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Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults

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(*Received* 4 *April* 1955)

The theoretical advantages of zone electrophoresis over free-boundary electrophoresis are well known: freedom from quantitatively important boundary anomalies, the possibility of preparing electrophoretically discrete proteins, and adaptability to small quantities of material. However, the two zone-electrophoresis methods at present in general use do not give a resolving power as great as the free-boundary method of Tiselius; the resolving power of the filter-paper-electrophoresis method of Kunkel & Tiselius (1952) is reduced by the difficulty of putting the sample to be investigated on the paper in a sufficiently narrow zone and by adsorption effects, and obligatory serial analyses limit the resolving power obtainable with the starch-grain method of Kunkel & Slater (1952) because of the difficulties of making sufficiently thin serial sections. A method of zone electrophoresis was therefore sought which would combine the advantages of the low adsorption characteristic of the starch-grain method with the convenience of protein detection by staining characteristic of the filter-paper method. At the same time, by introducing the sample in a sufficiently narrow zone, it was hoped to obtain a resolving power as good as that obtained in the classical Tiselius method.

The method evolved comprises zone electrophoresis with a starch gel as the supporting medium, and proves to have a resolving power in many cases superior to that of the Tiselius method. The method has been successfully applied to crystalline β-lactoglobulin, which can be resolved into its two main components (Smithies, O., unpublished work), to cabbage-enzyme preparations (Dixon, G. H. & Smithies, O., unpublished

work) and to the serum proteins of normal adult humans. As indicated in a preliminary publication on the serum-protein separations obtained by the present method (Smithies, 1955), sera from normal human adults are found to fall into three groups, according to the presence or absence of various previously undescribed components.

The present paper describes the general experimental procedures of this new type of zone electrophoresis, and gives the specific conditions used in investigating the serum proteins. The serum results are considered in detail in the discussion, and the method as a whole is discussed.

EXPERIMENTAL METHODS

General. A starch gel containing the desired buffer is prepared in a suitable plastic tray. The sample is introduced into a vertical slit in the gel at right angles to the greatest length of the gel. Electrical contact is made to the ends of the gel with filter-paper wads, soaked in a suitable buffer solution, which dip into vessels containing the same solution. Filter-paper bridges in turn connect these vessels to the electrode chambers. Current is passed for the required time. The gel is then removed from the tray and sliced along its length in a horizontal plane, and the slices obtained are stained with a protein dye. After washing with dye solvent the stained, separated proteins may be observed. Fig. 1 shows the general layout of the apparatus.

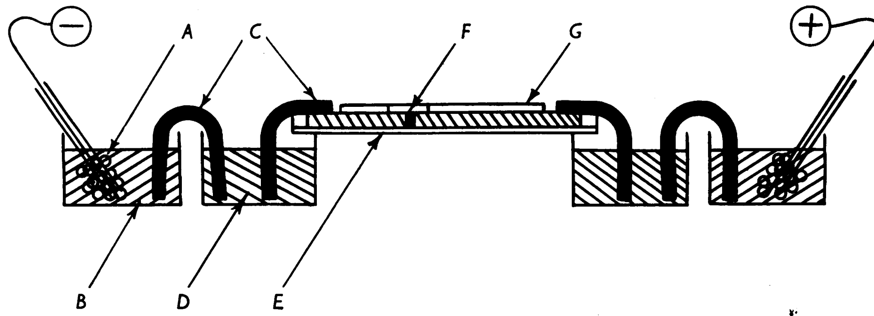

Fig. 1. General layout of apparatus. *A*, Ag/AgCl electrode; *B*, concentrated NaCl solution in electrode compartment; *C*, filter-paper bridges soaked in bridge solution; *D*, compartment containing bridge solution; *E*, starch gel contained in plastic tray; *F*, position of sample insertion; *G*, seal to prevent loss of water during electrophoresis.

Composition and preparation of the gels. The starch used in the present work was reagent-grade soluble starch supplied by Merck and Co. Ltd., Montreal, Quebec, Canada. Gels can be made from this starch at concentrations of from about 10 g. of starch/100 ml. of buffer up to at least 16 g./100 ml. A concentration of 15 g. of starch/100 ml. of buffer gives a final gel of considerable strength, yet the hot solution is not too viscous to handle during preparation. This concentration was most frequently used. Further supplies of this soluble starch proved erratic in their gelling properties. As an alternative, soluble starch with the required characteristics has been made as follows: 300 g. of B.D.H. (British Drug Houses Ltd.) potato starch was suspended in 600 ml. of acetone–HCl (1 vol. of concentrated HCl to 100 vol. of reagent-grade acetone) at 38·5°. After standing for 45 min. without further mixing, 150 ml. of aqueous M sodium acetate was added to stop the reaction. The starch was then filtered off on a Büchner funnel and washed thoroughly with distilled water. To remove any traces of acetate still remaining, the starch was resuspended in distilled water, allowed to stand overnight, washed again with distilled water on a Büchner funnel, dehydrated with acetone and finally dried thoroughly at 45–50°.

The temperature coefficient of the hydrolysis is considerable, so that the temperature should be carefully controlled. Potato starches from different manufacturers vary in their properties: some could not be made satisfactory for serum electrophoresis and others required different hydrolysis times. Small differences in the electrophoretic behaviour of different batches may be removed by varying the starch and/or buffer concentrations. The changes produced in these ways are considered in the discussion of the factors controlling the spreading of albumin during the electrophoresis. A starch prepared in the manner described here gave satisfactory results when used at 14 g./100 ml. of borate buffer (in place of the usual 15 g./100 ml.), with a concentration of 0·025 M total borate (in place of 0·03 M total borate).

The buffer solutions used to make the gel can be varied widely; gels with pH values ranging from 3 to 9 have been successfully employed. Because of traces of acid in the starch the pH of the gel is not the same as that of the buffer employed to make it, but if necessary this pH change can be reduced by washing the starch grains before use with suitable buffer, followed by distilled water, and dehydration with acetone. In the present work the starch as supplied by the manufacturers was used and the initial buffer pH was adjusted to give a gel of the pH required. If low ionic-strength buffers are employed, relatively high voltage gradients may be used during the electrophoresis without appreciable heating.

To a weighed amount of the soluble starch the requisite amount of buffer is added, and the mixture is heated over a naked flame in a conical flask with constant and vigorous swirling. The contents of the flask are brought to a point, just short of boiling, when the starch grains are ruptured

and a viscous homogeneous solution is obtained. The flask is then removed from the flame. The swirling is continued for a few moments to ensure a uniform temperature and, in order to remove small air bubbles, a negative pressure is then applied with a filter pump until the whole contents boil vigorously. The time of boiling is kept as short as possible (1–2 sec.) so that loss of water will not appreciably affect the composition of the gels. Standard conditions are essential at this point if the gels are to be reproducible, and are more easily obtained if the gels are prepared in fixed quantities in suitably sized flasks (e.g. 100 ml., made in a 500 ml. flask).

