# USE OF A STABLE TETRAZONIUM SALT TO SENSITIZE ERYTHROCYTES WITH PROTEINS FOR THE LOCAL PASSIVE HEMOLYSIS TEST

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A method of sensitizing sheep's erythrocytes with proteins based on the use of a stable tetrazonium salt is suggested. After fixation of protein to them by means of a covalent azo-bond, the erythrocytes can be used in the local passive hemolysis test for the detection of rabbit or mouse spleen cells secreting antibodies against protein antigens. A new type of hemolysis known as "aggregate hemolysis," is also described.

Local hemolysis, which has been suggested for the quantitative enumeration of cells secreting hemolysins [11, 12], has also proved useful for the detection of cells forming antibodies against certain polysaccharide-containing protein antigens [5-8, 10, 13]. However, the technique of sensitizing erythrocytes with protein antigens requires simplification and standardization. Azo-coupling with the aid of the stable tetrazotate of 4,4'-diaminodiphenylamine was chosen as a simple and reliable method of fixation of protein to erythrocytes in this investigation. The effectiveness of the use of a stable tetrazonium salt was demonstrated previously in the writers' laboratory during synthesis of an antibody-sorbent [1] and during the development of the aggregate-hemagglutination method [2-4].

The object of the present investigation was to determine the optimal conditions for protein sensitization of the erythrocytes used in the local passive hemolysis test in model experiments and with lymphocytes of immune rabbits and mice.

The product Diazol Black S (DBS), the active principle of which is 4,4'-diaminodiphenylamine tetrazotate, was used as the stable tetrazonium salt. This is a paste, stabilized with zinc chloride, manufactured by the Dorogomilovo Chemical Factory, Moscow, and it is equivalent to Echtschwarz B. [6]. Before use the DBS paste is dried at 18-20°C, during which it is covered with a sheet of chromatographic paper and a sheet of black paper, and then it is carefully ground a little at a time in a dry porcelain mortar, taking care to avoid local overheating. The resulting fine green powder is stored in a dark, dry place away from heating devices in a dark bottle.

To 50 mg DBS in a dry, cooled tube 5 ml of cold (4°) physiological saline was added, the suspension was shaken vigorously to mix it, and in order to remove insoluble particles it was filtered through one layer of chromatographic paper in a cooled tube. The transparent yellow filtrate, with an acid reaction, was used not later than 5-10 min after preparation. In an alkaline medium the color of the filtrate becomes red, and in the absence of an object for azo-coupling the tetrazotate is precipitated. To ensure stability of the conditions, it is advisable to take a constant weight of DBS and volume of solution and to use a filter of identical size and quality.

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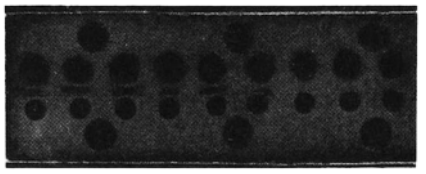

Fig. 1. Determination of sensitivity of the local passive hemolysis test in gel by the complement–antibody counter-diffusion method. Top row: wells contain complement (1:2); bottom row: serial 1:1 dilutions of rabbit antiserum against HGG added from left to right to wells. Gel on slide contains sheep's erythrocytes sensitized with HGG. Translucency of opaque gel between wells corresponds to zones of hemolysis. The wells on extreme top and bottom contain physiological saline.

Human serum albumin (HSA), separated by precipitation with alcohol, or horse $\gamma$ globulin (HGG), isolated by chromatography on DEAE-cellulose*, was used as antigen. To immunize rabbits and mice the antigens were used either in the soluble form or as the insoluble product obtained by polycondensation of the protein by means of DBS. Freshly thawed guinea pig serum was used as the source of complement. Native sheep's erythrocytes were kept in Alsever's solution at 4°. An ass antiserum against rabbit globulins was heated to 56° for 30 min and then absorbed with sheep's erythrocytes; after addition of merthiolate (1:10,000) it was used in a dilution of 1:300 (2.5 ml per dish). All the work was done with the same batch of antiglobulin serum, obtained in the early periods after immunization.

