Qualitative Analysis of Gel Precipitates with the Aid of Chemical Color Reactions. (20909)

BERTIL BJÖRKLUND. (Introduced by Joseph L. Melnick.)

From the State Laboratory of Bacteriology, and the Department of Virus Research, Caroline Institute, Medical School, Stockholm, Sweden.

In Ouchterlony's method of immunoprecipitation(6-13) antigen and antibody are allowed to diffuse towards each other in an agar gel. The result usually is a precipitation pattern consisting of a number of lines. In principle each line consists of a compound formed by a single antigen and its specific antibodies(1,2,9), which means that a separation of the different antigenic components of the material has been achieved. If these components after combination with antibody retain some of their chemical characteristics it should be possible to gain valuable information about the chemical nature of the different antigens in a complex system by combining the gel diffusion method with certain chemical and physical methods of analysis.

In the preliminary experiments here to be described, some color reactions were tried.

Materials and methods. For immunoprecipitation experiments 3 different serological systems were used. The first was a diphtheria toxin preparation and the corresponding horse antiserum(1). The second was a powder of rabbit bone marrow prepared by ether extraction(2,4) and a mucopolysaccharide fraction (4) of the crude material. The corresponding hyperimmune serum was obtained from a horse immunized during a period of 4 months with a total amount of 33 g of the bone-marrow powder(4). The third system consisted of purified type specific polysaccharides from pneumococcus type II(5) and the corresponding horse antiserum. All diffusion experiments were performed by the 2-dimensional technic in plates of 1.5% agar gel(1).

Experimental. Equivalent amounts of diphtheria toxin and antitoxic serum at concentrations varying from 100 to 700 LfU/ml were allowed to diffuse towards each other in a gel (Table I). In a parallel experiment 700 LfU/ml of toxin were diffused against concentrations of antitoxin varying from 100 to 600 LfU/ml, and 700 LfU/ml antitoxin against

TABLE I. Arrangement of Gel Diffusion Experiment with Diphtheria Toxin (Dt) and Anti-toxin (At).

Dt	At	Dt	At	Dt	At
700	700	700	600	600	700
600	600	"	500	500	"
500	500	"	400	400	"
400	400	"	300	300	"
300	300	"	200	200	"
200	200	"	100	100	"
100	100				

concentrations varying from 100 to 600 LfU/ml toxin. When the precipitation patterns were fully developed, the plates were washed in physiological saline for 3 days and then treated with increasing concentrations of ethyl alcohol up to 70%. The plates were then placed in a basic fuchsin solution (4 g basic fuchsin + 10 ml acetic acid + 1000 ml 70% ethyl alcohol) for 2 hours, washed again in decreasing concentrations of alcohol and finally in saline.

Of the numerous precipitates obtained with the diphtherial antigen-antibody system, one line had acquired a distinct red color, while the other lines were unstained (Fig. 1). The site of the colored line as well as that of the other lines was dependent upon the relative

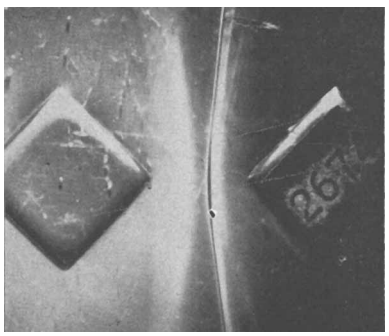

FIG. 1. Stained precipitation spectrum of 100 LfU/ml of diphtheria toxin and 100 LfU/ml of antitoxin. The plate is illuminated obliquely by reflected light from a Hg-lamp. The colored precipitate does not reflect the light and stands out as a distinct dark line.

concentrations of toxin and antitoxin. With equivalent concentrations the site of the lines was constant, regardless of the absolute concentrations. The strength of the color varied with the concentrations of the reactants. These observations indicated that the capacity of retaining the dye was associated with one particular antigenic component which means that the color reaction under these conditions was a specific one.

In the next studies antigens of rabbit bone marrow and a purified mucopolysaccharide fraction thereof containing 4 distinct antigenic components were used. The purified material was subjected to paper electrophoresis at pH 7 (phosphate buffer). After development with Mayer's mucicarmin(15) 4 spots were observed, 2 brightly red whereas 2 were more faintly stained. An unstained filter paper from the same run was cut into 7 sections, each of which was extracted with distilled water and the extract concentrated by lyophilization. The fractions thus obtained were dissolved in water and tested in gel plates against anti-bone-marrow serum. Precipitates were obtained only with fractions corresponding to the 4 spots. When treated with mucicarmin all 4 precipitates stained brightly red.

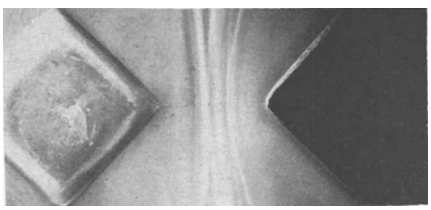

FIG. 2. Unstained precipitation spectrum of bone-marrow.

