

## Antigenic Relationships on the Diphtheria Toxin Molecule: Antitoxin Versus Antitoxoid

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We used the mouse to produce antisera to native diphtheria toxin and diphtheria toxoid. With these antisera it was possible to distinguish between toxin and toxoid. By gel diffusion analysis, antitoxin detected antigenic determinants on toxin which were not available on toxoid, indicating that some determinants had been lost or altered by formalin treatment. Antitoxoid, on the other hand, showed reactions of identity between toxin and toxoid in gel diffusion. The toxin neutralization titers measured in tissue culture were the same for both antisera. Only antitoxin neutralized the adenosine 5'-diphosphate ribosyl-transferase activity of fragment A, but surprisingly both antisera had significant anti-fragment A titers when tested by passive hemagglutination. It is suggested that some of the anti-fragment A activity in antitoxin affects the enzyme active site, whereas that in antitoxoid does not, implying the existence of a least two independent antigenic regions on fragment A.

Diphtheria toxin has a mass of 62,000 daltons and is toxic for sensitive animals and intact cells but is enzymatically inactive (8, 9, 12). Treatment of toxin with trypsin in the presence of reducing agents results in the formation of two nontoxic fragments: fragment A (24,000 daltons) and fragment B (38,000 daltons). Fragment A, the amino-terminal half of toxin, is an enzyme that catalyzes the coupling of adenosine 5'-diphosphate (ADP) ribose from nicotinamide adenine dinucleotide (NAD) to elongation factor-2. The latter is involved in protein synthesis that is blocked when elongation factor-2 is ADP-ribosylated (16, 17). Fragment B, the carboxy-terminal half, appears to be necessary for binding of intact toxin to the cell that leads ultimately to free fragment A in the cytoplasm (2, 32, 33).

After the description of diphtheria antitoxin by Behring and Kitasato in 1890 (4, 5), diphtheria toxin-antitoxin reactions served as a model for antigen-antibody studies. The use of toxin in very small doses was supplanted by mixtures of diphtheria toxin and antitoxin as immunogen (26), but no immunochemical studies were done with the antibodies elicited. After the introduction of formal toxoid (14, 15, 28) it soon became the standard diphtheria immunogen and immunochemical studies have been restricted to studies of antitoxoid antibodies. More recently Pappenheimer et al. (25) expanded such studies to include comparisons of antitoxoid antibodies with those elicited

against purified fragment A and two nontoxic serologically related mutant proteins, Crm<sub>45</sub> and Crm<sub>197</sub>. Evidence was presented (25) that the avidity of antitoxoid antisera was inversely related to their content of anti-fragment A antibodies. Bazaral et al. (3) reported that human antisera to diphtheria toxoid contained a substantial fraction of anti-fragment A antibody and suggested that it was unlikely that the anti-A activity had resulted from free fragment A in the immunizing toxoid because "cross-linking ... occurs between fragment A and fragment B during formaldehyde treatment." This interpretation would be contrary to that of Pappenheimer et al. (25), who proposed that most of the antigenic determinants of fragment A are masked in the intact molecule, which would tend to reduce immunogenicity of fragment A unless after injection it somehow became exposed. However, since neither investigation employed toxin as the immunogen, the relationship of antigenic determinants of fragment A to the intact molecule remains unclear.

The mouse is relatively resistant to diphtheria toxin (8, 11). We have taken advantage of this fact to produce antisera in the mouse against native diphtheria toxin and against diphtheria toxoid to begin comparative studies on the antigenic relationships of various portions of the toxin molecule and to determine the effect of formaldehyde treatment. The latter studies were in fact suggested by Pappenheimer et al. (25).

## MATERIALS AND METHODS

**Toxin, toxoid, and toxin fragments.** Diphtheria toxin lot D279 (1,600 flocculating units per mg of nitrogen) was purchased from Connaught Medical Research Laboratories, Toronto, Ontario, and purified according to the procedure of Cukor et al. (10) as described previously (19, 21). The purified toxin contained 10 guinea pig lethal doses per  $\mu\text{g}$  of protein and was homogeneous when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Toxin was prepared from this toxin by formalization according to the procedure of Linggood et al. (22). Diphtheria toxin fragment B was prepared according to the method of Pappenheimer et al. (25). Fragment B was identified by virtue of lack of enzymatic activity (9) and its migration as a 38,000-dalton protein on sodium dodecyl sulfate-polyacrylamide gels. Fragment A was the kind gift of R. J. Collier, University of California at Los Angeles. All of these proteins were stored at  $-65^\circ\text{C}$  in small aliquots and thawed only once just prior to use.