The hot starch solution is poured into a suitable plastic tray described below and immediately covered with a flexible plastic sheet (1·3 mm. polyethylene sheet was used) whose surface has been previously coated with a thin film of mineral oil. Air bubbles are avoided by lowering the sheet from one end. Samples of the gel are then taken from the same flask for pH measurements by two-thirds filling the small cylindrical vessels used with the Beckman pH meter and immediately sealing with Parafilm sheet (Marathon Corporation, Menasha, Wisconsin, U.S.A.). Excess of gel in the tray is then squeezed out by pressing on the polyethylene with a rigid plastic sheet, and the gel is allowed to cool. Gels are generally used on the same day as they are prepared. If the polyethylene seal is maintained intact they are found to be satisfactory when made 1 or 2 days previously, although their electrophoretic behaviour is then found to be similar to that of fresh gels containing less starch (see below for a description of the effect of the starch concentration on serum electrophoresis).

Introduction of the sample. Excess of gel is trimmed from the edges of the tray and the gel is exposed by stripping off the polyethylene seal. The sample may then be introduced into the gel in several ways:

(*a*) *On filter paper.* In this method a transverse cut is made in the gel, usually a third of the length from the left-hand end, with a rigid razor blade broken so as to be just a little smaller than the width of the gel (single-edged razor blades were found most suitable for this and other gel-cutting purposes). The razor blade is then used to compress the gel to the left away from the cut to form a slot about 5 mm. wide, and the blade is withdrawn. A piece of filter paper cut accurately to the cross-section of the gel and soaked in the sample is then introduced into the slit and allowed to adhere to the undisplaced cut surface of the gel. Air bubbles between the gel surface and the paper can be avoided if a little excess of liquid is present. The displaced section of the gel is then caused to close up to the other surface of the paper by returning it to its original position with a spatula introduced between the edge of the gel and the tray. Air bubbles should again be avoided.

This method of sample introduction is ideal for material available only in small quantities, provided that it does not show marked adsorption on filter paper, since practically no wastage of material occurs. As little as 0·02 ml. of sample on Whatman paper (no. 3) has been successfully used with small gels. It is also very well adapted to comparing different samples: up to four samples have been compared on a single 25 mm. gel with small pieces of filter paper each soaked in a different sample and arranged across a single slit. In this way very slight differences between samples can be detected, as the components common to all samples give uninterrupted bands across the gel, while any additional (or absent) components show as bands only opposite the respective papers. It is essential that the paper(s) fit the gel exactly, otherwise edge-effects will be present.

(*b*) *In a simple slit.* A slit is made in the gel with a razor blade which is then withdrawn. A thin celluloid spacer, in which is cut a rectangular hole a little smaller than the cross-section of the gel, is introduced into the slit. This holds open in the middle of the gel a space which is sealed along its edges by the cut gel surfaces in contact with the spacer. The top of the spacer is then slightly displaced so that the sample can be run into this space from a drawn-out glass tube. Excess of liquid is removed. This technique is useful chiefly to demonstrate that the supporting material [filter paper, or inert powder—see (*c*)] does not affect the results. It suffers in that edge-effects are usually present and, if the protein solution is at all concentrated, electrodecantation effects may also be present, leading to non-vertical boundaries in the protein zones.

(*c*) *In a slot cut in the gel and filled with inert supporting material.* A suspension of an inert supporting medium is made in the protein solution to be studied; this is drawn up into a Pasteur pipette and allowed to settle with the pipette in a vertical position. A slot is then cut out of the gel by making two parallel transverse cuts in the gel with two razor blades mounted together and separated by a distance equal to the width of the slot required (1–5 mm. slots have been used). The small block of gel so cut off is removed with a bent flattened probe. The gel is not displaced in the longitudinal direction in any way during the cutting out of the slot, so that no protein solution can run under the gel or along its edges. The partially settled suspension is then run into the slot and any excess of liquid remaining after the suspension settles completely is removed with filter-paper wicks applied to the lateral extremities of the slot. More suspension is added if necessary, until a uniformly packed block of the suspension is obtained. The slot is carefully sealed with a glass microscope slide, previously coated with a uniform film of petroleum jelly, so that no air bubbles are trapped in the slot yet no excess of protein solution lies on top of the gel. This method is the best procedure for preparative work as it gives zones which have vertical boundaries without any edge effects. However, results fairly satisfactory in this respect may also be obtained with the filter-paper technique if care is taken to fit the paper accurately and if the slit is sealed with a microscopic slide in the same way as for suspensions.

Various supporting substances were tried: microscopic glass spheres of the type used for traffic signs, microscopic polymethacrylate spheres, and starch grains. The glass spheres showed indications of adsorption phenomena with serum and the suspension of plastic spheres had a high electrical resistance. Starch grains were found to be completely satisfactory and gave results with serum essentially equivalent to a simple slit. For the most precise work starch grains of a narrow size distribution were used, so that the packing throughout the vertical extent of the slot should be uniform. These were prepared from 30–40 g. of crude potato starch by suspending the starch grains in acetone in a 250 ml. measuring cylinder and discarding the supernatant after settling for 2 min. The residue after repeating this process three more times was dried at about 45° and was used for the sample insertion.

Satisfactory introduction of the sample is of primary importance for obtaining good results, so that considerable

care should be exercised at this stage of the procedure. The author found that a little practice with ink in place of the sample greatly improved his technique in this respect.

Protection and cooling of the gels. During electrophoresis the gels must be protected from loss of water by evaporation. Two methods have been used: A layer of mineral oil can be poured on the exposed surface of the gel after introducing the sample and making the filter-paper bridge connexions, or liquefied petroleum jelly can be poured on the exposed surface and allowed to solidify. In the latter method the temperature of the petroleum jelly should be kept as low as possible and contact of the warm liquid with the sample should be avoided. (The microscopic slide seal prevents this.) In either case the gels should be left exposed to the air for the minimum time necessary to introduce the sample.

Heating difficulties are slight with the buffers and voltage gradients used in the present work with serum. Thus with a gel of 20 mm. × 7 mm. cross-section at 6 v/cm. the running temperature is only about 2·5° above that of the surroundings if the gel tray is allowed to rest on a copper plate with free air circulation. To minimize this temperature rise the bottoms of the trays should be made thin (about 1·5 mm.) and the thickness of the gel should preferably be kept under 10 mm.

Electrical connexions. Electrical contacts to the gels are made with filter-paper wads soaked in a suitable buffer solution of approximately the same pH as that of the gels. In working with serum it was found that the anodic end of the gel tends to swell if the bridge solution (see Fig. 1) is too dilute, and to shrink if it is too concentrated. However, under the conditions finally selected the active region of the gel is maintained at a constant electrolyte concentration and pH during the electrophoresis, judging by the constancy of the current required to maintain a given overall voltage gradient and by the absence of any change in colour of suitable indicators incorporated into the gels.

To prevent pH changes in the gels which might be caused by electrode products, reversible Ag/AgCl coiled-coil electrodes are used. (These electrodes may be prepared from silver wire by electrolysis in 0·5 M-HCl.) Each electrode is immersed in concentrated NaCl solution contained in a suitable container and filter-paper bridges soaked in the bridge solution are used to connect the electrode compartments to the rest of the system.

