# AN INVESTIGATION OF PURIFIED TETANUS TOXOID BY THE METHOD OF PRECIPITATION IN AGAR

I. M. Khabas and O. A. Fadeeva

From the Scientific Research Institute of Vaccines and Sera (Director – Docent M. P. Belov; Scientific Director – Prof. A. V. Ponomarev), Leningrad

(Received June 19, 1957. Presented by Active Member Acad. Med. Sci. USSR, G. V. Vygodchikov)

The method of precipitation in agar has been widely adopted in recent years for the analysis of preparations of various antigens and immune sera.

In the present investigation we used this method for the study of purified tetanus toxoid.

## EXPERIMENTAL METHOD

Tetanus toxoid, prepared in Ramón's medium, was purified and concentrated by a method devised by ourselves and described earlier [1], consisting of two main stages: precipitation with sulfosalicylic acid and dialysis in a collodion bag. The preparation was further purified in the cold (–10°) by precipitation with ethyl alcohol and subsequent centrifugation. Alcohol was added until its concentration was 55%. The mixture was allowed to stand for 50 minutes, after which the precipitate was removed by centrifugation and dissolved in a phosphate buffer (pH = 7.4), and the concentrate thus obtained was then dialyzed in a cellophane bag against tap water.

Titration by a standard method in white mice showed that the preparation contained 1300 units of combination (CU) per ml. Its specific activity was 1680 CU per mg nitrogen.

This preparation was subjected to further fractionation by gradual salting out with a phosphate mixture (pH = 7.4), containing 83.3% of dipotassium hydrogen phosphate $K_2HPO_4$ and 16.7% of potassium dihydrogen phosphate $KH_2PO_4$ [4]. The fractionation was carried out as follows. To a measured sample of toxoid was added a weighed portion of the phosphate mixture, and the whole was mixed and allowed to stand for 18 hours at 18°. The precipitate was collected on filter paper and dissolved in distilled water. To the filtrate was then added the next portion of phosphate mixture, and so on. Altogether 7 fractions were obtained.

## EXPERIMENTAL RESULTS

As may be seen from the table, the bulk of the antigen (77%) was salted out between the limits of 0-25% of phosphate mixture. The fractions "15-20" and "20-25" showed the highest activity.

In order to carry out the precipitation reaction, we used the method of counterdiffusion (a modification of Ouchterlony's method [3]).

The agar was cleared in the following manner [2]: it was dissolved in 20 parts of distilled water and to it was added a 20% solution of calcium chloride to give a concentration of 0.5% after coagulating, the agar was cut up finely and washed for 3 days in tap water. The washed agar was melted, mixed with 2 volumes of a hot 1.15% solution of sodium chloride and filtered through cotton wool, after which 0.02% of merthiolate was added.

The agar, prepared in this manner, was poured in a layer of 3 mm into a rectangular dish which was placed perfectly horizontally. The dish consisted of a glass plate, to the edges of which pieces of glass tubing were secured by means of plastic glue.

Using a stencil, rows of small holes were cut in the layer of solidified agar, 15 mm in diameter and 10 mm from each other. On both sides of these holes, parallel to them and at a distance of 15 mm, grooves were cut at right angles to each other, 5 mm wide. Into the holes was poured 0.5 ml of the toxoid preparation to be tested, and into the grooves a tetanus antiserum, purified and concentrated by the Diaferm-3 method, of two different series. The dish was covered with glass and allowed to stand at room temperature for 10 days.

