STUDIES ON THE REACTION OF DIPHTHERIA TOXOID WITH ITS HORSE ANTISERA BY THE CROSSING ELECTROPHORESIS.

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Antigen-antibody reactions of the diphtheria toxoid and horse antiserum have often been studied by many investigators. The distribution of antibodies contained in the antiserum was studied first by Tiselius and Kabat[1]. In hyperimmune antisera the antibody against diphtheria toxin was found to be associated with "T-fraction" which migrated between β-and γ-globulins. But the study of the distribution of antibodies in the fractions of antisera was not always easy: either the antiserum was fractionated and the antibody content of each fraction was determined or the antiserum was analyzed electrophoretically before and after the absorption of antibodies by the antigen, to find out the decrease in the antibody containing fractions.

The immunoelectrophoresis of Grabar[2] has brought about a profound improvement in the technique to elucidate the distribution of antigens and antibodies. It is efficiently utilized in the study of the distribution of protein antigens in human sera. The crossing paper electrophoresis of Nakamura et al.[3-6], especially the two-dimensional technique, can be utilized to the same purpose. But the technique of immunoelectrophoresis has an advantage over the crossing paper electrophoresis, in that the antigen-antibody reaction takes place in agar gel by the mutual diffusion of antigen and antibody and thus the lines of precipitate can be differenciated very finely. On the other hand the crossing electrophoresis shows the advantage, either when the precipitate is dissolved by the excess of antigen or antibody, or when the reaction product is not precipitated at all.

METHODS

For the paper electrophoresis two sorts of apparatuses were used as described by Nakamura et al.: a horizontal type of Grassmann[7] and a hanging paper type of Durrum[8]. The former type was so enlarged, and the latter was so modified as to enable a sheet of filter paper of 30×30 cm to be set in the apparatus. Diagrams of the apparatuses used will be presented in the paper of Zaizen[9].

The filter paper used was No. 52 of Toyo, a somewhat heavier type than usual in order to facilitate handling.

A barbiturate buffer of pH 8.6 and ionic strength 0.05 was uesd.

Procedure of the two-dimensional crossing paper electrophoresis: The two-dimensional crossing paper electrophoresis was carried out according to the procedure of Nakamura et al.[5] As shown in Fig. 1, first, for instance, the antiserum

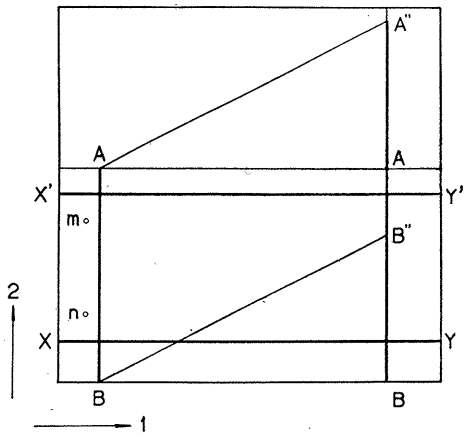

Fig. 1. An example of plans of lines in two-dimensional electrophoresis. Explanation, see text.

was applied on the line AB and the first electrophoresis was carried out in direction 1. When necessary, a drop of bromophenol blue solution was applied behind the line in order to follow the serum albumin. Then the current was stopped and the other reactant, the toxoid, was applied on the line XY, and the second electrophoresis was carried out in direction 2.

The change of the direction of electrophoresis was performed either by turning the filter paper sheet with the rack in the case of horizontal type apparatus, or by detaching the paper from the rack and turning it, in the case of hanging paper type apparatus. By the first electrophoresis the antiserum is separated into its electrophoretic components. By the second electrophoresis they encounter the line of antigen. Thus the latter will be depressed at the points of crossing with the lines of fractions which contain antibodies, to form a "crossing diagram". The peaks appeared on the line of antigen roughly correspond to the concentration distribution of antibodies contained in the fraction of antiserum. Hence the "crossing diagram" obtained in this case may be called the "crossing diagram of antibody or antiserum". As is obvious from this, a "crossing diagram of antigen" can be obtained on the line of antibody containing fraction by reversing the order of the two runs of electrophoresis: first antigen and then antiserum.

