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EXPERIMENTS ON ELECTROPHORESIS IN SEGMENTAL SYSTEMS COMPOSED OF POLYURETHANE FOAM

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SUMMARY

The use of foamed polyurethane sponge as a supporting medium in electrophoresis has been investigated. Segments of sponge were cut to fill horizontal and vertical cells which are described. The use of the described apparatus is recommended because of the ease of recovery of a product free from contamination and because of the latitude afforded in investigating migration through heterogeneous pathways. Thus, these cells were used in studies on zone electrophoresis and isoelectric migration employing serum, albumin and preparations of human prostatic acid phosphatase. The applicability of the segmental sponge system to electrophoretic studies requiring either one or simultaneously several gradients such as density, pH and/or ionic strength has been demonstrated.

INTRODUCTION

Preliminary efforts to isolate human prostatic acid phosphatase by electrophoresis with starch gel as the supporting medium gave a product heavily contaminated with

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soluble starch and protein. In an attempt to avoid such contamination it was decided to investigate the possibility of substituting an inert flexible plastic foam for the starch gel. Besides avoiding the introduction of extraneous matter, the spongy matrix offered the additional advantage of permitting electrophoresis in a segmental system. Such a system composed of a series of contiguous segments of sponge could be used to study migration not only within a medium of uniform composition but also along a pathway of heterogeneous composition. With a cell so constructed, it should be possible to study without difficulty the influence of variables like pH, ionic strength and density separately or simultaneously. The fact that an uncontaminated product might be easily recovered by squeezing out the contents of each segment separately opened these attractive possibilities to investigation. Accordingly experiments have been done to explore these possibilities and the results which demonstrate the potentialities of polyurethane foam in such investigations are reported in detail. Preliminary reports of this work have appeared[1,2].

MATERIALS AND METHODS

Polyurethane foam

The plastic foam used in these experiments was preferably of the smallest pore size stocked by the National Foam and Rubber Co., Boston, Mass. Rectangular segments could be cut by hand most conveniently from the dry plastic with a Stadie blade. Disks of foam were stamped out of sheeting by the Palais Die Cut-Outs Company, Boston, Mass.

Horizontal cell

Several different horizontal cells were employed. The standard cell (Fig. 1, A) was made from five strips of 0.318 cm thick Plexiglass of the following dimensions: one
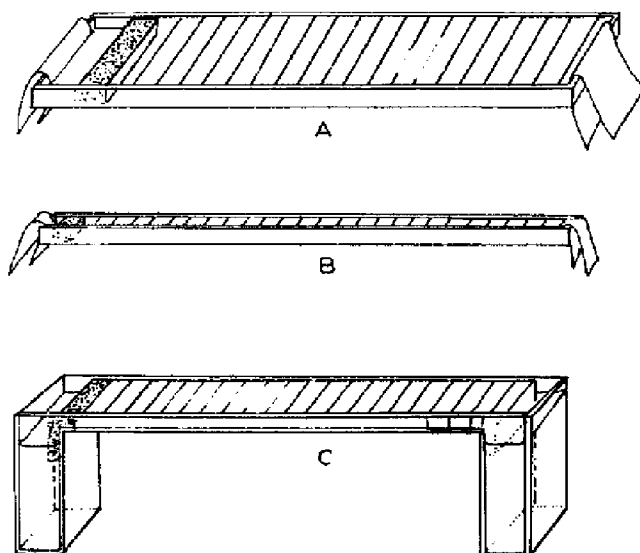

Fig. 1. Horizontal cells.

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piece 30.4 × 5.08 cm, two pieces 30.4 × 1.27 cm, and two pieces 5.08 × 1.27 cm. The above pieces were cemented together with Plexiglass Cement (Transplastics Co. of Boston) to form a trough measuring 30.4 cm long × 4.34 cm wide × 1.27 cm high. The two small pieces on the end were attached 0.2 cm above the level of the base of the trough so as to accommodate a loop of filter paper (Whatman 3). The wet paper served as a bridge to the buffer in a 250-ml beaker containing a silver chloride electrode (Perkin-Elmer). In addition to the electrode each buffer vessel contained 20 ml of a solution of 12 % sodium chloride in water which was layered beneath 200 ml of buffer solution. Some experiments were made using a cell with a trough 40 cm long × 4.05 cm wide × 1.27 cm high (Fig. 1, C). The latter cell was of one piece with its electrode vessels. The end sections of sponge were cut in the shape of the letter L, one end of which dipped into the buffer solution of the electrode vessels, thus replacing the filter paper bridges. For work with micro amounts of protein, a horizontal cell (Fig. 1, B) with a trough measuring 30.4 cm × 0.5 cm × 1.0 cm was also constructed*.

