# ANTIGENIC RELATIONSHIPS ON THE DIPHTHERIA TOXIN MOLECULE: A STUDY OF STRUCTURAL RELATIONSHIPS AND ITS FATE DURING TOXOIDING

by

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A THESIS

Presented to the Department of Microbiology

and the Graduate Division of the University of Oregon Medical School

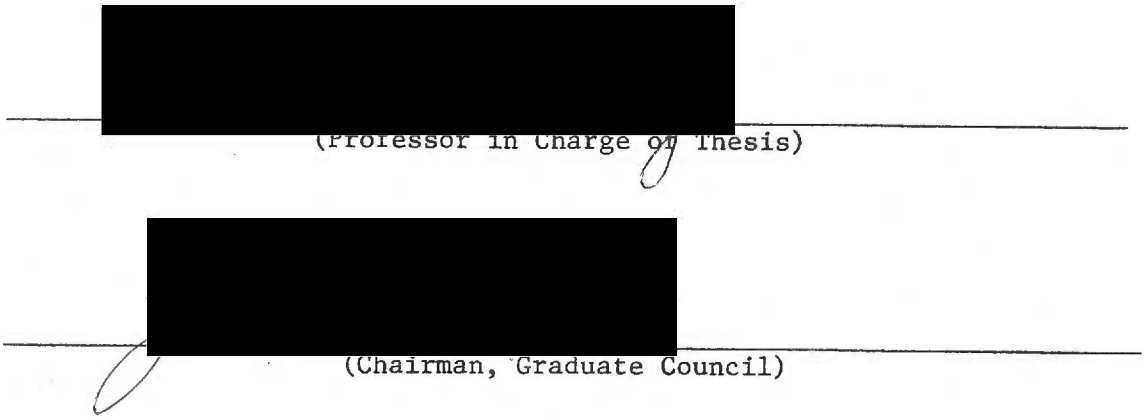
in partial fulfillment of

the requirements for the degree of

Masters of Science

September 1976

APPROVED:



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I would like to thank Dr. B. Iglewski for her interest in this work and valuable counselling.

I would also like to thank Dr. G. A. Leslie for his contributions and suggestions.

I especially would like to thank Dr. M. B. Rittenberg for his excellent guidance. His intellectual level and scientific insight are qualities that are possessed by few individuals. The demands of working with him are great but the rewards are beyond expectations.

Lastly, I would like to deeply thank my wife Pam and my daughter Sarah who's support and love were invaluable for my will to continue.

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## STATEMENT OF THE PROBLEM

There are a number of questions about the diphtheria toxin molecule that remain unanswered. It is known that the molecule is composed of two fragments, fragment A and B, each of which contributes in some way to the ability of the toxin to kill cells and susceptible animals (64,12). However, the question of the three dimensional structural relationships that exist between these two fragments in the toxin's native conformation is not certain. Pappenheimer *et al.* (63) suggest that, in the native state, the conformation is such that the hydrophobic portion of the molecule (fragment B) is completely exposed while fragment A is buried within the molecule. Such a conformation would explain their data which showed that anti-fragment A antibodies would precipitate only a small proportion of a labelled toxin preparation, since most of the antigenic determinants would have to be masked or unexposed in order for this result to occur (63). Also, the fact that the intact toxin and the nicked toxin are both enzymatically inactive seems to support the contention that antigenic sites of fragment A are not exposed (14).

Work done with toxoid and toxin have shown a number of interesting results regarding changes that might occur to the toxin after treatment with formaldehyde. For example, Masouredis (51) and later Baseman (2) have shown that the fate of toxoid and toxin *in vivo* are quite different. Others have shown that toxoid is not able to compete with native toxin for binding sites on susceptible cells (3). Linggood *et al.* (49) showed that toxoiding altered the electrophoretic migration pattern of toxin. Blass also suggests that formaldehyde

treatment results in the cross-linking of lysine residues with tyrosine or histidine residues and/or the formation of ε-amino bridges between the lysine molecules (9). Clearly alterations do occur in the toxin molecule upon treatment with formalin, not all of which are clearly understood or defined.

Immunochemical studies of toxin are one means to determine the three dimensional relationships in the molecule. However, the reason for much of the uncertainty about the conformation is that immunochemical studies done so far have been done with antitoxoid antisera. Considering the number of changes that occur during toxoiding (see above), the conclusions from such studies may be invalid. This research was initiated to resolve this problem. If one were to use toxin as an immunogen, the following types of questions could be answered: 1) What types of structural relationships exist between the respective fragments of the toxin that are detectable with an antitoxin but not an antitoxoid; 2) If there are differences in specificities, could these differences lend insight into the types of structural changes that occur during the toxoiding process; 3) If differences exist, would these differences also be reflected in the respective abilities to detect the fragment B portion of the toxin molecule, a portion thought to be a structural protein of the β-phage (20); and 4) Would the two antisera differ significantly in their neutralization capacity and/or avidity for the toxin.

# INTRODUCTION

Diphtheria toxin is an extracellular protein released from some strains of Corynebacteria diphtheria. Its existence was first postulated by Loeffler in 1884 (50) when he suggested that the disease diphtheria was due to a substance released from the infecting organism, since much of the pathological damage was at a site distant to the primary infection.

In 1888 Roux and Yersin (74) confirmed Loeffler's suspicions demonstrating that culture filtrates caused similar pathological changes in experimental animals as described in the natural infection. Two years later, Behring and Kitasato demonstrated the existence of antibodies to this extracellular substance (6,7). The true nature of the toxin, however, was not to be known until fifty years later.

## THE NATURE OF DIPHTHERIA TOXIN

In 1936, Eaton first purified and concentrated diphtheria toxin from culture filtrates (19). The next year Pappenheimer also purified the toxin and demonstrated it to be a protein (61). It is now known that diphtheria toxin is a protein of MW= 62,000 daltons that is released intact as a single polypeptide from the appropriate strain of C. diphtheria (25,13,18,83). The toxin is easily isolated from the culture supernatants when toxigenic strains of C. diphtheria are grown under conditions of limiting iron (69,31).