Technique of the two-dimensional crossing electrophoresis in agar gel: As discussed by Nakamura and his coworkers, the technique of the two-dimensional crossing electrophoresis can be carried out also in agar gel. The technique of the electrophoresis is the same as usual agar gel electrophoresis except that a square plate

instead of a strip is used: On a glass plate of 15 × 15 cm a frame of plastic plate was placed and a 1% solution of agar in buffer was poured into the area of 13 × 13 cm to 1 mm depth. After the agar has hardened, a fine strip of filter paper of 1 × 20～30 mm was placed on the appropriate position on the agar gel plate. The first reactant was applied on the filter paper strip and first electrophoresis was carried out. The second reactant was applied on the other fine strip of filter paper of 1 × 100～120 mm, placed on a appropriate position after the first electrophoresis was stopped. Then the second electrophoresis was carried out in the direction perpendicular to the first one. After the electrophoresis, protein spots were stained with amido black 10B.

Materials: Diphtheria toxin, toxoid and antiserum were delivered from the Takeda-Yakuhin-Kogyo Co. Protein concentrations of the toxin, the toxoid and the antiserum were 1.4, 1.0, and 8.4 per cent, respectively.

RESULTS

1. One-dimensional crossing electrophoresis of diphtheria toxoid and antiserum.

Fig. 2. shows the electrophoretic patterns of the diphtheria toxoid and one of the

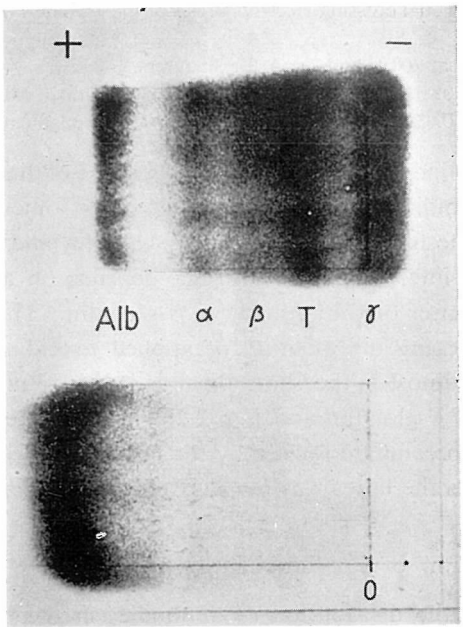

Fig. 2. Electrophoretic patterns of the diphtheria toxoid and of the antiserum (one of the used sera).
a) Diphtheria antiserum, 0.02 ml/4 cm.
b) Purified toxoid, 0.02 ml/4 cm. Electrophoresis at 60 V and 5 mA for 8 hours. Veronal buffer, pH 8.6, ionic strength 0.05. Paper, Toyo No. 52. Stained with bromophenol blue.

used sera. The toxoid contained one main component which migrated toward anode with a velocity somewhat larger than that of serum albumin. As can be seen from the figure, the antiserum contained the so-called "T-fraction" and increased β-and γ-globulins.

An example of the one-dimensional crossing electrophoresis of the diphtheria toxoid and the antiserum was shown in Fig. 3a. The antiserum applied on the line AB and the toxoid applied on the line XY, migrated both toward anode. With the progress of electrophoresis the line of toxoid came up with the line of antiserum at the nearer ends of the both lines. At least two lines of precipitate can be seen in the zone of β-and γ-globulin of the antiserum as a result of antigen-antibody reactions.