**Production of antisera.** Adult female C3H mice were obtained from Simonsen Laboratories, Gilroy, Calif., and housed six to eight per cage. Twenty mice each were immunized with diphtheria toxin or toxoid. The antigens were emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and injected subcutaneously into the back of the neck. Each injection contained 3  $\mu\text{g}$  of toxin or toxoid in 0.1 ml of 0.15 M phosphate-buffered saline (pH 7.2) mixed with 0.1 ml of Freund complete adjuvant. The injections were repeated again on days 10, 17, and 42 after the initial injection. One month after the last subcutaneous injection each animal received 1.0  $\mu\text{g}$  of either toxin or toxoid intravenously in 0.5 ml of phosphate-buffered saline. Each animal was assessed individually for the development of precipitating antitoxin and antitoxoid antibodies by gel diffusion analysis using varying concentrations of each antigen. The antisera used in this report were obtained 20 days after the intravenous injection. Blood was collected from the retro-orbital plexus in capillary tubes (29). Based on gel diffusion results, individual mice were identified as strong or weak precipitin producers, and the sera of the strong producers in each group were pooled. The antitoxin antiserum used here was pooled from the sera of 10 mice and the antitoxoid antiserum from 13 mice.

Rabbit anti-fragment A and anti-fragment B antisera were prepared by injecting 0.2 mg of fragment B or 1.4 mg of fragment A in 0.02 M phosphate buffer (pH 6.8) containing 6 M urea and 1.0 mM dithiothreitol emulsified in an equal volume of Freund complete adjuvant into the foodpads and backs of adult male New Zealand rabbits (six injection sites per rabbit). Two weeks later the animals were given booster injections in the rear foodpads of 0.093 mg of fragment B and 0.29 mg of fragment A in Freund complete adjuvant. Antiserum was obtained from blood samples drawn 3 weeks after booster injection.

**Gel diffusion analysis.** Gel diffusion (24) patterns shown were obtained in 0.85% Ionagar (no. 2; Colab Laboratories, Glenwood, Ill.) in 0.04 M barbital

buffer (pH 7.8) containing 1 M glycine and 0.14 M NaCl; similar results were obtained using 0.75% agarose (L'Industrie Biologique Francaise, S.A.) in 0.01 M phosphate buffer, pH 7.5, containing 0.5 M glycine and 0.14 M NaCl.

**Passive hemagglutination.** Tanned sheep erythrocytes (SRBC) were prepared according to the method of Stavitsky (31), except that treatment with 0.005% tannic acid was done at  $37^\circ\text{C}$  for 10 min according to the method of Campbell et al. (7). The SRBC were standardized at a final concentration of 2.5 to 3.0% according to the spectrophotometric method of Jacobs and Lunde (20). The normal rabbit serum used in the diluent was inactivated at  $56^\circ\text{C}$  for 30 min and absorbed with equal volumes of SRBC and human erythrocytes before use. Toxin, toxoid, and fragment A were used at a final concentration of 65  $\mu\text{g}$  of SRBC per ml for conjugation. The toxin and toxoid used to sensitize the cells were the batches that had been used to produce the antisera. Titrations were carried out in round-bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.) using 0.025-ml volumes of antiserum and SRBC. Titrations are reported as the reciprocal of the highest dilution showing confluent agglutination across the bottom of the well. The plates were read at 3 h and again after overnight at room temperature. The titers were identical at 3 and 24 h.

**Enzyme assay and inactivation by antibody.** Aminoacyl transferase-containing enzymes were prepared from crude extracts of rabbit reticulocytes as described by Allen and Schweet (1) and modified by Collier and Kandel (9). The NAD transferase activity of fragment A was measured according to the procedure of Collier and Kandel (9). The assay mixture in a total volume of 65  $\mu\text{l}$  contained 50 mM Tris-hydrochloride, pH 8.2; 0.1 mM ethylenediaminetetraacetic acid; 40 mM dithiothreitol; 25  $\mu\text{l}$  of reticulocyte enzymes; 0.367  $\mu\text{M}$  NAD ( $^{14}\text{C}$ ]adenine), 136 mCi/mmol (Amersham/Searle Corp., Des Plaines, Ill.); and 0.01  $\mu\text{g}$  of fragment A. After a 5-min incubation at  $25^\circ\text{C}$ , 65  $\mu\text{l}$  of 10% trichloroacetic acid was added, and the precipitates were collected, washed, and analyzed in a low-background counter as previously described (18). Preliminary experiments in which fragment A was incubated in either normal serum or saline indicated that samples containing serum showed greater enzyme activity than the sample in saline. Since the normal serum contained no detectable NAD transferase, we attributed the increased activity to a protective effect by normal serum. Subsequent enzyme neutralization studies were conducted in the presence of 5% normal mouse or rabbit serum.

