SEPARATION OF ANTIGENS OF LOW ELECTROPHORETIC MOBILITY BY THE METHOD OF IMMUNOFILTRATION

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A study of the antigenic structure of viral, bacterial, or tissue systems requires the application of methods which enable the specific antigens characteristic of these systems to be revealed and separated. One such method is immunofiltration [1]. It is based on the principle by which antigens and antibodies are caused to meet in agar by electrophoresis, and therefore it may be used only to study antigens whose electrophoretic mobility differs appreciably from that of the antibodies, i.e., from that of the γ-globulins.

In electrophoresis in agar of a mixture of antigens having an anodic mobility, only that portion of the components whose rate of migration exceeds the electro-osmotic flux directed from anode to cathode will move towards the anode. In electrophoresis of serum in the normal sorts of agar, only the albumins and the α-globulins move towards the anode, whereas the β- and γ-globulins are carried by the electroosmotic flux to the cathode.

If the antigen and substances attached to it have an anodic mobility in agar, it is then easy to create a system in which the γ-globulin fraction of the serum containing antibodies to the substances attached to the antigen but not to the antigen itself will be carried to meet the antigen in the electroosmotic flux. The antigen moves freely through such a "filter", whereas the impurities bound to the antibodies drop off as a precipitate, or else greatly change their electrophoretic mobility (immunofiltration).

This method may be used to separate by electrophoresis in agar only antigens having an electrophoretic mobility equal to or greater than the mobility of albumins and α-globulins.

In this connection the need arose to apply the method of immunofiltration to antigens having an electrophoretic mobility close to the mobility of β- and γ-globulins. The use of special sorts of agar of the type ion-agar No. 2, in which the electroosmotic flux to the cathode involves only γ-globulins, enabled the method of immunofiltration to be used for antigens having an electrophoretic mobility equal to that of the β-globulins. For antigens having the mobility of the γ-globulins the method of immunofiltration cannot be used without special modifications. For immunofiltration of such antigens it has been proposed to use antibodies conjugated through a diazo-bond with arsanilic acid [4]. Such antibodies lose their precipitating properties, but they retain the ability to combine with antigen [4, 6, 7]. The residue of the arsanilic acid increases the negative charge of the antibodies, and so increases their electrophoretic mobility. In electrophoresis in agar such antibodies have the mobility of the α-globulins.

As antigens we used β- and γ-globulin fractions separated by previous electrophoresis in agar from mouse, rabbit, and human serum as described previously [3]. In many experiments we used a preparation of human γ-globulin prepared at the I. I. Mechnikov Institute of vaccines and sera.

In analysis by immuno-electrophoresis preparations of rabbit and human γ-globulin gave only one precipitation band with the homologous antiserum. The β-globulin fractions contained a small amount of α- and γ-globulins as impurities.

For the immunofiltration, as the "filter" we used immune rabbit sera or γ-globulin fractions separated from them by previous electrophoresis in agar.

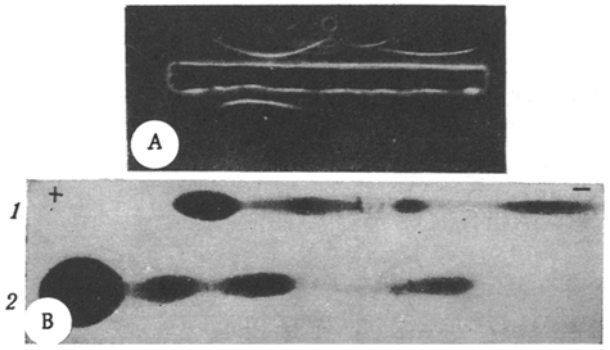

Fig. 1 (below). Distribution of the serum proteins in "difko" agar (1) and in ion-agar No. 2 (2).

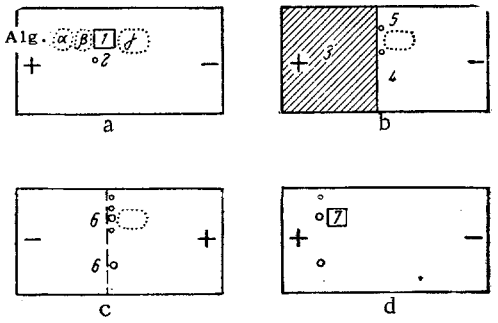

Fig. 2. Diagram illustrating the process of immunofiltration. 1) Reservoir for the "filter"; 2) hole for the pyronine; 3) zone of agar removed from a plate; 4) a line of cut in agar; 5) points indicating the cathode edge and width of the reservoir for the "filter"; 6) holes for the antigen; 7) reservoir for "azoglobulins" in "difko" agar.