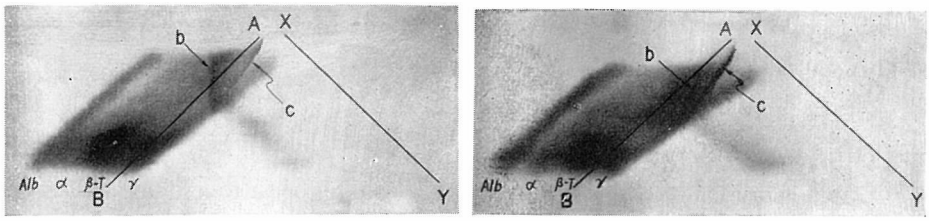

Fig. 3. One-dimensional crossing electrophoresis of the diphtheria toxoid and the antiserum.
Electrophoresis at 200 V and 7 mA for 3.5 hours.
a) Antiserum 0.03 ml/8 cm on line AB. Toxoid, 0.03 ml/8 cm on line XY.
b) Antiserum 0.03 ml/8 cm on line AB. Toxoid, 0.02 ml/8 cm on line XY.

One sharply defined line c, begins near the upper end of line AB and ends at the crossing point of γ-globulin with the toxoid. The other somewhat diffuse but dense one, line b, begins at the front of the zone of β-globulin and ends at the rear side of it. Thus it may be inferred that the former depends on an antibody contained in γ-globulin and the latter on an antibody in β-globulin. This can be more clearly demonstrated by decreasing the quantity of applied toxoid, as shown in Fig. 3 b. Here line c appeared almost in the same form as in Fig. 3 a. But line b appeared only at the rear side of β-globulin and is parallel to the latter. This has resulted from the fact that the precipitate formed by the antibody in β-globulin was not dissolved by the toxoid, as the latter was not in excess.

2. "Crossing diagram" of diphtheria antiserum.

As described above, the distribution of antibodies in the fractions of antiserum can be roughly determined by the one-dimensional crossing electrophoresis. But an exact determination of the concentration distribution of antibodies can be only achieved by the formation of the "crossing diagram" of antiserum by the two-dimensional crossing electrophoresis.

In Fig. 4 is shown an example of crossing diagrams of diphtheria antiserum against

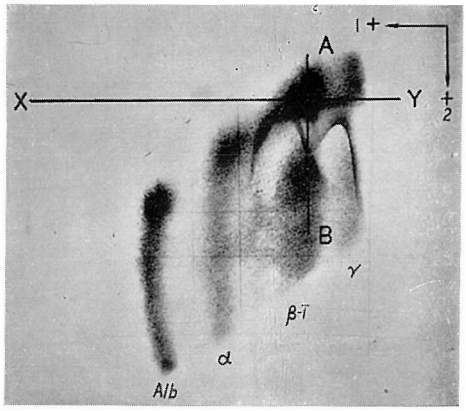

Fig. 4. An example of crossing diagrams of diphtheria antiserum against toxoid (I).
First electrophoresis in direction 1:0.03 ml/8 cm of antiserum alone were applied on line AB. 100 V and 7.5 mA for 8.5 hours. Second electrophoresis in direction 2:After the first one, 0.03 ml/16 cm of toxoid were applied on line XY. 50 V and 4.5 mA for 15 hours.

toxoid. The first electrophoresis was carried out in direction 1 after the antiserum alone was applied on the line AB. Then the toxoid was applied on the line XY and the second electrophoresis was carried out in direction 2. By the first run of electrophoresis the antiserum was separated into its components. By the second run they were crossed over by the line of toxoid. As can be seen from the figure, the line of the toxoid was depressed and formed a peak at the crossing with β-globulin and one with γ-globulin of the antiserum. The peaks formed on the line of the toxoid roughly correspond to the concentration distribution of antibodies contained in β-and γ-globulins of the antiserum.

Fig. 5 shows another example of the crossing diagram of diphtheria antiserum obtained by varying the proportion of the applied quantities of the toxoid and the antiserum. As can be seen from the figure two peaks appeared in the region of β-globulin of the antiserum.

