

# AGAR ELECTROPHORESIS

## Part I. A Simple Clinical Method for the Analysis of Serum Proteins

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### SUMMARY

A technique of agar electrophoresis of serum proteins is described, which possesses the advantages of simplicity of equipment, better resolving power without adsorption, simplicity in staining procedure and easily adaptable to densitometric measurements, elution and radioactive tracer analysis. It is suitable for routine analysis of serum protein fractions in any clinical laboratory.

The technique lends itself also to other fields of research, *e.g.*, enzymes, polysaccharides, hormones, antibiotics, alkaloids and a number of other high molecular substances.

A technique of agar electrophoresis for the separation of serum proteins was described briefly in earlier reports from this laboratory (Giri, 1956, *a, b, c* and *d*). The technique was successfully applied to the separation of haemoglobins (Giri and Pillai, 1956 *e*). The practical value of the method was shown by a series of analysis of normal and pathological samples of sera (Giri, 1956 *b*). In the present paper details of the procedure for the analysis of serum proteins by this technique are described. Methods for quantitative evaluation of the serum protein patterns by densitometric and elution analysis and for radioactive counting are given. The advantages of the agar electrophoresis technique over other types of zone electrophoresis are discussed.

### MATERIALS AND METHODS

**Agar.**—Agar fine powder (B.D.H.) is used exclusively without subjecting it to any purification process, as it does not contain coloured impurities to interfere with photometric analysis. A 2% agar gel is prepared by autoclaving 2 g. of agar in 100 ml. of water at 15 lb. pressure for 30 minutes and kept at 50–60° C. in an incubator. The buffered agar gel is prepared fresh by mixing equal volumes of the 2% agar and 0.10 ionic strength veronal-acetate buffer of pH 8.6 and used as medium for electrophoresis. This mixture is kept at 50–60° C. before use.

**Buffer.**—Veronal-acetate buffer pH 8.6, ionic strength 0.05, is used for each electrophoresis run. A stock solution of the buffer containing 29.43 g. of veronal sodium, 19.43 g. sodium acetate trihydrate and 180 ml. of N/10 HCl and made up to three litres, is prepared. The stock solution is diluted 1:1 with distilled water to prepare the buffer of 0.05 ionic strength.

*Dye Solution (Amido black 10 B).*—The dye solution used for staining the protein components after electrophoresis is prepared by dissolving 5 g. of Amido black 10 B in one litre of the solvent mixture of the composition—methanol-acetic acid-water (40:10:50). The dye solution can be kept for long time at room temperature, the decrease in volume after repeated staining being replenished by adding small quantity of fresh dye solution.

*Apparatus.*—The apparatus consists of two main component parts:—

(1) *Power Supply Unit.*—Any convenient regulated D.C. voltage power supply can be used. The present investigations have been carried out by using the Kelab Electrophoresis Apparatus. In the author's laboratory a simple and inexpensive equipment has been built consisting of a constant voltage transformer with a rectifier or a series of 45 volts radio batteries as source of potential, two glass jars or perspex rectangular vessels containing platinum wire electrodes connected to perspex sheets and a sensitive milliammeter which is placed in the circuit in series with the agar gel plate. This inexpensive and simple unit has given satisfactory results comparable with those obtained by using commercial and expensive instruments. Any voltage between 180–300 can be used (Giri, 1956 *b*).

A schematic diagram of the general layout of apparatus which can be assembled in any laboratory is shown in Fig. 1.

