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AGAR ELECTROPHORESIS

Part V. Preparative Agar Electrophoresis of Serum Proteins

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ABSTRACT

Agar electrophoresis developed by Giri has been adapted for preparative purposes. The method consists in preparing agarbuffer gel in a perspex trough in the centre of which a slit is made for the protein solution under test. About 1 to 2 c.c. of human serum has been subjected to preparative agar electrophoresis and separated into four distinct fractions albumin, α_2, β and γ-globulin, which have been isolated in a homogeneous state. The protein is eluted from the agar gel by freezing at-20°C for 3 hours and thawing it at room temperature. The resulting solution is filtered free of agar on a sintered glass funnel, dialysed free of buffer salts and then concentrated under *vacuo* over phosphorus pentoxide in cold. The isolated fractions retain their original mobility without getting denatured. The method can be used for the isolation of protein components in the native state on a semi-preparative scale. The recovery of protein from the agar gel is of the order of 95 per cent as shown by recovery experiments done with pure albumin.

One of the very fruitful outcomes of zone electrophoresis is its adaptability as a preparative tool. Consden *et al.* [1946] used silica gel for the first time to separate amino acids. Since then various stabilizing media have been tried by a number of workers [Gordon, *et al.*, 1950, Kunkel and Slater, 1952 *a*, *b*, Smithies, 1955 and Bernfeld and Nisselbaum, 1956]. Paper as the stabilizing medium has limited application for preparative electrophoresis for reasons such as the irreversible adsorption of proteins to some extent, the difficulties in obtaining a narrow

site of origin for the application of protein solution and the increased resistance offered to the migration of protein particles. Kunkel and Slater [*loc. cit.*] have developed preparative electrophoresis using starch as supporting medium with considerable degree of success. The agar electrophoresis technique developed in this laboratory [Giri, 1956] which has been used extensively for analytical purposes has now been applied for preparative work, particularly in view of some advantages, it possesses, which is discussed *et sequel*. The present paper deals with the details of the method for carrying out preparative agar electrophoresis of serum proteins.

MATERIALS AND METHODS

The preparation of agar buffer mixture has been described in the previous communication [Giri, *loc. cit.*]. For preparative electrophoresis 1% agar was used.

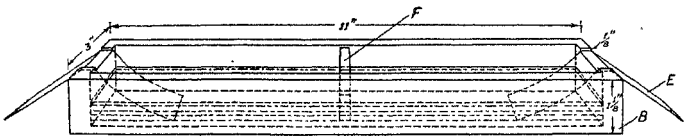

Fig. 1

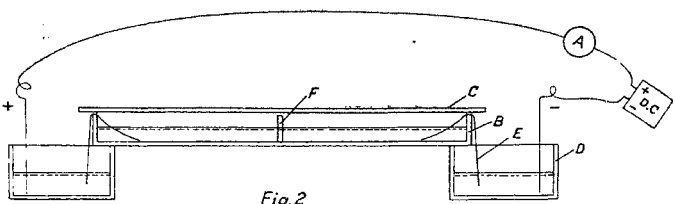

Fig. 2

Apparatus:—The apparatus used is illustrated diagramatically in Figs. 1 and 2. It consists of an electrophoretic cell and the power unit.

The electrophoretic cell [B] is in the form of a trough [$11'' \times 3'' \times 1\frac{1}{8}''$] made of perspex sheet [1/8" thickness]. On either sides of the trough a depression [1/8"] is made to accommodate the filter paper strips [E] which maintain contact between the cell and the buffer in the electrode vessels [D]. A small rectangular piece of perspex [F] [$2'' \times 7/8'' \times 3/32''$], is kept at the centre of the trough so as to give an uniform slit when the agar solidifies.

Direct current of variable voltage [200 – 300 volts] is obtained through a rectifier unit containing a constant voltage transformer, connected to the perspex electrode vessels containing platinum wire electrodes through a milliammeter [A].

Preparation of agar gel:—The buffered agar gel is conveniently prepared by mixing a freshly prepared 1% agar solution [50 c.c.] with an equal volume of veronal sodium acetate buffer [pH 8.6 and 0.1 μ]. Whatman No. 3 filter strips [$5\frac{1}{2}'' \times 1\frac{1}{8}''$] are introduced at either ends at an angle of about 60° [Fig. 1]. A rectangular piece of perspex [F] is placed at the centre of the trough without touching the sides of the trough. The agar gel is allowed to set which takes 20 – 30 minutes and the thickness of the resulting gel is about 9 mm. The trough is now carefully placed as a bridge on the two electrode vessels containing veronal buffer. The filter paper strips are allowed to dip into the buffer in the electrode vessels and evenly wetted with buffer by means of a capillary pipette. A glass plate [$12'' \times 3\frac{1}{2}''$] is placed over the trough. The side of the plate exposed to agar is covered full length with two layers of Whatman No. 3 filter paper and fastened tightly by means of a number of rubber bands. The paper serves to absorb the evaporated moisture from agar during electrophoresis and incidentally reduces sweating by maintaining a water saturated atmosphere.

