A SUPERSENSITIVE RADIOIMMUNODIFFUSION METHOD OF DETERMINING ANTIGENS

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A method of combined electrophoresis and precipitation in polyacrylamide gel which increases the sensitivity of the gel diffusion test by 2000-5000 times without loss of resolving power is described. The principle of the method is to increase the concentration of the test antigen by 30 times by electrophoresis in polyacrylamide gel, to detect the antigen by a test system giving invisible precipitation bands in the gel, to develop the bands with I^{125}-labeled antibodies against immune serum γ-globulin, and then to carry out autoradiography. The method is suitable for use to determine α-fetoprotein, when its sensitivity reached $3 \cdot 10^{-4}$ to $6 \cdot 10^{-4}$ μg/ml.

The most specific methods of determining antigens and antibodies are the various modifications of the gel diffusion test using modified test systems. However, these methods have comparatively low sensitivity in antigen determination – not less than 1 μg/ml. The sensitivity of immunodiffusion reactions is limited by the minimal quantity of precipitate to form a visible zone in the gel. By using radioactive anti-γ-globulin, labeled with I^{125} or I^{131} it is possible to detect precipitates invisible to the naked eye and, in this way, to increase the sensitivity of the immunodiffusion test to 0.05 μg/ml [1, 6, 9, 10]. Even in this modification, however, the sensitivity of the determination is much lower than that of the radioimmunological method, by means of which it is possible to detect amounts as small as 0.5-1 ng/ml of antigen [7, 11]. However, the radioimmunological method does not possess such resolving power and its specificity is completely determined by the degree of purification of the antigen used in the test system.

A variant of the method which combines the resolving power of immunodiffusion with the sensitivity of the radioimmunological test is described in this paper.

Principle of the Method and Its Technique

The method consists of three stages. In the first the antigen is concentrated 30 times and simultaneously incorporated into a finely porous polyacrylamide gel (PAG). In the second stage immunodiffusion development of the antigen is carried out with a monospecific precipitating test system. The third stage is detection of the precipitation bands by staining or by treatment with I^{125}- (or I^{131}-) anti-γ-globulin followed by autoradiography of the unstained precipitates.

Electrophoresis in PAG [4] is carried out in a flat disc of gel measuring 130 × 90 × 1 mm (Fig. 1A). The chamber for electrophoresis* consists of two sheets of glass: a thick (5 mm) sheet, to which glass sidepieces 1 mm high are glued, and a covering sheet 1.5 mm thick. The side pieces are smeared with vacuum grease and the cover glass pressed firmly against them. The bottom opening into the chamber is blocked with waterproof tape and the chamber is two-thirds filled with a 7% solution of Cyanogum 41 (Serva, Heidelberg) in Tris-HCl buffer, pH 8.9 (0.75 g Tris buffer, conc. HCl ~ 0.4 ml, water to 100 ml, 0.1 ml TEMED, 1 mg/ml ammonium persulfate). After polymerization of the 7% gel it was covered

*The apparatus made by A. I. Gusev was used.

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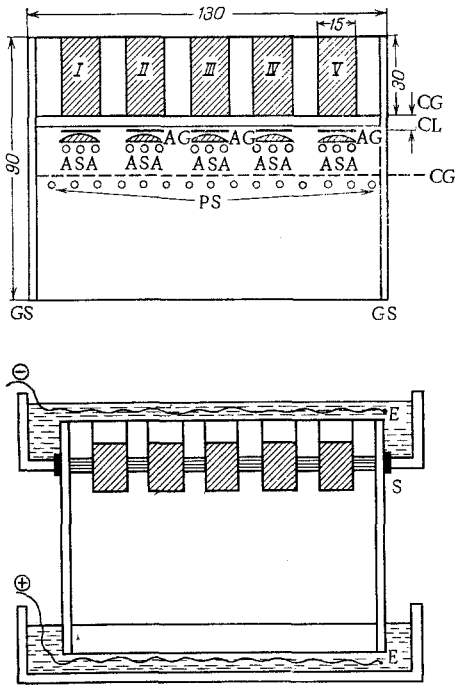

Fig. 1. Apparatus for electrophoresis and precipitation in PAG. Above: chamber of apparatus. I-V) Reservoirs for test antigen; CL) concentrating layer of gel; AG) zone of antigen in 7% PAG; A, C) reservoirs for antigen and serum of test system respectively; PS) reservoirs for physiological saline; GS) glass sides; C) line along which gel is cut. Below: apparatus for electrophoresis. E) Platinum electrodes; S) rubber seal.