The intact molecule contains two disulfide bridges, one of which spans an arginine-rich region of about 40 amino acid residues (14). This region is extremely sensitive to proteolytic cleavage and upon

mild treatment of toxin with trypsin, a preferential cleavage is observed at one of the three closely spaced arginine residues in this loop (26). If one subsequently reduces the disulfide linkage, the molecule can be separated into two fragments; fragment A, MW= 24,000 daltons and fragment B, MW= 38,000 daltons (18). Michel et al. (54) were the first to show that fragment A was the N-terminal end of the molecule and fragment B was the C-terminal end.

Fragment A has extremely different physical-chemical properties from fragment B, and its enzymatic activity is responsible for the toxic effects of diphtheria toxin. It is highly stable and can withstand temperature extremes of 100 degrees centigrade at neutral pH for up to ten minutes without an appreciable loss of enzymatic activity (44,26,18). Recently the amino acid sequence of fragment A was elucidated by DeLange et al. (16). Fragment A enzymatically catalyzes the ADP-ribosylation of Elongation Factor II (EF-2), a major protein needed in Eucaryotic protein synthesis; although much is known about the enzymatic activity, little is known about the active site. Optical studies by Kandel et al. (44) suggest that one of the two tryptophan residues interacts with the nicotinamide moiety of the bound $NAD^+$. Other studies by Collier indicate that the enzymatic activity is lost by the destruction of a single tryptophan residue (14). Conversely, Beugnier et al. (8) have shown that the nitration of a single tyrosine residue results in a 75% loss of enzymatic activity. There is also little known about the structural configuration of the native toxin molecule, and the relationship of fragment A to that configuration. This will be discussed below.

Fragment B on the other hand is a highly unstable protein

fragment, and it denatures and precipitates spontaneously after separation from fragment A. It is reported to contain a large number of hydrophobic amino acid residues, which accounts for its tendency to denature and to form aggregates in all but dissociating solvents (25). Fragment B has no known enzymatic activity, and the difficulty of working with such a hydrophobic molecule will severely hamper studies of this nature. A variety of studies have shown that fragment B is probably responsible for the binding of the toxin molecule to susceptible cells, facilitating the entry of fragment A into the cell interior (33,20,47).

## EFFECT OF TOXIN ON EUCARYOTIC CELLS

The first clue to the action of diphtheria toxin was reported by Strauss and Hendee (79) using a cell culture system. The results indicated that the toxin acts to shut down protein synthesis resulting in cell death. Later work showed that the cessation of protein synthesis was a direct effect of the toxin, not an indirect result of the cessation of some other important cellular system (45,80). Finally, the ultimate target of the toxin in the eucaryotic cell was identified as Elongation Factor II (12).

The enzymatic reaction that occurs in the cell involves the transfer of an ADP-ribosyl group on the $NAD^+$ cofactor, to EF-2. The ADP-ribosyl group is covalently bound and renders the EF-2 inactive. The reaction is reversible and is written as follows:

$$\text{EF-2} + \text{NAD}^+ \xrightarrow{\text{fragment A}} \text{ADPR-EF-2} + \text{Nicotinamide} + \text{H}^+$$

This reaction has an absolute requirement for $NAD^+$ *in vivo*, although

some unnatural pyridine nucleotides can substitute *in vitro* (31,44).

Although the ability of diphtheria toxin to cause cell destruction is dependent upon the enzymatic activity of fragment A, the relationship of enzymatic activity *in vitro* and toxicity *in vivo* are not as straight forward as it might appear. For example, the intact toxin molecule in its native state is highly toxic for susceptible animals and cell cultures, but it lacks any *in vitro* enzymatic activity (14). However, isolated fragment A is enzymatically active *in vitro*, but when given to susceptible animals is not toxic (64). Even in cell culture, a large molar excess must be given before cessation of protein synthesis occurs (55). Recently, Moehring and Moehring showed that on an equivalent molar basis with the intact toxin, fragment A is as toxic to the cells as intact toxin once it gains entry to the cell interior (55). The increased amounts required for toxicity reflect the inability of fragment A alone to enter the cells. Therefore, at some time during the intoxication of cells, both the binding of fragment B to the cell surface (which facilitated the entry of fragment A into the cell) and the nicking and reduction of the toxin molecule to release free fragment A, are necessary events. These results also suggest that the enzymatic active site of the diphtheria toxin molecule may not be exposed in the toxin's native configuration, and that the *in vivo* nicking and reduction is necessary to expose the active site (63).

## CORYNEBACTERIOPHAGE β

The ability of any particular isolate of *C. diphtheria* to cause a clinical case of diphtheria is directly correlated with toxigenicity.

However, many isolates from routine throat cultures appeared to be exactly the same as the "pathogens", except they lacked the ability to produce toxin. Finally, in 1951 Freeman (23) discovered that a bacteriophage, Corynebacteriophage β, was responsible for the conversion of a non-toxigenic strain to a toxigenic strain. Groman later confirmed this work in controlled population studies (33). Later work demonstrated that the $tox^+$ gene was indeed carried on the phage genome, and if one treated the isolated β-phage with selected mutagens and then infected the bacterium, altered toxin molecules would result (82). Such altered proteins have been used advantageously by investigators to help delineate structure-function relationships of the toxin molecule. These relationships will be discussed below.

The relationship of the toxin protein to the intact phage has also been a subject of controversy among investigators. Barksdale and Pappenheimer (4) suggest that the toxin is not an important structural protein of the phage, because antiserum against diphtheria toxoid which is protective against intoxication does not neutralize the phage infectivity of the host bacterium. Elwell and Iglewski (20) and later Elwell (21) suggest that fragment B of the toxin molecule is a structural protein of the phage, presenting immunological and physical-chemical evidence to support this contention. However, this relationship has not yet been confirmed.

Lastly, the sensitivity of various species of animals to diphtheria toxin is highly variable (14). The guinea pig is one of the most sensitive animals, requiring only 25 ng of toxin injected subcutaneously to cause death within 4 to 5 days (MLD). Intradermal

injections of 0.025 ng of toxin in either guinea pigs or rabbits can produce visible areas of edema (22). Other animals such as fowl, monkeys, rabbits and even man show approximately the same sensitivity to diphtheria toxin as does the guinea pig when calculated on the basis of dose per kg body weight (14). Rats and mice have been shown to be extremely resistant to diphtheria toxin requiring approximately three orders of magnitude higher doses per kg body weight to cause death (24). This differential sensitivity is also reflected in cell culture systems (24) but does not seem to be due to differences in the susceptibility of the protein synthetic systems (56). Also, it has recently been reported that tumor cells (39,10) and virus transformed cells (41) show increased sensitivity to diphtheria toxin over normal cells. Whether these susceptibility relationships are due solely to some unknown receptor for which Fragment B must bind, or whether they are due to differences in some other mechanism is not known at this time and remain to be elucidated.

