A MODIFIED METHOD FOR PROTEIN SEPARATION BY ZONE ELECTROPHORESIS ON A STARCH GEL*

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In the present paper (1) a method of preparative zone electrophoresis (cf. 2, 3) is described in which a stiff starch gel with only 7 to 8 per cent of solid constituents is used as a supporting medium. Two simple devices will be described, one for the fractionation of 2 ml. of protein solution and the other for processing up to 50 ml. of solution, containing 5 to 10 per cent of protein mixture. Both devices have been used successfully in our laboratory, and some of the results obtained with them, in particular the fractionation of plasma proteins from tumor-bearing mice, will be presented in this communication.

EXPERIMENTAL

Mouse Plasma—Citrated blood from Sarcoma 180-bearing mice of the C57BL/6 strain was collected as described elsewhere (4). The plasma obtained from pooled blood was lyophilized, and the solid residue was redissolved in an amount of water equal to about half of the volume of the original plasma. This concentrated plasma contained 60 to 80 mg. of protein per ml. and was dialyzed at 0° for 48 hours against Tris (tris(hydroxymethyl)aminomethane)-citrate buffer, pH 8.8, and ionic strength 0.03, prepared by dissolving 18.3 gm. of Tris and 2.1 gm. of citric acid monohydrate in 2000 ml. of water. The buffer solution was renewed several times during the dialysis.

Starch Gel—The quantities indicated in this paragraph are those used for the preparation of the supporting medium in the small scale apparatus. 20 times the given quantities were used for the large size cell. To a boiling mixture of 30 ml. of water and 100 ml. of a Tris-citrate buffer, pH 8.8, ionic strength 0.06 (containing 18.3 gm. of tris(hydroxymethyl)aminomethane and 2.1 gm. of citric acid monohydrate per liter), a concentrated slurry of recently prepared corn amylose containing a total of 3 gm. of

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carbohydrate was added. The amylose had been prepared according to Schoch et al. (5, 6), and the carbohydrate content of the slurry had been determined on each batch of amylose by measuring the dry weight at 110° (between 5 and 10 per cent). Immediately after the addition of amylose, enough water was added to the mixture to complete the volume to 190 ml.; then 6 gm. of Hyflo Super Cel and a suspension of 6 gm. of a commercial corn-starch in 10 ml. of water were introduced slowly with moderate stirring. After boiling for 10 minutes, the hot mixture was poured into the electrophoresis cell and was allowed to cool overnight. During this time the starch paste hardened to a stiff, elastic gel.

Small Cell—The hot starch paste was poured into a rectangular cell made of acrylic resin (30 X 5 cm. base, 1.3 cm. high). After cooling and hardening of the medium, a 0.5 X 4.5 cm. slot was cut transversely in the gel, about 5 cm. from one end. The slot was filled with 2 ml. of protein solution, containing up to 150 mg. of protein, previously dialyzed against buffer of 0.03 ionic strength. Both ends of the cell were connected through filter paper strips to beakers containing reversible silver-silver chloride electrodes. The cell was covered with a glass plate to avoid evaporation, and the entire apparatus was placed in an ice box at 13-15°. An electric field of 6.7 volts per cm. (200 volts difference of potential between the electrodes, 15 to 20 ma.) was then applied for 18 hours. The protein zones could be seen under ultraviolet light; with human plasma as the protein mixture, albumin appeared as a light band at a distance of about 20 cm. from the slot and β-globulins as dark areas at a distance of 10 cm. The latter could be seen as red and yellow areas under ordinary light.

In order to collect the protein fractions, the entire gel was cut transversely into 1 cm. sections. Each section was eluted with 3 ml. of water by triturating the gel in a test-tube with a mechanical stirrer, followed by centrifugation of the mixture. The elution was repeated twice more, and all eluates from the same section were combined. The liquid remaining in the slot was pipetted out before cutting the gel and was later combined with the eluate of the section to which it belonged. The protein content of the eluates of each section was determined on aliquots by means of the method of Lowry et al. (7), with crystalline chymotrypsin as the reference protein. Since the starch mixture itself gives a slight reaction with the phenol reagent, it is necessary to subtract a correction value from the protein content found in each section. The correction value was obtained from the eluate of a section of the starch gel not containing protein, i.e. from the sections on both extremities. The recovery of applied protein calculated from the sum of all fractions was generally near 100 per cent.

1 Care was taken not to cut through the sides of the cell in order to prevent the material, which was later placed in the slot, from leaking out between the gel and the walls of the cell.
A plot of the protein content per section, as a function of the distance of the section from the point of application of the protein, yielded patterns which were analogous to those obtained with the same plasma by the method of moving boundary electrophoresis, and which showed the distribution of protein on the gel. The eluates from the starch gel were then pooled into a small number of fractions (six to eight), according to the protein distribution on the gel. Thus, one fraction for each of the electrophoretically recognizable plasma constituents (albumin, α-, β-, and γ-globulins) and for some intermediate fractions was obtained.