Fig. 6 shows another example of the crossing diagram of diphtheria toxoid. Here appeared two peaks in the region of γ-globulin, as well as the two in the β-globulin zone. Thus it is obvious that the antiserum contained each two antibodies in β-and γ-globulins. As discussed above, the peak corresponds roughly to the distribution of an antibody, although the shape of a peak does not necessarily conform to a Gaussian error curve. But it will be formed, at least, symmetrically, if the corresponding antibody is a single entity and sufficiently separated from others. The peaks appeared in Fig. 6 are nearly symmetrical and hence appear to be derived from simple and single antibodies. But this can be further examined by the "crossing diagram of toxoid".

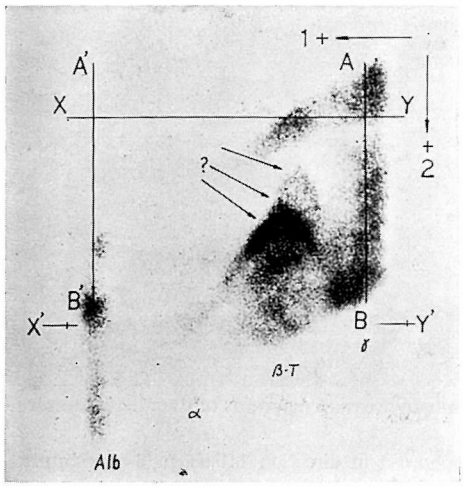

Fig. 5. An example of crossing diagrams of diphtheria antiserum against toxoid (II). 1st electrophoresis in direction 1, with 0.06 ml/8 cm of antiserum alone at 50 V and 5 mA for 12 hours. 2nd electrophoresis, with 0.03 ml/16 cm of toxoid, at 60 V and 5 mA for 8 hours.

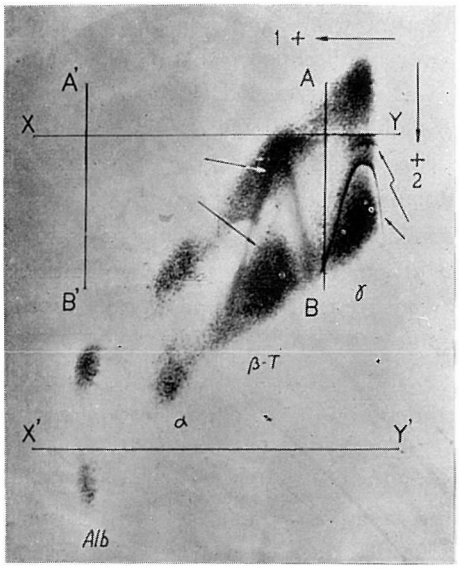

Fig. 6. An example of crossing diagrams of diphtheria antiserum against toxoid (III). 1st electrophoresis in direction 1, with 0.06 ml/8 cm of antiserum alone, at 50 V and 5 mA for 12 hours. 2nd electrophoresis in direction 2, with 0.01 ml/11 cm of toxoid, at 60 V and 5 mA for 12 hours.

3. Crossing diagram of "diphtheria toxoid".

In the experiment shown in Fig. 7, the diphtheria toxoid alone was first applied on the line XY and the electrophoresis was carried out in direction 1. Then the antiserum was applied on the line AB and the second electrophoresis was carried out in direction 2. The toxoid was separated in the first electrophoresis into its fractions, of which only the main fraction appeared clearly. In the second electrophoresis, the fractions of the toxoid came up and crossed with the lines of fractions of the antiserum. Thus a crossing diagram of the toxoid was obtained. As can be seen from the figure, peaks appeared in the region of β-and γ-globulin of the antiserum, respectively. The peak in the region of β-globulin corresponds to the main fraction of the toxoid. The peak in the region of γ-globulin is flat and extends almost from the start line to the main fraction of the toxoid.