The individual segments of sponge were filled by holding them below the surface of the liquid and repeatedly squeezing them to remove as much trapped air as possible. When a number of segments were to be filled with the same buffer, it was more convenient to put them into a beaker of buffer solution and heat the mixture to boiling. After the air had been driven out by this means, the sections of sponge readily filled with liquid on cooling. In assembling the cell, first the paper bridges wetted with buffer solution were looped over the end pieces and then the central section was built up by filling the trough with the loaded segments of flexible polyurethane foam. The segment containing the solution of protein was generally added last after temperature equilibrium had been reached.

Next, the horizontal cell was placed on a test tube rack in the refrigerator, the bridges of filter paper were dipped into the buffer vessels, and the central section holding the sponges was covered with a piece of glass. Leads from an external constant voltage power supply (Heathkit Co. or Research Specialties Co.) were clipped to the silver chloride electrodes and the temperature of the refrigerator was kept at 5°.

To empty the horizontal cell at the end of the run the individual sponges were lifted out with a spatula and slid into a syringe. The contents of the sponge were then squeezed out into a numbered graduated conical centrifuge tube. The last trace of the contents could be removed by rinsing the section with fresh solution. A small amount of debris torn from the sponge readily settled on centrifugation.

Vertical cell

The vertical apparatus (Fig. 2) consisted first of a 250 ml Erlenmeyer flask which served as one electrode vessel. The flask was fitted with a two-holed rubber stopper in one hole of which was a silver chloride electrode sealed with Pyseal Cement (Macalaster). The second hole contained a glass tube topped by the inner connection of a standard taper ground glass joint (size 29/26). The inner connection had been fitted originally with a perforated plate. The central portion of this plate had been cut out with a vibrating tool to leave only an inner rim which served to support the column of sponges.

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* The micro cell was used by O. S. PETTENGILL of our laboratory in the purification of urinary β-glucuronidase of mouse.
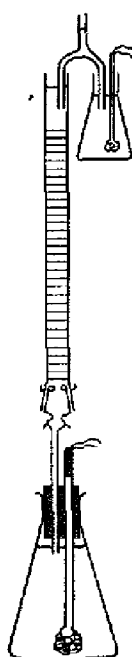
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Assembly of the cell was begun first with the lower electrode vessel. Thus, buffer solution was put into the Erlenmeyer flask so that with the stopper in place liquid was just forced upward into the glass tube. Then 20 ml of 12 % NaCl were introduced through the joint with a long needle reaching to the bottom of the flask. If the level of liquid now did not cover the sponge supporting rim, after the contents had been cooled to 5°, additional buffer solution was added as required.

Next, a glass column 23 cm long with a matching 29/26 standard taper joint was placed securely on the inner piece and then filled with disks of sponge containing liquid of the desired composition. The column of buffer extending from the interior of the flask to the top of the inner connection served as a bridge between the lower electrode vessel and the sponges.

Certain precautions had to be observed in assembling and disassembling the column of sponges in the vertical cell. Because the vertical cell is basically only a column of liquid, it was necessary to add additional liquid after inserting each disk of sponge in order to raise the level of liquid in the column to the upper edge of the disk. The additional liquid was needed to compensate for the space not filled by the sponge and its contents as well as for whatever liquid was lost from the sponge as it brushed against the inner wall of the glass column during insertion. In

Fig. 2.
Vertical cell.

this connection, it was also necessary to wipe off the inner wall occasionally in order not to contaminate succeeding sections that were to be placed higher up in the cell. At the end of the run the contents of each disk were collected separately. Most of the liquid from each section was expressed within the column *in situ* so that the segment of sponge was moderately dry before withdrawal. This procedure was adopted to avoid losses that always occurred when a liquid-laden section touched the inner wall of the glass column as the sponge was being withdrawn. The expressed liquid remaining in the column was aspirated by a syringe fitted with a long needle and was added to whatever residual liquid could be squeezed from the withdrawn segment. A convenient tool used both in setting up and taking down the column was a piece of glass tubing 50 cm long, one end of which was closed off as a small sharp hook for manipulating the disks while the other held a wad of absorbent paper for drying the inside wall of the column.