Enzyme inactivation by antibody was determined by assay after preincubation of fragment A with antiserum. Fragment A (0.01  $\mu\text{g}$ ) in 5  $\mu\text{l}$  of saline containing 5% normal serum was mixed with an equal volume of antiserum or normal serum (as control) and incubated for 5 min at  $37^\circ\text{C}$ . The reaction mixture was then cooled rapidly in an ice bath and assayed immediately for NAD transferase activity by adding the buffered reticulocyte enzyme mixture and then mixing and adding labeled NAD; the assay was completed as described above. The

final dilutions of antiserum in the preincubation mixture ranged from 1:2 to 1:100. Inactivation is expressed as the percentage of control activity.

Neutralization of toxin-induced cytotoxicity by antitoxin or antitoxoid antiserum. Toxin neutralization titers of the various antisera were determined by the cell culture cytotoxicity test (30). HEP-2 cells (ATCC no. CCL-23 which may be HeLa cells [23]) were grown in 150-cm<sup>2</sup> plastic tissue culture flasks (Corning no. 25120) in the presence of Eagle basal medium (no. 611, GIBCO, Grand Island, N. Y.) plus 10% fetal calf serum (growth medium). Trypsinized cells were suspended at  $2.5 \times 10^5$  cells/ml in growth medium and 2-ml quantities were distributed into plastic tissue culture plates (35 mm) (Falcon no. 3001, Oxnard, Calif.). The culture plates were incubated for 24 h at 37 C in humidified 5% CO<sub>2</sub> plus 95% air. The plates were washed twice with 2 ml of Hanks balanced salt solution and overlaid with 1 ml of maintenance medium (Eagle basal medium plus 2% fetal calf serum). After inoculation with 0.2 ml of toxin-normal serum mixture or 0.2 ml of toxin-antiserum mixture (see below), the cultures were reincubated as above. The cultures were examined at 24 and 48 h for cytotoxic changes.

The minimal cytotoxic dose of toxin was deter-

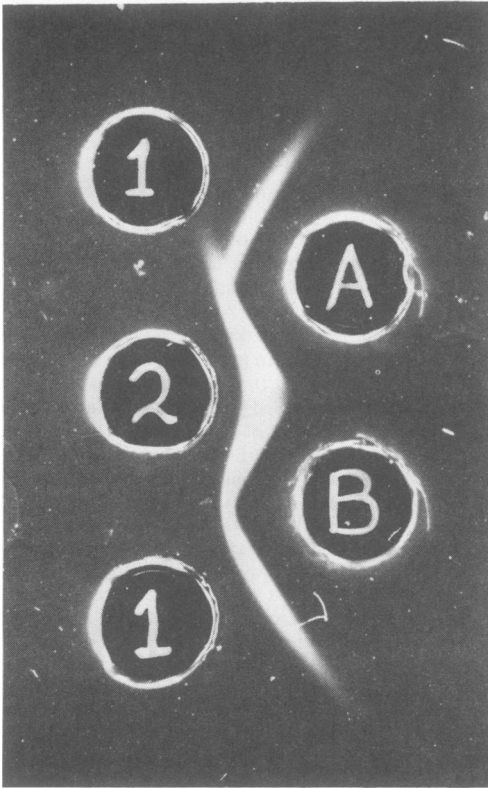


FIG. 1. Gel diffusion patterns comparing antitoxin (A) and antitoxoid (B) antisera against diphtheria toxoid (1) and diphtheria toxin (2). Toxoid concentration was 100  $\mu$ g/ml; toxin concentration was 250  $\mu$ g/ml.

TABLE 1. Passive hemagglutination using antitoxin or antitoxoid antiserum

Antiserum	Titer <sup>a</sup>			
	Protein coated on erythrocytes			
	Toxin	Toxoid	Frag- ment A	None
Antitoxin <sup>b</sup>	25,600	1,600	1,280	0
Antitoxoid <sup>b</sup>	51,200	12,800	640	0
Anti-fragment A <sup>c</sup>	10,240	640	5,120	0
Normal mouse serum <sup>d</sup>	0	0	0	0

<sup>a</sup> Reciprocal of last dilution showing confluent agglutination across bottom of well. We do not consider a one-tube difference in end point significant.

