FURTHER STUDIES ON THE REACTIONS OF DIPHTHERIA TOXOID WITH ITS ANTISERA BY THE CROSSING PAPER ELECTROPHORESIS.

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As already reported, the crossing electrophoresis developed by Nakamura and his associates[1] is a new approach to the elucidation of the distribution of antibodies in the antiserum and antigens in biological fluids. The reactions of diphtheria toxoid with its horse antisera were first demonstrated by Nakamura et al.[2] and Katsuno[3] on filter paper by their technique. They showed at least four antigens in the toxoid and a corresponding number of antibodies, two in β- and two in γ-globulin of the antiserum on the "crossing diagrams" developed in this method. The present communication reports the results of further studies on the distribution of the antibodies in diphtheria antisera.

METHODS

The methods and materials were the same as the report described by Nakamura et al.[2] and Katsuno.[3] The apparatuses used were those of horizontal type and of hanging paper type which accommodate a 30×30cm in size of filter paper. Filter paper of No. 52 (Toyo Roshi Co.) and a barbiturate buffer of pH 8.6 with an ionic strength of 0.05 were used.

The two-dimensional electrophoresis both for paper and agar gel electrophoresis was carried out as described by Nakamura et al.[2] Protein spots were stained with bromophenol blue or with amidoblack 10 B.

The diphtheria toxoid, diphtheria toxin and their antisera were made at the Takeda-Yakuhin-Kogyo Co. as well as the Kagaku-Kessei-Ryoho-Kenkyusyo, Inc. Standard antitoxic units of antisera were measured by the usual method*. Nitrogen was measured by the micro-Kjeldahl method.

The preparation of the fractions from the antiserum was carried out by the procedure of continuous paper electrophoresis as described by Grassmann and Han-

*) We are indebted to Dr. J. Kaneko of the Takeda-Yakuhin-Kogyo Co. for the determination.

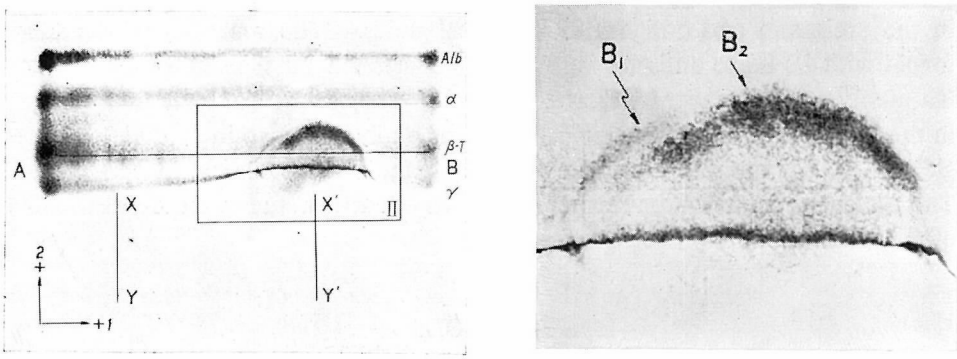
ning.[4] A phosphate buffer of pH 7.0 with an ionic strength of 0.05 was used. The isolated fractions were concentrated by lyophilization if necessary.

RESULTS

1) The Nature of the Peaks Appearing in the β-Globulin Zone in the Crossing Diagram of Toxoid.

(a) Antibodies in γ-Globulin Corresponding to Antigens Contained in the Main Fraction of the Toxoid.

Nakamura et al.[2] have already reported that one peak appeared in the region of β-globulin of antiserum and two very flat and broad ones in the γ-globulin zone. However, a careful reexamination of the diagrams disclosed that the foot of the peak in the β-globulin zone stands out of the γ-globulin (Fig. 1). Thus, it became



(a) Total view. (b) Partial view.

Fig. 1. Crossing diagram of diphtheria toxoid against antiserum showing peaks in β-globulin zone.
The first electrophoresis: 0.06 ml/4 cm of diphtheria toxoid was applied on line XY, electrophoresis in direction 1 at 50 V and 6 mA for 15 hours.
The second electrophoresis: 0.06 ml/16 cm of antiserum was applied on line AB, electrophoresis in direction 2 at 40 V and 5 mA for 6 hours. Filter paper, Toyo No. 52. Stained with bromophenol blue. Explanation, see text.