After the requisite number of sponges had been inserted, the rest of the column was filled with buffer solution. At this point in the procedure the apparatus was placed in the refrigerator and clamped to a ring stand for support. A second Erlenmeyer flask containing buffer and salt solutions and a silver chloride electrode was placed adjacent to the top of the column and supported on a second stand. The bridge between the liquid at the top of the column and that in the upper electrode was established by simultaneously drawing buffer solution into two arms of a U-tube from the column on one side and from the flask on the other. When the intersection of the U-tube had filled with buffer solution and the bridge was formed, the third arm was clamped shut thus holding the liquid bridge in place.

Experimental material

Studies were carried out with pooled human serum, bovine crystalline albumin

(Armour) and preparations of human prostatic phosphatase at various levels of purity. The better preparations containing phosphatase were 50 to 100 times more active per mg than the initial extract of prostate gland from which they had been isolated[3]. Solutions of proteins were dialyzed against the appropriate buffer solution for at least 18 h before electrophoresis.

The buffer solutions listed below were used in the experiments with serum. The composition of the buffer solutions used in other experiments is given most conveniently in the description of the relevant experiment.

Veronal-sodium citrate, 0.1 ionic strength, pH 8.7

One liter of buffer solution contained 12.37 g of sodium barbital, 1.47 g of sodium citrate·2 H_2O and 0.521 g of citric acid·1 H_2O and water. This buffer was employed for the standard boundary electrophoresis (Figs. 9 and 10).

Tris-citrate, 0.1 ionic strength, pH 8.8

One 1 contained 30.33 g tris-(hydroxymethyl)-aminomethane, 3.5 g citric acid·1 H_2O and water*. This buffer diluted 3 parts to 7 of water was employed in the sponge electrophoresis of serum (Fig. 3). Protein was determined turbidimetrically by the method of KUNITZ[1] and acid phosphatase by the phenol liberated from phenyl phosphate under conditions described elsewhere[3].

Horizontal zone electrophoresis of serum

The distribution of protein from pooled human serum at the end of a run lasting 21.5 h is shown in Fig. 3. The voltage was gradually lowered during the first 10 h of the run in order to maintain a current of 7 mA. For this reason, the strength of the field is expressed in terms of the V-h unit which amounted to about 5800 in this case. The current rose to 50 mA by the end of the run, probably because of diffusion of salt into the cell proper from the electrode vessels.

The protein pattern was reminiscent of the one generally seen with normal human serum in boundary electrophoresis. Although no proteins migrated into the electrode vessels some moved onto the anodic filter paper bridge and accounted for the peak in advance of the major one.

Other experiments on the migration of serum in the horizontal cell indicated that the distribution of protein was determined by a combination of influences. For instance, under the same conditions as those obtaining in the experiment of Fig. 3 more protein could be retained near the cathodic side simply by disturbing the salt layer in the cathodic vessel and thus hastening diffusion of salt into the cell. The resulting increase in conductivity tended to retard migration of the protein in that vicinity. Similar effects could be induced at the other end of the cell by stirring up the salt solution in the anodic vessel.

In addition peaking of protein has been observed under other circumstances. When two contiguous segments of sponge were of different porosities and neither was completely filled, liquid was drawn by capillary action into the segment with the greater porosity (smaller pores). Although two such contiguous segments may have contained solutions at the same concentration of protein, more protein was found in the