Characteristics of the Fractions of Purified Tetanus Toxoid Obtained by Salting Out with a Phosphate Mixture (pH= 7.4)

| Percentage of phosphate mixture | Titer (in CU/ml) | Yield of antigen (in %) | Specific activity (in CU/mg of nitrogen) |
|---|---|---|---|
| 0 - 15 | 400 | 10.3 | 1 270 |
| 15 - 20 | 1 300 | 33.3 | 3 300 |
| 20 - 25 | 1 300 | 33.3 | 3 270 |
| 25 - 30 | 250 | 6.0 | 428 |
| 30 - 35 | 100 | 2.0 | 510 |
| 35 - 40 | < 100 | — | — |
| 40 - 45 | < 50 | — | — |

When the reaction was performed by the method described, precipitation lines were formed in the layer of agar in the shape of an arc. If neighboring holes contained common antigens, the respective arcs merged together into a continuous, undulating line. Where the antigen which was contained in the other preparations being tested at the same time in neighboring holes was either absent or present in too low a concentration, the precipitation lines were interrupted.

In order to photograph the precipitation lines we used the contact method, using contrat paper for reflex printing. To remove spots in the dish before filming, a thin layer of water was poured on.

As may be seen from the photograph shown (Fig. 1), the purified toxoid formed 3-4 lines. It should be mentioned that in experiments performed at the same time, the number of lines may vary slightly as a result of more or less perfect separation of the lines, especially of those in very close contact with each other.

It was not possible to determine from the intensity of the precipitation lines which of them was due to the specific antigen – to the toxoid itself – since the degree of purification of the preparation was relatively low and some of the ballast antigens present could exceed the concentration of the specific antigen.

We were able to distinguish the line corresponding to the specific antigen by comparing the immunograms of the original, purified toxoid and its fractions, taking into consideration the results of estimations of the antitoxin combining power in white mice.

Let us compare the immunograms of two preparations: purified toxoid (No. 47) and fraction "20-25" (No. 46). According to the results of the biological experiment, both these preparations contained considerable and equal amounts of specific antigen (1300 CU/ml). Theoretically, in this case, we should have expected to see a continuous line of moderate intensity, due to the specific antigen, the arcuate portions of which would have been arranged symmetrically in relation to the corresponding holes in the agar. The lines closest to the holes, for example, conform to these requirements. If it is formed by the specific antigen, it must not be present, therefore, in the immunograms of the fractions not containing this antigen or containing it in too low a concentration.

In fact, in the immunograms of fractions "30-35" (No.51), "35-40" (No.53) and "40-45" (No.56), this line was absent. At the same time these fractions, as can be seen from the photograph, contained other antigens, presumably ballast, which were detected also in the original purified toxoid. In one fraction ("30-35") there were two such antigens, and in the others ("35-40" and "40-45") – predominantly one.

As an additional criterion of differentiation of the precipitation line of the specific antigen it was possible to make use of its relatively higher thermolability. It was shown by preliminary experiments on white mice that tetanus toxoid almost completely lost its antitoxin combining power after being heated for 20 minutes at 80°.

On the basis of these observations it might have been thought that a preparation treated in this way would not give a specific line of precipitation. In fact this line, clearly marked on the immunogram (No.46) of the "20-25" fraction, was absent from the immunogram (No.45) of the same fraction after heating, whereas the second line, corresponding to some other antigen, was clearly shown.