The method of obtaining the immune sera has been described previously [2]. The serum against mouse serum gave the precipitation reaction in agar with mouse β-globulin at a dilution of 1:32, the serum against human serum gave the precipitation reaction at a dilution of 1:64. To demonstrate the results of immunofiltration we used antisera to mouse serum and to human serum; we also used antisera to human serum obtained from the Scientific Research Institute of forensic medicine.

Conjugated γ-globulins with arsanilic acid were produced as follows [7]: 200 mg of atoxyl were dissolved in 20 ml of distilled water to which was added 5 ml of 1 N HCl. At the same time we prepared a 0.5 N solution of $NaNO_2$. Both solutions were cooled to 2-4°, and while the atoxyl was added to the solution drop by drop we added 1.3 ml of $NaNO_2$ over a period of 10-15 min. The appropriate course of the reaction was followed in terms of the transitory blue color acquired by Congo red paper. The mixture was then kept in the cold for 30 min. The completion of the diazo reaction was determined from the color of starch-iodine paper. We added 200 mg of urea, and retained the mixture for a further 10 min.

From the freshly prepared solution of the diazonium salt we took the required amount, calculated as 1 mg of atoxyl per 7 mg of protein, and added it drop by drop while carefully mixing it into the cooled solution of the γ-globulin fraction over a period of 40 min. The pH of the mixture was kept constant at between 8.0 and 9.0 by means of a 1 N solution of NaOH. The solution of "azoglobulins" obtained was acidified to pH 4.0-5.0, and the proteins, which formed a sediment, was washed 3-4 times by physiological saline brought to pH 4.0-5.0. The washed precipitate was dissolved in the required amount of veronal-medinal buffer having an ionic power of 0.05, and was then dialyzed against this buffer for 24-48 h.

The corresponding immunofiltration of the β- and γ-globulins was carried out in the apparatus for immunoelectrophoresis, or with the apparatus for the high-voltage electrophoresis in agar under a layer of petroleum ether, using the method of Wieme [8].

Immunofiltration of β-Globulins in Ion-Agar

For electrophoresis we used ion-agar No. 2 made by the firm Oxoid; the concentration was 0.5% in veronal-medinal buffer at pH 8.6; ionic power 0.025. The distribution of the serum proteins in this case is shown in Fig. 1 (inset).

Immuno-filtration of the β-globulins in ion-agar No. 2 was carried out by a special method. Glass plates measuring 7.5 × 5 cm were covered with a layer of melted agar 1.5-2 mm thick. A square hole measuring 5 × 6 mm was cut in the agar near to the anode edge (Fig. 2a). In our experiments, and as a variation from the method of Wieme [8] the glasses were placed in the apparatus for the high-voltage electrophoresis of agar upwards. The agar on the glass plate joined with the agar in the drippings from the heated agar.

The square hole was filled with rabbit serum against mouse serum which had previously been dialyzed against a buffer heaving an ionic power of 0.025. At the anode edge of the square hole an opening was made for pyronine. The pyronine, whose electrophoretic mobility is very close to that of γ-globulin is a convenient "witness" for the uptake of γ-globulin in agar. The electrophoresis was performed by use of a potential gradient of 20 v/cm, and at a current strength of 35-40 mA.

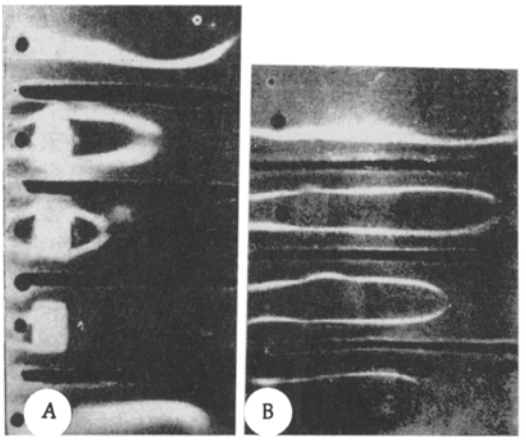

Fig. 3. Choice of dose of antibodies for "filter". a) Separation of the antigen, "filter" constant; b) various sizes of "filter" for constant amount of antigen.

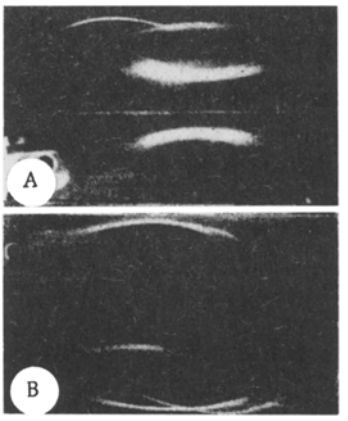

Fig. 4. Immunofiltration of β-globulins in ion-agar No. 2 (a) and in "difko" agar (b).