equivocal that this peak belonged to the β-fraction. It had to be studied that this peak was primarily in the γ-fraction but was dissolved and projected into the β-globulin zone as a result of overcrossing by an excess of the antigen contained in the main fraction of the toxoid. Such problem might be solved if the crossing of the toxoid was restricted to the extent that it remains in the γ-globulin zone not to run into the β-globulin zone. Fig. 2 represents an illustrative diagram of diphtheria toxoid against antiserum obtained with restricted crossing. Here, the restriction of crossing was effected as follows: After the toxoid had been separated into its components by the first electrophoresis, the second electrophoresis was performed

without the antiserum, in the direction perpendicular to the first run and opposite to the third run. The second run was carried out to the extent that a sufficient separation of antiserum in the third run would be secured. The antiserum was then applied and electrophoresed (3rd run) in the direction reverse to that in the second, to the point where the main component of the toxoid crossed halfway over the γ-globulin zone.

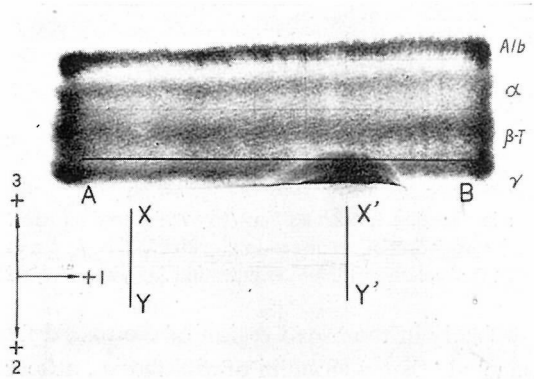


Fig. 2. Crossing diagram of diphtheria toxoid against antiserum.
The 1st electrophoresis: 0.04 ml/4 cm of toxoid on line XY, in direction 1, at 50 V and 6 mA for 15 hours. The 2nd electrophoresis: without antiserum, in direction 2, at 40 V and 5 mA for 3 hours. The 3rd: 0.06 ml/16 cm of antiserum on line AB, in direction 3, at 40 V and 5 mA for 6 hours.

As will be seen in Fig. 2, two peaks appeared in the area on γ-globulin zone where it crosses the main fraction of the toxoid, one peak overlapping the other. The lower one is sharply demarcated, whereas the outer one is diffuse and indiscrete, as were those which appeared in the β-globulin zone in the previous diagram. Thus γ-globulin contains at least two antibodies which have their corresponding antigens in the main protein fraction of the toxoid. These two peaks would be developed through the γ-globulin into the β-globulin zone and mistaken for those which belong to the latter fraction, if the crossing was overdone. This situation may be clarified if the toxoid was crossed with γ-globulin alone to an excessive extent. An experiment was carried out in which γ-globulin that had been separated free of β-globulin by the continuous electrophoresis was crossed with the main toxoid fraction. The result (Fig. 3) clearly showed that the peak formed by the reaction between the toxoid fraction and γ-globulin penetrated through the entire γ-globulin zone and appeared in the region where β-globulin would otherwise be found.

As demonstrated by Nakamura et al.[2], γ-globulin of diphtheria antiserum contains two antibodies which make very flat and broad peaks in the crossing diagram with toxiod. Hence, they are different from the ones which were demonstrated in this study, because they must have the corresponding antigens outside the main

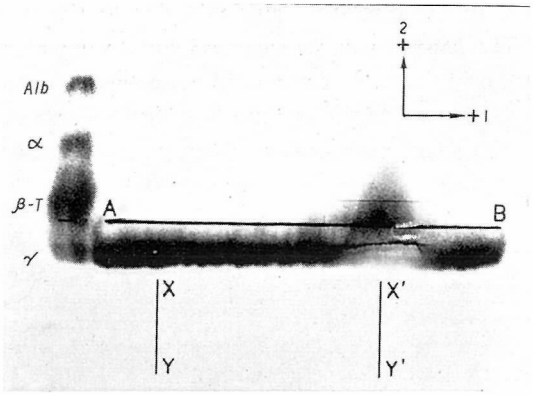


Fig. 3. Crossing diagram of diphtheria toxoid against purified fraction of antiserum.
The 1st electrophoresis: in direction 1, with 0.02 ml/5 cm of toxoid on line XY at 50 V and 6 mA for 15 hours. The 2nd electrophoresis: in direction 2, with 0.005 ml/1 cm of crude antiserum and 0.30 ml/16 cm of γ-globulin fraction (protein concentration 2%), at 40 V and 5 mA for 10 hours.

fraction of toxoid, probably in the broad region of the toxoid fractions. Therefore, it is reasonable to conclude that γ-globulin of diphtheria antiserum contains at least four antibodies.