Current [200 V ; 10-12 ma] is allowed to pass through the agar gel for about 30 minutes when a constant ampearage is attained. Now the sample to be analysed is introduced.

Introduction of sample:—The perspex rectangular piece kept in the centre of the trough is very carefully removed and a narrow uniform slit so obtained is now filled with the serum [about 0.8—0.9 c.c] either by means of a tuberculin syringe or a finely drawn out capillary pipette. Care is taken to see that there are no air bubbles.

Electrophoresis run:—The electrophoresis carried out at 200 volts [10-12 milliamps] for 24 hours at room temperature [22-26°] is found optimum in case of human serum. At the end of 24 hours, an yellow band of bilirubin corresponding to albumin and two faint brown bands in the position of α_2 and β-globulins are seen distinctly on the agar layer.

Location of separated fractions:—In order to locate the position of the separated fractions, a test print is taken on a piece of Whatman No. 1 filter paper. The paper is cut to suitable size and the centre is marked with a pencil. This is now layered on the agar gel such that the pencil mark rests on the point of application of serum sample for two to three seconds and then removed and dried before a fan. This is now stained with amido schwarz 10 B [0.5% in 5 : 1 : 4 methanol : acetic acid : water] for 15 minutes and then washed three to four times with fresh solutions of the same solvent mixture but in 10 : 1 : 5 proportion. The fractions appear as dark blue bands on a colourless background. The zones due to albumin, α_2, β and γ-globulins can be easily identified.

Elution and recovery of separated fractions:— By keeping the test strip before the agar layer, the area of each fraction on agar is cut and carefully transferred

into separate, clean, dry test tubes by means of a spatula and triturated well with a glass rod. A portion of agar not containing any protein is also taken in a similar manner to serve as a control. These tubes are left at -20°C for three hours and then removed to room temperature. The solution that thaws out of agar is filtered through a sintered glass funnel [S.F. 6A 2 pyrex], into a clean dry test tube. Thus all the fractions are collected in respective test tubes and their volume is noted. A suitable aliquot [0.1 c.c.] is taken from each test tube and the protein content is estimated by means of colouration with Folin-phenol reagent according to the method of Lowrey *et al.* [1951] using crystalline serum albumin as standard.

Recovery of crystalline serum albumin after preparative agar electrophoresis:— Following experiment was carried out to find out the percentage recoverability of serum albumin from agar after preparative electrophoresis.

Crystalline serum albumin [19 mg.] was dissolved in 0.8 c.c. veronal-acetate buffer [pH 8.6; 0.05 μ] and was quantitatively transferred to the agar gel. The experiment was conducted for 18 hours in the usual manner [200 V, 10-12 ma]. The site of albumin on agar was identified by means of a test strip and the portion of agar containing albumin was removed into a clean test tube, frozen and thawed as mentioned before. The solution was filtered through sintered glass funnel and the whole volume of filtrate was made to 25 c.c. with the same buffer. 0.2 c.c. of this solution was subjected to protein estimation by Lowrey's [*loc. cit*] method. 18.125 mg. of albumin could be recovered in the filtrate accounting for 95.4% of the original protein. Some loss of albumin results on account of adsorption on test strip.

Effect of isolation of serum protein fractions by agar electrophoresis on their electrophoretic mobility:—In order to test whether the different fractions obtained after elution retained their original electrophoretic mobility, they were concentrated to a volume of about 1 c.c. over phosphorus pentoxide, in a desicator in cold [0-5°C] under vacuum. These solutions were subjected to the analytical agar electrophoresis technique. Big plates [$12'' \times 3\frac{1}{4}''$] were used, since, in addition to the isolated serum fraction, normal serum sample was also spotted on agar one below the other, in the same line. After completion of electrophoresis, these were dried and stained with amido schwarz. It was found that albumin, α_2, β and γ-globulins tested, showed the same electrophoretic mobility as those obtained from normal intact serum [Plate I].

RESULTS

In Table I the results of a typical experiment conducted using 0.9 c.c. of an apparently healthy human serum is given.

Since the separation of α_1-globulin and albumin as also β_1 and β_2-globulins are not absolute, for purposes of estimation they are taken together. The percentage distribution indicated in the above table varies from the figures obtained by the usual analytical technique using only 10 μl of serum because of

some losses occurring during the process of recovery of these fractions by freezing and thawing. In Table II, the recovery of different fractions isolated from a few apparently normal human sera is given.

