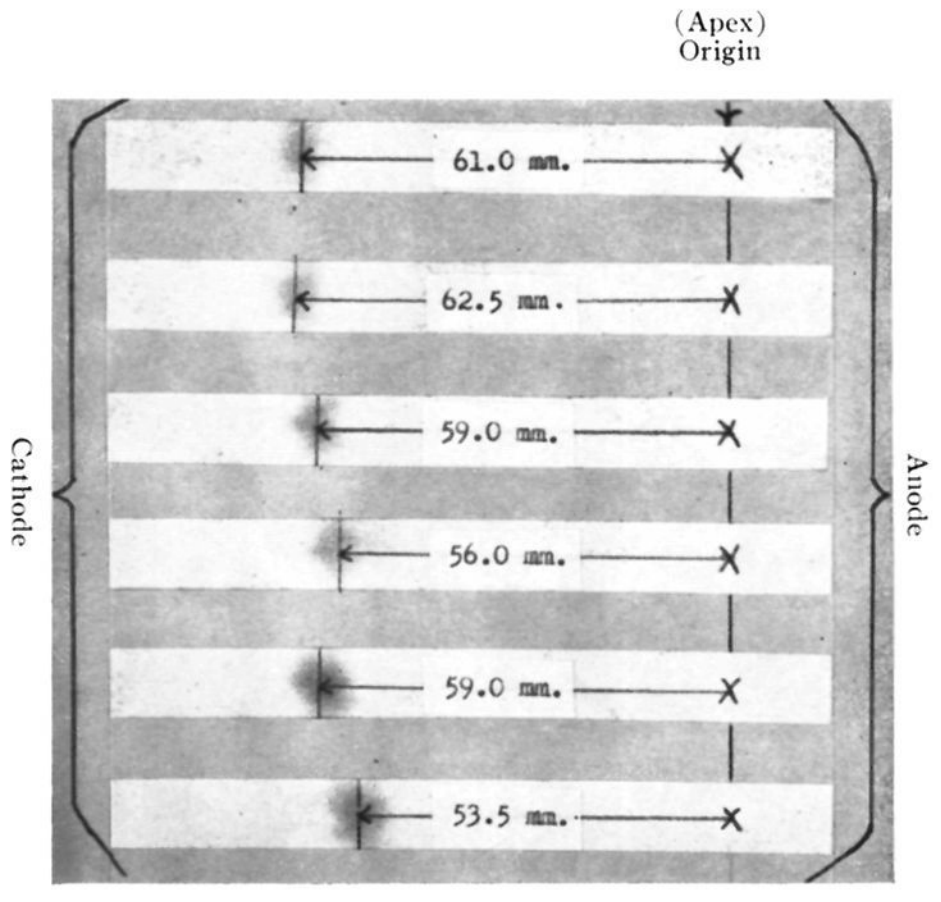


Fig. 6.—Reproducibility of parallel runs with phenylalanine (Experiment VI): electrolyte, 5 *N* acetic acid (*p*H 1.7); duration, 120 minutes; current, initial 1.8 ma./6 cm., final 1.7 ma./6 cm. (see text); potential, 580 volts; paper, Whatman No. 2 (6 1-cm. strips in parallel); apex height, 11.5 cm.

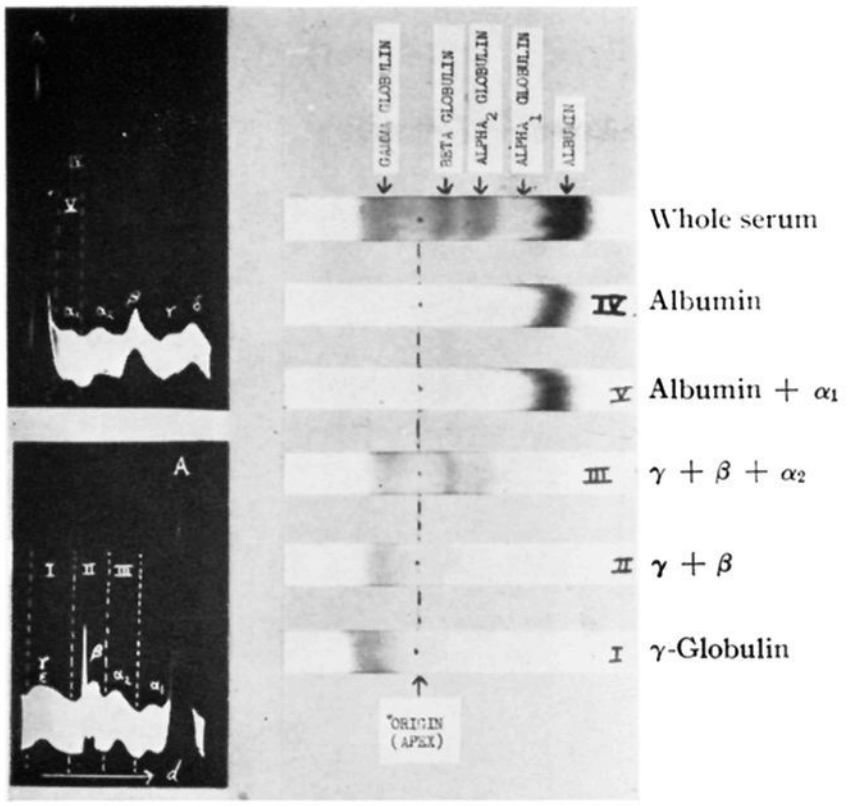


Fig. 8.—Comparison of separated components with whole serum (Experiment VIII): electrolyte, barbiturate buffer (*p*H 8.6), 0.05 *M* sodium diethylbarbiturate, 0.01 *M* diethylbarbituric acid; duration, 180 minutes; current, 0.50 ma./cm. (width); potential, initial 300 volts, final—not recorded; paper, Whatman No. 2; apex height 13.5 cm.