For the local hemolysis test on Petri dishes, 750 mg of agarose powder was mixed in a conical flask with 100 ml Hanks's medium, prepared from a concentrate of 10 times the normal strength, and melted on a boiling water bath. The flask was closed by a stopper fitted with an air condenser consisting of a glass tube 50 cm long. To neutralize the completely liquefied agarose 2-3 drops of 5% sodium bicarbonate solution were added until the medium was pale pink in color. Liquid agarose was poured into Petri dishes (Orion) in volumes of 4 ml, and the dishes were kept on a horizontal surface until the liquid had set. Erythrocytes were then added to the required volume of 0.75% agarose (made up in Hanks's solution, pH 7, by melting and then cooling to 45° on a water bath) at the rate of 70 million cells (or 0.035 ml of a 20% suspension) to 1 ml of the agarose solution. The mixture was poured into test tubes, 2 ml into each, on a water bath at 45°. To each tube 0.1 ml of a suspension of spleen cells was added and the contents were carefully mixed and rapidly poured out on to the solid layer of agarose in the Petri dishes or on to a slide. To prevent spontaneous lysis the sensitized erythrocytes must be kept at 45° for not more than 30 min. Dishes with a solidified upper layer containing erythrocytes and the cells to be tested were incubated for 1 h at 37°C and, if necessary, for a further 1 h with antiglobulin serum, which was washed off with physiological saline, and then incubated for 1 h at 37° with 2.5 ml complement (1:10). Local hemolysis in medium without gel [8] was carried out in a shallow chamber, the edges of which were sealed with petroleum jelly and a mixture of paraffin and wax.

To determine the sensitivity of the hemolysis test in the model experiment on Petri dishes,† drops of serial 1:1 dilutions of antiserum were applied to the layer of agarose containing sensitized erythrocytes (without lymphocytes), and incubation was carried out for 1 h at 37°. The surface of the gel was washed once with physiological saline, and after incubation with complement (1:10) the antiserum titer giving clarification of the gel was determined. To determine sensitivity in test tubes, 2 ml of 1:1 dilutions of antisera in Hanks's medium without bicarbonate was treated with 0.1 ml of freshly thawed complement (1:2) and 0.04 ml of a 15% suspension of sensitized erythrocytes. After incubation for 1 h at 37° the degree of lysis was measured on a photoelectric colorimeter fitted with a red filter.

By varying the conditions of treatment of the erythrocytes and aiming at a combination of maximum sensitivity of the hemolysis test and stability of the resulting erythrocytes, the following method of sensitization was chosen.

A cold freshly prepared solution on DBS was added in increasing amounts to a series of centrifuge tubes each containing 1 ml of 1% protein solution (HGG or HSA) in 0.11 M phosphate buffer, pH 7.4, containing 0.9% sodium chloride solution (temperature about 20°). Depending on the activity of the DBS batch, from 0.05 to 0.5 ml of a 0.5-1% solution of DBS was taken; a dose leading to opalescence of the protein solution is not used. The minimal dose of DBS to give maximal sensitivity of the hemolysis test, which is

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† The procedure of the model experiment in Petri dishes was recommended by L. A. Pevnitskii, to whom the writers are grateful.

subsequently determined in a model experiment undertaken either on dishes, in tubes, or on a slide (see below), is taken as optimal. In experiments with batch No. 7 of 1964 DBS from the Dorogomilovo Chemical Factory the desired dose was 0.1 ml of a 1% solution of DBS to 1 ml of 1% HGG solution or 0.2 ml of 1% DBS solution to 1 ml of 1% HSA solution. After addition of the DBS to the protein and mixing for 30 sec, 0.2 ml of the residue of native sheep's erythrocytes (preliminarily washed three times with physiological saline) was added to the mixture in each centrifuge tube, the contents were mixed and incubated for 90 min at 37°, after which the brown residue of erythrocytes was washed five times with 8-10 ml physiological saline. Next, in a model experiment, the optimal dose of DBS was chosen, and a large batch of sensitized erythrocytes was prepared from it by the method specified above. The erythrocytes were then preserved in Alsever's solution. To do this, the residue of erythrocytes, washed with physiological saline, was suspended in 20 volumes of Alsever's solution and used during a period of 10 days while being stored at 4°, until a zone of spontaneous hemolysis appeared above the residue of the preserved erythrocytes. Before use the erythrocytes were washed three times with physiological saline and made up into a 20% suspension in Hanks's medium or in medium No. 199.