Power supply and voltage control. In the earlier stages of the work 220 v d.c. mains were used as a source of power, the current being controlled by a 20 000 Ω wire-wound rheostat in series. In the later stages a variable voltage regulated power supply with a maximum output of 70 mA at 300 v (The Heath Co., Benton Harbor, Michigan, U.S.A., Model PS-2) was used.

The voltage gradients in the gels are measured directly with a high-resistance voltmeter (20 000 Ω/v), using probes inserted into the gels a known distance apart and clear of the working parts of the gels. When the sample used is undialysed and contains electrolytes the current may need adjustment in the early stages of a run, if an absolutely constant voltage gradient is required, but at later stages the overall voltage gradient will remain constant without outside control. It may here be noted that, by inserting a grid of wires at regular intervals along the gel and then measuring the voltage drop between adjacent pairs of wires during the electrophoresis, the migration of these low-molecular-weight ions may be 'observed'. This is possible because of local decreases in the voltage gradient caused by the contribution of these ions to the conductivity. With serum these non-protein-staining electrolytes can be seen to resolve into at least two parts as the electrophoresis proceeds. The major portion migrates some 15–20 times more rapidly than albumin.

Protein detection. After electrophoresis has proceeded sufficiently long the seal of mineral oil or petroleum jelly is removed by blotting the top of the gel with adsorbent paper, and the gel is then turned out on a suitable cutting block (see Fig. 2). To do this the gel is freed from the edges

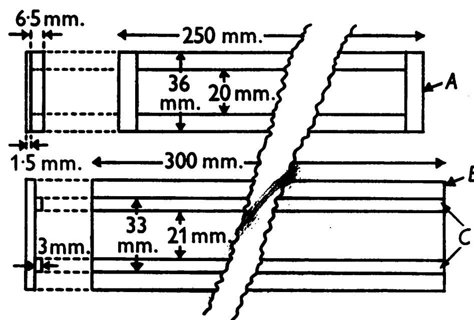

Fig. 2. Construction and dimensions of the plastic apparatus required for starch gels used in general analytical work. *A*, Plastic tray in which the gels are cast; *B*, cutting block for slicing gels before staining; *C*, guide strips on which the cutting blade is supported during the slicing of the gel, which is placed longitudinally between these strips.

of the tray with a spatula, when it can then be caused to fall out on the cutting block by inverting it over the block and starting movement from one end of the gel with the spatula. In order to observe the protein distribution in regions of the gel where no edge-effects are present the gel is then split along its length in a horizontal plane before staining, by means of a thin rigid cutting blade (a single-edged razor blade removed from its holder) and making the cut parallel to the bottom of the gel. The cut should be made in a single movement with the blade supported on either side of the gel by the flat guiding strips of the cutting block. The two gel slices so obtained are rapidly separated (to avoid interdiffusion of the proteins in case of relative displacement) and transferred to a dish for staining with the cut surfaces uppermost. The protein detecting dye is then poured over them, allowed to remain in contact with the gels for the requisite time and decanted for re-use. The gels are then washed with solvent until the background is colourless.

The dye employed in most of the present work was a saturated solution of Amido-Black 10B in solvent of composition: methanol–distilled water–glacial acetic acid (50:50:10, v/v). About ½ min. was sufficient to stain the proteins in the gel. Three changes of the solvent were usually found to be adequate for washing. The gel was left in the last for at least 2 hr. Covered vessels were used during the washing. No appreciable fading occurred, using this dye to stain the serum proteins, even after more than 6 months when the stained gels were stored in rubber-stoppered test tubes under the solvent containing a trace of

the free dye. They should not, however, be exposed to direct sunlight for long periods.

Bromophenol blue has been used for proteins, e.g. insulin, which do not give a stable dye–protein complex with Amido-Black. It gives qualitatively the same results as Amido-Black with serum, but is less sensitive and storage of the gels is less satisfactory.

Preparative experiments

As indicated above, it is essential in preparative experiments that the boundaries of the protein zones be vertically plane, otherwise cutting out any required zone free from contamination with other zones will not be possible. Consequently, for the best possible results the starch-grain method of introducing the sample should be employed. At the completion of the run the gel surface is marked by shallow transverse cuts at suitable distances and vertical cuts are then made along the length of the gel to enable two slices from the edges of the gel to be removed for staining. The bulk of the gel can then be used for preparative work with the stained slices as guides. The gels shrink 5–10% on staining, so that the transverse cuts are necessary guides in determining the cutting points.

The protein can be removed from the gel by diffusion after maceration in a suitable solvent or by digestion of the starch with amylase. However, a more convenient method, which has proved effective with all proteins tested, is to freeze the section of gel concerned. The gel is found on thawing to have broken down to a sponge-like structure from which the solvent containing most of the protein can be removed by low-speed centrifuging. The sponge is kept away from the bottom of the centrifuge tube with a mushroom-shaped glass rod with its end flattened out to a diameter just short of that of the centrifuge tube. Washing with the solvent, followed by recentrifuging, can be carried out as often as necessary. It was found with cabbage-enzyme preparations (Dixon & Smithies, unpublished work) that the frozen gel sections should not be stored for long periods before protein removal, otherwise the frozen starch in this case adsorbs the protein irreversibly, although without loss of enzymic activity. No data are at present available on the quantitative recoveries possible by these procedures.

If low-ionic-strength buffers are used in making the gels their buffering power will be small. Consequently it is preferable to dialyse samples of high protein concentration or considerable volume which are to be employed with such gels.

Dimensions of apparatus

A number of Plexiglas (Perspex) trays were used in which to cast the gels. Sizes tried ranged in internal dimensions from 4 to 10 mm. in depth, 15–80 mm. in width, and 200–450 mm. in length. The tray found most convenient for general analytical work and the corresponding cutting block are illustrated in Fig. 2: depth of tray 6·5 mm., width 20 mm., length about 250 mm. Gels much deeper than this prove awkward to handle and are difficult to keep cool during electrophoresis, but the width and length can readily be increased if required. The trays should be made of uniform cross-section with thin bottoms to minimize heating effects. If serial sections are to be prepared from the gels, as for example in enzyme-analysis procedures, it is convenient to make the width of the trays such that a multi-blade cutter constructed of single-edged razor blades can be used (39·5 mm. for the 'PAL hollow ground' single-edged blades which were found most suitable).

Conditions for starch gel electrophoresis of serum proteins

Preparation of human sera. To avoid haemolysis the following procedure has been adopted. About 10 ml. of whole blood are withdrawn from a suitable arm vein with a no. 20 needle. The blood is gently expelled from the syringe after removing the needle, and allowed to run down the side of a centrifuge tube, care being taken to avoid the expulsion of the air bubbles unavoidably present in the syringe. The internal surface of the centrifuge tube was coated previously with a thin layer of petroleum jelly so that the clot produced could shrink away from the walls without tearing. After clotting for about 1 hr. at room temperature the sample is left overnight at about 5° for clot-shrinkage to occur. Occasionally it proves necessary to add traces of filter paper to induce clotting. The serum is decanted after centrifuging at a low speed for 5 min. in a horizontal centrifuge. The decanted specimen is then further centrifuged for 10 min. at 15 000 r.p.m. in a Serval refrigerated angle centrifuge. The samples so obtained never showed any signs of haemolysis, as judged by their colour.