(2) *Agar Plate Unit.*—Two pieces of plate glass (2 mm. thickness) cut into 30 cm. length and 5.2 cm. width are used, one for supporting the buffered agar gel and the other to be used as cover. Two frames of marginal width of 0.9 cm. are cut from a perspex sheet (1.2 mm. thickness), the outer dimensions being exactly the same as the two plate glasses. These frames serve to enclose the agar gel. The width of the enclosed space provided by the frames can be increased by reducing the marginal width of the perspex frames. By increasing the width of the enclosed space, the serum sample can be applied by means of a paper strip of greater length thereby improving the resolution of the components into more compact bands. One of the frames is placed over the plate glass. Two Whatman No. 3 paper strips cut to the size 8 cm. × 4 cm. are inserted at each end between the edges of the frame and the plate glass and about  $\frac{1}{2}$  cm. of the paper extending into the space provided by the frame. The frame and the glass plate with the paper strips are kept well clamped by means of spring clips and placed on a suitable support, arranged for proper levelling. A small spirit level can be used to adjust it to a horizontal position. Ten c.c. of the buffered agar gel (1%; pH 8.6; ionic strength 0.05) while still warm is poured carefully with 10 ml. pipette on the plate glass into the space provided by the frame. The gel is allowed to cool at room temperature. After the gel is set, the spring clips are removed and another perspex frame is placed over the one already kept on the plate glass. The plate glass with the frames is placed on the lips of the two electrode vessels of the Kelab Paper Electrophoresis equipment or any other suitable electrode vessels with the ends

of the filter paper strips at each end dipping into the buffer solution (about 300 c.c. of veronal-acetate buffer, pH 8.6; ionic strength, 0.05 in each vessel) in the electrode vessels. The distance between the buffer level and the agar plate is adjusted to about one cm.

*Application of sample.*—The application of serum to the agar gel layer as a narrow and uniform zone is of primary importance for obtaining good results. Considerable care should be exercised at this stage of the procedure. Usually 10  $\mu$ l of serum may be used. If the extinction values for albumin fall outside the range of the measuring instrument, the amount of serum should be reduced. The serum sample to be measured is carefully drawn into a 10  $\mu$ l micropipette by means of a rubber tube with a glass mouth-piece attached to one end, and the other end being connected to the pipette. The tip of the pipette is wiped clean with a piece of soft paper and the level of the serum is brought to the upper division mark on the pipette by touching the tip gently to a filter-paper. The serum is streaked evenly on a small strip of Whatman No. 1 filter-paper (20 $\times$ 2 mm.). If the width of the space provided by the perspex frame is more, a paper strip of the dimension 30 $\times$ 2 mm. can be used for application of the serum. This can be carried out conveniently by holding the paper strip with a forceps in the left hand and touching it with the tip of the pipette in the right hand and blowing gently. The paper strip containing the serum is placed at the centre of the agar gel layer perpendicular to the long axis of the plate without disturbing the surface of the gel leaving a space of about 2 mm. on each side of the gel layer. This technique of paper strip application ensures separation of the protein components into sharp zones. If the sample is applied directly on the agar gel as suggested in the earlier report (Giri, 1956 a), it is found that the zones are not always sharp and poor results due to blurring will be observed. After the application of the filter-paper strip containing serum, the other plate glass is placed on the perspex frame and the current is switched on. Two agar plate units are used for each electrophoretic run (Fig. 2).

*Electrophoresis run.*—The power supply is adjusted to 200 or 300 volts. A voltage of 300 volts, with the set up used in these investigations usually furnishes a current of 8–9.5 ma. giving adequate separation in approximately 3 hours. If a lower voltage of 200 is used the duration of the run is increased to 4 hours. The current is never allowed to exceed 10 ma. Optimum separation is usually obtained between 3–5 hours run. Longer runs (over 5 hours) usually produced patterns of greater width and the agar gel loses water. The ionic strength of the buffer used (0.05) is sufficiently low to minimise the heating of the gel by the current and at the same time maintaining the buffering capacity. The electrophoresis is always carried out at room temperature (22–25° C.). The maximum voltage permissible in any given experiment depends on the conductance of the gel, which varies with the thickness of the gel, the conductivity of the buffer and the rate of heat dissipation. Under the experimental set-up provided in the present investigation a voltage of 180–300 has given satisfactory results.

It is found that reversible electrodes or agar bridge between electrode vessels are not necessary for routine purposes provided the buffer in the vessels is replenished after each experiment. It is possible to carry out 4 or 5 electrophoresis experiments with one filling of buffer solution. The flow of current should be reversed by alternating the connections with the positive and negative poles in order to minimise the pH changes.

Owing to the sweating of agar gel during electrophoresis, moisture collects on the bottom surface of the top plate glass. The droplets of water collected do not fall on the agar layer. If the moisture collected on the plate glass is too high depending on the laboratory temperature, the top plate can be removed to wipe off the droplets of moisture and electrophoresis is continued again after replacing the plate.