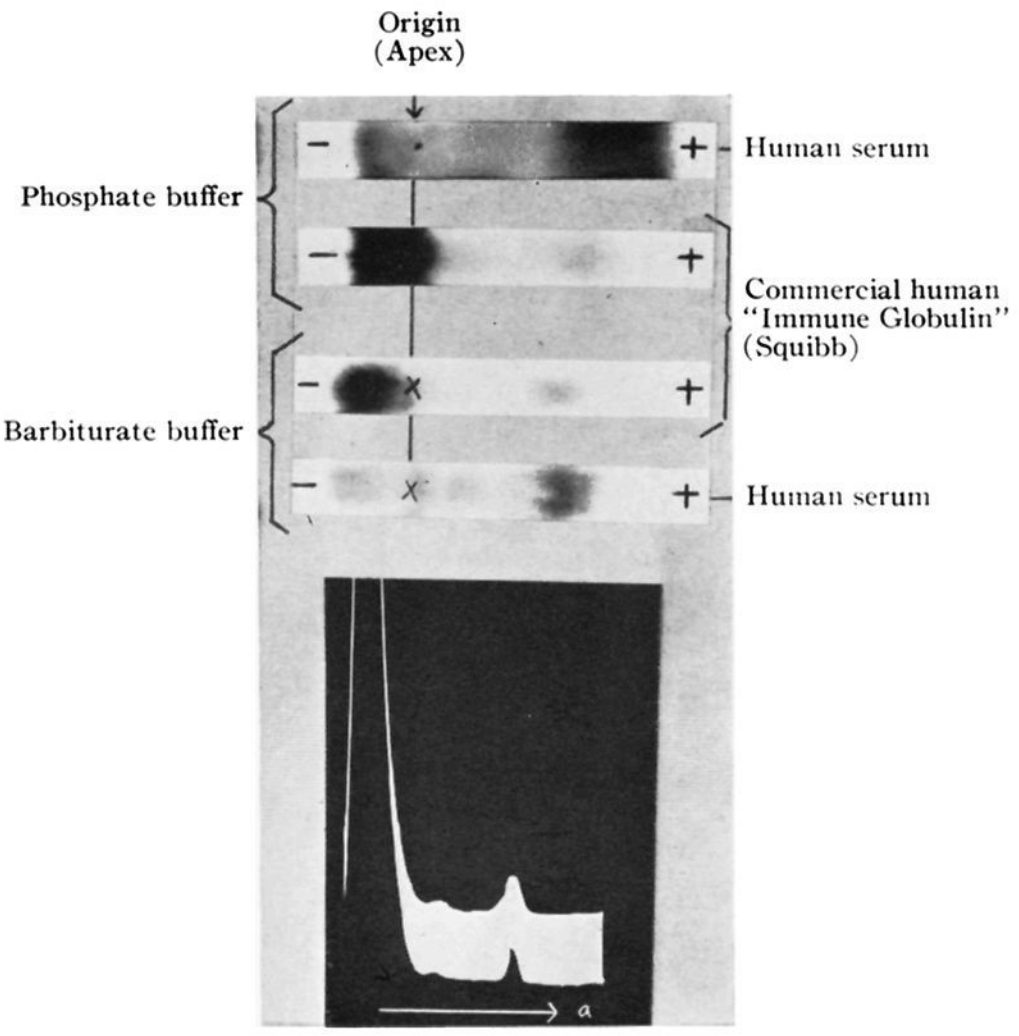


Fig. 9.—Separation of "Immune Globulin" (Experiment IX):

Electrolyte:	Phosphate buffer (*p*H 7.6), 0.02 *M* dibasic and monobasic sodiumphosphate	Barbiturate buffer (*p*H 8.6), 0.05 *M* sodium diethylbarbiturate and 0.01 *M* diethylbarbituric acid
Duration:	120 minutes	180 minutes
Current:	0.5 ma./cm. (width), initial 400 volts, final 340 volts	0.5 ma./cm. (width), initial 310 volts, final 220 volts
Paper:	Whatman No. 2 (2 strips in parallel in apparatus of Fig. 1)	Whatman No. 2 (2 strips of 5 run in parallel in apparatus of Experiment III)
Apex height:	14.5 cm.	11.5 cm.