## IMMUNOLOGY

The immunological studies that have been associated with diphtheria toxin and antitoxin are numerous and varied. Behring and Kitasato first described the existence of antibodies to diphtheria toxin in 1890 (6). Subsequently, bacteriologists and immunologists began to use the diphtheria toxin-antitoxin reactions as a prototype reaction to which all other bacterial toxins could be compared. The standard immunizing agent used during the first few decades was toxin-antitoxin mixtures, usually using horse anti-diphtheria toxin antisera. These mixtures were used as a prophylactic immunization measure for pro-

tection against the occurrence of clinical diphtheria. However, this method of immunization began to be recognized as somewhat hazardous. Evidence began to accumulate that hypersensitivities to horse serum proteins could develop over the course of immunization (36). There were intermittant reports of deaths during immunization due to what was later identified as incomplete neutralization of the toxin in the immunizing toxin-antitoxin mixture (17). There were also questions about the ability of the toxin-antitoxin mixtures to raise sufficient protection, since results of such immunizations were extremely variable as judged by the Schick test (65). When immunization was effective, the antibodies raised were of high avidity (63).

In 1921, Glenny and Sudmerson showed that formaldehyde treatment of culture supernatants would result in the detoxification of the molecule (28). Later Ramon confirmed these results demonstrating that the preparation was still antigenically similar to the native toxin (70). Ramon used his preparation to immunize humans, finding that the toxoid was able to stimulated protective antibodies with relatively few side effects. Glenny (29) confirmed these studies and subsequently, the toxoid became the antigen of choice for prophylactic immunizations against diphtheria (65,36). The introduction of the formol toxoid also led to a limitation of the immunochemical studies of diphtheria toxin to antitoxoid antibodies.

Early immunochemical studies done with antitoxoid antibodies suffered from problems of purity of the immunizing preparations. Until 1936, the only preparations available for making toxoid were crude supernatants of the culture growth medium. Eaton (19) and later Pappenheimer (61) succeeded in purifying the toxin, but both

groups encountered problems in separating the toxin from large numbers of non-specified components in the culture medium. In 1940 Mueller (57) introduced the first chemically defined medium with none of the components larger than the molecular weight of most amino acids. Subsequently, it was shown that toxin of high potency could be consistently produced on this medium (58). The development of the defined culture medium aided in the purification of toxin and also helped eliminate all of the non-specific immunizing antigens in the toxoid preparations. Finally, crystalline toxin was achieved (68). Along with the increased purity of the toxin preparations came a decrease in the stability of the toxoid preparations. In 1963, Linggood et al. (49) developed a standardized method of preparing toxoid using purified toxin which maintained good immunogenicity and was stable for long periods of time. The method is still used today as the standard method of preparing toxoid.

With the introduction of toxoid the availability of antibody of high titer and/or avidity increased dramatically. Antitoxoid antibodies were used in many immunochemical studies which include quantitative precipitation reactions, flocculation reactions used for standardization, and avidity studies (43). More recently, antitoxoid antibodies have been used for immunochemical studies related to the toxin molecule and structure-function relationships.

## ALTERATIONS DURING TOXOIDING

The alterations that occur to the toxin molecule during toxoiding are at best only conjecture. The method serves to detoxify the molecule, but other molecular changes are uncertain. Early workers using

crude toxin preparations felt that the formaldehyde treatment resulted in the disappearance of greater than 50% of the free amino groups of the toxin, which the authors suggested were the amino groups that were important for intoxication (38). Later workers suggested that not only the loss of free amino groups, but also toxin aggregation and oxidation-reduction reactions of an undefined nature occurred during formalin treatment. The combination of these processes resulted in the loss of toxicity (38). Again, these early workers experienced problems in purity of their reaction mixtures.

After purified toxin was introduced, Woiwood and Linggood (87) showed that toxoiding resulted in the loss of the tyrosine spot in an amino acid spot analysis. They therefore suggested that somehow the tyrosine was an important amino acid to the toxin for its ability to exert toxicity. This suggestion is supported by Beugnier (8) but has not yet been confirmed. Later, work from Linggood's laboratory was published detailing an extensive study of the toxoiding process and showing a reproducible way of making a stable toxoid preparation from pure toxin (47). The process they used involves dialyzing the toxin against a basic amino acid, preferably lysine, and then treating the mixture with 0.5% formalin. The work not only demonstrated the importance of using a basic amino acid, but also that the molecule seemingly incorporated the amino acid into its structure.

In 1960, Masouredis (51) and later Baseman (2) showed some interesting results regarding the fate of labelled toxin or toxoid in vivo. Following the intravenous injection of toxin or toxoid labelled with $I^{125}$ or $I^{131}$ respectively, they found that the two

antigens behaved very differently. Toxoid was taken up preferentially by the tissues of the reticuloendothelial system and eliminated rapidly from the system. On the other hand, toxin is not taken up preferentially by any one tissue, and appears to follow a clearance pattern similar to a non-toxic foreign protein bovine serum albumin. As the blood levels of toxin decreased, there is a corresponding increase of label that could be detected in a variety of tissues including the brain, the heart, and the liver, etc. Therefore, formalin treatment of the toxin results in a protein that behaves markedly differently in vivo than the untreated molecule. Later work by Raymond demonstrated that formaldehyde reacts primarily with ε-amino groups of the lysine causing cross-linkages, but it may also form methylene bridges between the lysines and either tyrosine or histidine (9). Detoxification of the molecule may be a result of masking of critical reactive sites on the molecule or possibly a stabilizing effect resulting from the cross-linkages. If the molecule is stabilized, fragment A may not be cleaved from the intact molecule in vivo and therefore remains enzymatically inactive (5). To date the evidence indicates that the toxoid lacks receptor blocking activity (3). There is also evidence that the nicked toxin cannot be dissociated by thiols or SDS after formaldehyde treatment (5,54). One can infer from these data that fragment B is inactivated or altered at least to some degree by formaldehyde treatment. The action of formaldehyde on fragment A is still unknown because no definite evidence exists about the chemical nature of the active site, nor about the three dimensional relationship of fragment A in the intact molecule. Beugnier and Zanen, however, have published

data that indicated the loss of toxicity during toxoiding is due to the inability of fragment A to be cleaved from the toxin *in vivo* (8).