Sera were used for the first electrophoretic tests the day after withdrawing the blood. All samples were stored for later experiments at about 5° in Parafilm-sealed tubes. It should be noted that many of the sera showed changes in the α_2-globulin on storage for periods in excess of about a week.

Electrophoretic conditions. The buffer finally selected for the electrophoresis of serum contains 0·03 mole of H_3BO_3 and 0·012 mole of NaOH/litre. It proved essential that this solution should be accurately made. The gels contain 15 g. of soluble starch/100 ml. of this buffer, giving a final pH of $8{\cdot}48 \pm 0{\cdot}03$. The potential gradient employed is 6 v/cm., applied for 6 hr. at room temperature, corresponding to a current of 4–5 mA for each gel of the size shown in Fig. 2. The reasons for selecting these conditions, which were rigorously adhered to, are considered in the discussion. In the first tests on a given serum the sample is introduced into a slot approximately 1·2 mm. wide, the starch-grain-suspension technique described above being used. In comparative experiments the filter-paper method of sample introduction is used (Whatman no. 3 paper). The serum need not be dialysed.

The bridge solution contains 0·3 mole of H_3BO_3 and 0·06 mole of NaOH/litre.

RESULTS AND DISCUSSION

Fig. 3 shows photographs of the electrophoretic patterns obtained with a typical serum from each of the three serum groups previously described for normal adults (Smithies, 1955). As far as the main components are concerned, the pattern for the group I serum looks similar to those obtained by conventional methods, and in the previous publication a preliminary identification of these components was suggested by comparing it with the

classical patterns. Further studies (still in progress) indicate, however, that this preliminary identification, based on comparison alone, was in part incorrect. Full details of this aspect of the new method, which is being investigated in collaboration with M. D. Poulik, will be published at a later date, but a brief account of some of the results follows in order that the present data may be discussed in terms comparable with those classically employed.

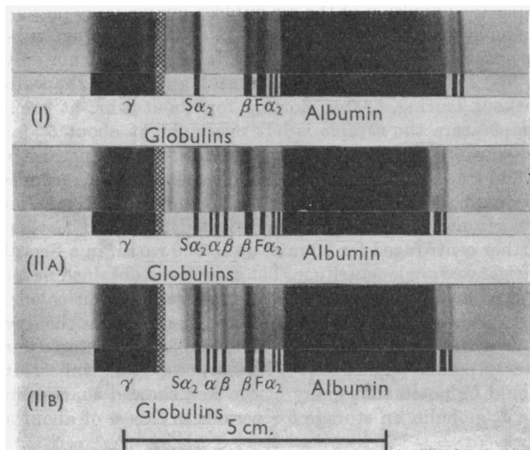

Fig. 3. Photographs and explanatory diagrams of the stained gels obtained with typical sera from groups I, II A and II B. Those components that are of variable occurrence even within a given group are not indicated in the diagrams. The starch-suspension method of sample insertion was used. The position of the sample slot is indicated by cross-hatching.

The most important method used to identify the starch-gel protein zones has been to separate the serum proteins first by electrophoresis on filter paper using a conventional barbiturate buffer. The moist filter-paper strip is then cut serially into 5 mm. sections in the region where the serum proteins are located. Each section is inserted into a starch gel, prepared in the usual way, alongside a piece of filter paper soaked in the whole serum. After electrophoresis, protein components common to both the whole serum and the serum fraction obtained from the filter-paper electrophoresis can be identified as uninterrupted bands across the whole width of the gels.

The results of these experiments show that albumin had been correctly identified, as would be expected from quantitative considerations. The protein zone migrating in a retrograde direction had also been correctly identified as derived from γ-globulin—an observation previously confirmed by studies on a clinical serum in which this zone was absent, and which also showed an absence of γ-globulin when investigated by the classical filter-paper method. However, the earlier identification of the remaining components has proved to be largely incorrect. In particular it is found that most of the new components demonstrated by the starch-gel method are derived from α_2-globulin, which proves to be a complex mixture of many different proteins. Some of these migrate in the starch gels in front of β-globulin and some behind.

The following system of nomenclature, which includes the more important identifications made as described, will be adopted in the present paper. In order to facilitate discussion of the results, trace components are named according to their positions relative to the major components which are indicated in the line drawings under the photographs in Fig. 3. The names given to these trace components do not imply any relations other than those of position.

The two components migrating more rapidly than the broad albumin zone are referred to as the pre-albumins $_1$ and $_2$ ($_1$ indicating the faster-moving component). The faint components migrating a little more slowly than albumin are referred to as the post-albumins. The protein zone (present in group I sera to a larger extent than in sera from the other two groups) which migrates behind the post-albumins proves to be due to a part of the complex group of proteins termed α_2-globulin in the classical nomenclature. In the following it will be referred to as F(fast)α_2-globulin. The next slowest band, common to all three serum groups, is β-globulin. The darkly staining component migrating between β-globulin and the starting position in all three serum groups proves to be part of the α_2-globulin complex and is referred to as S(slow)α_2-globulin. The three components characteristic of sera from groups II A and II B which migrate between Sα_2-globulin and β-globulin are termed the $\alpha\beta$-proteins; it is emphasized that the $\alpha\beta$-proteins of group II A sera are not the same as those of group II B sera (see below). These $\alpha\beta$-proteins are again part of the α_2-globulin complex of the classical methods.

Other components, present usually in small amounts, have been observed, but are apparently much more variable in occurrence than those mentioned above. The following are notes on these components which, in general, will not be discussed further at present: (*a*) A component migrating more slowly than β-globulin, but faster than the first $\alpha\beta$-protein, is usually seen in group II A and II B sera, but again is not the same in these two groups (see below). This post-β-globulin is only infrequently observed in sera from group I. It is

derived from the α_2-globulin complex. (*b*) A component is sometimes seen migrating a little faster than the Sα_2-globulin but slower than the slowest $\alpha\beta$-protein. This component has been rarely seen except in sera from group II B. (*c*) Components are frequently seen migrating between the position of sample insertion and Sα_2-globulin. Usually there are two such components. The slower of the two most often appears as a very narrow band (see the group II B serum in Fig. 3). The faster one varies in position in different sera (compare the positions in the group I and group II B sera in the figure). However, these components are only clearly seen when the starch grain method of sample insertion is used. For this reason they are not regarded as so well established as the other components, which, when present, can be seen irrespective of the method of sample insertion. They appear to be derived from γ-globulin.

It will be noted that α_1-globulin does not appear as a definite band between α_2-globulin and albumin in starch gel electrophoresis. The majority of the protein staining material in the α_1-globulin position on filter paper is found to migrate in the starch gels in the same region as albumin, although it is possible that pre-albumin$_2$ is derived from α_1-globulin.

Before the significance of the results obtained by the application of the present method to sera may be discussed, it is necessary to establish that the new components are not artifacts. Three possibilities must be eliminated: it must be demonstrated that the additional components (*a*) are not caused by unsuspected anomalies of this new type of electrophoresis (this applies particularly to those components migrating close to albumin), (*b*) are not introduced by the clotting mechanism, and (*c*) are not the result of undetected haemolysis occurring during the serum preparation.