<sup>b</sup> Starting dilution 1:100.

<sup>c</sup> Starting dilution 1:10.

<sup>d</sup> Starting dilution 1:20. Saline was also tested in place of serum but caused no hemagglutination.

mined by mixing equal volumes of toxin dilution with a 1:5 dilution of normal mouse or rabbit serum. The diluent in all cases was phosphate-buffered saline. These mixtures were preincubated at 37 C for 1 h and from each dilution three culture plates were each inoculated with 0.2 ml of the mixtures. The minimal cytotoxic dose was taken as the lowest concentration of toxin destroying >50% of the cells in all three plates at the end of 48 h (30). With this toxin preparation the minimal cytotoxic dose was 0.02  $\mu$ g/ $5 \times 10^6$  cells plated.

The neutralizing titers of various antisera were determined by mixing 0.4 ml of diluted antiserum with an equal volume of toxin containing either 2 or 10 minimal cytotoxic doses. The mixtures were preincubated and added to triplicate culture plates as described above. The plates were examined at 24 and 48 h; the titer represents the reciprocal of the highest dilution of serum giving complete cell protection after 48 h.

## RESULTS

The results in Fig. 1 indicate that immunizing mice with toxin as opposed to toxoid resulted in antisera with distinguishing specificities. Whereas the antitoxoid antiserum formed a line of identity between toxin and toxoid, the antitoxin antiserum revealed only partial identities. The spur extending toward the toxoid well (Fig. 1) suggests that the toxoid preparation lacks antigenic determinant(s) detectable with antitoxin.

The passive hemagglutination titration supported this interpretation as shown in Table 1. The antitoxin and antitoxoid antiserum pools had approximately equivalent end points when titrated against toxin-coated erythrocytes but differed significantly when tested against toxoid-coated cells; the antitoxoid activity of the antitoxin preparation was only one-eighth that of the antitoxoid pool. The titrations shown were repeated twice using other batches of coated erythrocytes with identical results. Table 1 also shows that both antitoxin and anti-

toxoid had approximately equal titers against fragment A-coated erythrocytes. In view of enzyme neutralization data discussed below, this latter observation was unexpected. However, identical results were obtained with a different batch of fragment A (also obtained from R. J. Collier). Dithiothreitol treatment of the sera did not alter the results of hemagglutination titrations against either toxin, toxoid, or fragment A-coated erythrocytes (data not shown); thus, neither antitoxin nor antitoxoid contained an appreciable amount of immunoglobulin M antibodies (27). The anti-fragment A antiserum showed high titers against fragment A and against toxin-coated erythrocytes but had a significantly lower titer against toxoid-coated cells.

Based on these results, we asked whether the antitoxin and antitoxoid antisera would differ when tested against the A and B fragments of toxin. We attempted to answer this question by gel diffusion analysis but with only partial success. We were not able to obtain gel diffusion reactions with fragment B despite testing over a wide range of concentrations using different agars and buffer systems including 0.5 M urea (25). We assume this failure to be due to the relative insolubility and tendency of fragment B to aggregate, which has been reported by others (8, 9, 12). In contrast there was good reactivity of fragment A with antitoxin as shown in Fig. 2a. The pooled antitoxin antiserum reacts strongly with fragment A; the latter shares partial identity with both intact toxin and with toxoid. The direction of the spur between fragment A and toxoid suggests that a majority of the toxoid determinants recognized

by antitoxin may be associated with the A fragment, since we have not detected spurring in the opposite direction. Figure 2b, as in Fig. 1, shows lines of complete identity between toxin and toxoid when antitoxoid is used as the antiserum. Unlike the antitoxin preparation, however, antitoxoid shows no detectable reactivity with fragment A; this was true over a 300-fold range of concentrations (not shown).

Given the readily detectable differences in fragment A reactivity between mouse antitoxin and antitoxoid antisera, we tested their relative abilities to inhibit the NAD transferase activity of fragment A *in vitro*. Figure 3 compares the ability of various antisera to inactivate the enzymatic activity of fragment A. Mouse antitoxoid antibody was ineffective. It failed to neutralize the enzyme even at the highest concentration of antiserum tested (dilution of 1:2), whereas antitoxin still had partial enzyme neutralizing capacity at the 1:80 dilution. This is in accord with the gel diffusion results shown in Fig. 2b but not with the anti-fragment A passive hemagglutination data shown in Table 1. The rabbit anti-fragment A antiserum inhibited the enzyme approximately 90% at a 1:4 dilution but was not inhibitory at 1:10. By this criterion the mouse antitoxin antibody was 10 times more effective against fragment A than the rabbit anti-A antibody and at least 40 times more effective than the mouse antitoxoid antiserum.