(b) Peaks Appearing in the β-Globulin Zone in the Crossing Diagram of Toxoid.

As discussed above, one of the peaks observed by Nakamura et al.[2] in the β-globulin zone in the crossing diagram of toxoid was unequivocally dependent upon the antibody contained in γ-globulin. Scrutiny of the diagram in Fig. 1 disclosed that the peak is split into two, B_1 and B_2 (Fig. 1 *b*). The outer peak becomes faint

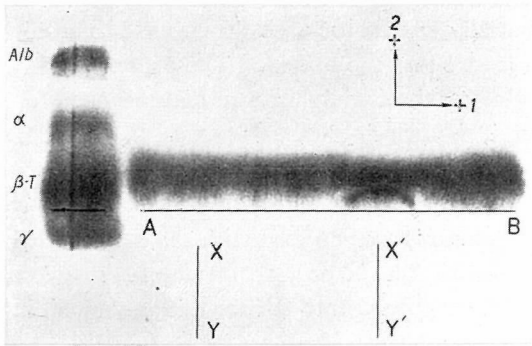


Fig. 4. Crossing diagram of diphtheria toxoid against purified fraction of β-globulin.
The 1st electrophoresis: in direction 1 with 0.02 ml/4 cm of toxoid on line XY, at 50 V and 6 mA for 15 hours. The 2nd electrophoresis: in direction 2 with 0.01 ml/1 cm of crude antiserum and 0.30 ml/16 cm of β-globulin fraction (protein concentration 2%) on line AB, at 40 V and 5 mA for 10 hours.

at the apex, whereas the limbs remain sharply defined. This may be interpreted that the outer peak is originated from the γ-globulin but has been driven into the zone of β-globulin by an excess of antigen and finally dissolved at its apex, while the inner peak is derived from the β-globulin. The validity of this interpretation may be proved by the use of a purified fraction of β-globulin. Fig. 4 illustrates the diagram showing the toxoid crossed with the purified fraction of β-globulin of the antiserum. Here, one peak appeared where the main fraction of the toxoid crossed.

2) Biological Potency and Antibody Composition of Purified Antisera.

In Japan, several preparations of diphtheria antiserum are commercially available at present. They are all in a purified form and authorized for therapeutic use. Two antisera, one prepared by the Takeda-Yakuhin-Kogyo Co. (Antiserum 1) and the other, by the Kagaku-Kessei-Ryoho-Kenkyusyo, Inc. (Antiserum 2), were used in this study. The concentrations of protein and antitoxin (in biological units) in these products are shown in Table 1.

TABLE I. Biological Activities of the Preparations of Diphtheria Antisera.

Antiserum	Concentration of N. mg/ml.	Antitoxic Units/ml.
1	11.7	1250
2	13.4	1200

It can be seen from the table that their protein and antitoxin contents are nearly the same. However, the electrophoretic patterns were distinctly different from each other. Antiserum 1 contained mainly β- or T-globulin, whereas antiserum 2 contained mainly γ-globulin (Fig. 5), indicating a difference in purification.

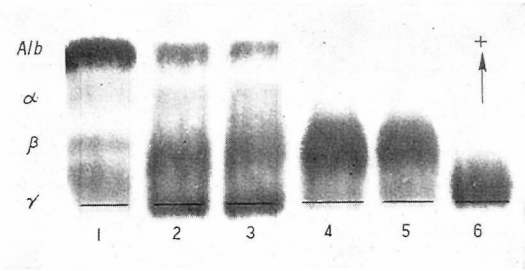


Fig. 5 Electrophoretic patterns of diphtheria antisera.

An aliquot of 0.01 ml/2 cm of each serum was applied on the line, electrophoresed at 60 V and 5 mA for 8 hours. 1: Bovine serum. 2: Diphtheria antiserum, No. 0737-II. 1,000 Lf/ml. 3: Diphtheria antiserum, No. 068-I. 470 Lf/ml. 4: Purified antiserum 1 (Takeda), No. 214. 1,200 Lf/ml. 5: Purified antiserum (Takeda), No. 208. 1,200 Lf/ml. 6: Purified antiserum 2 (Ka-Ketsu-Ken), 1,250 Lf/ml.

The difference in the relative concentrations of fractions containing antibodies means that the two antisera differ in their antibody contents. The distribution of antibodies was therefore studied on different preparations by the two-dimensional crossing electrophoresis. Fig. 6 *a* shows a crossing diagram of antiserum 1 against diphtheria toxoid. Two peaks appeared, one in the zone of β-globulin and the other in that of γ-globulin. As shown in Fig. 6 *b*, when the relative quantity of toxoid applied was increased, the peak in γ-globulin region disappeared and two peaks appeared in the region of β-globulin. Therefore it is apparent that antiserum 1 consisting mainly of β-globulin contained also antibodies which belong to γ-globulin.