An anti-bone-marrow serum and an extract of the crude bone-marrow powder were then allowed to diffuse towards each other in gel plates (Fig. 2). The resulting multiple precipitates seen on the photographs were treated with mucicarmin. Of the several lines, only 4 retained the stain, 2 strongly, 2 less so (Fig. 3).

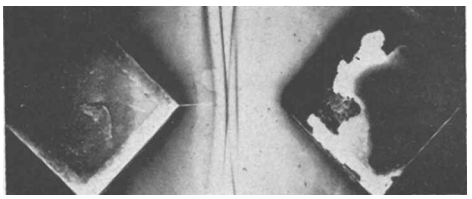

FIG. 3. Precipitation spectrum of bone-marrow. Plate stained with Mayer's mucicarmin.

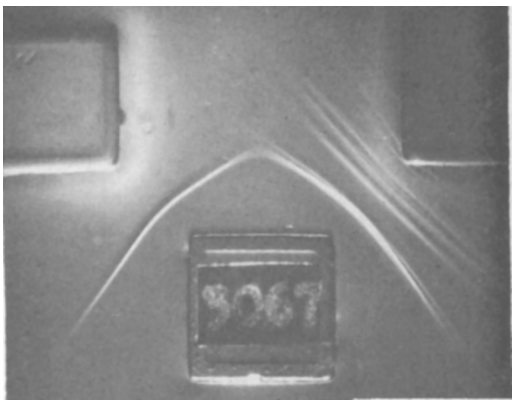

FIG. 4. Precipitation pattern of type specific polysaccharides from pneumococcus type II (left) and the same antigen in mixture with rabbit serum protein (right).

In the third system studied (Fig 4.) a mixture of anti-pneumococcus type II and anti-rabbit-serum-protein horse serum in the bottom basin (Fig. 4) was diffused against pneumococcus type II polysaccharide in the left basin and a mixture of pneumococcus type II polysaccharide and rabbit serum in the right basin. On both sides the pneumococcus system gave rise to precipitates which showed the reaction of identity. Furthermore, on the right side a number of precipitates due to the rabbit serum developed. These lines were not deflected towards the left indicating that they did not correspond to the antigens in the left basin. One plate of this kind was treated with mucicarmin and another with basic fuchsin. In both cases the precipitation lines corresponding to the pneumococcus polysaccharide were brightly stained and easily distinguished from the rabbit protein lines (Fig. 5).

Discussion. An antigen will diffuse through the gel until it meets the corresponding antibodies. When these two reactants meet in optimal proportions a precipitate will occur. On one side of the precipitate, only antigen can exist in a free state and on the other side only antibody. Antigen, diffusing through the precipitate, will suddenly meet the corresponding antibody and vice versa. In this

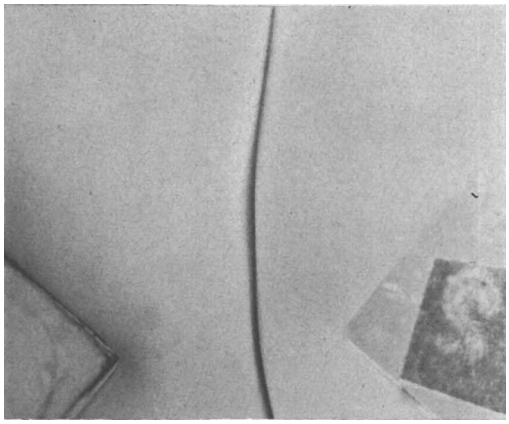

FIG. 5. Detail of the precipitate of pneumococcus type II polysaccharide. The line is stained with basic fuchsin.

way the precipitate is built up progressively during the continued diffusion from both diffusion centers. The precipitate, however, does not seem to hinder the diffusion of other components not involved in the reaction, thus acting as a selectively semipermeable layer.

Even if eventually the bulk of the reactive antigens and antibody protein will accumulate in the precipitates the medium in between will probably never be completely exhausted. Thus, in the gel surrounding the antigen basin, all of the antigenic components will be demonstrable in addition to all non-reactive constituents of the antigen as well as of the serum preparation. Moving from this region towards the serum basin one will, on crossing the first precipitate line, notice a qualitative change in the composition of the medium which beyond this line is poorer by the antigen participating in the reaction but richer by the particular antibody involved. Similar stepwise changes will occur each time a precipitate line is passed; one antigen will disappear and one new antibody will appear (Fig. 6). Consequently in order to obtain a clean separation of the reactive components, one has to remove all of the soluble material by washing, leaving only the spatially distinct insoluble precipitates.

Although the antibody globulins in the precipitates undoubtedly display structural differences associated with their immunological specificity, chemical means of detecting such differences are not yet available. The antigen components of the precipitates, on the other hand, can be expected to show a wide variation in chemical characteristics, and a demonstration by means of chemical reactions of such differences and thereby a differentiation between the various lines in a precipitate spectrum should be feasible once a separation of the components has been achieved. The simple staining reactions applied in the present experiments were chosen because they could be expected to single out components of a polysaccharide nature.

In the experiments described the results concerning the diphtherial antigen system showed: 1. That one precipitation line was selectively stained; 2. the colored line behaved as a homogeneous immunoprecipitate.