The first of these possibilities was regarded as excluded by the following observations carried out on several samples of group I serum. The presence of pre- and post-albumins could be demonstrated over a wide range of conditions. They were observed in buffers other than borate (barbiturate and aminotrishydroxymethylmethane buffers) in the presence of ethylenediaminetetraacetic acid, at pH values ranging from 7·7 to 8·7; at potential gradients of from 4·5 to 10 v/cm. and at temperatures from 7·5° to 40°.

More direct proof was obtained by preparing small amounts of the pre-albumins and of one of the post-albumins in electrophoretically pure states from isolated slices of a gel after electrophoresis, by means of the freezing and centrifuging technique described above. The serum used in these experiments had a relatively high post-albumin$_2$ concentration with very little post-albumin$_1$, so that only one of the post-albumins was investigated. The separated samples were dried from the frozen state and redissolved in a minimum of distilled water, and were then compared with the original serum. Fig. 4 shows diagrammatically the resulting stained gels (drawn from the originals).

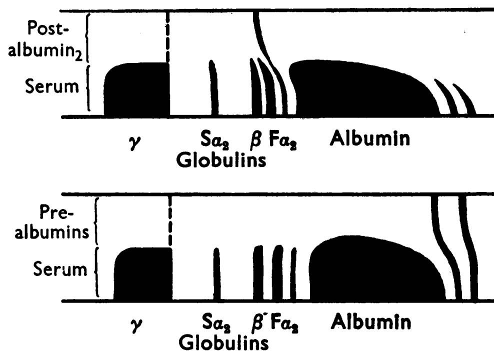

Fig. 4. Results (drawn from the original gels) of preparative experiments to establish that the components migrating close to albumin are not artifacts. In the lower part of the figure the pre-albumins are compared with the serum from which they were prepared. In the upper part a post-albumin is similarly compared. The filter-paper method of sample insertion was used.

The stained bands representing the components are continuous with the corresponding bands in the complete serum; they demonstrate effectively that the purified components are real and are not artifacts produced from albumin by electrophoretic anomalies. The lack of straightness in the bands is due to local differences in the potential gradients in the two halves of the gel produced mainly by albumin (see below), but this does not affect the conclusions drawn from their continuity.

That the pre- and post-albumin components are not introduced by the clotting process was shown by comparing plasma with serum on single gels. Plasma was prepared from whole blood after addition of oxalate or oxalate plus fluoride. With gels made from suitable buffers containing 0·005 M sodium oxalate to prevent possible clotting during the electrophoresis no differences in these components could be observed when oxalated plasma was compared with serum. Heparinized plasma gave similar results (with gels prepared from buffers containing 10 mg. of sodium heparin/100 ml.), although heparin was found to modify the solubility of the dye–protein complexes, so that no permanent records could be obtained in this case. In none of these experiments was conclusive electrophoretic demonstration of the fibrinogen of plasma obtained; probably the large and markedly

asymmetric fibrinogen molecules cannot enter the gels. In support of this is the observation that when filter paper was used for insertion of the plasma samples its anodic side showed quite marked staining at the completion of the experiment. However, since oxalate or heparin in the gels at the concentrations used will prevent clotting it is concluded that the components observed are not products of the clotting process.

That none of the protein-staining components in group I serum are dialysable was demonstrated by finding no change in the results following dialysis of the serum for 24 hr. against 0·03 M borate buffer of pH 8·50. Hoch & Chanutin (1953) have previously described a trace component in serum which migrates faster than albumin and which is not dialysable. Probably pre-albumin$_1$ and their fast-moving component are identical.

Haemolysis was excluded as a source of the additional components in sera from any of the groups by experiments in which increasing amounts of haemolysed red cells were added to samples initially showing no visible haemolysis. These experiments are considered in detail below.

When it had been established that the trace components present in group I sera were not artifacts, various experiments were performed to show that the three serum groups are the reflexion of real differences in the protein compositions of the respective sera. Of these the primary method was to compare sera from the different groups on the same gel so that inadvertent differences in the pH and composition of the gels or in the potential gradients were excluded as the cause of the observed differences. Fig. 5 shows photographically and diagrammatically a typical experiment of this type. On a single gel are compared the sera from three individuals, one from each group. The serum in the upper third of the gel in the figure differs from the lower two in the absence of the $\alpha\beta$-proteins and in the presence of additional protein-staining material migrating in the Fα_2-globulin position. The lower two sera differ from each other in that the $\alpha\beta$-proteins are not identical.

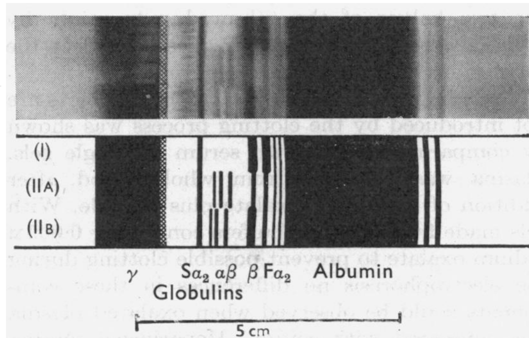

Fig. 5. Photograph and explanatory diagram of typical sera from the three groups compared on a single gel; the filter-paper method of sample insertion was used. The position of the filter papers is indicated by cross-hatching. The streaks appearing between the starting position and the $S\alpha_2$-globulin appear to be due to adsorption effects associated with the filter paper used in the sample insertion, since they do not occur except when filter paper is used (cf. Fig. 3).

Since groups II A and II B differ only slightly, all sera of these groups were further checked as follows: two sera were selected as representative of the two groups and the non-equivalence of their $\alpha\beta$-proteins was demonstrated by comparing them on the same gel. All sera classified as II A on the basis of the results obtained with separate gels were then compared with the II A standard and the equivalence of their respective $\alpha\beta$-proteins was confirmed. Similarly, all II B sera were compared with the II B standard. Finally the two standards were compared again (to exclude any ageing effects) and were shown to be different.

Other less pronounced differences may be observed in sera from the three groups. In group I sera the maximum number of post-albumins which can be resolved is two, but frequently in group II sera three may be seen (for example the II B serum in Fig. 3). When a post-β-globulin component is observed it is found to be different in the groups II A and II B. In II A sera it migrates only a little more slowly than β-globulin and may scarcely be resolved from it. The II A serum in Fig. 3 shows a poorly resolved post-β-globulin. The II A serum example photographed for the preliminary publication (Smithies, 1955) shows a resolved post-β-globulin. Post-β-globulin, when present in II B sera, is always well resolved and migrates appreciably more slowly at a position midway between β-globulin and the leading $\alpha\beta$-protein. These differences in the post-albumins and post-β-globulins are, however, of secondary importance as indices of the three groups as the proteins concerned are not always present.