*Drying.*—After completion of the electrophoresis, the top plate glass and the top perspex frame are removed carefully without the drops of moisture condensed on the bottom surface of the plate falling on to the surface of the gel. The plate glass containing the agar gel with the other perspex frame is removed from the electrode vessels. The two filter-paper strips at both ends are detached from the gel layer. The small filter-paper strip of serum is carefully removed from the surface of the gel by means of forceps without disturbing the surface of the gel. The plate glass supporting the gel and the perspex frame are kept intact by means of rubber bands fixed at both the ends of the plate and kept overnight (12–16 hours) at room temperature for drying. By keeping the agar plate in front of an electric fan the gel can be dried within eight hours. It is necessary that the agar gel layer is completely dried, as the presence of moisture seriously prevents the washing of the dye, which is strongly adsorbed at those regions containing moisture. In earlier experiments (Giri, 1956 *a*), the agar gel layer was not dried before staining and as such it took more than four hours to completely wash the free dye from the surface of the gel. After drying, the agar forms a thin transparent layer on the plate.

*Location of protein components.*—Three methods have been used for the location of the components:—

(1) The protein components may be made visible by coagulation with trichloroacetic acid. On adding 10 c.c. of 10% trichloroacetic acid to the surface of the gel before drying, the proteins coagulate and turbid bands appear on the gel, which can be seen clearly against the clear background. A rough evaluation of the variation in the concentration of the components can be made by visual observation of the turbid bands.

(2) By examination of the gel layer before drying under ultra-violet light with Wood's filter, the albumin band can be clearly seen. The decrease in concentration of the albumin band in pathological sera can be observed visually. The increase in  $\gamma$ -globulin band in pathological sera such as cirrhosis and kala-azar can also be observed under ultra-violet lamp.

(3) The usual staining procedures used in paper electrophoresis are applicable to agar electrophoresis. In the author's laboratory the Amido black dye is used for staining.

*Staining.*—After drying, the protein components separated on the agar gel are stained as follows:—

In earlier investigations (Giri, 1956 *a*), a saturated solution of the dye Amido black 10 B in methanol-acetic acid solution prepared according to Grassmann and Hannig (1952) was used for staining. This saturated dye solution is not suitable for staining the agar plate after drying, as the dye is tenaciously adsorbed on the edges of the dry agar layer, which cannot be removed completely by washing with methanol-acetic acid solution. The aqueous methanol-acetic acid dye solution of the composition described in this paper is better suited for staining the protein bands without leaving any marginal stain after washing. This dye solution has the further advantage that the time of washing is reduced considerably. About two minutes washing with methanol-acetic acid solvent removes completely the free dye, while it takes more than four hours, to remove the free dye completely from the wet agar gel when the saturated dye solution prepared according to Grassmann and Hannig (1952) is used as in the procedure described previously (Giri, 1956 *a*).

In the present improved staining procedure, the dry agar plate is immersed for about 20–30 minutes in the dye bath contained in a tall jar of one litre capacity. It is then removed from the dye bath and washed with a previously used methanol-acetic acid (90 parts of methanol and 10 parts of glacial acetic acid) solvent in an enamelled tray for 1–2 minutes followed by a second wash in a fresh solvent mixture for the same period. The agar plate is then kept for drying at room temperature. By this procedure the protein components show as blue bands against clear and transparent background, without any free dye adsorbed on the agar layer.

*Quantitative evaluation.*—The quantitative evaluation of the stained protein components can be made by (*a*) direct photometry of the bands and (*b*) elution and colorimetry of the dye.

The optical density of the separated protein fractions is determined with the photovolt Electronic densitometer, Model 525. The plate glass containing the electropherogram is covered with another plate glass, with a millimetre graph paper cut to 1 cm. width inserted between the two plate glasses at one of the edges lengthwise. The two plate glasses are then fastened together by means of an adhesive tape at the two ends. The plate glasses are passed across a 1 mm. light slit, through a carrier specially made of perspex. It is slowly moved until the movement of the meter pointer indicates the edge of the stained area of the albumin bands. This position is marked zero reading. The plate glass is then moved slowly by steps of one mm. The optical density is noted at every millimetre distance starting from the forward edge of the albumin zone. The readings thus obtained are plotted against the distance in millimetres on a graph paper and the resulting curves are analysed by planimetry. The relative areas can also be determined by cutting

the profiles from the paper along the lines and weighing the pieces of paper. By determining the weight of the paper of known area, the area of the curve in question can be calculated from the weight of the cut-out curve. This technique is, however, laborious. The use of planimeter is more rapid for routine measurements.