We then compared the relative abilities of these antisera to neutralize the cytopathogenic effects of diphtheria toxin. The neutralizing capacity of the antisera was titrated in HEp-2 cells using either 2 or 10 minimal cytotoxic

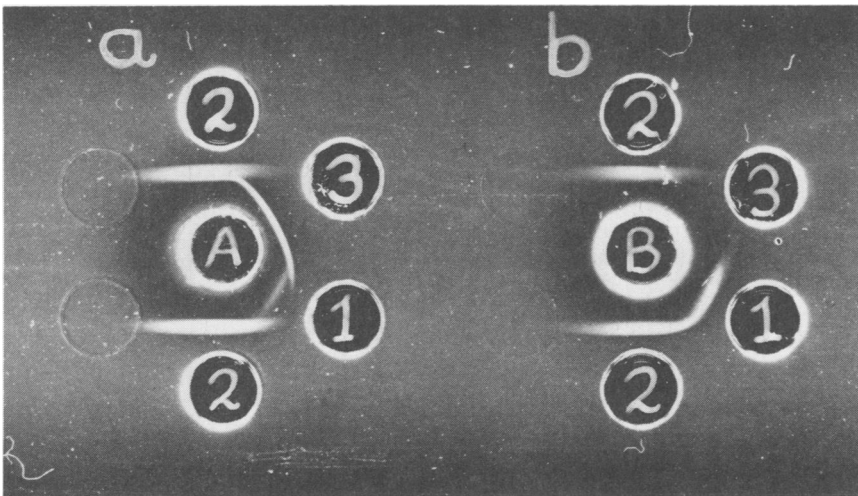


FIG. 2. Gel diffusion patterns showing activities of (a) antitoxin (A) and (b) antitoxoid (B) against toxin (1), toxin (2), and fragment A (3). Toxin concentration, 150  $\mu\text{g/ml}$ ; toxoid, 300  $\mu\text{g/ml}$ ; fragment A, 75  $\mu\text{g/ml}$ .

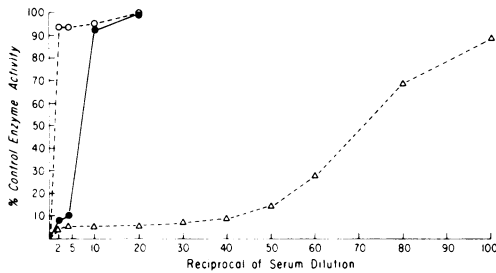


FIG. 3. Neutralization of the ADP-ribosyl transferase activity of fragment A by various antisera. Symbols: (●) Rabbit anti-A; (○) mouse antitoxoid; and (△) mouse antitoxin. See text for methods.

doses as described in Materials and Methods. At both toxin concentrations tested the neutralization titers of the mouse antitoxin antiserum were comparable to those of the antitoxoid antiserum (Table 2). The anti-fragment A antiserum had little, if any, neutralizing activity.

## DISCUSSION

It is clear that in addition to destroying its toxic potential, treating diphtheria toxin with formalin to produce toxoid alters immunogenicity. Pooled serum from mice immunized with toxin can be distinguished from mouse antiserum to toxoid. This conclusion is drawn from several different types of evidence. Gel diffusion analysis (Fig. 1) indicates that antitoxin can detect antigenic determinants on toxin that are no longer available on the toxoid molecule, giving rise to the toxin spur extending toward the toxoid well in the reaction with antitoxin. Mouse antitoxoid, on the other hand, appears unable to distinguish between toxin and toxoid based on the reaction of identity shown by the two antigens in the same figure. On this basis we may conclude that formalin treatment is more apt to destroy antigenic determinants on diphtheria toxin than it is to create new ones through altered conformation, even though it is known that formalin treatment both blocks amino groups and forms cross-links between lysine and tyrosine or histidine via methylene bridges (6). The latter in particular might be expected to give rise to new antigenic determinants depending on the degree of cross-linking (13). The increased stability and resistance to proteolysis of toxoid compared to toxin have been attributed to the cross-linking effects of formalin treatment (3, 25).