Effect of added haemoglobin

Further evidence of the discreteness of the three types of sera is obtained from the experiments in which haemolysed red cells were added to sera from the three groups. The left-hand section of Fig. 6 shows diagrammatically the changes in the protein pattern of a group I serum on adding progressively increasing amounts of haemolysed red cells. The haemoglobin concentrations quoted are approximate in absolute magnitude (based on the assumption that a haemolysed red cell preparation contains 33 % of haemoglobin) but their relative magnitudes are correct. The cells were prepared from the same blood as the serum. At a haemo-

globin level of about 16 mg. of haemoglobin/100 ml. of serum a faint red band is seen migrating at approximately the same rate as β-globulin. At 31 mg./100 ml. this red band is more definite and it is apparent that the amount of protein in the $F\alpha_2$-globulin position is decreased. At 125 mg./100 ml. this decrease is maximal. However, protein-staining material is still present in the $F\alpha_2$-globulin position in approximately the same amount as in the sera of groups IIA and IIB. Furthermore, it is apparent that three post-albumins are present; presumably the third post-albumin was previously masked by the protein(s) removed from this region by haemoglobin. On further increasing the amount of haemoglobin a second red band appears which, by comparison with haemoglobin in the absence of serum, was shown to correspond to free haemoglobin. This protein is observed to migrate faster than β-globulin but a little slower than the material originally present in the $F\alpha_2$-position. No coloured bands are ever observed in the region between $S\alpha_2$- and β-globulins in group I sera on the addition of haemoglobin.

The changes on adding haemolysed red cells to a group IIA serum are shown in the centre section of Fig. 6. They may be summarized as follows: at 31 mg. of haemoglobin/100 ml. of serum the $\alpha\beta$-proteins have begun to bind haemoglobin; at about 125 mg./100 ml. the binding is maximal and three red bands can be seen in the region between the $S\alpha_2$- and β-globulins (but at positions not equivalent to the 'uncomplexed' $\alpha\beta$-proteins) with a fourth red band migrating at the position corresponding to free haemoglobin. On further increasing the haemoglobin concentration the only change is an increase in the amount of free haemoglobin present. Neither a decrease in the amount of material at the $F\alpha_2$-globulin position nor a coloured band at the β-globulin position, such as are observed with group I serum, are seen with group IIA serum in the presence of haemoglobin.

When a group IIB serum is similarly studied (right-hand section of Fig. 6) it is clear that the $\alpha\beta$-proteins present in this type of serum are different in their properties when 'complexed' with haemoglobin from those present in group IIA sera. Although three red bands are again seen they are neither in position nor in quantity similar to those present in group IIB sera after the addition of haemolysed red cells. The leading one is so faint as to be only just observable in the unstained gels. Once again neither a decrease in the amount of material at the $F\alpha_2$-globulin position nor a red band in the β-globulin position is observed.

In a series of experiments in which haemolysed red cells from individuals of each of the three groups were added to sera of the other two groups it was established that these results are due to differences in the sera. The source of haemolysed red cells appears immaterial.

The changes on adding haemolysed red cells to sera from each of the three groups are thus sufficiently different as to allow the characterization of a given serum on the basis of this effect alone, and the differences are so marked that sera can be classified, in the presence of haemoglobin, even

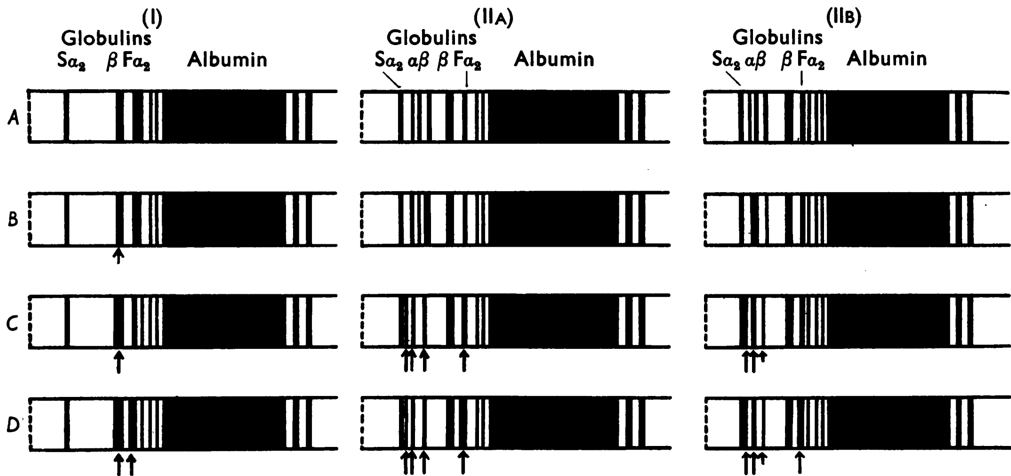

Fig. 6. Changes occurring in the serum-protein patterns on the addition of progressively increasing amounts of haemolysed red cells to sera of the three groups. Approximate haemoglobin concentration: *A*, 0; *B*, 31; *C*, 125; *D*, 500 mg./100 ml. of serum. The regions where red bands can be seen are indicated by arrows; a short arrow indicates a very faint band. The portions of the gels containing the γ-globulin are not shown.

before staining the gels. As a check on the reliability of this type of distinction, haemolysed red cells to the extent of about 660 mg. of haemoglobin/100 ml. of serum (0·02 ml. of haemolysed cells/ml. of serum) were added to all previously collected sera from over forty normal adults. In every case the changes observed were of the type expected from the known serum groups. This result is of practical value in that any serum samples which are inadvertently haemolysed may still be classified into their correct groups by observing the patterns obtained in the presence of excess of haemoglobin. Furthermore, in some individuals the $\alpha\beta$-proteins are present at a level low enough to make their characterization somewhat difficult, but after the addition of haemolysed red cells this difficulty is found to be removed since the bound haemoglobin increases quite considerably the amount of protein-staining material in the characteristic regions. On the basis of these various experiments it is concluded that the three serum groups reflect a difference in the serum proteins of the individuals concerned.

The haemoglobin experiments also lead to several conclusions about the nature of the proteins characteristic of the three serum groups and the interrelation of the three groups. They suggest that the excess of protein-staining material migrating in the $F\alpha_2$-globulin position in group I sera does not represent a quantitative change in the $F\alpha_2$-globulin but is due to the presence of a protein (or proteins) absent in group II sera. Apparently in group I sera $F\alpha_2$-globulin is present to about the same extent as in sera from the other two groups, but at least one qualitatively distinct protein characteristic of group I sera and notable for its haemoglobin-binding properties is also present with a mobility, under the conditions described, so close to that of $F\alpha_2$-globulin as to be unresolved from it. Hence the characteristic proteins of all three serum groups have a common property, not shared by any of the other serum proteins, of binding haemoglobin at the pH and electrolyte concentration employed.

Serum protein distribution in individuals

The present results lead to the suggestion that a fundamental serum-protein distribution exists from which all three serum groups are derived. In this fundamental distribution no $\alpha\beta$-proteins are present and the protein-staining material migrating in the $F\alpha_2$-globulin position is at the low level seen in group II sera. The detailed characteristics of the additional proteins present, over and above those in the fundamental distribution, determine the serum group that will be observed. Such a fundamental distribution, in which the serum-group-characterizing proteins are absent, has not been observed in any of the sera from over fifty adults studied so far, but it has been found in a number of young children. In such sera, as would be expected, the addition of haemolysed red cells produces no change other than the appearance of a single coloured band corresponding to free haemoglobin.