In the second method for the quantitative evaluation of the protein components, the stained bands can be scraped off with a razor blade from the plate glass into a test-tube and extract the colour with N/20 sodium hydroxide for colorimetric measurement. The elution technique used in paper electrophoresis by cutting the paper into strips of small width and extracting the dye for colorimetric measurements cannot be adapted to agar electrophoresis carried on glass. Furthermore, it is a general limitation of this technique that it cannot be adapted easily to radio-active tracer studies by counting technique, as in the case of paper electrophoresis, without further improvement. A simple procedure in which the above limitations are eliminated has been developed recently in this laboratory and the description of this technique has been reported in earlier publications (Giri, 1956 *c, d*). A brief description of this technique is given below:—

*Agar electrophoresis on cellophane and polyester films.*—A thin film of cellophane or any other suitable material, which is transparent, uniform in thickness, easily amenable to cutting into small strips and does not exhibit pronounced self-absorption of the radiation will fulfil the requirement.

Thin sheets of cellophane (0.0009 inch thickness) or “Mylar” polyester film\* (0.00025 inch thickness), cut to the size of the plate glass, are used as support for the agar gel. The cellophane sheet is first wetted with water and placed over the plate glass. The moisture is removed by blotting with a filter-paper and the sheet is pressed gently and evenly on the plate so as to obtain a smooth surface without the formation of wrinkles. The perspex frame is placed on the cellophane and the paper strips are introduced at each end. The cellophane, the perspex frame and the paper strips are kept intact by means of spring clips. The agar gel containing the buffer is layered on the cellophane sheet, and the electrophoresis is carried out in the same manner as described before. After the run, the plate glass with the cellophane containing the agar gel is removed from the assembly, dried and stained. The washing of the free dye takes about 30–45 minutes. After washing, the cellophane can be peeled off from the glass, washed again with a fresh methanol-acetic acid solvent and dried at room temperature. Similar procedure can be adopted for the ‘Mylar’ polyester film. The time taken for washing of the dye, in this case, is however short (3–4 minutes). The polyester film, being water repellent, the agar film can be peeled off from the film after drying and cut for elution and colorimetric estimation of the dye bound to the proteins. The same film can be used again for subsequent experiments. The electropherograms obtained on these films can be used for densitometric evaluation of the patterns by the scanning instruments used for direct photometry of paper electropherograms.

\* Manufactured by E. I. Du Pont de Nemours & Co. (Inc.), Wilmington 98, Delaware, U.S.A.

As cellophane sheets are readily available, less expensive and the electropherograms can be preserved for future reference, it is more convenient to carry out the routine analysis of serum proteins on cellophane instead of glass. In addition, the technique is useful for investigations of radioactive compounds, as the radioactivity of the substances separated on the thin film of agar can be easily determined. It can be used in any continuous radioactive scanning device used for paper strips.

#### RESULTS

The electrophoretic patterns of normal and pathological sera, and the corresponding densitometric curves, as obtained by the methods just outlined, are illustrated in Fig. 3. Nearly more than hundred samples of sera have been subjected to agar electrophoresis. Five main components—albumin,  $\alpha_1$ ,  $\alpha_2$ , beta and gamma globulins are readily visible in all cases investigated.  $\alpha_1$  component is always very faint,  $\alpha_2$  and beta globulins separate as compact bands, while gamma globulin separates as a diffuse band. Albumin appears as a compact and intensely coloured band. In many of the normal serum patterns and some of the pathological samples investigated there is observed a faint component having a mobility between beta and gamma globulins, which is here referred to as the  $\beta_2$  component, while the faster moving one is referred to as  $\beta_1$  component. Owing to electro-osmosis, the gamma and  $\beta_2$  globulins always occupy the position towards the cathode side of the starting point. Usually the width of the patterns is always about 9 cm. when the electrophoresis is run at 300 or 200 volts for 3 or 4 hours respectively. The width of the pattern increases with the increase in the time of electrophoresis. Duplicate runs showed the patterns to be exactly reproducible, provided the same volume of agar gel is used.

