METHODS

A CAPILLARY MICROMETHOD FOR THE PRECIPITATION REACTION IN AGAR

O.E. Vyazov, B.V. Konyukhov and L.L. Lishtvan

From the Laboratory of Immunology of Embryogenesis (Head – Candidate Med. Sci. O.E. Vyazov) of the Institute of Experimental Biology (Director – Prof. I. N. Maiskii) of the AMN SSSR, Moscow

(Received March 10, 1958. Presented by Active Member AMN SSSR N.N. Zhukov-Verezhnikov)

The precipitation reaction in gel, carried out in tubes, was introduced by Oudin [8, 9, 10], who showed that the number of rings of precipitate formed in agar and gelatin was equal to the number of antigen-antibody systems. Ouchterlony [7] and Elek [3] suggested a method of double diffusion in agar plates. Later, Oakley and Fulthorpe [6] used the principle of double diffusion in order to carry out the reaction in tubes. This method is essentially as follows: the antigen and antibody diffuse in a column of gel towards each other, and where they meet a precipitate is produced in the form of a ring.

Different antigens, under the same conditions, possess different coefficients of diffusion, and they therefore meet their corrresponding antibodies at different levels of the column of gel. During the analysis of complex mixtures of antigens, several rings of precipitate are formed in the gel. The level at which the antigen and antibody meet depends also on the relative concentration of antigens and antibodies. The intensity of the rings of precipitate in the gel depends on the absolute concentrations of antibodies (mainly) and antigens.

The theory of this subject has been described in detail in several papers [5, 7, 11, 12, 13 and others].

In our investigations of the tissue antigens of early embryos, we were confronted with the necessity of using very small quantities of antigenic material. The methods previously described required relatively large quantities of reagents (as much as 0.25 ml of serum and tissue extract per tube). We therefore devised a capillary method of carrying out the precipitation reaction in agar, requiring negligible quantities of serum and extracts.

Independently of us, Preer [12] also suggested a method of carrying out the precipitation reaction in agar in small quantities. This method does, however, require larger volumes of reagents and is more difficult and complicated than our own.

In performing the test, we used antigens from the crystalline lens and sera from the blood of a chick. Antisera against the antigens of the crystalline lens were prepared by immunization of three rabbits of the chinchilla species – males weighing 2.5-3.0 kg – by the method put forward by P.N. Kosyakov [2]. Antisera against antigens of the chick's blood serum were obtained from the Institute of Forensic Medicine. The antisera were diluted with physiological saline in proportions of 1:4, 1:8 and 1:16.

Antigens were prepared from the crystalline lens as follows: the lenses of chicks were freed from their capsules, ground up in a porcelain mortar and mixed with 9 volumes of physiological saline. The suspension was shaken up in an agitator for 2 hours and centrifuged at 3000 rpm for 20 minutes. The supernatant fluid (saline extract) was used as the original solution of crystalline lens antigens. As an original solution of serum antigens we used whole serum from chick's blood. The original solutions of antigens were diluted with physiological saline to 1:20, 1:50 and so on.

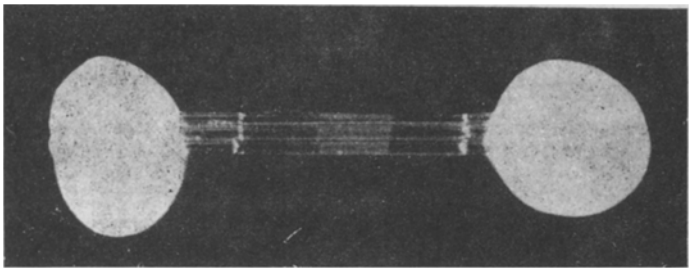

Fig. 1. Glass slide with capillary tubes whose ends are sealed with paraffin wax.

The antisera against the crystalline lens antigens were highly specific; they gave a reaction in the ring precipitation test, when carried out in the usual manner [1], with extract of the crystalline lens diluted 1:20,000, but did not react with extracts of other organs. Antisera against the antigens from the blood serum reacted with serum of chick's blood diluted 1:10,000.