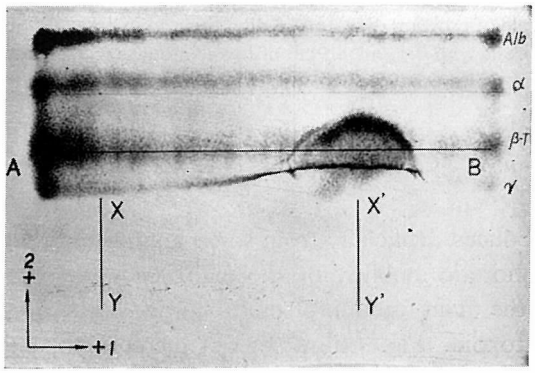

Fig. 7. An example of crossing diagrams of diphtheria toxoid against antiserum (I). 1st electrophoresis in direction 1, with 0.06 ml/4 cm of toxoid alone, at 50 V and 6 mA for 15 hours. 2nd electrophoresis in direction 2, with 0.06 ml/16 cm of antiserum, at 40 V and 5 mA for 6 hours.

In Fig. 8 another example of the crossing diagrams of the toxoid using another antiserum is shown. Here two flat peaks appeared in the region of γ-globulin, in addition to the peaks in the β-globulin zone.

Thus it is clear that the antigens which have their corresponding antibodies in γ-globulin of the antiserum are electrophoretically not homogeneous. They contain fractions of varying electrophoretic mobility, ranging from a very slow one to that of the main fraction of the toxoid.

4. Crossing diagram of diphtheria toxin.

In regard to the two antigens which correspond to the two very flat peaks in the region of γ-globulin, it was suspected whether they were contained as such in the

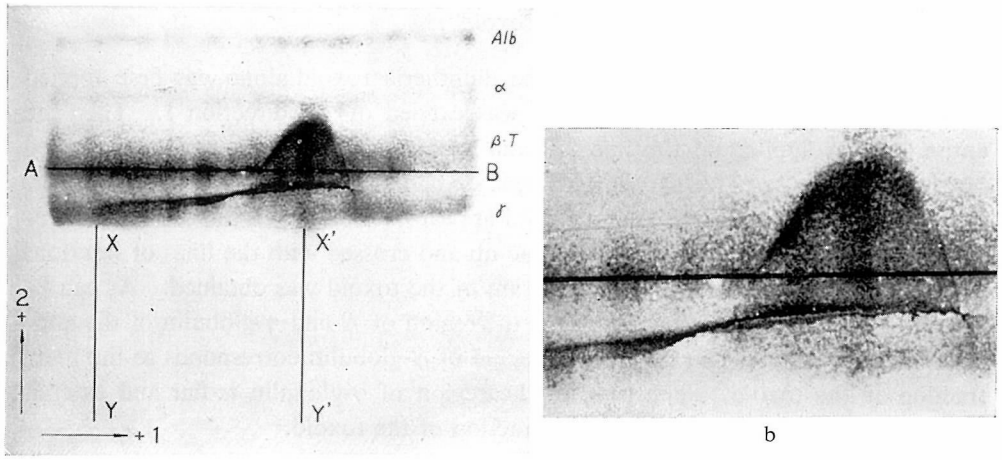

Fig. 8. An example of crossing diagrams of diphtheria toxoid against antiserum (II).
1st electrophoresis with 0.06 ml/6.5 cm of toxoid alone, at 50 V and 5 mA for 10 hours. 2nd electrophoresis with 0.06 ml/16 cm of antiserum at 60 V and 5 mA for 5 hours.
a) Total view.
b) Partial view. Two flat and broad peaks in γ-globulin zone are to be attended.

original toxin or produced artificially from some homogenous antigens in the toxin. In fact the electrophoretic mobility of the main fraction of toxin was evidently slower than that of the main fraction of the toxin, indicating some change occurred in the formation of toxoid. Fig. 9 shows one of the crossing diagrams of the toxin.