The relatively high capacity of the antitoxin to inactivate the ADP-ribosylating activity of fragment A (Fig. 3) was in accord with the gel diffusion data. The enzyme neutralization data

suggested that the activity detected against toxin that was not detected by antitoxoid was anti-fragment A activity particularly since, as shown in Fig. 2, only antitoxin precipitated fragment A in gel. Consequently, we were surprised to find that when fragment A was used to coat SRBC for passive hemagglutination, both antisera had significant anti-A titers (Table 1). Thus it is not possible to view the differences between antitoxin and antitoxoid solely on the basis of the presence or absence of anti-fragment A antibodies.

The loss of determinants through formalin treatment is borne out by the results of passive hemagglutination. Antitoxin and antitoxoid had similar end points when tested against toxin but differed when titrated against toxoid-coated cells; in this instance the antitoxoid preparation gave a much higher titer (12,800 versus 1,600, Table 1), suggesting inability of antitoxin to recognize portions of the toxoid molecule. This difference in titer could also suggest antitoxoid recognition of some new formalin-induced antigenic determinants as discussed above even though such determinants could not be detected by precipitation reactions in gels.

There are several explanations possible for the paradox arising from the differences between antitoxin and antitoxoid with respect to their anti-fragment A activity. The first is that the two antisera differ greatly in antibody quantity, but this seems unlikely in view of the toxin neutralization titers (Table 2) and the hemagglutination titers against toxin (Table 1) even though the latter method is only semi-quantitative. Furthermore, both sera are totally resistant to reduction by dithiothreitol (data not shown), indicating that neither had a significant immunoglobulin M component (27). A second possibility is that only the antitoxin anti-fragment A antibodies are of sufficient

TABLE 2. Neutralization of cytopathogenic effect of diphtheria toxin in HEP-2 cell cultures

No. m.c.d's	Neutralization titer <sup>b</sup>		
	Anti-toxin	Anti-toxoid	Anti-fragment A
Expt 1: 2 m.c.d (0.04 μg)	4,000	2,000	20
Expt 2: 10 m.c.d. (0.2 μg)	200	200	<10

<sup>a</sup> m.c.d., Minimal cytotoxicity dose.

<sup>b</sup> The titer represents the reciprocal of the highest dilution of serum giving complete cell protection after a 48-h incubation.

avidity to inactivate the enzyme under the test conditions used. Antitoxoid anti-fragment A antibodies, being of lower avidity, could dissociate during the enzyme assay and fail to precipitate fragment A in gel for the same reason, although the latter seems unlikely since the antitoxoid is of sufficient avidity to hemagglutinate fragment A-coated erythrocytes.

A third and more interesting possibility is that only one antiserum (antitoxin) has antibodies that affect the enzyme active site either through specificity for the active site itself or for some other site-affecting determinant. According to this interpretation the anti-fragment A activity in the antitoxin preparation may be subdivided into active-site-related activity and one or more (a required correlate if precipitation with fragment A involved only monomeric A) non-site-related determinants. In turn antitoxoid would contain antibodies to only a limited number (one?) of the nonactive-site-related determinants and thus be unable to form a stable lattice necessary for precipitation; such antibodies could be detected by hemagglutination through the multivalency provided by fragment A-coated erythrocytes. We hope to resolve this question through the use of appropriate immunoabsorbent columns, particularly since antibody that affects the ADP-ribosylating site of diphtheria toxin could prove useful in further elucidating the structure-function relationships of this important microbial toxin.

Rabbit and horse antitoxoid antisera which inactivate the ADP-ribosylating activity of fragment A have been described (25), and antibodies that bind to fragment A are produced by humans immunized with toxoid (2). Whether specificities distinguishing between toxin and toxoid similar to these reported here would be encountered in other species cannot be predicted. We deliberately chose mice because their relative insensitivity permitted immunization with purified toxin in immunogenic doses (3  $\mu$ g) which would be lethal for many other species. Since we have used pooled sera from at least 10 mice for each antiserum, it is unlikely that the distinguishing antibodies resulted from individual variations in immune recognition, but we cannot rule out the possibility that the immunogenic distinction between toxin and toxoid is species related. Whether the anti-fragment A activity was raised to A determinants while still a part of the intact toxin molecule or after some *in vivo* unfolding or cleavage remains to be determined.

The fact that both antitoxin and antitoxoid antisera have similar toxin neutralizing titers but differ markedly in their capacity to neutral-

ize the enzymatic activity of fragment A suggests that the inverse relationship between avidity and anti-A activity may be more complex than previously proposed (25).

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