The patterns relating to tuberculosis and cancer show the usual evidence of increase in the  $\alpha_2$  component with corresponding decrease in albumin and increase in gamma globulins. The patterns of nephritis cases show the characteristic decrease in albumin and globulin with the splitting of the gamma-globulin component into two or more components. This has been observed in seven cases of nephritis investigated. The patterns of cirrhosis cases show the usual changes in albumin and gamma-globulin components.

#### DISCUSSION

The agar electrophoresis method described above is simple and easy to manipulate and the method brings electrophoresis within the reach of the smaller institutions and hospitals. The equipment required for this technique can be easily assembled with small cost from the materials available in any hospital or laboratory. A small rectifier unit or a radio battery can serve as source of power supply. A laboratory technician can easily carry out the analysis of serum samples in short time. Some skill is required to learn the proper paper strip technique of application of the sample and to layer the agar gel as a uniform surface. With some practice the procedure can be easily mastered.

The examination of pathological changes in the electrophoretic patterns is as easy as any other method of zone electrophoresis. An increase or decrease in the intensity of the colour of the globulins and albumin bands in the case of pathological samples of serum is immediately seen qualitatively on the agar plate, and the approximate percentage can be estimated quantitatively by rough computation and comparison with the pattern obtained with normal serum sample. As the colour of the bands do not fade, the patterns furnish a permanent record of an experiment for future reference. The densitometric curves are more informative from quantitative aspect.

By increasing the width of the agar plate to 8 cm. two samples can be run side by side thereby facilitating the comparison of the changes in the concentration of the components present in the samples of serum.

The technique affords the advantages over paper electrophoretic method of shorter experimental time, easier staining procedure, transparency of the dry agar film which facilitates photometric measurements, absence of trailing of proteins and very small resistance offered by the gel to the movement of the proteins. Furthermore, the defects inherent in paper electrophoresis, namely, the anisotropy due to fibres and the reticular structure of the paper which makes the protein follow a tortuous path in its passage through the paper thereby necessitating the introduction of a factor for determining the mobilities (Kunkel and Tiselius, 1951), are not present in the agar electrophoresis technique.

One of the important advantages of agar gel over the starch and paper as supporting media is that agar gel has a very low supporting medium—liquid ratio, which is an important factor in increasing the capacity of the system, without offering much resistance to the movement of large size molecules. The other supporting media used in electrophoresis, namely, paper and starch occupy half or more than half of the total volume (Kunkel and Tiselius, 1951; Kunkel and Slater, 1952).

Another characteristic feature of the agar electrophoresis technique is the better resolution of the component, without adsorption. The resolution of beta components in most of the sera examined and the gamma component into two or more components in nephritis cases have been observed. These resolutions could not be observed even in boundary electrophoresis, using the Kern Micro-electrophoresis apparatus.

The cooling system introduced by Bussard and Perrin (1955) is found to be superfluous in this arrangement. For obtaining better resolution of the components, the 'sweating' of agar which takes place as a result of the passage of the current need not be suppressed by coating the surface with a plastic pellicle as recommended by Bussard and Perrin. The small amount of moisture collected on the bottom surface of the top plate glass can be allowed to remain till the end of the run to provide the required humidity in the cell. If, in any experiment, it is found that the drops of moisture collected on the plate is likely to fall on the agar gel



during electrophoresis, the top plate can be removed and replaced again after wiping off the moisture.

Although there is a significant electro-osmotic flow through the agar gel, it remains constant during electrophoresis provided the electrical field strength and the concentration of the electrolytic solution are kept constant. It does not vary with the different lots of the same agar gel, unlike paper in which the flow varies with different lots of paper (Engelke, Strain and Wood, 1954). The electro-osmotic flow does not interfere too much with the resolution of the components. Our experience with agar electrophoresis has shown the patterns to be exactly reproducible provided the same experimental conditions are maintained. Further advantage of agar over paper is that the water content in agar is maintained almost constant than in paper (Bussard and Perrin, 1955\*).