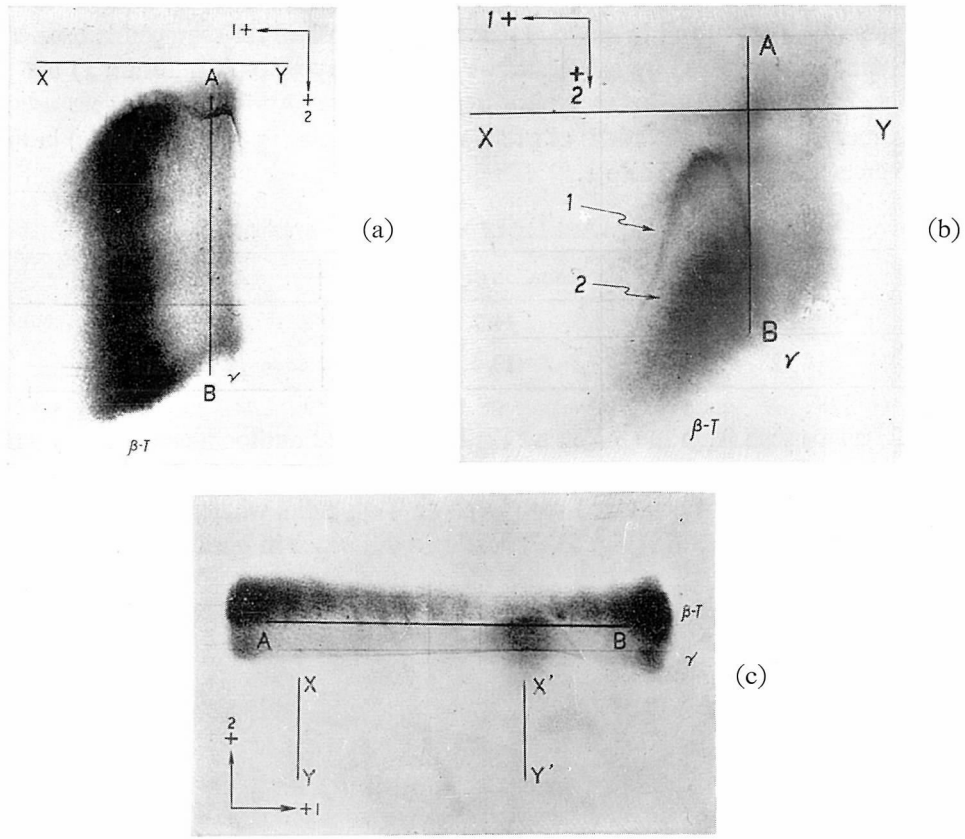


Fig. 6. Crossing diagrams of purified antisera against diphtheria toxoid.
The 1st electrophoresis: at 100 V and 7.5 mA for 8.5 hours with purified antiserum 1. The 2nd electrophoresis: at 50 V and 4.5 mA for 15 hours with toxoid.
a) Antiserum 0.06 ml/8 cm, toxoid 0.005 ml/16 cm.
b) Antiserum 0.04 ml/8 cm, toxoid 0.03 ml/16 cm.
c) Crossing diagram of diphtheria toxoid against purified antiserum 1.
The 1st electrophorsis: 0.03 ml/4 cm of toxoid, at 50 V and 6 mA for 15 hours.
The 2nd electrophoresis: 0.06 ml/16 cm of antiserum 1, at 40 V and 5 mA for 8 hours.

Fig. 6 *c* illustrates a crossing diagram of the toxoid against antiserum 1. Peaks similar to those obtained with crude antiserum appeared in the zone of γ-globulin. Thus, the nature and the number of antibodies contained in antiserum 1 seem to be the same as those in the crude antiserum.