In the interests of economy with complement and to obtain a clear end point of titration when determining the sensitivity of the hemolysis test, a new technique was developed for the model experiment: counterdiffusion of complement and antibodies in gel containing sensitized sheep's erythrocytes (Fig. 1). This technique enables clear circles of translucency to be obtained in the gel, like precipitation lines in the immunodiffusion method, while reducing the consumption of complement.

A separate series of preliminary experiments showed that sheep's erythrocytes, after sensitization with aggregated antiserum proteins, undergo lysis in the presence of homologous antigen and complement. A positive result was obtained in model experiments with erythrocytes coated with proteins of ass anti-rabbit serum, polycondensed [2] with 4,4'-diaminodiphenylamine tetrazotate, when normal rabbit serum was used as the source of antigens. Additional (before complement) treatment of the gel containing antigen and sensitized erythrocytes with native, nonpolycondensed, ass antiserum increased the sensitivity of the hemolysis test. The action of ass antiserum in this case is evidently like the effect of antiglobulin. The variant of the passive hemolysis test described above may evidently be suitable for the detection of individual cells secreting different antigens.

Activity of the erythrocytes sensitized with antigen was tested, not only in model experiments, but also in experiments with suspensions of spleen cells of immune animals. Spleen cells taken from rabbits on the fourth day after reimmunization gave 3-12 plaques per $10^6$ nucleated cells with native sheep's erythrocytes (the background). The number of plaques with cells of the same spleens, when the test was carried out with erythrocytes sensitized with HGG, without incubation with antiglobulin, was from 30 to 900 per $10^6$ cells for different rabbits, and with incubation with antiglobulin [9, 14] serum it was from 400 to 62,500 per $10^6$ cells. The number of immune cells secreting antibodies against HGG thus reached 6% of the total number of nucleated spleen cells in some rabbits. The specificity of the test was confirmed by inhibition of plaque formation on addition of homologous antigen. Specific plaques also were obtained in the case of immunization of a rabbit simultaneously with HSA and HGG. Similar results also were obtained if the dishes were replaced by slides or by shallow chambers, but the addition of antiglobulin to the shallow chamber simultaneously with complement inhibited plaque formation. In tests with mouse spleen cells specific plaques also were obtained, but they were fewer in number and there was considerable scatter in the number of active cells.

The use of a stable tetrazonium salt for sensitization of erythrocytes thus enables stable sensitive erythrocytes, suitable for use in the local passive hemolysis test, to be obtained very simply and under easily controllable conditions.

## LITERATURE CITED


1. A. M. Olovnikov, Dokl. Akad. Nauk SSSR, 158, No. 5, 1202 (1964).
2. A. M. Olovnikov, Dokl. Akad. Nauk SSSR, 169, No. 5, 1180 (1966).
3. A. M. Olovnikov, Immunochemistry, 4, 77 (1967).
4. A. M. Olovnikov, Zh. Vses. Khim. Obshch. im. D. I. Mendeleeva, 13, No. 4, 445 (1968).
5. L. A. Pevnitskii, M. I. Chernysheva, I. I. Dubrovskaya, et al., Zh. Mikrobiol., No. 7, 52 (1969).
6. A. G. E. Pearse, Histochemistry [Russian translation], Moscow (1962).
7. V. V. Solov'ev, L. N. Fontalin, L. A. Pevnitskii, et al., Byull. Éksperim. Biol. i Med., No. 5, 119 (1966).

8. A. J. Cunningham, Nature, 207, 1106 (1965).
9. D. W. Dresser and H. H. Wortis, Nature, 208, 859 (1965).
10. E. S. Golub, R. I. Mishell, W. O. Weigle et al., J. Immunol., 100, 133 (1968).
11. J. S. Ingraham, C. R. Acad. Sci. (Paris), 256, 5005 (1963).
12. N. K. Jerne and A. A. Nordin, Science, 140, 405 (1963).
13. S. Kishimoto, I. Tsuyuguchi, and Y. Yamamura, Internat. Arch. Allergy, 34, 544 (1968).
14. J. Sterzl and I. Riha, Nature, 208, 858 (1965)