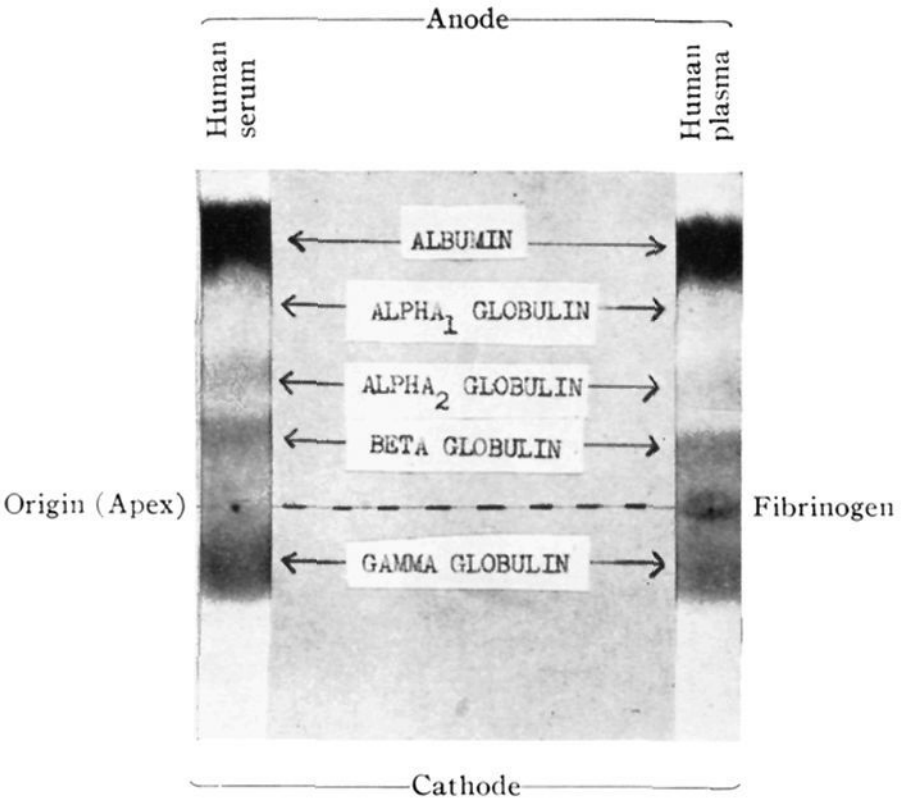


Fig. 10.—Comparison of human plasma and serum patterns (Experiment X): electrolyte, barbiturate buffer (*p*H 8.6), 0.05 *M* sodium diethylbarbiturate, 0.01 *M* diethylbarbituric acid; duration, 180 minutes; current, 0.5 ma./cm. (width); potential, initial 320 volts, final 220 volts; paper, Whatman No. 2 (2 1-cm. strips in series in 2 cells), apex height, 13.5 cm.