The most recent immunochemical studies using antitoxoid antisera have been done by Bazaral *et al.* (5) and Pappenheimer *et al.* (63). Bazaral *et al.* examined human antibodies to diphtheria toxin, following toxoid immunization, using a radioimmunoassay technique. They made comparisons of avidity and antibody titer in a large number of human sera, noting a large variability in the amount of antibodies to fragment A present in any one serum sample. He suggested that the anti-fragment A antibodies in the samples were not a result of free fragment A being present in the immunizing preparations, as suggested by Gill and Dinius (25). Bazaral and colleagues suggested that the anti-fragment A antibodies were more likely a result of antibodies being formed to fragment A in its native configuration in association with fragment B (5).

Pappenheimer *et al.* also used antitoxoid antibodies for immunochemical studies of toxin (63). These antibodies showed partial identity between fragment A and the intact toxin, and partial identity between fragment B and toxin. They also noted a large variation in the content of anti-fragment A antibodies among the various antisera tested.

In addition to the antitoxoid antisera, Pappenheimer *et al.* immunized animals with two cross reacting molecules, CRM 197 and CRM 45 to test the antisera elicited for the amounts and specificity of antibodies. The CRM proteins are molecules that are produced by mutant bacteriophages. CRM 197 for example, is a 62,000 dalton protein that is non-toxic but is immunologically identical to toxin. It is

thought to be inactive due to a missense mutation at or near the active site of fragment A (82). CRM 45 is a 45,000 dalton protein, with an intact fragment A but has a deletion of a 17,000 dalton fragment in the B portion of the toxin molecule. CRM 45 is also non-toxic probably due to its inability to bind to susceptible cells through the altered fragment B (82). Antisera prepared to both of these CRM proteins have a high proportion of anti-fragment A antibodies (63). This result is not surprising when CRM 45 is used as the immunizing antigen, since it does contain a smaller fragment B than the native toxin or CRM 197. However, the high proportion of anti-fragment A antibodies in the anti-CRM 197 serum was unexpected, especially since it is immunologically indistinguishable from the native toxin. If the CRM 197 was formalin treated before being used for immunization, the antiserum elicited did not contain the high proportion of anti-fragment A antibodies.

A third important observation presented in this work was the correlation of avidity of an antiserum with the proportion of antibodies directed against the fragment B portion of the toxin molecule. The data showed that the avidity of a particular antiserum against diphtheria toxin was directly dependent upon the proportion of antibodies directed towards the C-terminal portion of the toxin molecule (fragment B) and was inversely correlated with the amount directed towards the N-terminal portion (fragment A). These data suggest that the antibodies directed towards the B portion of the toxin, that portion which is responsible for the binding and efficient entry of toxin into the cell, are the most important antibodies in determining the avidity of the antiserum preparation.

On the basis of these three pieces of evidence, Pappenheimer et al. proposed that the A fragment of toxin is buried within the molecule in its native configuration. They agree with the explanation of Gill and Dinius (25) regarding the existence of anti-fragment A antibodies in the antiserum, suggesting that these antibodies arise from free fragment A in the immunizing preparation. This idea is contrary to that of Bazaral et al. (5). Pappenheimer et al. explain the high proportion of anti-A antibodies in the anti-CRM 197 antiserum to be due to the higher susceptibility of this molecule to proteolytic cleavage. Formaldehyde treatment stabilized the CRM 197 molecule thereby preventing the formation of the high amounts of anti-fragment A antibodies produced against the non-formalin-treated CMR 197.

The conformational relationship of fragment A to the native toxin may not be as easily determined as suggested by Pappenheimer et al. work. The major problem responsible for most of the uncertainty about this conformational relationship is that the immunochemical studies done to date have used antibodies elicited by diphtheria toxoid. From the information that exists regarding the changes undergone by proteins during toxoid formation (formaldehyde treatment), one would suspect that the types of antibodies elicited by the toxin compared to the toxoid may be quite different. Formaldehyde treatment could easily create new antigenic determinants through modification, or destroy other existing antigenic sites. Such a result was seen in the data obtained with the CRM 197 protein (63). The native molecule elicits an antiserum that differs drastically from those elicited to the formaldehyde-treated protein in the relative amounts of antibodies directed to fragments A and B of the toxin.

Therefore, there must be some further studies done on diphtheria toxin to determine first of all its true three dimensional conformation and secondly the relationship that exists between the A and B fragments of the toxin. Also, there must be more work done to determine the effect of formalin treatment on this three dimensional structure, and/or the specific actions on the A and B fragments.

# MATERIALS AND METHODS

## Buffers

Phosphate Buffered Saline (PBS) - This buffer was used for making the dilutions of diphtheria toxin or toxoid. The following were the stock solutions:

Solution A = 0.15 M $KH_2PO_4$

Solution B = 0.15 M $Na_2HPO_4$

The diluting buffer was prepared with 78 ml of solutions A and 22 ml of solution B, bringing the total volume to 1 liter with physiological saline (0.85%). The pH was adjusted with dilute NaOH to pH = 7.2 and the final solution was 0.01 M.

Passive Hemagglutination Buffers - PBS of two pH values were prepared according to the method of Stavitsky (77). Stock solutions were prepared as above and used as follows:

1. pH 7.2 Buffer - This was used for diluting the erythrocyte suspensions and was prepared by mixing 119.5 mls of solution A + 380 mls of solution B + 500 mls of physiological saline. The pH was adjusted with dilute phosphoric acid.

2. pH 6.4 Buffer - This was used for the coupling of the proteins onto the tanned sheep erythrocytes. The buffer was prepared by adding 338.5 mls of solution A + 151 mls of solution B + 500 mls of physiological saline. The pH was adjusted as above.