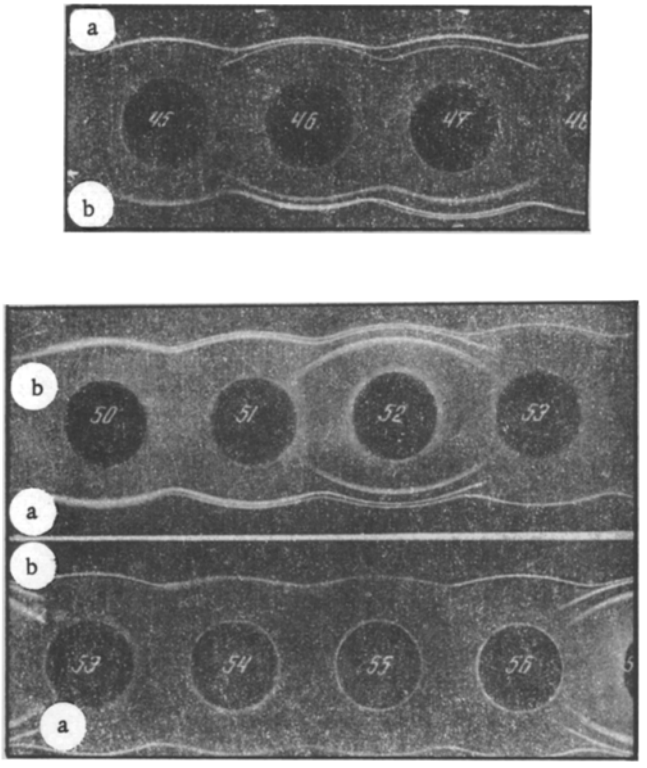


Fig. 1. Precipitation in agar of purified tetanus toxoid (series 1) and of certain of its fractions obtained by salting out with a phosphate mixture (pH = 7.4).
47 and 52) Original purified toxoid; 46 and 45) "20-25" fraction, before and after heating; 53 and 54) "35-40" fraction, before and after heating; 56 and 55) "40-45" fraction, before and after heating. Experiment carried out with tetanus antisera from horse, purified by the Diaferm-3 method, of series 438 (titer 2000 antitoxin units) and series 436 (titer 1400 antitoxin units). a) Serum 438; b) serum 436.

From the results of the precipitation reaction in agar, not all ballast antigens showed equal thermostability. After being heated in the manner described above, certain of them underwent some degree of damage. This was shown by the weakening of the corresponding precipitation line, or by its greater diffusion (immunograms Nos. 50, 54 and 55).

We give for comparison a photograph of the immunogram (Fig. 2) of purified tetanus toxoid of the second series, before and after heating. This preparation contained 4200 CU/mg of nitrogen.

The photograph clearly shows the disappearance of one of the precipitation lines as a result of heating the toxoid, whereas the other lines were preserved to a greater of lesser degree.

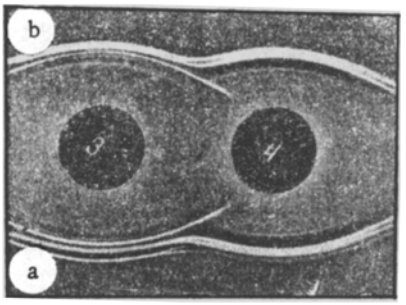


Fig. 2. Precipitation in agar of purified tetanus toxoid (series 2).
3) Original purified toxoid; 4) the same toxoid, heated for 20 minutes at 80°. a) Serum 438; b) serum 436.

Two of these lines, as may be seen from the immunogram, obtained by means of serum of series 438, appeared to split up into two diverging branches at one point.

This interesting phenomenon may possibly be accounted for by the formation of two forms of the same antigen, differing slightly in their physicochemical properties as a result of partical denaturation by heating.

The results described in this paper show that the method of precipitation in agar, when combined with other methods, may be used with success to investigate the antigenic composition even of such complex preparations as purified tetanus toxoid.

## SUMMARY

Several antigens were detected in the purified tetanus antitoxin by the method of precipitation in the agar. These antigens gave different distribution in the fractions obtained by fractional precipitation of this antitoxin by the phosphate mixture (pH = 7.4). Precipitation of specific antigen (antitoxin) was differentiated by comparison of the data obtained by reaction of precipitation in the agar with the data of antitoxin-fixating reaction obtained in experiments on white mice. Relatively greater thermolability of the specific antigen compared with the admixed ballast antigens served as an auxiliary factor in this test.

## LITERATURE CITED

[1] I. M. Khabas and O. A. Fadeeva, Interchange of Experience of Departments of Research Vaccine and Serum Institutes, 63-68, (Moscow, 1956). [In Russian].

[2] B. Bjorklund, Proc. Soc. exp. biol. med., 79, 319-324 (1952).

[3] O. Ouchterlony, Acta pathol. microbiol. Scand., 25, 186-191 (1948).

[4] C. G. Pope, Internat. arch. allergy, 5, 115-140 (1954).