A crossing diagram of antiserum 2 against the toxoid is shown in Fig. 7 *a*. One broad peak appeared in the zone of γ- and β-globulin combined. When the quantity of toxoid applied was further increased, the peak became shouldered in the region of β-globulin (Fig. 7 *b*). This was probably due to dissolution of the precipitate formed in the β-globulin zone by an excess of the toxoid and to further migration of the peak. Besides, two peaks appeared in the β-globulin region (b_1 and b_2) indicating the presence of two antibodies. With increassing quantities of the toxoid applied, the peak became narrower and the shoulder in the β-globulin zone disappeared

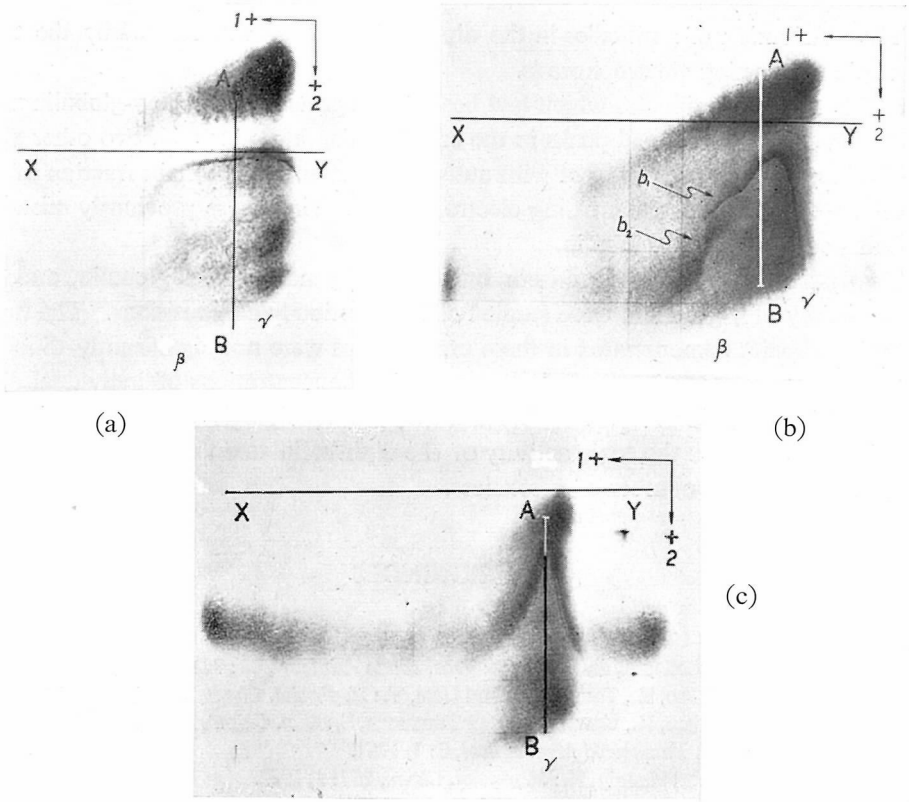


Fig. 7. Crossing diagrams of antiserum 2 against the toxoid with varying ratios of quantities.
The 1st electrophoresis: with antiserum 2, at 50 V and 5 mA for 15 hours.
The 2nd electrophoresis: with toxoid of following quantities at 75 V and 8.5 mA for 8.5 hours.

Applied quantities	a	b	c
Antiserum 2 (ml/8 cm)	0.06	0.03	0.03
Toxoid (ml/16 cm)	0.005	0.03	0.09

(Fig. 7 *c*), several new peaks appearing in the γ-globulin region at the same time.

The presented evidence will suffice to demonstrate that both antisera 1 and 2 contained probably the same kinds of antibodies, yet their relative concentrations were quite different. If the toxicity of the diphtheria toxin were to be ascribed to a single antigen, or specific "toxin", thence one of the antibodies being the specific antitoxin to counteract the specific toxin[5], such antibody would be present in the same concentration in any antisera that have the same toxic potency. Our data contradict this assumption indicating that this would not be the case. Inference may be made, therefore, that toxicity of diphtheria toxin is attributed not to a single, but to a number of substances.

SUMMARY

The distribution of antibodies in the diphtheria antisera was studied by the two-dimensional crossing electrophoresis.

Beside the two antibodies which had been found previously in the γ-globulin zone to form very flat and broad peaks in the crossing diagram of toxoid, two other antibodies in γ-globulin, which react with antigens contained in the main fraction of the toxoid, were found by the crossing electrophoresis. They were previously mistaken as being contained in β-globulin.

Two purified commercial antisera, one consisting mainly of β-globulin, and the other mainly of γ-globulin, were studied of their antibody compositions. The number of antibodies demonstrated in these preparations were not significantly different from that in crude antiserum. Yet, the relative concentrations of individual antibodies differed considerably. The fact that they possess biologically nearly equivalent activity suggests that the toxic activity of the diphtheria toxin may not be ascribed to a single, but to a number of substances.

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