Borate Buffered Saline - This buffer was used during the ammonium

sulfate precipitation of the immunoglobulins. The buffer was prepared according to the method of Campbell (11).

Boric Acid- - - - - - - - - - - - - - - - - - - 6.184 g
Sodium Tetraborate ($NaB_4O_7 \cdot 10\ H_2O$)- - - - - 0.536 g
Sodium Chloride (NaCl)- - - - - - - - - - - - - 4.384 g

Add distilled water to 1 liter and adjust the pH to 8.4 with dilute acid or base. The final buffer was made by adding five parts (5) of the above buffer to 95 parts of physiological saline.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Three buffers were used during this procedure:

A. Running Buffer - This was used for the electrophoretic run and consisted of a 0.1 M sodium phosphate buffer with 1% SDS. It is prepared as follows:

$Na_2HPO_4 \cdot 7\ H_2O$ - - - - - - - - - 19.2 g

$NaH_2PO_4 \cdot H_2O$ - - - - - - - - - - 3.86 g

SDS - - - - - - - - - - - - - - - -10.0 g/1

Adjust to pH = 7.2 with dilute acid or base.

B. Elution Buffer - This buffer was used to elute the protein from the SDS-PAGE fractions. The buffer is buffer number 33 A of Williams and Chase (86).

solution A - - - - - - - - - - 7.8 mls
solution B - - - - - - - - - - 12.2 mls
Dithiothreitol - - - - - - - - 0.0309 g
EDTA - - - - - - - - - - - - - - 0.0074 g

Distilled water is added to a final volume of 200 mls.

C. Dialysis Buffer - This buffer was used for the dialysis of fragment A, to remove the SDS. It was also used as the standard diluting buffer of fragment A in all of the enzyme assays. The buffer is a 0.1 M Tris buffer pH = 8.0.

Tris-HCl (Sigma) - - - - - 7.62 g
Trizma Base (Sigma)- - - - 1.42 g

Distilled water is added to 6 liters and the pH adjusted with dilute acid or base.

Gel Diffusion Analysis - Gel diffusion (60) patterns shown were obtained in two gel systems. Either 0.85% ionagar (No.2 - Colab Laboratories, Glenwood, Ill.) in 0.04 M sodium Barbital, pH = 7.8 containing 1 M glycine, or 0.85% Agarose (53) (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 was used.

Passive Hemagglutination - Tanned sheep erythrocytes were prepared according to the method of Stavitsky (77) with a few minor alterations in the procedure (73). Sheep blood was obtained from the Prepared Media Labs (Tualatin, Oregon) in Alsever's solution. Sheep erythrocytes (SRBC) were spun at 2200 RPM for 5 minutes and then washed three times in physiological saline (0.85%). The last spin was done at 3000 RPM for 10 minutes. The volume of packed SRBC was measured and resuspended in PBS pH = 7.2 (see Buffers). Every 1 ml of packed SRBC's was resuspended in 40 mls of PBS pH = 7.2. The washed cells were used within 24 hours. The suspension was standardized spectrophotometrically by the method of Jacobs and Lund (42). One ml of the SRBC suspension was lysed by placing the cells into 5 mls of distilled water. The absorbance at 580 nm was kept between 0.4 - 0.6 on a Beckman Spectrophotometer. A stock tannic acid solution of 1.0% in saline was prepared using reagent grade tannic acid (Merck) and stored at $5^{o}$ C. The stock solution was diluted 1:200 in

physiological saline for use. The tanned SRBC suspensions were prepared by adding 1 ml of a standardized SRBC suspension to 1 ml of 0.005% tannic acid. The mixture was incubated according to the method of Campbell at 37° for 10 minutes (11). The cells were washed two times with PBS pH = 7.2 spinning at 1500 RPM and then were resuspended in the original volume of PBS pH = 7.2. These cells were used within twelve hours of preparation.

Antigen Sensitized Erythrocytes - These were prepared by adding (in order) 4 mls PBS pH = 6.4, 1 ml of antigen, and 1 ml of the tanned SRBC suspension. This mixture was kept at 20° (room temperature) for 10 minutes. The cells were then spun at 1500 RPM and then washed two times with a 1:100 solution of normal rabbit serum (NRS) in PBS pH = 7.2 which had been previously inactivated at 56° for 30 minutes and absorbed at 37° for 30 minutes with an equal volume of packed sheep and human erythrocytes. The sensitized cells were resuspended in their original volume with 1:100 NRS and kept on ice until used. These cells were used within four hours of preparation. The antigens used were either diphtheria toxin, toxoid, or fragment A and all were conjugated at a final concentration of 65 μg per milliliter. Titrations were done in round bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.) using 0.1 ml volumes of antiserum and 0.025 ml of antigen sensitized SRBC. Titers were read at three hours and again after overnight at room temperature. No differences were noted between the three hour reading and the overnight readings. The titer is reported as the reciprocal of the highest dilution showing confluent agglutination across the bottom of the well.

For the passive hemagglutination inhibition assays, 0.01 ml of the competing antigen was added per well and mixed prior to the addition of the sensitized erythrocytes. The concentrations of the competing antigens are expressed in μg/ml.

Dithiothreitol treatment of the mouse antisera was done according to the method of Pirofsky and Rosner (66). Briefly, the antisera were placed in test tubes and treated with 0.01 M dithiothreitol in saline at 37° for 30 minutes. The serum was then rapidly diluted in the wells of the microtiter plates and the sensitized erythrocytes were added immediately. The titers were read and expressed as before.

Antigen Preparations

Toxin - Diphtheria toxin, lot D279 (1600 Lf/mg nitrogen) was purchased from Connaught Medical Research Laboratories and purified according to the procedure of Cukor et al. (15) as described previously (73,39,47). The purified toxin contained 10 guinea pig lethal doses/μg of protein and was homogeneous when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The toxin was kept in small aliquots at -70°, and thawed just prior to use. The final protein concentration of the toxin preparation was 26 mg/ml. Later work was done with a second toxin preparation which contained 60 mg/ml of protein. This toxin was also purified by the method of Cukor et al. (15). This preparation was kept in 0.01 ml aliquots stored at -70°, which were thawed just prior to use.