The precipitation reaction in agar gel was performed as follows: a 1% solution of agar (washed free from contaminants) was prepared in physiological saline. To suppress the growth of microorganisms in the agar, 0.1% phenol was added*. The agar was autoclaved and stored (not longer than 7 days) at room temperature. Before carrying out the test, we heated the agar on a water bath and centrifuged it at 3000 rpm for 3 minutes to precipitate the insoluble particles. The end of a capillary tube with an internal diameter of 0.6-0.8 mm and external diameter of 0.7-0.9 mm (the capillary part of specially selected Pasteur pipettes) was immersed in the hot agar and a column of agar of a strictly determined length was taken. The column was moved so that it left a space of 15-20 mm at the end of the tube. The capillary tube containing the coagulated agar column was cut from the rest of the Pasteur pipette by means of a corundum disk. With a tuberculin syringe, connected by a rubber tube to another glass tube, drawn out into a capillary, a layer of antiserum was applied on the agar at one end, and a layer of antigen solution at the other (the height of each column of liquid was 4-8 mm). The capillary tubes of each series of experiments were placed on separate glass slides, and their ends were sealed with molten paraffin was (Fig.1).

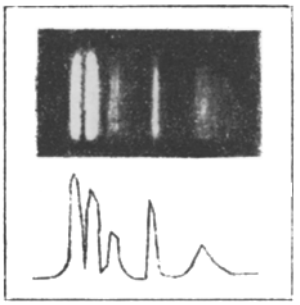

Fig. 2. Rings of precipitate in a capillary tube(x 12) and the corresponding curve obtained by photometry.

The slide with the capillary tubes was incubated at 37°, in the horizontal position.

Great care was necessary when adding the layers of antiserum and the solutions of antigens to avoid the formation of bubbles. When bubbles formed at the boundary between the agar and liquid it was necessary to agitate the tube gently in order to raise the bubbles to the surface, when they could be pricked with the point of a hot needle.

The first rings appeared after 2-48 hours, depending on the length of the column of agar and the concentration of antibodies and antigens. In short columns of agar (3-4 mm) and at high concentrations of antibodies and antigens, the rings usually appeared early (after 2-4 hours), but they were very close to each other, which made it difficult to distinguish them. Capillary tubes with agar columns 3-4 high were therefore suitable only for cases when there was no question of making a detailed analysis of the antigenic structure of the tissue extract under study, and it was only necessary to establish the presence of very small quantities of the anitgen or antibodies which were being sought. Parallel experiments with

* Preliminary experiments showed that phenol, in this concentration, only very slightly moves the pH of the solution towards the acid side (pH = 6.4) and has no perceptible effect on the formation of rings of precipitate in agar.

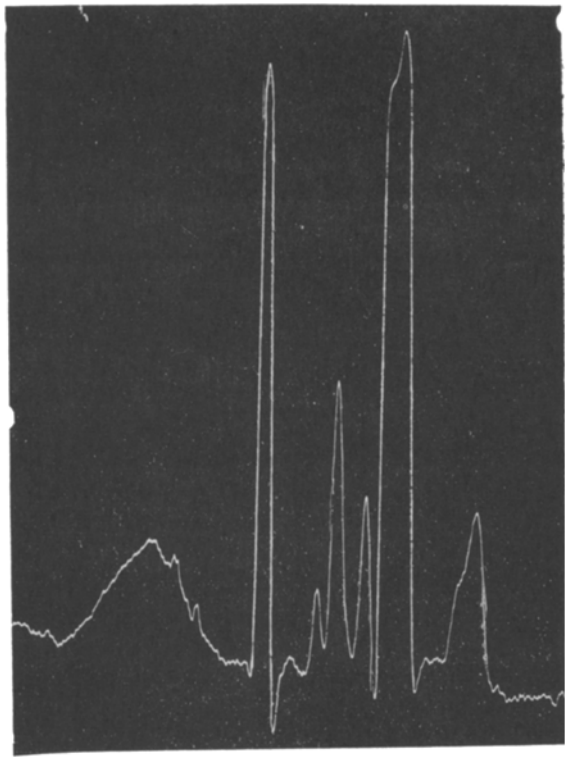

Fig. 3. Curve obtained by photometry of a capillary tube with 7 rings with a recording microphotometer.

the ring precipitation test and precipitation in agar in capillary tubes showed that the precipitation reaction in agar (using agar columns 3-4 mm high) was the more sensitive.

For a detailed analysis of the antigenic structure of a tissue extract the optimum length of the agar column was 10-15 mm (with a dilution of 1:8 of antisera and of the original crystalline lens antigen solution 1:50, and serum antigen solution 1:500). Under these circumstances the rings of precipitate were formed later but were further apart from each other. The largest number of rings appeared, roughly, between the 14th and 17th days. On later days, no new rings usually appeared and at the end of the period of observation (1 month) blurring and disappearance of some rings took place. With antigens from the crystalline lens 7 rings were formed, and sometimes very weak 8th and 9th rings appeared. The use of saline extracts of the other organs of the chick (brain, liver, heart) as antigens, just as in the ring precipitation test, gave no precipitation rings with antisera against the antigens from the crystalline lens of the chick.

