A MICROMETHOD OF IMMUNODIFFUSION

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A method of counterimmunodiffusion using a technique of horizontal micromanipulation is described by which it is possible to detect 1-10 ng protein in 0.3-1.2 μl of test fluid.

KEY WORDS: immunodiffusion; micromethod.

In some cases the quantity of antigen or antiserum is the factor limiting the number of immunochemical experiments in a series. Several micromodifications of immunochemical procedures requiring much smaller volumes of samples for analysis have accordingly been suggested in recent years [1, 4]. The possibility of producing a worthwhile miniature version of the double immunodiffusion technique is based on the following arguments. The sensitivity of the method is ultimately determined by the density of the "flow" of antigen and antibodies at the point of their interaction:

$$\rho = K\frac{m}{S},$$

where ρ is the density; K the constant of the immunoprecipitation reaction, determined experimentally for immunoglobulins, antigen, and arrangement of the wells of the chosen type; m the quantity of antigen (antibodies) introduced into the well; S the area of the immunoprecipitate in the gel. For wells of cylindrical shape the equation assumes the form:

$$\rho = K_1\frac{\pi r^2 hC}{2\pi R\cdot h} = K_1\frac{r^2}{2R}C,$$

where r is the radius of the well for the antigen (antibodies); R the distance of the immunoprecipitate arc from the center of the well; h the thickness of the layer; C the concentration of antigen (antibodies), and K_1 the equilibrium constant of the antigen–antibody reaction. It will be evident that if K and C are constant, the density of flow ρ will be independent of the volume of the reacting components, and will depend only on the radius of the well and the relative distance of the diffusing "flows" of antigen and antibodies. Consequently, by reducing the radius of the well and the distance between the wells proportionally, the value of ρ can be kept constant, even though microvolumes of samples are taken for analysis.

The method of determination is as follows. Defatted slides measuring 76 × 26 mm were first covered with a 0.3% agarose layer and dried. A 1% solution of agarose (Sigma, A grade) was made up in physiological saline, buffered with 0.1 M potassium phosphate buffer, pH 7.1, and boiled on a water bath until it had entirely dissolved. The agarose was poured on the slides held on the micromanipulation table. The thickness of the layer was 1 mm. To prevent evaporation from the surface, microdrops of buffer were applied to the gel which was kept in a moist chamber. Holes 1.2 mm in diameter were made in the agarose with a glass capillary tube held strictly vertically in the arm of the MM-1 micromanipulator. Agarose was withdrawn from the capillary tube by means of a microsyringe under the control of a binocular loupe. The distance between the central and peripheral wells was 2-3 mm. The volume of the well was 1.1 μl. Antiserum was introduced into the peripheral wells from a special micropipet 30-40 μ in diameter, connected to a microsyringe. All procedures were carried out under the control of the binocular loupe (magnification 14×). With another micropipet, the antigen solution was introduced into the central well 15 min after introduction of the antiserum. Drops of 1% merthiolate made up in physiological saline were applied to the surface of the agarose in a few areas. The whole procedure of adding the antigen and antiserum to the wells took 10-15 min. The slides were then placed

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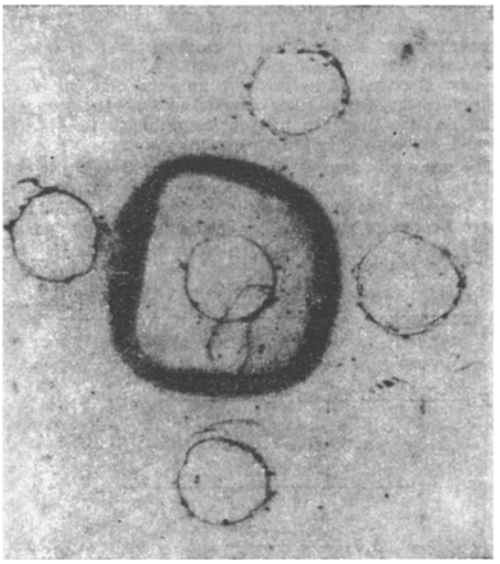

Fig. 1. Photograph of microimmunoprecipitation arc taken by camera attached to the MBS-2 binocular loupe. Central well contains 10^{-9} g antigen (IgG). Photograph taken after gel had been dried and stained with Amido Black; magnification 16×.

in a moist chamber to prevent evaporation. After 20-30 min, 0.7-0.8 μl of sterile physiological saline was added to all the wells, for partial evaporation of the microvolume of fluid from the wells could not be prevented even by keeping the material in the moist chamber. This evaporation of the microvolumes evidently caused no visible disturbances of the surface or of the structure of the wells in the gel, as was verified visually by means of the binocular loupe.

Slides with antigen and antiserum in the wells were incubated at room temperature in the moist chamber for 24-48 h. Excess of unreacted protein was washed off with cold physiological saline in the course of 24 h. The slides were then wrapped in a strip of wet filter paper, soaked with glycerol, and dried in an incubator at 37°C. The dried strips were stained with 0.1% Amido Black (Merck, West Germany) in 7% CH_3COOH. The excess of dye was removed with a mixture of 7% CH_3COOH and 40% ethanol.

A commercial preparation of human immunoglobulin G (from Sevag) with an initial protein concentration of 1.8 mg/ml was used as the marker antigen. The serum of a rabbit immunized with human immunoglobulin G was used as the test antiserum.

Antiserum in a volume of 1.1 μl was introduced into the peripheral wells and different dilutions of the original antigen in the same volume into the central wells. Arcs of immunoprecipitate became clearly visible under the binocular loupe with side illumination after 5-30 min if the antigen was added in a dilution of 1:10-1:50. If the antigen was diluted 1:100 (about 2×10^{-9} g protein per well) the arcs were identifiable visually with side illumination after 48 h. On staining the dried strips of gel with Amido Black clear immunoprecipitation arcs were detected with a dilution of 1:200 (about 1×10^{-9} g antigen per well, Fig. 1). The approximate quantity of antigen taking part in the formation of one immunoprecipitation arc can be calculated by the equation given above.

The suggested micromodification of the immunochemical method of identification of protein in microvolumes of fluid is not inferior in sensitivity to optical methods of quantitative measurement of protein in microvolume (capillary spectrophotometry and capillary microdisk electrophoresis [2, 3]), but it is considerably less sensitive than the micromethod of detection of antigens in conjunction with autoradiography [1].

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