In order to remove amylose which was eluted from the starch gel and which slowly precipitated from the solutions, each fraction was centrifuged at 20,000 × g in an International centrifuge with a high speed attachment. The clear supernatant solutions were concentrated to a protein content of 5 to 10 per cent by lyophilization and by redissolving the residue in small volumes of distilled water. They were then freed from soluble starch by exposing them to an electric field of 15 volts per cm. in a standard 6 ml. Tiselius cell of a Perkin-Elmer electrophoresis apparatus, after dialysis against buffer (Tris-citrate buffers, ionic strength 0.02, pH 3.5 for γ-globulin, pH 8.8 for all other fractions). Thus, the protein was allowed to migrate away from the soluble starch and the pure protein solution could be drawn off easily after closing the cell. An attempt to remove the starch by digestion with α-amylase and subsequent dialysis was not satisfactory. Dry powders from the starch-free fractions were obtained by lyophilization of the solutions after dialysis against 0.01 M NaCl solution.

Large Cell—The cell had the dimensions of 30 × 30 cm. and was adjustable in width from 1 to 5 cm. It was constructed of cast aluminum, and the surfaces were dip-coated with a thin layer of rubber for electrical insulation. Both walls were hollow, and cold water from a commercial water cooler was circulated through them, directed by a system of baffles. The cell was mounted on a heavy base by two swivel joints which allowed it to be turned to a horizontal position.

The electrode vessels were made of acrylic resin blocks which were fixed to the ends of the cell; the joints were made water-tight by soft rubber gaskets. The electrode compartments were 30 cm. high, 2 cm. deep, and 1 cm. wide, separated from the cell by cellulose membranes. The electrodes were electrolytic silver rods, 31 cm. long and 0.5 cm. in diameter. They were made reversible by coating them with silver chloride and packing them in a heavy suspension of finely ground anion exchange resin2 charged with chloride.3 A concentrated solution of sodium chloride could not be

2 The high capacity resin Amberlite IR-4B, Rohm and Haas Company, was used.
3 Any silver or silver chloride which was peeled from the electrodes was allowed to remain mixed with the ion exchange resin, and the mixtures were used again with opposite polarity.
used as a source of chloride, since the salt would diffuse rapidly into the gel, increasing its conductivity and thus markedly reducing the mobilities of the proteins.

The hot starch paste was poured into the cell with the electrodes in place. A piece of acrylic plastic, 32 cm. high, 0.5 cm. thick, and 0.4 cm. narrower than the width of the cell, was inserted vertically into the hot paste, about 5 cm. from the cathode and 0.2 cm. from each wall. The cell was cooled, and the paste was allowed to harden to a gel overnight, with the plastic piece fastened in place. The plastic piece contained a longitudinal hole through which the protein solution was introduced, as the plastic was slowly withdrawn. This technique helped to avoid collapse of the gel.

The electrophoretic migration of protein could be followed by observation under ultraviolet light. When plasma was employed, the current was interrupted after the albumin, noticeable as the fastest moving protein, had migrated about 20 cm. The liquid from the slot was withdrawn, and the cell was turned to an almost horizontal position. The top wall was removed, and the gel was cut as described.

To elute the protein from the gel and simultaneously to remove soluble starch, the gel pieces were placed in suitable containers, overlaid with buffer solution, and an electric field was applied to the system. The protein thus migrated out of the starch and into the overlaying buffer solution which was then siphoned to yield starch-free protein eluates.

**Electrophoretic Analyses**—The composition of the fractions was studied by using a 2.5 ml. Tiselius cell of a Perkin-Elmer apparatus with Veronal-citrate buffer, pH 8.6, ionic strength 0.1, in an electric field of 10 volts per cm. as described previously (8). Salt anomalies were reduced by the addition of appropriate amounts of water to the dialyzed protein solution (9).

**Measurement of Electroosmosis**—0.5 ml. of a 20 per cent solution of crystalline bovine serum albumin in Tris-citrate buffer, pH 8.8, and ionic strength 0.06, was mixed with 0.5 ml. of a 20 per cent solution of hydrogen peroxide. The slot in a starch gel contained in the small scale apparatus was filled with 0.8 ml. of this mixture. In contrast to the standard conditions observed during electrophoretic fractionations, this slot had been cut into the gel at about 10 cm. from one end and had the dimensions of 0.15 × 4.5 cm. A difference of potential of 150 volts at the electrodes was maintained for 18 hours. At the end of this time, the position of the albumin zone was observed under ultraviolet light, and its

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4 This liquid was later freed from soluble starch in an electric field and then combined with the eluate of the section to which it belonged.