Antisera against the blood serum antigens usually gave 9 rings with the antigens from the blood serum of the chick, the majority of them being separated by wide intervals along the whole length of the agar column.

Many experiments were performed in order to discover if there was any difference between the formation of rings of precipitate in the capillary tubes and the formation of rings in larger tubes (with an internal diameter of 8 mm). Antigens and antibodies were used in different concentrations, and also columns of neutral agar of different lengths in the capillary tubes, and also by Oudin's method [11]. It was found that the formation of rings in the capillary tube method is no different from the formation of rings in test tubes. It was much easier, however, in the capillary tubes to identify the rings and to study their displacement because of the small diameter of the tube and the high translucency of the agar column.

We identified the rings as a rule under the hand lens (× 9). This method did not, however, permit an objective assessment to be made of the thickness and density of the rings of precipitate formed. In 1956, Glenn [4] proposed for this purpose an apparatus (SAMA) based on the use of a photron reflectometer. We constructed an apparatus along similar lines, but it did not enable us to differentiate rings of precipitate which were close together. For determination of the density and the number of rings of precipitate in the capillary tubes, we therefore used the optic system of a microscope (objective 8 ×, ocular 10 ×), connected to a selenium photoelectric cell, amplifier and galvanometer (sensitivity 10^{-8} A). In the eyepiece of the microscope was inserted a diaphragm having an aperture 0.1-0.2 mm wide, which was directed perpendicular to the long axis of the capillary tube. The glass slide with the capillary tubes was placed on a cruciform stage, and a strictly collimated and centered beam of light was passed through one of the capillary tubes (to prevent variations in the intensity of illumination, a voltage stabilizer was used). To avoid strong scatter of the light, the capillary tubes were mounted in immersion oil and a cover slip placed over them. When the stage was moved longitudinally it was possible to observe simultaneously the galvanometer readings and the scale rule, used in constructing the curve. A fall in the reading of the galvanometer pointer corresponded to a zone of turbidity of the agar (a ring). As an illustration we give the curve obtained as a result of photometry of a capillary tube with 5 rings (Fig. 2).

Clearer results were given by photometry of the capillary tubes by means of a recording microphotometer using the MF-4 system. As an illustration, we give the curve obtained by photometry of a capillary tube with 7 rings with this apparatus (Fig. 3).

The capillary tube method of performing the precipitation reaction in agar is thus technically simple and highly sensitive, and requires very small amounts of reagents (0.001-0.004 ml). This method enables the study of "turbid" antigens and even tissue suspensions and so dispenses with the need for centrifuging and filtration, which causes loss of material. Another essential advantage of the method is the possibility of objective evaluation of the results by using the optical system of a microscope, connected to a photoelectric cell and galvanometer, or by means of the MF-4 microphotometer.

SUMMARY

The authors developed a method of precipitation reaction in agar in capillaries with an internal diameter of 0.6–0.8 mm. This enables the use of very small amounts of reagents (0.001–0.004 ml). The following methods have been suggested for the objective evaluation of the number and the thickness of the precipitate rings: that of capillary photometry involving a microscope optic system linked with a photoelement, amplifier, galvanometer, and another one, with the aid of an MF-4 microphotometer.

LITERATURE CITED

[1] B.V. Konyukhov, Byull. Eksptl. Biol. i Med., 42, No.9, 55-60 (1956).*

[2] P.N. Kosyakov, The Antigenic Substances of the Body and Their Importance in Biology and Medicine, Moscow, 1954 [In Russian].

[3] S.D. Elek, Brit. J. Exper. Path., 1949, v.30, p.484-500.

[4] W.G. Glenn, J. Immonol., 1956, v.77, p.189-193.

[5] L. Korngold, J. Immonol., 1956, v.77, p.119-122.

[6] C.L. Oakley, A.J. Fulthorpe, J. Path. a. Bact., 1953, v.65, p.49–60.

[7] Ö. Ouchterlony, Acta path. et microbiol. scandinav., 1949, v.26, p.507-515.

[8] J. Oudin, Compt. rend. Acad. Sc., 1946, v. 122, p.115.

[9] J. Oudin, Ann. Inst. Pasteur, 1948, v.75, p.30-51.

[10] J. Oudin, Ann. Inst. Pasteur, 1948, p.109-129.

[11] J. Oudin, In the book: Methods in Medical Research. Chicago, 1952, v.5, p.335-378.

[12] J.R. Preer, J. Immunol., 1956, v.77, p.52-60.

[13] M.W. Wilson, B.H. Pringle, J. Immunol., 1954, v. 73, p. 232-243.

*Original Russian pagination. See C.B. Translation.