Toxoid - Diphtheria toxoid was prepared according to the method of Linggood et al. (49) from the 26 mg/ml toxin preparation. Aliquots of 0.5 mls were frozen at -20° and thawed just prior to use.

All toxoid used for this work was from this preparation.

Fragment A - Most of the experimental work was done with a highly purified preparation of fragment A kindly supplied by Dr. R. J. Collier, University of California, Los Angeles. Some of the later work required the preparation of new fragment A. This preparation was made using a highly nicked aliquot of purified diphtheria toxin, as assessed on sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The toxin was then boiled according to the method of Cukor et al. (15). The supernatant of the boiled toxin preparation (pH = 6.4) was collected and 0.01 ml samples, which contained 150 µg of protein, were run on SDS-PAGE according to the method of Weber and Osborn (85). The gels showed a staining pattern of five protein bands, after staining with 1% comassie blue. Two millimeter slices of the gel were cut and placed into 10 x 75 mm test tubes -- two slices per tube. The gels were crushed with applicator sticks and 1 ml of an elution buffer was placed into each tube (see Buffers). The protein was allowed to elute overnight in the cold and then the supernatant of each tube was assayed for the in vitro NAD-transferase activity by the method of Collier and Kandel (13). Those fractions showing enzymatic activity were pooled and lyophilized on a Virtis Unitrap (model 16-100) overnight. The lyophilized material was resuspended in two mls of 0.01 M Tris buffer, pH 8.0 and a 0.01 ml sample was rerun on SDS-PAGE. The eluted and concentrated protein migrated as a single band. The concentrated protein was extensively dialyzed against the Tris buffer. After dialysis, the material was assayed for the amount of enzymatic activity present in the sample. The final protein concentration as

judged by enzymatic activity and the intensity of the stained band in the gel after electrophoresis, was approximately 65 µg/ml.

Fragment B - This fragment was prepared according to the method of Pappenheimer et al. (63) and was identified by virtue of its lack of enzymatic activity (13) and its migration as a 38,000 dalton peak on SDS-PAGE. Aliquots were kept at $-70^{o}$ and thawed just prior to use.

## Antiserum Preparations

Antitoxin and antitoxoid were prepared in two ways, serum antibody or ascites antibody:

Serum Antibodies - Adult female $C_3H$ mice were obtained from Simonsen Laboratories, Gilroy, California and housed 6-8/cage. Twenty mice each were immunized with either diphtheria toxin or toxoid from the 26 mg/ml preparation. The antigens were emulsified in an equal volume of Freund's Complete Adjuvant (FCA Difco Laboratories) and injected subcutaneously in the back of the neck. Each injection contained 3 µg of antigen in 0.1 ml of emulsion. Injections were given on day 0, 10, 17 and 42. One month after the last subcutaneous injection, each animal received 1 µg of the appropriate antigen intravenously in 0.5 ml of PBS. The mice were checked periodically by gel diffusion analysis, and on this basis were assigned a designation of either a strong or weak precipitin producer. The antisera used for these studies were pools of the strong precipitin producers obtained either on day 20 following the intravenous boost or a pool of sera collected on days 15 and 27 following the boost. Blood was collected from the retroorbital plexus in capillary tubes (72). The antitoxin serum was a pool of 19 high precipitin producers and the antitoxoid was a pool of 13 high producers.

Ascites Antibody - In an effort to obtain larger samples of antibodies, ascites were induced in mice by two separate methods. The first method employed the procedure of Hermann (37) injecting Erlich Lettres Ascites (ELA) tumors into the high responder mice of the above groups. Five mice from both the toxin and toxoid immunized groups were injected on day 1 with $2 \times 10^7$ ELA cells intraperitoneally in a total volume of 0.2 ml . The viability of the tumor preparations was about 70%. The tumors were allowed to develop and after the ascites fluid had accumulated sufficiently, the mice were tapped and the fluid collected. The ascites fluid was a combination of liquid and cells, so the fluid was spun at 2500 RPM for 30 minutes and the supernatant was removed. Each individual sample was tested in gel diffusion analysis for the presence of precipitating antibodies, and then frozen at $-20^{\circ}$ until used in the clarification procedure (see below).

The second method of obtaining ascites antibodies employed the method of Tung et al. (81). Toxin or toxoid was injected intraperitoneally into adult female $C_3H$ mice. Each injection consisted of 3.0 µg of antigen emulsified in either Freunds Complete Adjuvant (FCA) or Incomplete Adjuvant (IFA) at a ratio of 9 parts adjuvant to one part antigen, in a total volume of 0.2 mls. Injections were given on day 0 in IFA and days 14, 21, 28, 35 and 51 in FCA. Each animal was boosted again on day 58 with 30 µg of antigen in FCA intraperitoneally. When fluid accumulation allowed, the mice were tapped and then tested in gel diffusion analysis for precipitating antibodies to either the toxin or the toxoid.

Both of the ascites preparations were clarified and ammonium

sulfate precipitated according to the procedure of Harris (35). The ascites fluid was stirred for 60 minutes in the cold with an equal volume of fluorocarbon (Freon TF, DuPont Chemical Co.). To the cleared ascites fluid, saturated ammonium sulfate (SAS) was added to a 27% saturation. This solution was then centrifuged at 2250 RPM for 15 minutes at room temperature and the pellet discarded. The supernatant was then brought to 50% saturation with SAS, and the fluid was recentrifuged for 30 minutes at 2250 RPM. The pellet was resuspended in borate buffered saline (11) to ¼ the original volume. This solution was dialyzed extensively against borate buffered saline and concentrated by vacuum dialysis. The final solution was then tested for antibodies in gel diffusions analysis, passive hemagglutination and neutralization of Fragment A enzymatic activity. If the solution showed no antibodies in the gel diffusion tests, it was further concentrated and tested again in gel diffusion.

Anti-Fragment A - Two preparations of anti-fragment A were made: 1) a rabbit antiserum prepared in adult male New Zealand rabbits; and 2) a mouse anti-fragment A prepared by the method of Tung et al. (81).