TABLE I

Percentage distribution of human serum proteins by preparative Agar Electrophoresis

Fraction	Protein content μg/0.1 c.c.	Total volume eluted (c.c.)	Total protein present (μg)	% Distribution protein
Alb + α_1	517.5	8.0	41,400	63.7
α_2	160	4.0	6,400	10.0
$\beta_1 + \beta_2$	190	4.0	7,600	11.5
γ	237	4.0	9,480	14.8

TABLE II

Percentage distribution of human serum proteins by preparative Agar Electrophoresis

Name	Alb + α_1%	α_2%	$\beta_1 + \beta_2$%	γ%
S. S.	56.8	9.0	17.7	16.5
H. R.	57.6	10.4	20.8	11.2
N.	52.3	12.1	15.6	20.0
D.	49.7	11.0	20.9	18.4
J. S.	63.7	10.0	11.5	14.8
K.	56.0	13.0	13.0	18.0
S.	50.8	9.4	13.4	26.4
Average	55.3	10.7	16.1	17.7

DISCUSSION

The preparative agar electrophoresis described above is very simple and can be easily done in ordinary laboratories. About 0.8 to 1 c.c. of undiluted serum can be handled when 100 c.c. of agar-buffer mixture is used. By increasing the volume of agar-buffer mixture to 200 c.c., quantities of serum upto 1.8 c.c. can be used. The separations obtained are clear and the process of elution by freezing and thawing is found to be quantitative and convenient.

It is evident from the experiment on the recoveribility of crystalline serum albumin described in the experimental portion that not only quantitative recoveries can be obtained but there is also no irreversible adsorption of albumin to agar.

Further the experiment on the mobility of isolated albumin, α_2, β and γ-globulins confirms the view that the isolated fractions do not get denatured in the presence of agar by the fact that they retain their original mobilities.

As already pointed out another advantage in using agar for preparative electrophoresis is that it offers a very low supporting medium, thereby not only increasing the capacity of the system but also preventing the possibility of introducing impurities which result on account of a high medium-liquid ratio as in the case of starch. The elution and recovery of the protein also becomes easy in view of the low medium-liquid ratio in case of agar. The total solids present in a supporting medium prepared by mixing equal quantities of veronal-acetate buffer and 1% agar solution is approximately 1.15%. That is, an agar-liquid ratio of 1 : 87 is obtained. By the method of Kunkel and Slater [*loc. cit.*] starch occupies 50% of the total volume of the supporting medium with a solid : liquid ratio of 1 : 1 while in case of the method adapted by Smithies [*loc. cit.*] 15% starch gel is used and Bernfeld and Nisselbaum [*loc. cit.*] have further decreased the total solids to about 8%.

By using 2% agar solution instead of 1%, it has been found that the recovery of the separated fractions is reduced. Using concentration of agar higher than 2% proves unsuitable as the agar gel gets hardened and breaks during the process of electrophoresis. Since the gelling properties of agar varies with different batches of ager used, it is necessary to determine by preliminary experiments the optimum concentration of agar which in this case happens to be the minimum concentration giving satisfactory gel.

The complete absence of protein adsorption to agar, the ease with which it can be eluted and concentrated without denaturation, and the low supporting medium that it offers makes preparative agar electrophoresis a very useful technique. The technique can be easily adopted to the isolation particularly of the minor fractions of serum proteins such as mucoproteins and also those that increase in certain disease conditions such as cancer and tuberculosis. The application of this method to studies on the above lines is in progress in these laboratories.

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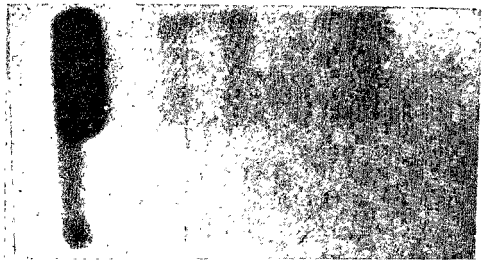
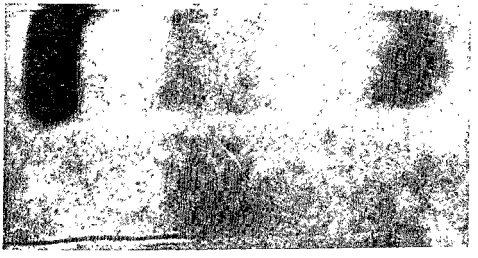


Normal Human Serum

Isolated albumin

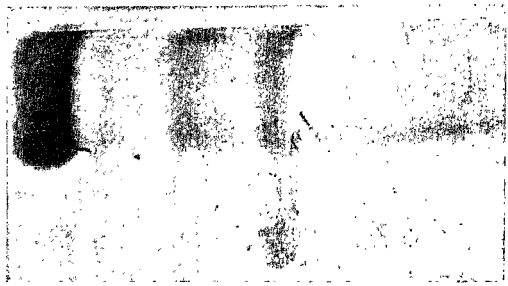


Normal Human Serum

Isolated α_2 globulin

PLATE I, A

Agar electrophorograms showing the comparison of the mobilities of the serum proteins isolated by preparative agar electrophoresis with the normal intact serum proteins



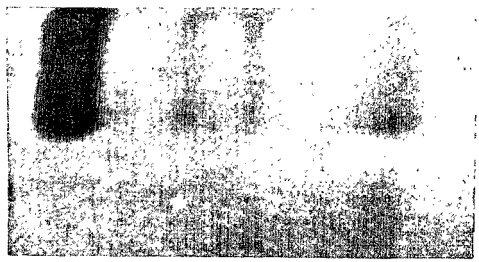


PLATE I, B

Agar electrophorograms showing the comparison of the mobilities of the serum proteins isolated by preparative agar electrophoresis with the normal intact serum proteins

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