The experiments on rabbit bone marrow and its mucopolysaccharide fraction demonstrated: 1. That the bone-marrow extract contains among others, 4 antigens which when separated electrophoretically, display a characteristic affinity for mucicarmin. 2. The immunoprecipitates of the purified components gave the same staining reactions. 3. Finally, among the several lines produced by the crude antigen, 4 only were stainable, showing the characteristics of the purified material.

The experiments on pneumococcus polysaccharide demonstrate that it is possible to detect components of a polysaccharide structure in a complex mixture of antigens.

From these results it may be concluded that chemical staining reactions of immunoprecipitates afford the possibility of studying to a certain extent the chemical nature as well as the immunological behavior of antigens in complex mixtures.

As shown by Ouchterlony *et al.*(7), Pope

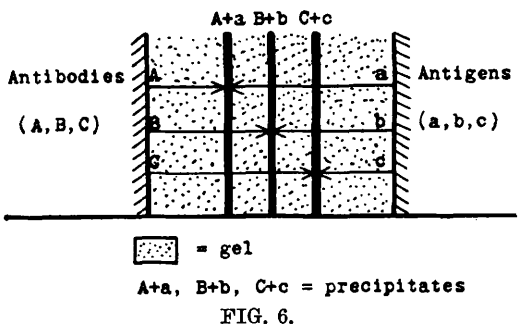

FIG. 6.

and his associates(14) and others, preparations of diphtheria toxin, by chemical and physico-chemical standards considered as pure, may display a considerable immunological inhomogeneity when analyzed in gel precipitation tests. This fact is probably partly explained by the sensitivity of the immunological method by which minute traces of impurities otherwise not demonstrable can be detected. This might not be the whole truth, however. According to the reports quoted above several major components could be distinguished in such presumably pure toxins. If so, the selectivity and resolving power of immunoprecipitation in gels must obviously considerably exceed that of the chemical methods. It is tempting, then, to apply the former method in attempts to further fractionate chemically purified, biologically active substances. Experiments along these lines are in progress.

Summary. Experiments on 3 immunological systems showed that antigens, when combined with antibodies in a gel retain their affinity for certain dyes. This affords the possibility of studying not only the chemical nature of certain antigens, but also their immunological behavior in complex mixtures.

1. Björklund, B., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 319.
2. ———, *ibid.*, 1952, v79, 324.
3. ———, *Int. Arch. Allerg. and Appl. Immunol.*, 1953, v4, 340.
4. ———, *ibid.*, 1953, v4, 379.
5. Kabat-Mayer: *Experimental Immunochemistry*, Thomas, Springfield.
6. Ouchterlony, O., Thesis, Karol. Inst., Stockholm, 1949.
7. Ouchterlony, O., Ericsson, H., Neumüller, C., *Acta Med. Scand.*, 1950, v138, 76.
8. Ouchterlony, O., *Ark. f. Kemi*, 1949, v1, 43, 55.
9. ———, *Ark. f. Kemi, Min. Geol.*, 1948, v26 B, 14.
10. ———, *Lancet*, 1949, v5, 256, 346.
11. ———, *Bull. Soc. Chim. Biol.*, 1951, v33, 758.
12. ———, *Acta Path. Microbiol. Scand.*, 1949, v24, 507.
13. ———, *ibid.*, 1952, v32, 232.
14. Pope, C. G., Stevens, M., Caspary, E. A., and Fenton, E. L., *Brit. J. Exp. Path.*, v32, 246.
15. Visscher, M., *Methods in Medical Research*, vol. 4, The Year Book Publishers, Chicago, 1951.

Received January 20, 1954. P.S.E.B.M., 1954, v85.

Leucocyte and Thymus Changes in the Pyridoxine-Deficient Young and Adult Male Rat.* (20910)

LILLIAN C. BUTLER† AND AGNES FAY MORGAN.
From the Home Economics Department, University of California, Berkeley.

The lymphopenic response is a well established index of the increase of 17-hydroxysteroids in the blood and of lymphoid tissue involution. During pyridoxine deficiency lymphopenia, usually accompanied by granulocytosis, has been seen in dogs(1,2), rats(2), monkeys(3,4), and mice(5). When pyridoxine was administered to pyridoxine-deficient animals, the lymphopenia and granulocytosis were reversed in some cases(2,5) but not in others(1,4,2). In adrenalectomized pyridoxine-deficient rodents, involution of the thymus(6) and lymphopenia(7) have been reported. These latter phenomena were presumably independent of the intermediation of the adrenal hormones and were attributed specifically to the lack of pyridoxine. Inanition could also induce thymic atrophy(8,9) and lymphopenia(10,11) in the intact animal, but these alterations did not occur during inanition in the absence of the adrenals(9,12). Some of the above findings were studied in this laboratory and have been confirmed.

Procedure. The total number of leucocytes in tail vein blood was counted from duplicate pipettes. One smear per animal was stained

* The authors wish to express their sincere appreciation to Dr. William O. Reinhardt for his valuable counsel.

† Public Health Research Fellow of the National Institute of Arthritis and Metabolic Diseases.