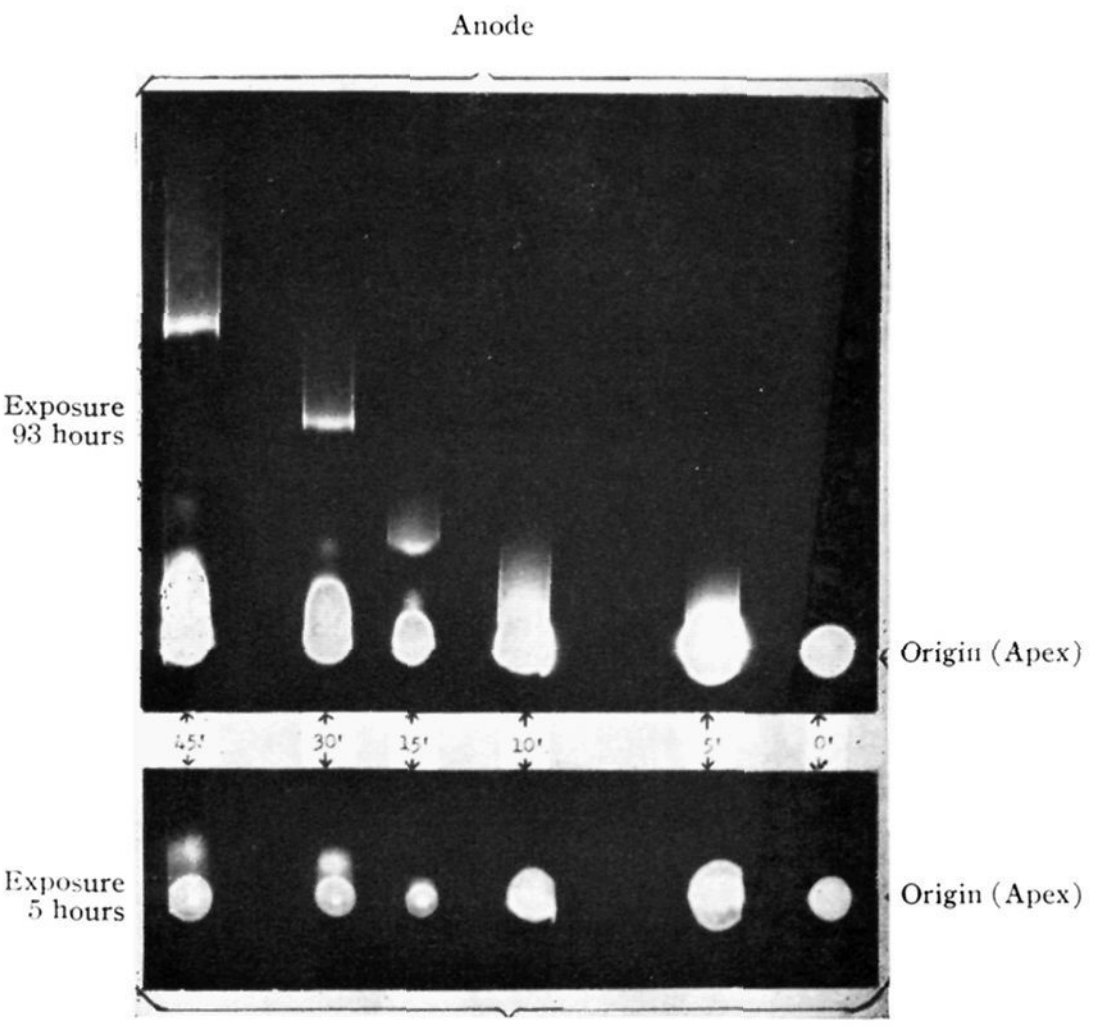


Fig. 11.—Autoradiographs of thyroid separations (Experiment XI): electrolyte, barbiturate buffer (*p*H 8.6), 0.05 *M* sodium diethylbarbiturate, 0.01 *M* diethylbarbituric acid; duration, as indicated above; current, 0.5 ma./cm. (width); potential, 310 volts (average); paper, Whatman No. 2; apex height, 13.5 cm.

paper which was then draped across the glass support rod with the ends dipping about 1 cm. below the surface of the buffer solution in the electrode vessels. The apex of the paper strip was 14.5 cm. above the solution level. When in position, the paper strip was saturated with buffer solution applied to the apex with a medicine dropper. This served to wash from the paper strip any traces of amino acids picked up from the hands in the course of previous manipulations. The top tumbler was put in place and the apparatus allowed to stand for about thirty minutes to permit excess buffer to drain from the paper. About 20 micrograms of amino acid mixture in the form of a dry powder was then applied to the paper strip at the reference mark.

A potential of 600 volts direct current (supplied by a well filtered full wave rectifier) was applied across the carbon electrodes in series with a milliammeter and rheostat. The current was maintained at 1.0 milliampere by frequent adjustment of the rheostat for a period of one hundred and twenty minutes. Then the paper strip was transferred to a glass drying rack using forceps to avoid finger marks and taking care to maintain the apex upward during drying in order to prevent excess buffer at the ends of the paper from running back toward the apex and "smearing" the amino acid zones. The paper strip was dried in an oven at 90° for five minutes, then removed and sprayed with a 0.25% ninhydrin solution in water-saturated butanol (Williams and Kirby[7]) and replaced in the oven for five minutes. The strip showed the following pattern (with all measurements to the center of the spot concerned): (a) toward the anode, 75 mm. from the reference mark (x) a bluish spot corresponding to aspartic acid; 60 mm. from the reference mark, a lavender spot corresponding to glutamic acid; (b) toward the cathode, 4 mm. from the reference mark a dense mauve spot corresponding to the monoamino-monocarboxylic acid group; 23 mm. from the reference mark a grayish spot corresponding to histidine[8] and at 43 mm. a lavender-rose spot corresponding to arginine and lysine which were not completely separated in this experiment. A photograph of the significant portion of this strip is shown in Fig. 2. The initial *p*H was measured with the glass electrode and found to be 5.93. After the experiment was completed (one hundred and twenty minutes), the *p*H of the anode vessel was found to be 5.91 and the cathode 6.01.

To separate a solution rather than dry crystals, the following variation in technique is employed: The paper strip to be used is draped on a glass drying rack after the reference pencil mark is made and washed down by directing several milliliters of distilled water at the apex. The strip is air-dried and, being handled with forceps, inserted into the apparatus as described above. Next, about 0.01 ml. of hydrolysate or protein solution, such as blood serum, is applied to the reference mark (at the apex). Then, very carefully, buffer solution is applied with a medicine dropper *below* the apex of the strip at equal distances from the apex on either side, permitting the buffer to flow upward to the drop position by capillarity. In this manner, the solution is prevented from running down the filter paper as has been found to happen usually if even a minute drop of solution is applied to presaturated though drained paper with a resultant lack of sharpness in the patterns obtained. When this variation is used, it is not necessary to wait more than about ten minutes before applying the potential.

The above variation of technique is illustrated in the following example.