The distribution of the serum groups in the two sexes (normal adults) is shown in Table 1. Although the results show an apparent difference in the distribution, a statistical test indicates that this is unlikely to be significant ($\chi^2=3\cdot3$, $P=0\cdot2$). If the results obtained on some clinical sera are also included in the analysis, even the apparent difference largely disappears, and the statistics then indicate that a real difference in the two sexes is very improbable ($\chi^2=1\cdot3$, $P=0\cdot5$). No correlation appears to exist between the serum groups and the blood group (O, A, B, AB, Rh+ and Rh−) of individuals.

Table 1. *Distribution of the serum groups in forty-nine normal adult humans of both sexes*

Sex	Serum group I	Serum group IIA	Serum group IIB	Totals
Male	7	10	5	22
Female	3	15	9	27
Totals	10	25	14	49

$\chi^2=3\cdot3$, $P=0\cdot2$.

Little can be said with certainty at present on the physiological significance of the serum groups, but several tentative observations may be made. From the results on over fifty adults it seems that the groups are discrete. No intermediary types have been found and all the adult sera so far examined fall into one of the three groups. It is probable also that the serum group of an adult does not change with time; thus the author's serum showed no change of group in 15 months, and sera from eleven other individuals showed no group change in shorter times (2 days to 11 weeks). Some support for this is found in the literature in the only reference known to the author on individual variations in the plasma proteins of humans. Bernfield, Donahue & Homburger (1953) describe 'considerable dissimilarities' in 'the contours and fine architecture of the electrophoretic patterns of human plasma in different individuals' which they demonstrate by the Tiselius method. They illustrate the constancy of the type of result obtained from a given individual for periods up to 14 months and quote results of periods of over 2 years. The variations they describe were particularly apparent in the α-globulins (which is in agreement with the present work), but they were unable to make any generalizations from their observations, probably because the electrophoretic conditions that they

used do not demonstrate the presence of $\alpha\beta$-proteins. The constancy of the patterns which they observed in individuals would, however, agree with the present results.

The results so far obtained in children show that the proteins characteristic of the three serum groups are not always present in sera from young children, and suggest that their occurrence may vary with the age of the individual. Hereditary factors may determine the serum groups of adults [cf. Thompson, Foster, Gowen & Tauber (1954), who describe experiments suggestive of inherited differences in the serum proteins of mature mice]. Further studies of these possibilities are in progress.

Advantages and limitations of the starch-gel method

The greater resolving power, compared with previous electrophoretic methods, of the starch-gel method of zone electrophoresis for separating the serum proteins is probably chiefly a consequence of the use of a supporting medium the pore size of which approaches the molecular dimensions of some of the proteins involved. It is found that proteins with closely similar mobilities in free-solution electrophoresis and filter-paper electrophoresis may have widely different mobilities in the starch gels and that two proteins which migrate in a given order in free solution may migrate in the reverse order in the gels. This suggests that proteins of comparatively small size are slowed relatively much less in the gels than proteins of larger size or greater disymmetry, even though in free solution they may have the same mobilities. The starch-gel electrophoretic method thus appears to combine resolution by 'true' (free solution) mobilities, with resolution according to molecular dimensions, at least over some of the ranges of molecular size present in the serum proteins.

The low ionic strengths present in the gels, besides allowing high potential gradients without troublesome heating-effects, may also be partly responsible for the good resolution, in that anion-binding by the various proteins will be reduced so that intrinsic differences in their charges at a given pH will not be partly masked by large numbers of bound anions. Another factor is the sharpening of the protein bands that will occur because of the greater mobilities of the proteins in the slot into which the sample is introduced compared with their mobilities in the gel. That this type of sharpening occurs is seen by the existence of some protein bands very much narrower than the sample slot (see, for example, the component closest to the origin in the top photograph of Fig. 3). A further factor in the serum work has been the variation in the potential gradient along the gel caused by albumin. The reason for this variation is not clear, but its extent and its practical use in sharpening certain bands are illustrated in Fig. 7. In this figure the protein patterns and associated potential-gradient changes observed in 12·5 and 15% starch gels are shown. (The potential differences were measured with a vacuum-tube voltmeter at 5 mm. intervals by using as electrodes a grid of copper wires inserted into the gels.) In the 15% gel the potential-gradient changes are such that the post-albumins will be electrophoretically sharpened because of their relation to albumin and a region of

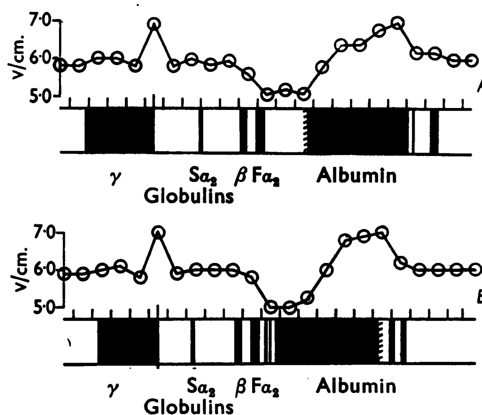

Fig. 7. Potential gradients observed at 5 mm. intervals along the gels and corresponding protein distributions obtained after 6 hr. electrophoresis of a group I serum in *A*, a 12·5% starch gel, and *B*, a 15% gel. The marks along the upper edges of the diagrams of the gels represent the electrode positions. The circles on the graphs show the mean potential gradient between the corresponding adjacent electrode-pairs. The cross-hatching indicates regions where the protein concentration changes slowly. The positions of the filter papers used for sample insertion are shown by a line extending above and below the diagrams.

rapidly changing potential gradient. In the 12·5% gel the relation of the post-albumins to albumin and the region of rapidly changing potential gradient is not such as to sharpen these bands and they are so diffuse as to be practically invisible. For similar reasons the presence of the post-albumins could not readily be demonstrated at potential gradients of less than 4·5 v/cm., but, as would be expected, a lower limit for the useful potential gradient was not marked for any of the other components.

The potential-gradient changes associated with albumin have one disadvantage, namely that the albumin spreads considerably during electrophoresis. After 1 hr. the albumin band is only about 5 mm. long, but after 6 hr. it is about 25 mm. long. The extent of this spreading is influenced by the amount of serum used in a given experiment

and, as is suggested by the results shown in Fig. 7, by small inadvertent variations in the percentage of starch used. Although these factors can be fairly well controlled, small variations in them will cause the position of the albumin front to vary a little in different experiments. However, the conditions of electrophoresis selected are such as to make these variations unimportant and at the same time to give the maximum sharpness to the greatest number of components.