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* Abbreviations used in this paper are Tris for tris(hydroxymethyl)-aminomethane and SA for specific activity (units of acid phosphatase activity/mg of protein).
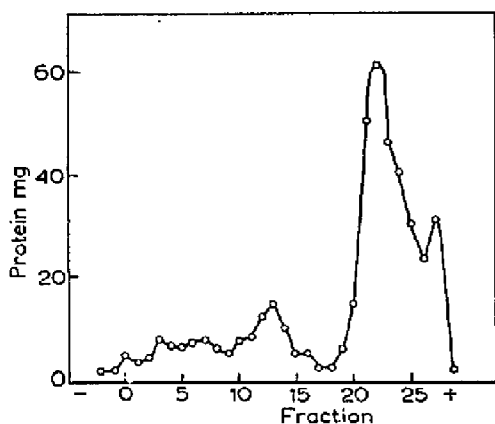
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Fig. 3. Horizontal zone electrophoresis of pooled human serum. 400 mg of protein in 5 ml of serum dialyzed against 0.03 ionic strength Tris–citrate, pH 8.8, were placed in a section of sponge 5 cm from the cathodic edge of the cell. The rest of the sponges contained 0.03 ionic strength Tris–citrate buffer. Total fields, 5800 V-h. Each fraction represents a segment 1 cm in width.
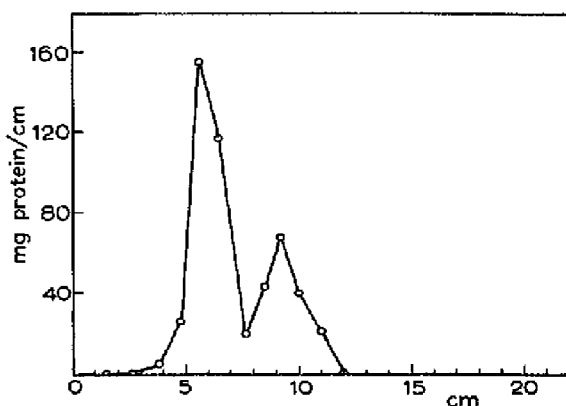
Fig. 4. Distribution of serum protein after electrophoresis on moist sponge. 4 ml of serum were placed in a segment at 5.9–6.5 cm from the anodic end of the horizontal cell. The rest of the sponges were moistened with 0.083-μ Tris–citrate, pH 8.8. 500 V were applied for 45 h at 2 mA.

segment with the smaller pores because it held the greater volume of liquid. This phenomenon would sometimes account for the peaks and depressions observed in a plot of total protein per sponge against mg of protein/cm and could be avoided by using sponge of uniform capillarity throughout the length of the cell. However, it is conceivable that the principle of this phenomenon may be used to introduce liquid constrictions into the cell without having to change the shape of the horizontal apparatus.

An additional feature that had to be taken into account especially in the electrophoresis of undiluted serum was the movement of material simply because of differences in density of solutions in contiguous segments. By using sucrose to equalize the density of the buffer solution with that of serum it was possible to decrease the spreading of dense solutions of protein often observed in aged sponge. In this connection, better control of spreading appeared possible in a vertical column stabilized by a density gradient, vide infra.

Diffusion from the starting segment of dense solutions of protein began immediately in old sponge. Such movement without the application of an electric field seemed to be inhibited in new or unused polyurethane foam probably because of the hydrophobic finish on the surface of the material. Microscopic observations have shown that after repeated use, the plastic material contained fewer cell facings and fewer residual, unfoamed particles of polyurethane than fresh foam. Also with reference to diffusion it was observed in electrophoresis of serum on new sponge that had been moistened but not filled with buffer that even after 45 h at 2 mA the spread of serum was minimal and protein remained in two sharp peaks close to the origin (Fig. 4). This shows that the protein would not move as a result of diffusion in unfilled sponges.

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Zone electrophoresis of acid phosphatase across a pH gradient

Migration horizontally along a pathway in which the concentration of hydrogen ion of the liquid varied from section to section was carried out using a preparation containing acid phosphatase (Fig. 5). The sections were filled with acetate buffers ranging from pH 4.0 to pH 6.0 but all 0.03 M in sodium acetate. Besides the pH gradient there was therefore, a second gradient of acetate concentration at the start of the run. The enzyme at the beginning was placed at pH 4.4 close to the region of maximum concentration of acetate which was at pH 4.0. The current was allowed to flow in the direction of increasing pH and decreasing concentration of acetate. Most of the protein migrated along with the activity away from the starting point at pH 4.4 but material found at high acetate concentration and at pH 4.15 was observed to have more than twice the initial specific activity (units/mg). It was also noted that the pH decreased across the gradient during the course of the run. Although the pH did not fall below 4.0 at any point, the range of the gradient narrowed during the run and at the end of experiment extended only from pH 4.0 to pH 5.2 within a region of the cell now occupied by protein. About 85% of the original activity was recovered.