Experiment II.—Separation of human serum: 0.01 ml. of serum was applied from a micro-pipet to the reference mark of a 1-cm. paper strip as described above. Immediately, a 0.05 molar sodium diethylbarbiturate buffer solution (*p*H 8.6) was applied. A potential of about 350 volts was applied through a rheostat for one hundred and eighty minutes. The current was maintained at 0.5 milliampere by frequent adjustment of the rheostat. At the end of the run the strip was removed and dried for five minutes in an oven at 100°, then immersed for five minutes in a saturated solution of mercuric chloride in 95% alcohol to which had been added 0.1 g./100 ml. of brom phenol blue (tetrabromophenolsulfonphthalein). The strip was next removed and washed for ten minutes in running tap water. The strip was then dried. Four distinct blue zones were visible, all located toward the anodal side of the reference mark: the first, 35 mm. from the reference mark, corresponding to albumen; the second at 25 mm., probably corresponding to alpha$_1$-globulin; the third at 15 mm., corresponding to alpha$_2$-globulin and the fourth, 6 mm. from the reference mark, corresponding to beta-globulin. A fifth zone was located 13 mm. toward the cathode corresponding to gamma-globulin. The establishment of identity of these protein zones is discussed later in this paper.

Experiment III.—Separation of a mixture of alanine, valine, proline and tryptophane the apparatus of larger dimensions: in this apparatus, 500 ml. of 5 *N* acetic acid was placed in each electrode vessel. The apex height in this experiment was 11.5 cm. above the fluid level. Five paper strips were supported, washed down and saturated with electrolyte as described above. On one strip, a few micrograms of a mixture of these amino acids were placed at the reference mark (x) and on each of the other strips, one of the amino acids of the mixture was placed at the reference mark. A potential of 580 volts was applied across the carbon electrodes. The initial current was 1.5 milliamperes per 5 cm. (width). After one hundred and twenty minutes, the current had risen to 1.7 milliamperes per 5 cm. At this time, the strips were removed, dried and sprayed with ninhydrin. Portions of the resulting strips are shown in Fig. 3.

Experiment IV.—Separation of a mixture of glycine, isoleucine, phenylalanine and hydroxyproline. In an experiment exactly analogous to Experiment III, a mixture of the above amino acids was separated as illustrated in Fig. 4. (The faint zone on the hydroxyproline strip represents accidental contamination with isoleucine.)

Experiment V.—Separation of glycylglycine from glycyl-*l*-leucine in the apparatus of Experiments III and IV. Five strips were employed, to one of which a mixture of glycylglycine and glycyl-*l*-leucine was applied and to each of the remaining strips only one of these substances. Glycine and *l*-leucine were added to separate strips for comparison. The separation attained at the end of two hours is illustrated in Fig. 5.

Experiment VI.—Reproducibility of parallel runs. The reproducibility of this method on parallel runs is illustrated by Fig. 6 which shows sections of the paper strips obtained in a simultaneous run when a few micrograms of crystalline phenylalanine was applied to the origin of all strips. In this experiment, the electrolyte was 5 *N* acetic acid, the initial current 1.8 milliamperes per 6 cm. and the final current (one hundred and twenty minutes later) 1.7 milliamperes per 6 cm. (Ordinarily, the current has been observed to increase during the course of the runs. Rather marked line voltage fluctuations are sometimes noted which perhaps explain why the final current was recorded lower than the initial value.) The mean position of the phenylalanine was found to be 58.5 mm. from the origin with a standard deviation of ± 3.27 mm. It is evident that the reproducibility of parallel runs is of sufficient degree ordinarily to permit selection of "matching pairs of acids" as, for example, is illustrated in Figs. 3, 4 and 5.

Experiment VII: Rate of Migration of Phenylalanine.—Figure 7 illustrates the findings in an experiment in which the migration of phenylalanine toward the cathode was measured as a function of time. Six strips were set up in parallel with the apparatus previously described, the electrolyte being 5 *N* acetic acid. At thirty-minute intervals

(7) Williams and Kirby, *Science*, **107**, 481 (1948).

(8) It has been observed that *p*H 5.9 gives a good separation of the histidine from the arginine-lysine zone and the monoamino-monocarboxylic zone. As the *p*H is increased, the histidine tends to migrate at a velocity closer to the latter group, merging with it about *p*H 6.6. As the *p*H is decreased, the reverse has been observed with the histidine zone merging with the arginine-lysine zone at about *p*H 5.2.

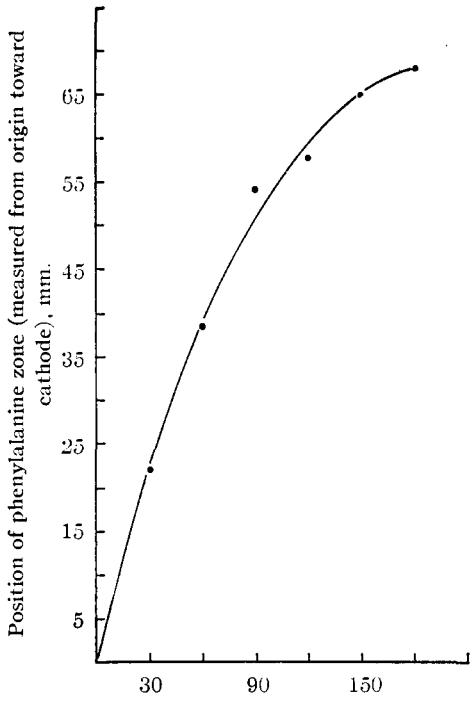


Fig. 7.—Migration of phenylalanine *vs.* time (experiment VII): 5 *N* acetic acid, Whatman No. 2 paper, 580-volt potential, 0.3 ma./cm. (width) average current, apex height 11.5 cm.

strips were removed. During the course of the experiment under a potential of 580 volts, the current per strip averaged 0.3 milliampere. It is evident that the migration of this amino acid down the paper under the conditions of these experiments is not a linear function of time.