One of the chief advantages of the agar electrophoresis technique is that the densitometric evaluation of the stained protein bands is rendered easy on account of the transparency of the dry agar film. The densitometric curves (Fig. 3), which are quite smooth without any irregularities are easily amenable to planimetry and the areas thus obtained can be used for calculating the percentage of each protein component. Further work on the quantitative evaluation of the densitometric curves obtained by this technique and comparison with the values obtained by moving boundary method is in progress, which will be reported shortly.

The use of cellophane and 'Mylar' polyester films affords a simple method of eluting the components separated on the gel by cutting the film into small strips at right angles to the direction of separation, as in the case of paper electropherograms, and extracting the components by suitable methods. The concentration of the substances can be determined by the usual methods of analysis. In the case of proteins stained with the dye, the concentration of the proteins can be determined by estimating the blue colour extracted with N/20 sodium hydroxide by colorimetry. The dye can be easily eluted from the cut film strips. As agar does not exhibit any absorption in ultra-violet light, the technique can be employed for the separation and quantitative determination of ultra-violet light absorbing substances by ultra-violet photometry. It is obvious that the technique of agar electrophoresis obtained on the films is applicable to investigations of radioactive compounds, as the self-absorption of the thin films will not be as high as paper.

The simplicity of the technique provides a convenient means for following the progress of attempts to separate individual protein components and in the purification of proteins.

For clinical purposes, quantitative evaluation by photometric scanning or elution analysis is not always necessary. A set of electrophoretic patterns of normal sera may be prepared against which the patterns of the experimental samples may

\* The paper by Bussard and Perrin appeared after this work was completed. The technique described by these authors is mainly intended for the determination of the mobilities of charged constituents including serum proteins.