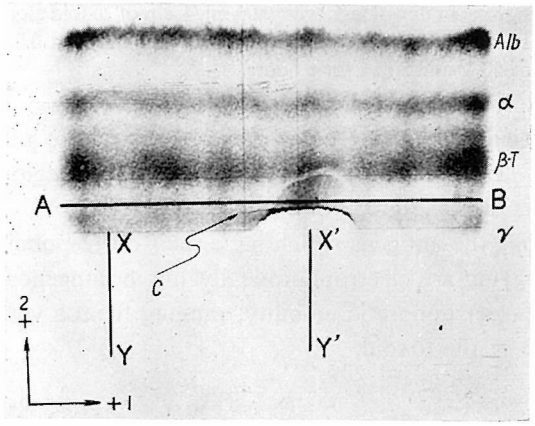

Fig. 9. An example of crossing diagrams of diphtheria toxin against antiserum.
1 st electrophoresis with 0.30 ml/4 cm of toxin alone, at 150 V and 9 mA for 8 hours. 2nd electrophoresis with 0.04 ml/16 cm of antiserum, at 150 V and 10 mA for 10 hours.

As can be seen from the figure, here also a faint flat peak, c, appeared in the region of γ-globulin, and two peaks corresponding to the main fraction of the toxin appeared. However, when the toxin was lyophilized and used in concentrated form, the crossing diagram obtained could not be distinguished from that of the toxoid. Thus it seems as if the flat and broad peak resulted from the change of antigen in the toxin owing to the lyophilization. But in some examples of the crossing diagrams of the toxoid the flat peak was scarcely visible. Therefore, it could not be determined whether the flat peak came from the change of antigen owing to the lyophilization, since the toxin could not be applied plentifully without lyophilization.

5. Crossing diagram of diphtheria antiserum in agar gel.

That the peaks in the crossing diagrams of diphtheria toxoid or of antiserum consist of precipitate formed by the reaction of antigen and antibody can be evidently demonstrated by the crossing electrophoresis in agar gel film. Fig. 10 shows an ex-

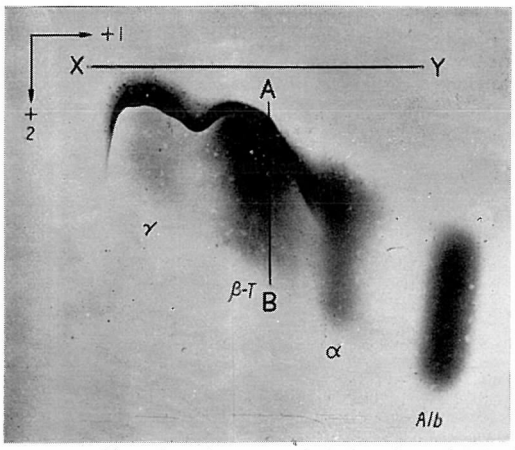

Fig. 10. An example of crossing diagrams of diphtheria antiserum in agar gel. lst electrophoresis with 0.01 ml/3 cm of antiserum alone, at 60 V and 10 mA for 15 hours. 2nd electrophoresis with 0.002 ml/6 cm of toxoid, at 150v and 20 mA for 8 hours. Stained with amido black 10 B.

ample of crossing diagrams of diphtheria antiserum in agar gel film. As the agar gel film is transparent, two peaks of precipitate can be seen on the black back ground. By staining with amido black 10 B, it is clear that the two peaks appeared in the regions of β-and γ-globulin, as shown in Fig. 10.

SUMMARY

Diphtheria toxoid contained a main component which migrated toward anode

with a velocity somewhat larger than that of serum albumin.

By the one-dimensional crossing paper electrophoresis two lines of antigen-antibody complex were demonstrated.

By the two-dimensional crossing electrophoresis, "crossing diagrams" of diphtheria antiserum and those of toxin and of toxoid were obtained. In the crossing diagrams of antiserum appeared each two peaks in the region of β-and of γ-globulin. In the crossing diagrams of toxoid appeared one peak in the region of β-globulin and two very flat peaks in the region of γ-globulin. In the crossing diagram of lyophilized toxin also appeared a similar flat peak in the region of γ-globulin. The antigens corresponding to these flat peaks appeared to be contained in the toxin.

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