1) Rabbit anti-fragment A - This antiserum was prepared by Dr. B. Iglewski. 1.4 mg of fragment A (J. Collier) in 0.02 M phosphate buffer pH = 6.8 containing 6 M urea and 1 mM dithiothreitol was emulsified in an equal volume of Freund's Complete Adjuvant (FCA). Six injections per rabbit were given in either the footpads or back. Two weeks after the initial injection, a booster injection was given in the rear footpads with 0.29 mg of fragment A in FCA. The antiserum was obtained from blood samples drawn three weeks

after the booster injection and aliquots were kept at -70° until use.

2) Mouse anti-fragment A - This antiserum was made according to the method of Tung et al. (81). Five adult female $C_3H$ mice (Simonsen Laboratories, Gilroy, California) were immunized intraperitoneally with antigen in either Incomplete Freund's Adjuvant (IFA) or FCA at a ratio of 9 parts adjuvant to 1 part antigen. Each mouse received approximately 1.5 µg of antigen on day 0 in IFA, and on day 14, 21, 28 and 35 in FCA. The animals were tapped when the ascites fluid accumulation permitted and the collected fluid was tested in gel diffusion for precipitating antibodies to fragment A. The antigen used for these immunizations was prepared by SDS-PAGE as described below.

Rabbit anti-β-phage (βv $tox^+$) - This antiserum was prepared by Dr. L. Elwell by injecting 3 mg of purified phage into the rear footpads and backs of two male New Zealand rabbits. The antigen was first emulsified in a mixture of equal volumes of antigen and FCA. Sixteen days after the primary injection, each animal was boosted with 1.4 mg of purified phage in FCA, in the rear footpads. The serum was obtained 21 days after the booster injection, and aliquots were stored at -20° until used.

Immunoabsorbent columns

The immunoabsorbent columns were prepared using either diphtheria toxin or toxoid of the 26 mg/ml preparation. CNBr-activated Sepharose 4-B (Pharmacia, Upsaala, Sweden) was weighed in 0.5 g amounts and separate preparations for the toxin and toxoid columns were washed for 20 minutes in $10^{-3}$ M HCl (Pharmacia, Upsaala, Sweden).

Each washed sepharose preparation was placed into 17 x 100 mm plastic tubes. The toxin reaction mixture consisted of the activated sepharose plus 3.2 mg of toxin in 5 mls of PBS pH = 7.2. The toxoid reaction mixture consisted of the activated sepharose plus 5 mg of toxoid in 5 mls of PBS pH = 7.2. The protein concentrations were initially determined by the method of Lowry (48) and then were monitored by the absorbance at 280 nm. The tubes containing the reaction mixture were rocked gently overnight in the cold ($4^{o}$). Then this mixture was poured into plastic drug screening columns (Evergreen Scientific) which had nylon wool in the base to retain the matrix material, to a final bed volume of 1 x 3 cm. The individual columns were then washed extensively in PBS pH = 7.2 and the washings were monitored for protein by the absorbance at 280 nm until the reading was less than 0.030; 1M ethanolamine (Eastman Chemicals) in distilled water was adjusted to pH = 8.1 with 0.5 M NaOH and then added to the column and allowed to react at $4^{o}$ for 2.5 hours. Then the columns were again washed extensively with PBS pH = 7.2. Before using the columns, both were treated two times with the eluting buffer, 3 M NaSCN (MCB), rinsing extensively with PBS pH = 7.2 between washes and after the last wash. The toxin conjugated to the column was determined by measuring the $OD_{280}$ units of the antigen before reacting with the sepharose, and then monitoring all protein washed off during the procedure. In this way it was determined that 85% of the toxin (2.72 mg) was bound to the sepharose and 70.1% of the toxoid (3.5 mg) was bound.

The serum samples were passed over the columns in 1 ml volumes, and the columns were washed with PBS pH = 7.2 until the absorbance

at 280 nm was less than 0.030. The fractions containing protein were pooled and concentrated by vacuum dialysis to the original 1 ml volume. This serum was labelled column passed serum. The columns were then treated with the eluting solution (3 M NaSCN) and the fractions collected were immediately pooled and dialyzed for one week against PBS pH = 7.2 with numerous buffer changes. These samples were labelled eluted serum and were concentrated by vacuum dialysis to the original 1 ml volume of the sample applied to the column. All samples were stored after column passage and/or elution at $-20^{\circ}$ until used.

Phage Assays

Stock Phage Corynebacteriophage β (βhv $tox^{+}$) was a gift of Dr. W. Iglewski. The phage was supplied as a crude lysate in citrate. The crude stock was prepared for use by spinning at 8000 RPM for 10 minutes. 1 M $CaCl_2$ was added to the supernatants to a final concentration of 5% of the final volume. This was allowed to sit for 30 minutes at $4^{\circ}$ and then spun at 8000 RPM for 10 minutes.

The phage was titered by the method of Groman (33). Corynebacteria strain $C_7(-)$ was grown overnight and innoculated into 50 ml of heart infusion broth (Difco). The cells were incubated on a shaker at $37^{\circ}$ until cell density was approximately $5 \times 10^8$ to $9 \times 10^8$ cells/ml. This corresponded to $OD_{590}$ reading of between 0.49 and 0.55. One ml of phage dilution was added to 0.15 ml of this log phase culture and allowed to adsorb at room temperature 3-5 minutes. Then this was mixed with 2.5 ml of a 1% heart infusion agar overlay and poured onto prepoured heart infusion agar (1.5%) petri dishes. The plates were allowed to harden at room temperature

(20°) for 20 minutes then incubated overnight.

Phage Neutralization By Antibody was assayed by mixing equal volumes of a 1:10 dilution of serum with a stock phage dilution of approximately $10^6$ pfu/ml. These were incubated at 37° C in a water bath and at various times aliquots were taken from the reaction mixture and immediately diluted 1:100 in heart infusion broth to stop the antigen antibody reaction. This 1:100 dilution was used directly for plating or used to make further dilutions. The phage titer was determined as above using 1 ml of the dilution desired with 0.15 ml of log phase $C_7^-$ culture. The dilutions were plated in triplicate and after overnight incubation at 32° the plaques were counted on a Quebec Colony Counter. Titers are the average of the triplicate plates.