As a consequence of finding that the protein richest in enzyme was located in the vicinity of high acetate concentration at pH 4.15, the electrophoretic separation
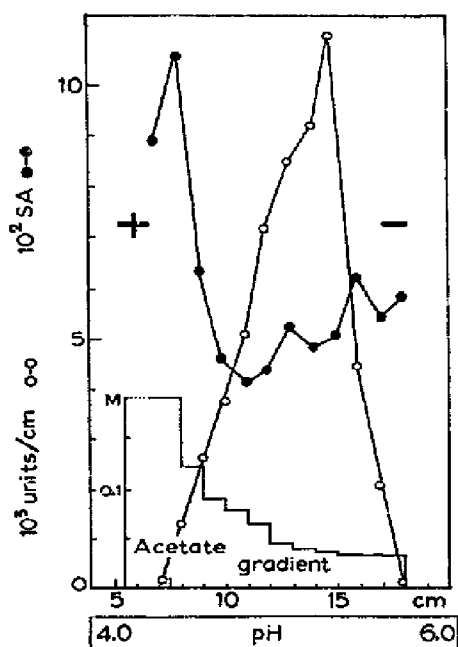

Fig. 5. Zone electrophoresis of a preparation containing prostatic acid phosphatase across a pH gradient made with acetate buffer solutions. 3.7 ml with 220 mg of protein at 310 units/mg at pH 4.4 were placed at 9.5–10 cm from the cathodic end. 160 V were applied for 18 h with current rising from 8 to 16 mA.
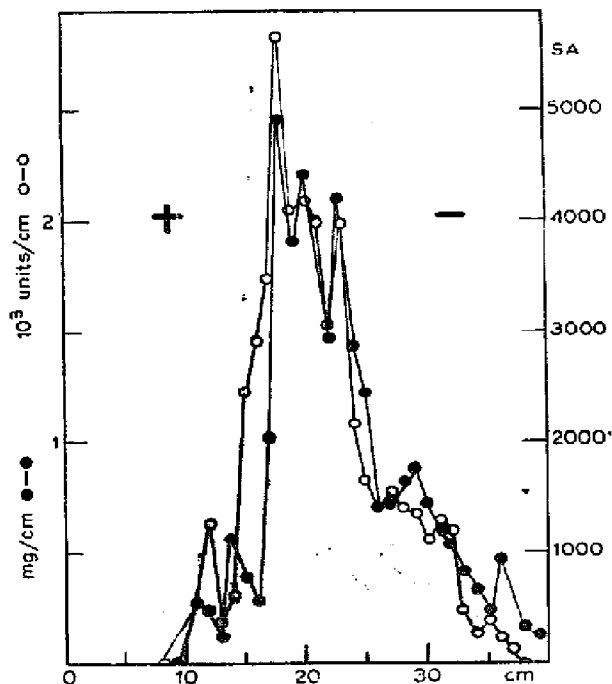
Fig. 6. A preparation of human prostatic acid phosphatase with 13.3 mg of protein at 1500 units/mg of protein was placed in a 0.5 cm wide section of sponge 11 cm from the anode. Sponges from 0 to 10 cm and from 31 to 49 cm contained 0.02 M acetate buffer solution at pH 4.15. All other sponges contained 0.2 M acetate buffer at pH 4.15. 300 V at an initial current of 2.2 mA were applied for 17 h. Final current was 4.0 mA. The shaded area indicates the specific activity (SA).

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of enzyme in such a buffer solution was next attempted. A preparation with 1510
units/mg of protein served as starting material for this experiment. The sponges were
filled with an acetic acid–sodium acetate buffer which was prepared by bringing a
mixture of 10 ml of 3 M sodium acetate and 140 ml of M acetic acid to 1 l with water.
Although here, also, much of the activity moved ahead with the non-enzymic
protein, a fraction was recovered containing protein with a specific activity of 5000,
a level of purity hitherto unequaled (Fig. 6). 83% of the activity was recovered.