with a layer of 4% Cyanogum solution in Tris-HCl buffer, pH 6.7 (0.75 g Tris-buffer, conc. HCl ~0.5 ml, water to 100 ml, 0.25 ml TEMED, 1 mg/ml ammonium persulfate), which polymerized to form a layer of concentrating gel 3 mm high. Five glass plates, each 15 mm wide, are fitted into the top of the chamber so that they lie on the concentrating layer and descend into the chamber to a depth of about 30 mm. The whole of the upper part of the chamber is then filled with a 4% solution of Cyanogum, pH 6.7, which is polymerized. After a gel has formed the glass plates are taken out, leaving five reservoirs for antigen measuring 15 × 30 mm. The reservoirs are filled with antigen solution, previously dialyzed against Tris-buffer, pH 6.7, and diluted 1:2 with 8% Cyanogum solution in the same buffer. Bromphenol blue, TEMED, and ammonium persulfate are added to the solution. After polymerization the antigen solution forms a continuous gel, of the same composition as the surrounding 4% PAG. The chamber is placed in the apparatus illustrated in Fig. 1B. The electrode vessels are filled with Tris-glycine buffer, pH 8.3 (1.5 g Tris buffer, 14.5 g glycine, water to 1 liter). Electrophoresis is carried out in a potential gradient of 5-6 V/cm and with a current of 10 mA until the zone of dye has migrated to a distance of 3-4 mm below the border of the 7% gel. All the protein fractions under these circumstances lie above the strip of dye in the finely porous gel, forming concentrated narrow bands (see Fig. 1A).

The chamber is placed in a bath containing 1% NaCl solution; the top sheet of glass is carefully removed, and the gel, firmly fixed to the bottom sheet, is left for 5-7 min in the NaCl solution. The excess of solution is carefully removed from the surface of the gel. Three wells for the test system are drilled at a distance of about 1 mm from the zone of the dye (diameter of wells 3 mm, distance between them 2 mm) as illustrated in Fig. 1. For each specimen of test antigen there are three wells. Antiserum is poured into the central well and the antigen of the test system into the side wells. The glass is left in a humid chamber for 2 days at room temperature. After the end of the reaction a strip of gel about 10 mm wide, containing the precipitation bands, is cut out, wrapped in Kapron gauze and immersed in a bath of 0.85% NaCl solution. Washing continues for 18-20 h at room temperature with mechanical stirring of the solution. The washed slabs of gel are immersed in a solution of antibodies against rabbit γ-globulin labeled with I^{131} or I^{125} (0.5-0.7 mCi/mg, 0.027 mg antibodies per ml) for 3-4 h, left overnight in the humid chamber, and then washed to remove unbound antibodies. The method of labeling the antibodies and treatment with the labeled antibodies were described previously [6]. The washed bands of gel are stained with 1% amido black solution in 7% acetic acid, washed with the 7% acid, and transferred to slides. The gel is covered with a melted 1% solution of agar-agar in distilled water, covered with wet filter paper, and dried at room temperature [8]. Autoradiography is carried out on RF-3 film for an exposure of 15-20 h [6].

Determination of α-fetoprotein. A method of determining α-fetoprotein (αFP) was developed. A series of consecutive dilutions of a purified preparation of mouse αFP from 10 μg/ml to 0.15 ng/ml was set up. The protein concentration in the original preparation was determined by Lowry's method, using serum albumin as the standard. All the dilutions were made in Tris buffer, pH 6.7, containing bovine serum in a dilution of 1:200 to prevent adsorption of the αFP when present in very low concentrations. Rabbit antiserum against mouse αFP [3] and the serum of newborn mice, taken in equivalent proportions and diluted until clear but thin precipitation lines formed in the agar were used as the test system. The concentration of αFP in the antigen of the test system was approximately 40 μg/ml. Preliminary experiments to study the incorporation of αFP-I^{125} into the gel showed that during electrophoresis from a reservoir 30 mm high the αFP is distributed in the 7% PAG in a zone 1 mm wide along the cathodal boundary of the serum albumin.