5 Obtained from Pentex, Inc., Kankakee, Illinois.
distance from the slot was measured. The gel was then cut into 1 cm. sections as described, and each section was eluted once with 2 ml. of 2 N H₂SO₄. The liquid contained in the slot had been pipetted before the cutting and was combined with the starch gel of its proper section before the elution. 2 ml. aliquots of each section were titrated with 0.1 N KMnO₄ to locate the position of H₂O₂ as an indicator of electroosmotic flow.

Fig. 1. Zone electrophoretic protein fractionation of plasma from tumor-bearing mice. Tris-citrate buffer, pH 8.8, ionic strength 0.03. Electric field, 6.5 volts per cm. (200 volt difference of potential at the electrodes, with a current of 6 ma.). Duration, 21 hours and 40 minutes; amount of protein applied, 142 mg.; amount of protein recovered, 138 mg. The direction of migration is indicated in the figure by an arrow. The origin, i.e. the location of the slot in the starch gel where the protein was applied, was at the line marked 0. The horizontal dotted line indicated the true base-line of protein. This correction was necessary due to a slight reaction with the protein reagent of non-protein material from the starch gel. The vertical dotted lines indicate how the eluates from all twenty-four starch sections were pooled into six protein fractions.

Results

When pooled plasma from tumor-bearing mice was subjected to zone electrophoresis on starch gel in the small scale apparatus, a protein distribution on the starch medium, as seen in Fig. 1, was found. The data in this chart were obtained from one typical experiment. Other experiments with the same plasma gave like results. The diagram in Fig. 1, representing a plot of protein recovery versus the position of protein in the medium, bears a marked resemblance to the diagram of the moving boundaries obtained in the Tiselius cell (see the last diagram in Fig. 2, a). Both show four main peaks, corresponding to albumin, α-, β-, and γ-globu-
lins, respectively. In particular, the comparison of the apparent mobilities of the protein fractions in the starch gel with those in free solution (Tiselius cell) shows that there is little if any electroosmotic flow.

The twenty-four eluates were pooled into six fractions as shown in Fig. 1. Thus, one fraction at each of the protein peaks was obtained (Fractions I, III, V, and VI). Since there were areas on the gel between the main peaks which contained mixtures of protein, the eluates from these areas were combined into intermediate fractions, e.g. Fractions II and IV.

The recovery of protein in all eluates was 58 mg. in a typical experiment when 60.5 mg. of plasma protein had been applied to the gel.

**Boundary Electrophoretic Analyses**—Figs. 2, a and 2, b show that Fraction I consists chiefly of albumin. Fraction III contains a-globulin as the main component and a small amount of a slightly slower moving protein. Since the mobility of the latter is faster than that of β-globulin, this protein is designated as α2-globulin, analogous to the slow moving a-globulin found in certain human plasmas. An α2-globulin with the mobility of that found in Fraction III can also be detected in the patterns of whole mouse plasma (last diagram of Fig. 2, a). Fraction V consists mainly of β-globulin, and Fraction VI is a mixture of β- and γ-globulins. Fractions II and IV are mixed fractions (see Fig. 2, b). The former contains albumin and α-globulin, the latter α- and α2-globulins.

Planimetric evaluations of the patterns in Figs. 2, a and 2, b yielded data indicating the content of albumin or of the respective globulins in each of
the fractions. The results of these evaluations are shown in Table I. This table also gives the amount of protein contained in each fraction, expressed in per cent of the total amount of protein eluted from the starch (sum of the protein contents of all twenty-four eluates) as calculated from the data of Fig. 1.

**Table I**

**Electrophoretic Analysis of Six Fractions, Obtained from Mouse Plasma by Zone Electrophoresis**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Protein content as per cent of total protein recovered</th>
<th>Protein content as per cent of total protein per fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>I</td>
<td>41.0</td>
<td>97.0</td>
</tr>
<tr>
<td>II</td>
<td>10.5</td>
<td>42.5</td>
</tr>
<tr>
<td>III</td>
<td>13.1</td>
<td>3.0</td>
</tr>
<tr>
<td>IV</td>
<td>8.4</td>
<td>10.25</td>
</tr>
<tr>
<td>V</td>
<td>15.3</td>
<td>18.8</td>
</tr>
<tr>
<td>VI</td>
<td>11.7</td>
<td>6.95</td>
</tr>
</tbody>
</table>

**Fig. 3.** Electrophoretic patterns of human γ-globulin in Veronal-citrate buffer, pH 8.6, ionic strength 0.1. The peaks represent, read in the direction of the arrows, salt anomaly, γ-globulin, and 3 per cent of a faster moving impurity.