Isoelectric concentration of protein in the horizontal cell, (Fig. 7)

The principle underlying separation in a combined pH and potential gradient is
that when the current flows in the direction of increasing pH, proteins within the field
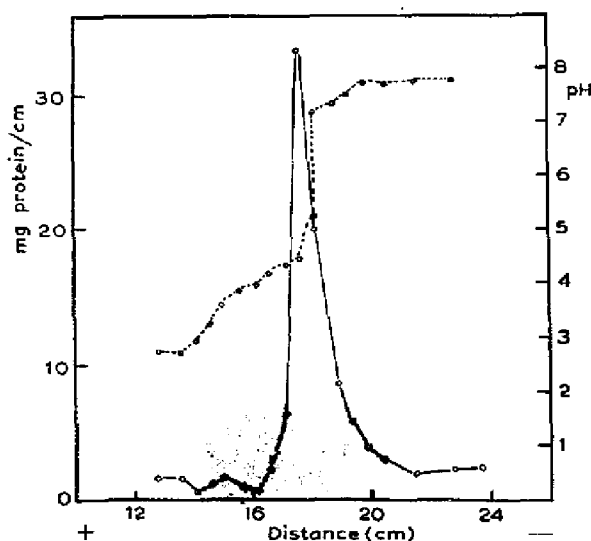migrate to and remain at the pH corresponding to their respective isoelectric points.

Fig. 7. Isoelectric concentration of albumin. Bovine crystalline albumin at 6.8 mg/cm (shaded area)
was placed at the start over a pH range of 4–8. The solid line shows the distribution of albumin
after 2 h at 250 V, 20–25 mA. The broken line shows the pH at the end of the run.

This technique has been applied by TISELIUS[5] and by KOLIN[6] for the separation of
mixtures in free solution and by HOCH AND BARR[7] and by McDONALD AND WILLIAMSON[8]
in filter paper.

In order to test this principle in the horizontal sponge apparatus experiments
were done with albumin. Thus in a typical experiment approximately 100 mg of
crystalline bovine plasma albumin (Armour) in 25 ml were dialyzed overnight at 5°
against distilled water. Fifteen 2-ml portions containing 7.12 mg of albumin were each
mixed with 1 ml of a combination of two buffer solutions of KOLIN (B and C) so as to
obtain solutions of 3 ml of albumin over a range of pH from 4 to 8. Solution B
contained 300 ml 0.1 N HCl, 60 ml H_2O and 100 ml of Solution A per liter. Solution A
consisted of 19.43 g sodium acetate·3 H_2O and 29.43 g sodium barbital in water in a
final volume of 1 l. Solution C was prepared by mixing 800 ml of Solution A with
200 ml of Solution B.

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The anode electrode vessel contained 200 ml of B and 20 ml of a solution of 12% NaCl in water; the cathode, 200 ml of A and 20 ml of 12% NaCl solution. Sponge segments of the first 14 cm of the cell on the anodic side contained Solution B. Segments of 0.5 cm from 14 cm to 21 cm were each filled with the albumin-containing solutions arranged in order of increasing pH. The remainder of the cell on the cathodic side was filled with segments containing Solution C. In such a system, the albumin moved from both sides of the pH range to peak at a position 17.8 cm from the anode. The pH of the solution at the position of the peak of protein was 4.4 (Fig. 7).

A similar experiment was carried out with the center section containing buffered solutions of a preparation of human prostatic acid phosphatase assaying 1730 units/mg of protein (Fig. 8). Under the conditions of this experiment employing the same buffers as in the previous experiment the protein and the activity were distributed in two major peaks. Protein of the highest specific activity (twice that of the starting material) was not associated with these two peaks.
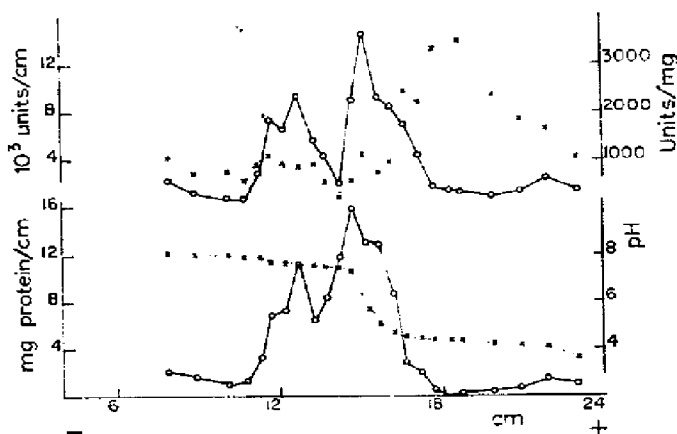

Fig. 8. Isoelectric fractionation of human prostatic proteins in veronal–acetate–HCl buffers. Protein was placed in the segments from 12 to 18 cm. 200 V were applied for 5 h. In the upper graphs the solid line shows the distribution of units; the dotted line, the specific activity. In the lower graphs, the solid line shows the distribution of protein; the dotted line, the final pH. Some protein was lost in an insoluble precipitate that concentrated at 16 cm.