From the movement of the pyronine beyond the cathode edge of the hole we followed the uptake of γ-globulins of the serum in agar. If it was required to repeat with a second or third dose of γ-globulins, then after the first operation of the "filter" the current was switched off and the residue of the serum and of the buffer was sucked out of the hole, which was then filled with a new serum, and the procedure again repeated. Under these conditions a single operation of the filter in agar occupied 7-12 min.

The position of the hole in the agar was indicated by dots; then a slit was made along the cathode edge of the hole and all the agar was removed from the anode half of the glass plate where all the serum fractions except the γ-globulin were present (Fig. 2b). The removed agar was replaced by a new melted portion which was made to lie level with the agar remaining on the plate.

If the next determination of the antigens is to be made with immune sera, then, instead of the agar being removed from the plate it is sufficient to pour on a square reservoir of melted agar. The serum proteins (albumin and α-globulins) remaining in the agar exert no influence on the subsequent appearance of the immunophoregram. In the agar opposite the zone containing γ-globulin we bored holes having a diameter of 2-3 mm for the antigen (Fig. 2c), and filled them with the β-globulin fraction of mouse serum, or with a mixture of mouse and human β-globulins. We then reconnected the supply, and switched on the current. The second stage of immunofiltration was continued until the pyronine had returned to its original position, or had removed even further towards the cathode. Then, by the method normally used in immuno-electrophoresis [5] we identified the zones of antigens by the corresponding sera, or we stained the agar plates for protein by amidoblack, by the method of Uriel.

In some cases, particularly in prolonged electrophoresis, before the glass plate is embedded in the agar, in order to avoid the agar sliding off the plate it is convenient to make on it an agar spoon, as has been recommended by Grabar [5].

Immunofiltration of β- and γ-Globulins by Use of "Azoglobulins"

Immunofiltration of the β- and γ-globulins was carried out in "difko" agar. In this case the square reservoir for the "filter" was placed in the pathway of migration of the antigens, and into it we poured "azo-antibody". The "azoglobulin" was heated on a waterbath to 45-48°, and was mixed with an equal volume of 2% agar prepared from distilled water and brought to the same temperature. This mixture was used to fill the reservoir in the agar rapidly to the level of the agar on the plate. In front of the filter and beside it we bored holes for the antigen, for control (Fig. 2d). In line with the antigen we placed a hole for the pyronine which in this case is the witness for the position of the antigens. Electrophoresis was continued until the pyronine had moved 10-15 mm from the cathode edge of the "filter". The antigens were then revealed in the same way as before.

Choice of Dose for the "Filter"

The relative amounts of antigen and antibodies required for complete retention of the antigen was determined empirically. For this purpose we used various dilutions of antigen and a "filter" of constant size, or else filtration

of a given antigen through a single, double, or triple "filter" (Fig. 3, inset). When filtration of a mixture of human and mouse β-globulins was made in "difko" agar, a double filter of "azoglobulins" completely retained the human β-globulins (Fig. 4, inset).

A mixture of human and rabbit γ-globulins served as a model of immunofiltration of antigens of the very lowest electrophoretic mobility. For the filter we used "azoglobulins" against human sera. A double "filter" of these azo-antibodies completely retained human γ-globulin if the amount in the mixture did not exceed 1.2 mg/ml.

To obtain definite and reproducible results the antigens must be dialyzed or prepared in the same buffer which is used for preparation of agar. This is particularly important if the diameter of the holes for the antigens is greater than 1.5 mm. If these conditions are not fulfilled breaks occur between the agar and the holes, and a lot of buffer leaks out leading to dilution and smearing of the antigen, with the result that the result presented by the experiment is blurred.

It is most important to exercise care in the dialysis of the serum or of the "azoglobulins" used as "filter". If native antisera are used they must be carefully dialyzed against a buffer having the same ionic power as the buffer in which the agar was prepared.

In cases when the serum or the "azoglobulins" were mixed with 2% agar in distilled water the dialysis should be made against a buffer having twice the ionic power. The serum or "azoglobulins" should be mixed with 2% agar in strictly equal volumes in order that the percentage content of the agar in the filter and on the working plate should be the same. A hole for the filter should be filled with the mixture to the level of the agar on the glass plate.

If these conditions are not observed many undesirable phenomena occur: clefts and cracks occur in the agar in the filtrate, buffer leaks on the surface, and part or all of the agar slides off the glass plate. The formation of clefts in the agar leads to a change in the mobility of the antigens in the experiment (in comparison with the control group), giving the illusion of some partial nonspecific retention of the antigen by the filter.

If the conditions enumerated above are observed it is easy to obtain clear cut and reliably reproducible results in immunofiltration of antigens having a low electrophoretic mobility.

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