Experiment VIII.—Comparison of zones derived from separated electrophoretic components with whole serum. Figure 8 illustrates an experiment in which electrophoretic components separated in a Tiselius apparatus are compared with the whole serum pattern from which these components were derived. In the electrophoretic apparatus of Moore and White,[9] a human serum sample was separated utilizing 0.1 *M* barbiturate buffer (*p*H 8.6, Longsworth[10]) into the following components: (a) albumin (from Zone IV ascending limb of Tiselius cell); (b) a mixture of albumin plus alpha$_1$-globulin (from Zone V); (c) pure gamma-globulin (from Zone I descending limb); (d) a mixture of gamma-globulin plus beta-globulin (from Zone II); (e) a mixture of gamma-, beta- and alpha$_2$-globulins (from Zone III).

Paper patterns of these fractions were prepared under the similar conditions enumerated in Fig. 8. The results of this experiment appear to establish the identity of all the components except alpha$_1$-globulin. It will be noted that in Fig. 8, paper patterns IV and V do not differ appreciably although alpha$_1$-globulin is presumably present in pattern V only. The probable explanation is that since this component is present in such low concentration it is scarcely visible on the whole serum pattern; it is then not surprising that it is not more evident in pattern V which material was diluted in the course of the prior Tiselius separation.

Experiment IX.—Comparison of zones derived from "immune globulin" and reference serum with electrophoretic patterns. A similar analysis of a sample of commercial human "immune globulin" (Squibb) appears to be valid. Figure 9 illustrates an electrophoretic pattern prepared with 0.02 phosphate–0.15 *M* sodium chloride buffer (*p*H 7.4) in comparison with paper strips prepared with 0.05 *M* barbiturate buffer, *p*H 8.6 and 0.02 *M* phosphate buffer, (without added sodium chloride) *p*H 7.6.

The component migrating most rapidly appears to be albumin from comparison with the serum patterns. Superior resolution and correspondence seems in this case to be found with the barbiturate buffer although a greater migration has occurred in shorter time with the phosphate buffer, undoubtedly due to the greater field strength in this experiment.

Experiment X.—Comparison of human plasma and serum patterns. Figure 10 illustrates the patterns obtained in an experiment in which a serum and a heparin-plasma derived from the same sample of human blood were separated on paper with barbiturate buffer. Control experiments have established that heparin does not stain with brom phenol blue under the conditions used in these experiments. Therefore, the zone present near the origin of the plasma pattern but not evident in the serum pattern may be regarded as being derived from fibrinogen. In interpreting this pattern it is difficult to decide with certainty whether the position of the fibrinogen zone is due to its having been coagulated at the point of application (origin), as suggested by the circular configuration and size which has about the same dimensions as the circle resulting from the applied drop of plasma (about 0.01 ml.) at the beginning of the experiment, or is simply an expression of its low rate of diffusion and inherent electrical mobility, since in this pattern the point of origin falls coincidentally at a point intermediate between the gamma and beta globulins. It is of course well known that the fibrinogen boundary falls between these same constituents in conventional electrophoretic patterns obtained with sodium diethylbarbiturate buffer (Longsworth[10]).

Experiment XI.—Separation of radioactive inorganic iodide from protein-bound iodine. A 230-g. Wistar strain rat was injected intraperitoneally with 87 microcuries of I^{131}. The animal was sacrificed 210 minutes later. The thyroid was removed and all possible connective tissue carefully dissected from it. The resulting thyroid was ground in a Ten Broeck tissue grinder together with about 10 drops of 0.9% sodium chloride solution. The resulting material was centrifuged and the clear supernatant fluid applied to the reference marks of strips of filter paper which were separated in 0.05 *M* barbiturate buffer, *p*H 8.6, for various periods of time (from 0 to forty-five minutes) as illustrated in Fig. 11. The resulting strips were dried in an oven for five minutes and then autoradiographs were made of the strips with exposures of five hours and ninty-three hours as indicated. The following points may be noted. In the experiments, a distinct band of radioactivity is visible migrating rapidly toward the anode. This undoubtedly corresponds to inorganic iodide ion and it is seen that, in a comparatively short time, the paper in the zones retaining protein (identified by its property of being coagulated and dyed) is completely "cleared" of inorganic iodide, the residual activity being associated with protein and/or amino acid fractions. The lower photograph (five-hour exposure) is included to show detail in the protein–amino acid zones which is obscured by the longer exposure necessary to show the migration of the iodide ion.

Discussion

In the technique employed in all the experiments illustrated, it is believed that the paper strip plays merely a passive role as a carrier of the electrolyte. It probably may thus be regarded as analogous in a limited sense to the Tiselius cell.

In the course of several hundred experiments, no evidence of adsorptive phenomena has been noted under the experimental conditions em-

(9) Moore and White, *Rev. Sci. Inst.*, **19**, 700 (1948).