Vertical zone electrophoresis of serum

With the vertical apparatus (Fig. 2) particular attention had to be paid to differences in density that exist at the start or that might develop owing to the concentrating of a component during the course of the run. Therefore, care had to be taken to set up the column in such a manner not only that solutions of lesser density were placed above denser ones, but that the gradient was maintained throughout the run. Because the design of the apparatus permitted placement of the test material anywhere in the column, the protein may be so placed as to migrate upwards through a negative or downwards through a positive density gradient. The subject of density gradient electrophoresis in free solution has been explored in detail elsewhere[9-11].

Fig. 9 shows the distribution of protein from pooled human serum which was placed near the bottom of the column and allowed to migrate upwards in the direction of decreasing density. In addition to the density gradient the ionic strength increased

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in the same direction so that the protein moved up through a region of increasing conductivity. The effect of employing a gradient of ionic strength which increased in the direction of migration was to retard the advance of the fastest components. At the end of the experiment, boundary patterns were obtained on the material isolated from the starting point and from the forward edge of the migration. The difference in the patterns indicated that true electrophoretic separation had occurred.
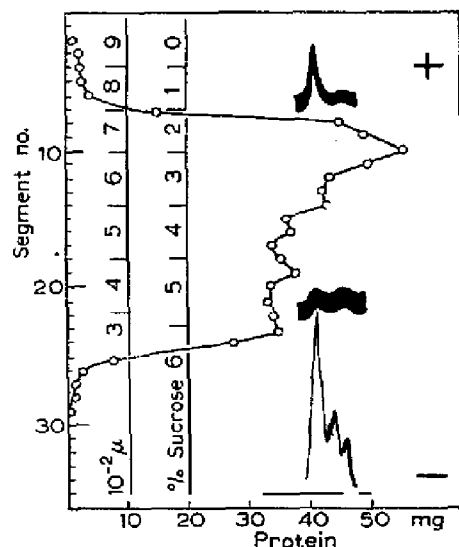

Fig. 9. Upward migration of serum in the vertical cell through gradients of ionic strength and density. Segment No. 23 originally contained 6.75 ml of pooled serum that had been dialy ed against 0.03-n veronal-citrate at pH 8.7. Applied voltage was from 365 to 400 V for 39 h (15,000 V-h); the current, 15-19 mA. Boundary patterns are shown for pooled human serum (bottom) and for the contents of segments No. 23 (middle) and No. 8 (top) at the end of the run.
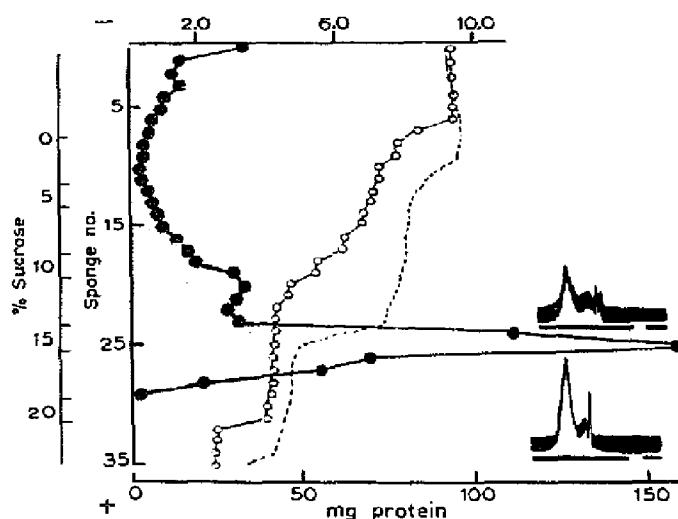

Fig. 10. Isoelectric fractionation of human serum in gradients of pH and density. Distribution of protein is indicated at the start by the shaded area and at end by the solid circles. Open circles indicate initial pH; dotted line equals final pH. Boundary patterns are given for the contents of segments No. 25 (lower) and 24 (upper).