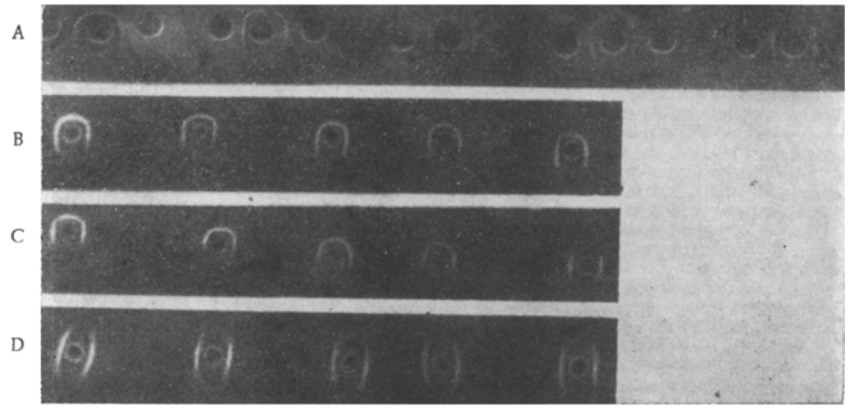

Fig. 2. Diffusion test in PAG (arrangement of antigens and antisera shown in Fig. 1A). A) visible reaction (staining with amido black). I-V) Double dilutions of test antigen from 1.2 to 0.075 μg/ml. B) Test system in dilution of 1:16. I-V) Double dilutions of antigen from 0.075 μg/ml to 4.7 ng/ml (autoradiography). C) Test system 1:64. I-IV) Antigen in dilutions from 9 to 1.1 ng/ml; V) control – bovine serum 1:200 (autoradiography). D) Test system control 1:16. I-V) Dilution of bovine serum from 1:4 to 1:64 (autoradiography).

In the zone of visible precipitation in agar the limit of sensitivity of the determinations was 1.0-1.5 μg/ml with a 1:2 dilution of the test system. By the method of immunoautoradiography in agar [6], it was possible to determine a minimum of 50-70 ng/ml αFP. By the combined electrophoresis and precipitation in PAG method as described above, 150 ng/ml, αFP was detected when the original test system was used, and as little as 40 ng/ml when a diluted (1:4) system was used. In both cases the precipitation bands were revealed by simple staining, without treatment with radioactive antibodies (Fig. 2A). When autoradiography was used, the sensitivity of the determinations was increased by a further 2-3 dilutions.

The sensitivity of the reaction in the invisible zone was increased in proportion to the dilution of the test sytem: in a dilution of 1:16 it revealed αFP clearly down to a concentration of 2.5 ng/ml, and in a dilution of 1:64 down to 0.3-0.6 ng/ml (Fig. 2B, C). With higher dilutions of the test system an ill-defined image was obtained on the film.

In adult male serum αFP was detected by means of the 1:16 test system down to dilutions of serum of 1:32. Bovine serum in the same dilutions gave a negative reaction (Fig. 2D).

In determinations carried out with the original sera or with their first dilutions (1:2-1:4) swelling of the fluid above the albumin zone was observed. In these cases the fluid must be accurately removed (15-18 h after the beginning of the reaction) or the wells of the test system must be located above the band of antigen. In the latter case electrophoresis is carried out so that the albumin zone migrates for a distance of 12-15 mm into the 7% gel.

By means of the suggested method the sensitivity of the gel diffusion test can thus be increased by 2000-5000 times without loss of its specificity. The method does not require preliminary purification of the antigen and it is suitable for use with all antigens with positive mobility in PAG. If the antigen does not migrate into the finely porous gel, it is possible to use a lower concentration of gel or 2% agarose, which does not have the "filtering" effect and which is perfectly suitable for electrophoresis in the modification described here. The use of electrophoresis in PAG for the determination of antigens and antibodies widens the scope of immunochemical analysis [2, 4].

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LITERATURE CITED

1. G. I. Abelev and D. A. Él'gort, Internat. J. Cancer, 6, 145 (1970).
2. L. V. Vorob'ev, Byull. Otkrytii, Izobretenii i Prom. Obraztsov, No. 34, 20 (1972).
3. A. I. Gusev and A. K. Yazova, Byull. Éksperim. Biol. i Med., No. 4, 120 (1970).
4. G. Maurer, Disk Electrophoresis. The Theory and Practice of Electrophoresis in Polyacrylamide Gel [Russian translation], Moscow (1971).
5. N. I. Khramkova and G. I. Abelev, Byull. Éksperim. Biol. i Med., No. 12, 107 (1961).
6. D. A. Él'gort and G. I. Abelev, Byull. Éksperim. Biol. i Med., No. 2, 118 (1971).
7. W. Hunter, in: D. Weir (editor). Handbook of Experimental Immunology, Oxford (1967), p. 608.
8. M. Reid and R. Bielsevski, Analyt. Biochem., 33, 347 (1968).
9. D. Rowe, Bull. Wld. Hlth. Org., 40, 613 (1969).
10. D. Rowe, Lancet, 1, 1340 (1970).
11. E. Ruoslahti et al., Internat. J. Cancer, 8, 347 (1971).