(10) Longsworth, *Chem. Revs.*, **30**, 323 (1942).

ployed, in either the case of protein[11] or amino acid separations. That is to say, (allowing for certain factors discussed below) the components seem to behave as they would be expected to in "free solution." Critical studies designed to answer this question have not been carried out and, therefore, the possible role of adsorption in the process must await elucidation. For this reason, it seems best for the time being to regard the separations described as ionophoretic or electrophoretic rather than "electrochromatographic" as are the separations described by Strain,[1a] or as "partition chromatography with applied voltage" as in the process described by Haugaard and Kroner.[5]

It will be realized that under the experimental conditions employed a relatively complicated equilibrium obtains which includes a number of simultaneously occurring processes which include at least the following: (a) migration of ions due to the electrical field; (b) diffusion; (c) electroendosmotic flow; (d) evaporation of water from the paper strip due to heating of the strip incidental to the current flow; (e) hydrodynamic equilibrium on the paper strip between capillary forces and gravitational forces; (f) electrical resistance changes along the length of the paper strip principally due to concentration effects secondary to factors d and e.

Sufficient data for a criticial evaluation of these factors are not available. However, some of the more obvious relationships which appear to explain some of the experimental findings will be discussed briefly. We may consider that the field strength equation which is applied to the Tiselius cell is applicable as a first approximation at least to thin cross sections of the paper at any given level above the electrolyte level at any given instant. Then, migration velocity is proportional to field strength X and

$$X = I/qk_s$$

where I = current; q = cross sectional area of the paper; and k_s = conductivity of the solution on the paper at the cross section under consideration.

Limiting consideration to the case where the current is held constant, the cross section of the paper is constant and, therefore, must at any given level after equilibrium is established "contain" a given quantity of electrolyte. But, since the amount of electrolyte contained along the length of the paper varies due to the hydrodynamic and distillation equilibria mentioned, the "effective cross section" of the paper may be regarded as increasing as the electrolyte level is approached, and as decreasing as the apex is approached, reaching its minimum "effective cross section" at the apex. Therefore, the field strength may be expected to be highest at the apex and to decrease as the electrolyte level in the electrode vessels is approached. The "drier" apex may be expected, therefore, to have more electrical resistance and, for a given current, would be expected to produce more heat than the "wetter" areas below. This factor would be expected to accentuate (or perhaps be principally responsible for) the "wetness gradient" down the paper. The above considerations appear to explain the lack of linearity of migration of ions with time as demonstrated for phenylalanine in Experiment VII (Fig. 7).

It is for the above reasons that the apex height has been recorded in experimental data, it having been observed that reproducibility of the method could not always be achieved unless this factor were carefully controlled, especially with protein separations where a certain optimal "degree of wetness" of the paper for a given current and buffer seems to be essential for satisfactory resolution.

Under the experimental conditions employed, due to the very large surface area-electrolyte volume ratio present in the paper strip, pronounced electroendosmotic currents toward the cathode would be anticipated. It is believed that this explains the apparent migration of the gamma-globulin toward the cathode as illustrated in the serum and plasma patterns (Figs. 8, 9 and 10). In conventional electrophoretic separations at *p*H 8.6, all the serum components are known to migrate to the anode. It is believed that this apparent migration of the gamma globulin toward the cathode can be explained by a displacement of the entire pattern toward the cathode due to this pronounced electroendosmotic current.

In view of the above considerations, a close correlation of the mobilities of protein constituents as measured in the Tiselius apparatus with these paper patterns is not to be expected.

It will be noted that the barbiturate buffer employed in these experiments is 0.05 M as compared with 0.1 M buffer often employed in conventional electrophoretic studies of human sera. It has been observed empirically that the more dilute buffer very much improves the degree of resolution attainable in human serum and plasma samples in the present technique. This may be due to the fact that the concentration of the buffer on the paper is increased above its value in the electrode vessels due to evaporation from the paper and, on the paper, thus approaches a concentration comparable with the optimum concentration observed in Tiselius separations.[12]

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(11) Professor A. Tiselius (personal communication), has evidence that the colored protein phycoerythrin from the alga *Ceramium rubrum* is adsorbed on paper under conditions similar to those described in this report.

(12) Since the present paper was submitted for publication, independent work by Wieland and Fischer, *Naturwissenschaften*, **35**, 29 (1948), has been brought to the author's attention. These workers report ionophoretic separations on filter paper saturated with acetate buffer and reported that at *p*H 5 they were able to separate a mixture of glutamic acid, alanine and histidine, at *p*H 7.5, a mixture of lysine and histidine, and at *p*H 3.7 glutamic acid and aspartic acid.

Summary

1. A micro-technique for the separation of amino acids, peptides and proteins has been developed.

2. The technique is carried out by applying an electrical potential across the ends of strips of filter paper saturated with electrolyte solution. At some intermediate position of these strips, the mixture to be separated is applied.

3. The course of separations is followed in the case of amino acids and peptides by ninhydrin treatment; in the case of protein separations by coagulation and selective dyeing *in situ* and in the case of radioactive components by autoradiography.