Isoelectric concentration and fractionation of serum proteins in the vertical cell

In the experiment shown in Fig. 10, a steeper density gradient was used in order to accommodate the high protein concentration expected at the point where the serum albumin might accumulate. The pH range of the buffers and their composition in this experiment were the same as had been used in previous horizontal isoelectric concentration experiments, *vide supra*, except that the buffer solutions here contained varying amounts of sucrose. Protein precipitated in the region where it was expected to and actually did concentrate. This precipitate dissolved easily upon addition of a few ml of 0.9% NaCl. Boundary electrophoresis patterns even of the adjacent fractions, 25 and 24, showed differences. No sponge contained protein corresponding to the serum gamma globulin fraction. It was suspected that the gamma globulin must have moved out of the cell into the cathodic electrodes vessel, which, in fact, was found to contain a substantial amount of protein.

DISCUSSION

These investigations were undertaken in an effort to avoid contamination that resulted from the use of starch gel as a supporting medium in the purification of enzymes by zone electrophoresis. Extracts of the gel alone gave a positive reaction in the Lowry[12] determination of protein as well as a positive iodine test for starch. The substitution of a supporting medium made of polyurethane foam eliminated the problem of contamination of the product with starch and foreign protein and at the same time introduced certain other advantages inherent in the properties of the plastic material especially when arranged in segments.

One major advantage stemming from the use of pre-cut segments of flexible foam is the ease of recovery of each fraction at the end of the run. In addition once a set of sponges had been cut to fit a cell, the set could be used repeatedly. The most attractive feature of working with this material, however, was the latitude it afforded in designing cells that varied in composition from section to section. Accordingly, it was possible to carry out electrophoretic experiments not only in the conventional manner in a medium of constant buffer composition throughout the cell but also in cells in which, for instance, the pH, ionic strength and density were varied at will along the path of expected migration. It thus became possible to apply the segmental cell to the study of the electrophoretic behavior of mixtures of proteins when subjected to the influence of one or more gradients of these three types.

The sponge system was especially useful for performing experiments based on the technique of isoelectric fractionation which requires a pH gradient. The rather considerable concentration of protein which occurs as a consequence of the isoelectric technique is an attractive feature where the objective is simply one of concentrating dilute protein solution.

From these experiments it is recommended that when dense solutions are investigated electrophoretically in the sponge system, the zones should be stabilized by employing a density gradient. This was done conveniently in the vertical cell. This objective is met also by the apparatus of Svensson[11]. It should be pointed out that the problem of droplets that form during zone electrophoresis in a sucrose density gradient (Brakke[10]) was not encountered in the sponge system.

The merits of foamed polymers other than the polyurethanes available to us

have not been explored here. Besides the wide variety of polyurethane foams, there are other materials such as polyether and rubber which might offer unique advantages in electrophoretic studies especially in view of the possibility of synthesizing them to conform to the special requirements of electrophoresis investigations. MITCHELL AND HERZENBERG[13] have reported on the use of foam rubber in zone electrophoresis and very recently WILLS[14] has reported on the purification of hog pancreatic lipase using zone electrophoresis on a flexible plastic foam.

It should be noted that electrophoresis through a supporting medium of plastic foam involving migration through the holes of a lattice is quite different from electrophoresis through a medium of packed particles (e.g., starch granules). The sorption effects so pronounced in the latter process which may or may not assist the resolution of the components in the mixture should be minimal in an essentially open system. It is conceivable, however, that with a sponge matrix of the appropriate chemical composition beneficial sorption effects may be reintroduced into the system without sacrificing other desirable ones stemming from the peculiar features of the sponge material in electrophoresis.

Finally it would appear that the segmental sponge system would offer some additional advantage should the investigation require the use of multiple gradients or especially one or more regions of marked discontinuity of composition within a density gradient. In this regard it is not unlikely that one might incorporate adsorbents or ion exchange materials within one of the segments of sponge of the column if such an inclusion promised to promote the isolation of a desired product.

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