Fraction VI from the plasma of tumor-bearing mice turned out to be very heterogeneous (Table I, Fig. 2, a). This is probably owing to the very low concentration of γ-globulin in the original plasma. Good γ-globulin preparations, however, can be obtained by zone electrophoresis on starch gel. The result of an electrophoretic analysis of normal human γ-globulin prepared in the large apparatus is shown in Fig. 3.

The electroosmotic flow in the starch gel appears to be negligible, since it is obvious that the relative distances from the origin of each plasma
protein fraction are the same, whether the migration took place on the starch gel (Fig. 1) or in free solution in a Tiselius cell (last diagram of Fig. 2, a). Further evidence for the practical absence of electroosmosis was demonstrated by an experiment in which a mixture of hydrogen peroxide and bovine serum albumin was exposed to an electric field in a starch medium at pH 8.8 (Fig. 4). The figure shows that albumin migrated toward the anode. The front of the albumin zone, observed under ultraviolet light, was 14 cm. from the origin (arrow). The consumption of permanganate in this area was due to the protein itself, measured with albumin alone in a control experiment. During the same time, hydrogen peroxide diffused into the gel on both sides of the origin. The distribution of peroxide in the gel indicates that the electroosmotic flow was less than 0.5 cm. towards the cathode. The recovery of peroxide in this zone was 80 percent.

**DISCUSSION**

The use of a gel as the supporting medium in electrophoresis, in particular agar jelly, has been described by several authors (10–12). Since agar is itself an electrolyte, it is liable to form complexes with proteins. Its use is, therefore, limited to those proteins which will not interact with it. A method of analytical electrophoresis in which a starch gel was used as the supporting medium has been reported by Smithies (13).
The starch gel described in this paper appears to have all the qualities necessary for a good carrier medium in zone electrophoresis, i.e. (1) it has a consistency permitting the easy removal of sections for the purpose of collecting fractions, (2) due to the low content in solid constituents (less than 8 per cent) the protein is able to migrate freely in this medium, (3) for the same reason, and in contrast with all other supporting media described, electroosmosis has been found to be negligible, and (4) no interaction between constituents of this medium and proteins has been observed. This starch gel contrasts, therefore, with supporting media composed of starch granules (14, 15), cellulose slabs (16), or glass powder (17), which are solids soaked with aqueous solution, and contain 50 per cent or more of solid constituents. Protein particles migrating in such media must travel irregular paths, since they encounter many barriers (18). The extremely high content of solid material in these media has also the disturbing effect of causing a considerable electroosmotic flow of the buffer (14), thus displacing all protein zones toward the cathode. This phenomenon generally interferes with the proper identification of the protein fractions according to electrophoretic mobility. In addition, the use of a modified starch gel as a supporting medium makes it possible to process rather large quantities of protein, since channel formation and non-uniform packing which cause uneven current distribution and irregular zones of protein do not occur. Further increase of the dimensions of the cell for fractionation of still larger amounts of protein should not present serious difficulties, if proper engineering of the apparatus provides an efficient cooling system.

The composition of the starch gel is of decisive importance for its mechanical properties. The addition of amylose is necessary for the formation of a suitable gel (19). A 3 per cent starch paste without added amylose would yield a highly viscous liquid. The use of a higher concentration of starch without addition of amylose, on the other hand, may lead to the formation of a solid gel. Such a gel has been found, however, to hinder free electrophoretic migration of proteins. The addition of Hyflo Super Cel to the mixture has the same effect as the addition of zinc oxide to rubber as a reinforcing filler. It increases the modulus of elasticity and improves the mechanical resistance of the product.

An apparent disadvantage in the use of a starch gel as a carrier medium is the solubility of the starch which must be removed from the protein solutions after the fractionation. This disadvantage is compensated for by the many advantages of the method, since it is possible to extract the proteins from the gel with simultaneous removal of soluble starch. Simply overlaying the separated section of starch gel with salt solution and applying an electric field to this system allow the protein to move away from both soluble and insoluble starch.
A starch gel consisting of 3 per cent of corn-starch, 1.5 per cent of additional amylose, 3 per cent of Hyflo Super Cel, and the proper buffer solution has been used as an improved supporting medium in zone electrophoresis for the fractionation of plasma proteins. This medium forms a stiff gel which can easily be cut into small sections for the purpose of separating the protein zones obtained in the electric field. Because of the low content of solids in this medium, the electroosmotic flow is negligible. The peculiar properties of the starch gel allow a considerable increase in the capacity of the cell and hence in the amount of protein undergoing fractionation. Two devices have been described in this paper, a small scale cell for the separation of 2 ml. and a large cell for fractionating up to 50 ml. of a 5 to 10 per cent protein solution. A detailed description of the method and its use in isolating six protein fractions from mouse plasma are given. A preparation of pure human γ-globulin is illustrated.

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