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[CONTRIBUTION FROM THE RADIATION LABORATORY, DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Some New β-Diketones Containing the Trifluoromethyl Group[1a]

BY J. C. REID[1b] AND M. CALVIN

In the course of studies on the properties of β-diketones, it was desired to investigate effects of the trifluoromethyl group in such molecules. Accordingly, a number have been prepared of the type $RCOCH(R')COCF_3$. These are described in Table I. Copper chelates and oximes which were prepared from them as characterizing derivatives, are also described there.

All the diketones but one were prepared by the Claisen condensation between ethyl trifluoroacetate[2] and a series of alkyl methyl and aryl methyl ketones, with excellent yields.

The exception, 1-acetyl-1-trifluoroacetylethane, was prepared by methylating the sodium salt of acetyltrifluoracetone with methyl iodide. The yield in this step was very low.

On four of the compounds, measurements of the rate and equilibrium constants and the heat of activation for the keto ⇆ enol process were made, using the bromine titration method to obtain the rate of enolization and extrapolating to zero time to obtain the equilibrium constant.

Experimental[3]

Synthesis of the Diketones

The Claisen condensation was carried out using standard technique.[4] To a suspension of 1.05 mole of commercial sodium methoxide in 100 cc. of dry ether[5] was added dropwise 1.00 mole of ethyl trifluoracetate with stirring, which was followed by 1.00 mole of the selected methyl ketone. The mixture was allowed to stand overnight to complete the reaction, then worked up as described below for the individual ketones.

Acetyltrifluoroacetone.[6]—This compound is not new; however, the procedure used for working up the reaction mixture is described here as it represents a modification of that used by Henne, *et al.*[6b] Ethyl trifluoroacetate was condensed with acetone and the reaction mixture was evaporated to dryness at reduced pressure (oil pump) then heated at 90° for twelve hours to remove alcohol from the sodium salt. If this was not done, an azeotrope was encountered in the subsequent distillation which reduced the amount of product obtained in pure form. The salt was then stirred with one mole of 10% sulfuric acid to liberate the diketone, which was drawn off. Product remaining in the water phase was extracted with ether. After drying with calcium chloride[7] and distilling, a yield of 80% of purified product was obtained. The diketone in the tailings could be recovered by making the copper chelate, purifying this and releasing the ketone either by steam distillation from 10% sulfuric acid or by treatment in ether solution with hydrogen sulfide.

Propionyltrifluoroacetone, isovaleroyltrifluoroacetone, 1-propionyl-1-trifluoroacetylethane and **2-naphthoyltrifluoroacetone** were prepared from ethyl methyl ketone, methyl isobutyl ketone, diethyl ketone and 2-naphthyl methyl ketone, respectively, in the same way as acetyltrifluoroacetone.

2-Naphthoyltrifluoroacetone, which is a solid was separated directly from the acidified sodium salt and purified by crystallization from dilute ethanol.

With diethyl ketone the reaction was slower than usual and forty-eight hours was allowed for completion.

That the condensation with ethyl methyl ketone occurred at the α-methyl carbon rather than at either carbon of the ethyl group was shown by demonstrating that the product formed a copper chelate and that this was different from the one obtained from the diketone formed by direct methylation of acetyltrifluoroacetone (see Table I).

Heptoyltrifluoroacetone, benzoyltrifluoroacetone, *p*-fluorobenzoyltrifluoroacetone and **2-furoyltrifluoroacetone** were prepared from *n*-hexyl methyl ketone, acetophenone, *p*-fluoroacetophenone and 2-furyl methyl ketone, respectively. The reaction mixture was worked up by releasing the diketone with one mole of 10% sulfuric acid and converting it to the copper chelate. After drying, the chelate was subjected to steam distillation to remove volatile impurities. Sulfuric acid was then added to bring the concentration to 10% and the diketone was obtained by further steam distillation. After separation from the water in the distillate it was dried with calcium chloride and distilled at reduced pressure if liquid or crystallized from dilute ethanol if solid.

(1a) Work described in this paper was sponsored by the Atomic Energy Commission.

(1b) National Cancer Institute, National Institutes of Health, Bethesda 14, Maryland.

(2) (a) Henne, Alderson and Newman, THIS JOURNAL, **67**, 918 (1945); (b) Henne and Trott, *ibid.*, **69**, 1820 (1947); (c) Reid, *ibid.*, **69**, 2069 (1947).

(3) All melting points are corrected except as noted.

(4) (a) "Organic Reactions," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1942, p. 266. (b) Henne, Newman, Quill and Staniforth, THIS JOURNAL, **69**, 1819 (1947).

(5) Benzene also serves well as a solvent.

(6) Only the oxime of this compound is new (see ref. 5b). The ketone and its chelate are listed for convenience.

(7) A considerable amount of water is not removed by the calcium chloride and forms a water–ketone azeotrope in the distillation, which lowers the yield of pure product. This azeotrope separates into two phases on condensation and when preparing large batches, it is advantageous to utilize this behavior to dry the product by using a separating distilling head which takes off the water-rich (upper) layer and returns the ketone-rich phase to the still.