Of the factors relevant to the albumin-spreading, small changes in the starch percentage produce the biggest effect; increases in pH at a fixed total borate concentration tend to cause patterns in which the albumin front is sharp, as do increases in total borate at an approximately fixed pH. Probably, therefore, the relative values of the starch concentration and the ionic strength are the fundamental determinants of the detailed pattern observed. Confirmation of this comes from the observation that when barbiturate buffer is used for the electrophoresis the albumin-spreading is similar to that in the borate experiments only if the ionic strength is made approximately the same. Although all components in the three serum groups can be demonstrated with barbiturate the results with borate are superior. Furthermore, total nitrogens can be performed on sections of the borate-containing gels, if required, and used as measures of the protein concentration without interference from the nitrogen of the buffer components. This consideration is particularly important in serum work as the local potential-gradient variations will cause a non-uniform distribution of buffer ions along the gel.

One limitation of the starch-gel method requires comment. As discussed above, the gels used for this work appear to have an effective pore size which prevents application of the method to high-molecular-weight substances. The failure to demonstrate fibrinogen in plasma (see above) suggests this, as does the observation that even with serum some protein-staining material fails to enter the gels. By observing the residual staining of the sections cut from the filter-paper electrophoresis experiments, and then used for starch-gel electrophoresis, it is found that high-molecular weight γ-globulin is probably the material involved. Indications of the same effect have been observed in other complex protein systems that are likely to contain high-molecular-weight proteins. Some of the cabbage-enzyme preparations studied by Dixon & Smithies (unpublished work) showed it, as did some samples of diphtheria toxin and toxoid. With lower-molecular-weight proteins of known purity (β-lactoglobulin and insulin) non-migrating material was never observed.

The retrograde movement of part of the γ-globulins should be noted. This is apparently not only a function of electro-osmosis in the gel as a whole but also of changes occurring in the region where the sample is introduced. Thus the γ-globulin is not so clearly defined when filter paper is used to insert the sample as when a starch suspension is used, and if a simple slot and spacer are used to insert the sample the γ-globulin appears to move backwards to a marked extent only in the regions where the spacer is touching the cut gel surface. Further indication of some unexplained behaviour in the γ-globulin migration is shown by failure under the conditions yet tested to obtain a true band for γ-globulin. Longer runs cause only a greater spread of the protein, never the appearance of a protein-free region between it and the site of introduction. Despite this, γ-globulin appears to show partial resolution into two fractions, as is seen in Fig. 3. No other protein systems tested have shown this effect, which may be merely a property of γ-globulin since only that part of the γ-globulin, which on filter paper travels most slowly, shows the effect.

In conclusion it is clear that the starch gel method cannot be regarded as capable of replacing other electrophoretic methods for all serum studies. The results here considered indicate, however, that the method when applied to the serum proteins will give a great deal of information that cannot be obtained by any other method now in use, even though the method is not yet in a form capable of giving quantitative data with serum.

SUMMARY

1. A method of zone electrophoresis in which starch gel is used as the supporting medium is described. Protein detection is by staining. The resolving power is in many cases superior to that obtained by the Tiselius method. As little as 0·02 ml. of sample can be used, if necessary, and the method is very well adapted to comparing closely related samples.

2. When the method is used for the electrophoresis of normal human sera several previously undescribed non-dialysable components may be demonstrated. These components can be seen with several buffers, including barbiturate. A borate buffer is normally employed. The period of electrophoresis is 6 hr.

3. Electrophoretic anomalies, products of the clotting process, and inadvertent haemolysis as sources of the new components are excluded.

4. Sera from over forty normal human adults of both sexes are found to fall into three groups on the basis of the occurrence of some of the new components. No difference in the distribution of the groups in the two sexes has been shown.

5. The components characterizing the three groups have a common property, not shared by any other of the serum proteins, of binding haemoglobin under the conditions used for the electrophoresis. They migrate in the α_2-globulin position when filter-paper electrophoresis is used.

6. The occurrence of the characteristic proteins may be related to age and to hereditary factors.

7. Possible reasons for the high resolving power of the new method and its limitations are discussed.

The author wishes to thank Dr D. A. Scott for his constant encouragement in the course of this work, Dr A. Sass-Kortsak and Dr F. O. Wishart for their assistance in obtaining blood, and Mr C. G. Smith for the photographic work. The help of Dr A. G. Ogston in the preparation of the manuscript is gratefully acknowledged.

Financial support for the work was provided by the Insulin Committee, University of Toronto.

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The Absorption Spectra of Porphyrin *a* and Derivatives

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(*Received* 7 *March* 1955)

In recent years, there has been a revival of interest in the problem of the structure of the prosthetic group of cytochrome *a* and cytochrome oxidase. Negelein (1933) and Roche & Benevent (1936) made the earliest attempts to isolate haemin *a* from heart muscle, but improvements in the preparative method by Rawlinson & Hale (1949) allowed of greater success. Warburg & Gewitz (1951) have claimed the isolation of crystalline 'cytohaemin', but publish no experimental details. Attempts to isolate haemin *a* from cytochrome oxidase and cytochrome *a* obtained in 'solution' by the use of sodium cholate have been reported by Dannenberg & Kiese (1952), and Falk & Rimington (1951) have studied the preparative methods for porphyrin *a*. Lemberg & Falk (1951) compared the spectroscopic properties of the porphyrin *a* prepared by Rawlinson & Hale with other carbonyl-porphyrins, and Lemberg (1953) has recently published more extensive results on porphyrin *a* and other porphyrins of heart muscle.

We have now shown that specimens of porphyrin *a* possessing identical spectra can be obtained under different experimental conditions. By applying a new method of porphyrin estimation (Oliver & Rawlinson, 1951), it has been possible to express the extinction data on a millimolar basis. The effects of solvents, oxime and copper-complex formation on the absorption spectrum of porphyrin *a* have also been examined.

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EXPERIMENTAL

Preparation of porphyrin a

Method A. The method is based on that of Rawlinson & Hale (1949).

(1) Six ox hearts freed of fat and ligaments were minced finely and extracted three times with 4% NaCl (total vol., 36 l.) at 0°. After draining, the pulp was treated twice with 2 vol. of cold ethanol; the pulp was pressed free from solvent each time. Extractions were then made with 2 vol. portions of acetone at 0°, until no more yellow pigments appeared in the extracts (usually three times). Excess of acetone was removed in a mechanical press.

(2) The dehydrated tissue was stirred with 2 vol. of chilled, acidified acetone (containing 2·5% v/v of 10N-HCl) and left standing at 0° for 1 hr.

(3) The dark-brown acetone solution was separated, filtered and added to an equal volume of peroxide-free ether. Addition of water (2 vol.) caused transfer of the pigments into the ether phase. Acetone was removed from the ether solution by washing with 2N-HCl. The ether solution contained both haemin *a* and protohaemin, as judged by the haemochrome test. The ether solution was filtered through a column of alumina (British Drug Houses Ltd., for chromatographic analysis) and the mixed haemins were obtained as a dark band at the top of the column.

(4) After thorough washing with fresh ether, the haemins were eluted with glacial acetic acid at 60–70°. The removal of iron by ferrous acetate and glacial acetic acid (Warburg & Negelein, 1932) gave a mixture of protoporphyrin and porphyrin *a*.

(5) After dilution with water, the mixed porphyrins were transferred to ether and washed free of acetic acid. Rapid extraction with 25% (w/v) HCl removed the porphyrin. By careful addition of solid Na_2CO_3 it was found that