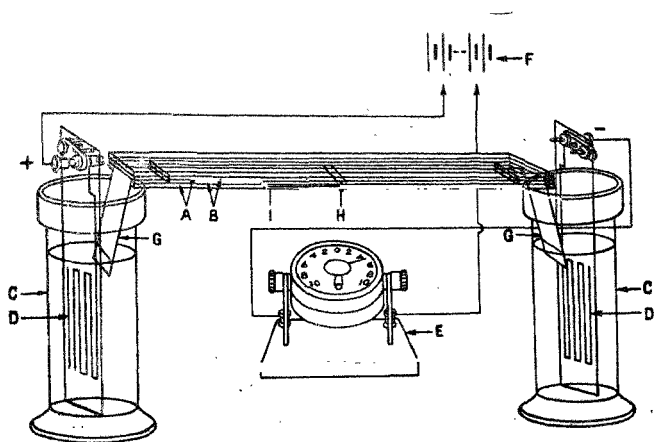


FIG. 1

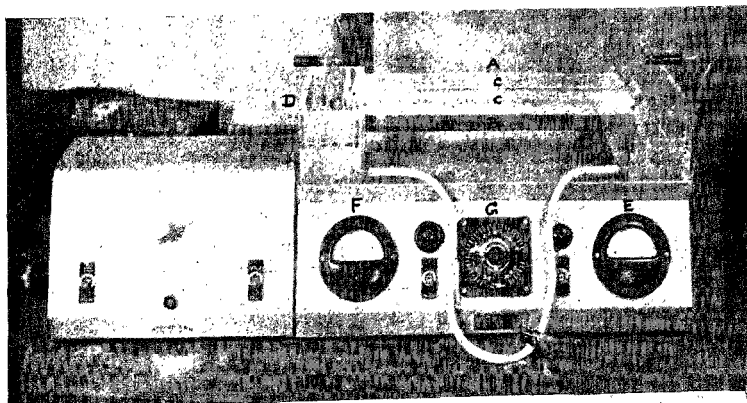


FIG. 2

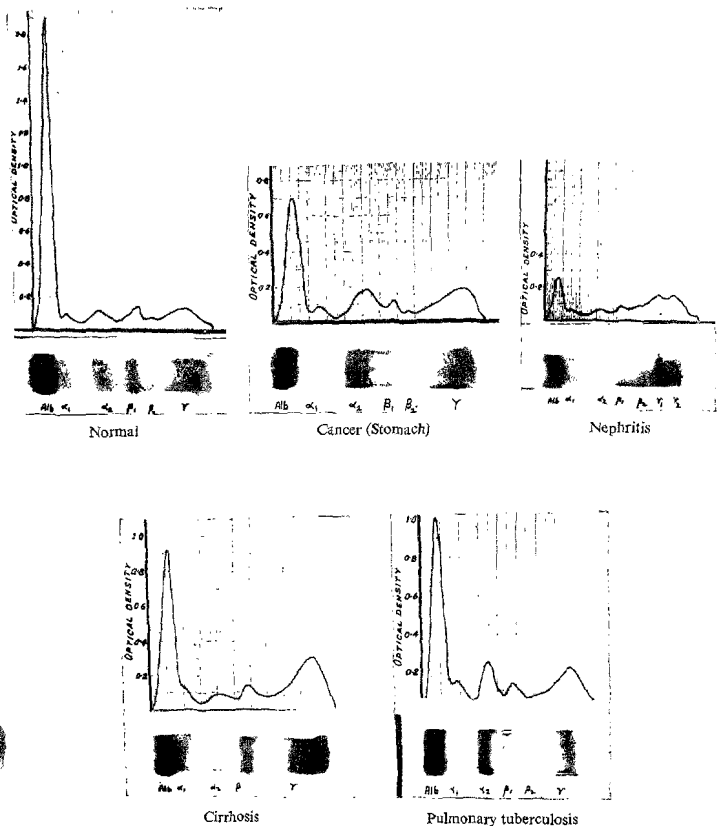


FIG. 3

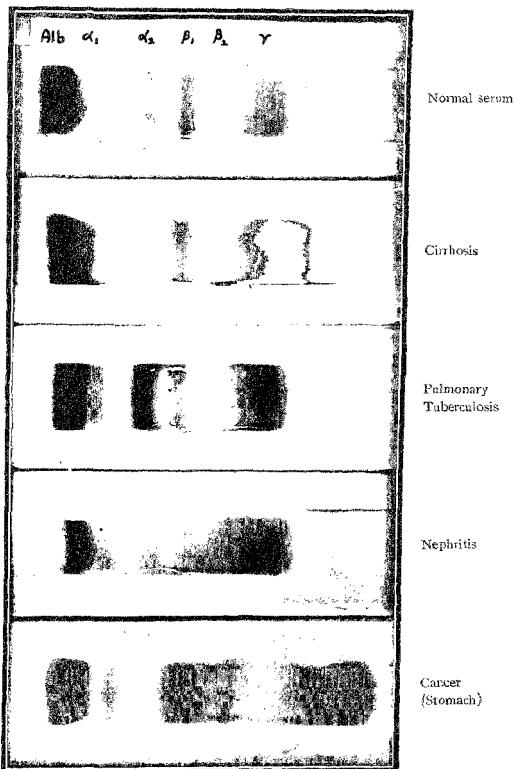


FIG. 4. Agar electrophoresis patterns of normal and pathological serum samples

be visually matched. After some experience, it is possible to assess the variation of the patterns from the normal by inspection alone, without comparison with the normal pattern. Since the major changes in the components of serum can be observed by simple inspection, and the quantitative analysis may be made by simple photometric scanning, the agar electrophoresis method offers distinct advantages as a simple clinical method for routine serum protein analysis.

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## EXPLANATION OF PLATES

- FIG. 1. Schematic diagram of the general layout of apparatus. A—Plate glasses; B—Perspex frames; C—Electrode vessels containing buffer solution; D—Platinum electrodes; E—Milliammeter; F—Source of potential; G—Filter-paper (Whatman No. 3) connecting the electrode vessel and the agar plate; H—Position of filter-paper strip for sample insertion; I—Layer of agar gel.
- FIG. 2. Kelab Paper Electrophoresis equipment used for agar electrophoresis. A and B—Electrophoresis cells containing buffered agar gel; C—Paper strip containing serum; D—Electrode vessels; E—Voltmeter; F—Milliammeter; G—Automatic time switch.
- FIG. 3. Agar electrophoresis patterns with the densitometric curves of serum proteins of normal and pathological cases.