In one experiment rabbit anti-mouse IgG (R anti-MIgG) was used to facilitate the reaction. In this experiment equal volumes of phage and 1:10 dilution of antisera were mixed and incubated at 37° for the appropriate time. This mixture was divided in half and 50 µl of a 1:10 dilution of either NRS or R anti-MIgG was added and incubated as before. The mixtures were treated in a manner similar to the other experiments to determine the phage titer.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gels containing 10% recrysatllized acrylamide (Eastman) in 0.1 M sodium phosphate buffer pH = 7.2, 0.1% SDS were prepared according to the method of Weber and Osborn (85). The gels were 0.8 cm x 80 cm. Boiled Fragment A (75 µl quantities) containing 150 µg of protein were mixed with 13 µl of 8% SDS and 0.12 µl of 8% dithiothreitol to give a final concentration of 1% SDS

and 1% dithiothreitol. To the mixture was added 1 crystal of highly purified sucrose (Schwarz-Mann) and after it had dissolved, the sample was applied to the top of the gels. The gels were electrophoresed for 7 hours at room temperature at a constant current of 5 mA per gel. The tracking dye was 0.002% bromophenol blue and ran faster than peptide fragments larger than 10,000 daltons. Gels were stained with 0.7% coomassie brilliant blue for 2 hours and destained overnight at 45° in 40% methanol.

Enzyme Assay And Inactivation By Antibody

Amino acyl transferase-containing enzymes were prepared from crude extracts of rabbit reticulocytes as described by Allen and Schweet (1) and modified by Collier and Kandel (13). The NAD-transferase activity of Fragment A was measured by the procedure of Collier and Kandel (13). The assay mixture in a total volume of 65 µl contained 50 mM Tris-HCl pH = 8.2, 0.1 mM EDTA, 40 mM dithiothreitol, 25 ml of reticulocyte enzymes, 0.367 mM NAD ($^{14}$C-adenine), 136 mC/mM (Amershan/Searle) and 0.01 µg Fragment A. After 5 minutes incubation at 25° C, 100 µl 10% TCA was added and the precipitate collected, washed and analyzed on a low background counter.

Enzyme inactivation by antibody was determined by assay after preincubation of Fragment A with antiserum. Fragment A (0.01 µg) in 5 µl saline containing 5% NMS (73) was mixed with 5 µl of antiserum or normal serum and incubated at 37° for 5 minutes. The mixture was cooled rapidly in an ice bath and assayed immediately for NAD-transferase activity by adding buffered reticulocyte enzyme mixture and labelled $NAD^+$. 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 % of control activity.

Reverse Radial Immunodiffusion Assays

Radial immunodiffusion plates were set up according to the method of Stiehm et al. (78) and Leslie et al. (46) except that the antigen, either diphtheria toxin or diphtheria toxoid differed. The standard antiserum samples were sheep antitoxoid. Quantitative precipitation assays were performed on the sheep serum obtained at various bleeding dates according to the method of Campbell (11). Slides were prepared using 0.75% agarose, in 0.01 M phosphate buffer pH = 7.5 which contained 0.5 M glycine and 0.14 M NaCl, to which the antigen was added. The solution was poured onto 3 x 3 glass slides, using 4 mls of agar per slide. The agar was allowed to harden in the cold. Holes were punched and 5 µl of the serum dilution was placed in each well. These slides were then incubated in the cold for 40 hours. Diameters of the precipitation bands were measured to the nearest 0.1 mm. The concentration of the antibody in the unknown samples was determined and expressed in mg/ml of serum.

Toxin Neutralization - Neutralization titers of the various antisera were determined by a cell culture cytotoxicity test (75). Hep-2 cells (ATCC #CCL-23) which may be HeLa cells (59) were used in the assays and grown in 150 $cm^2$ plastic tissue culture flasks (Corning #25120) in the presence of Eagles basal medium (Gibco #611) + 10% FCS. Trypsinized cells were suspended at a concentration of 2-5 x $10^5$ cells per ml in growth medium and 2 ml quantities were innoculated into 35 mm plastic tissue culture plates (Falcon #300). These plates

were incubated for 24 hours in 5% $CO_2$ + 95% air and then were washed 2 times with 2 mls of Hanks BSS and overlayed with 1 ml of maintenance medium (Eagle's basal salts + 2% FCS). After inoculation with a 0.2 ml mixture of either toxin-normal serum or toxin-antiserum the cultures were reincubated as before. The cultures were examined at 24 and 48 hours for cytotoxic changes.

The minimal cytotoxic dose was determined by mixing equal volumes of a toxin dilution with a 1/5 dilution of normal mouse serum (NMS) or normal rabbit serum (NRS). The diluent used was PBS pH = 7.2. These toxin-serum mixtures were preincubated for 1 hour at 37° and from each dilution 3 plates were inoculated with 0.2 ml of the mixtures. The minimal cytotoxic dose (m.c.d.) was taken as the lowest concentration of toxin destroying greater than 50% of the cells in all three plates at the end of 48 hours (69). With this toxin preparation the M.C.D. was 0.02 µg toxin per 5 x $10^6$ cells plated.

The neutralization titer was determined by mixing 0.4 ml of antiserum dilutions with an equal volume of toxin containing either 2 or 10 m.c.d. The mixtures were preincubated as above and added to triplicate plate cultures. The plates were examined at 24 and 48 hours. The titer was taken as the reciprocal of the highest dilution of serum giving complete protection of the cells at 48 hours.

Avidity Measurements - Avidity of the antisera was measured according to the method of Jerne (43) utilizing the intracutaneous method of Fraser (22) to determine the amount of free toxin present in the antigen-antiserum mixtures. Adult New Zealand white rabbits were clipped and the hair removed by either shaving or applying Nair for 4 minutes and then washing thoroughly. Small areas, approxi-

mately 2 $cm^2$ were marked off on the back using a crystal violet solution. Diphtheria toxin was prepared at two different concentrations, diluted with PBS pH = 7.2 containing 0.7% normal mouse serum (NMS). Antiserum dilutions were prepared and 0.15 ml quantities of both the antiserum dilutions and the toxin were mixed in test tubes and incubated at 37° for 1.5 hours. Controls were either antigen mixed with a dilution of NMS or PBS pH = 7.2 containing 0.7% NMS mixed with a dilution of the antiserum. After incubation, 0.1 ml quantities of the mixture were injected intradermally into the backs of the rabbits using a 27 guage needle. The reaction diameters were read at 44 hours and recorded to the nearest millimeter.

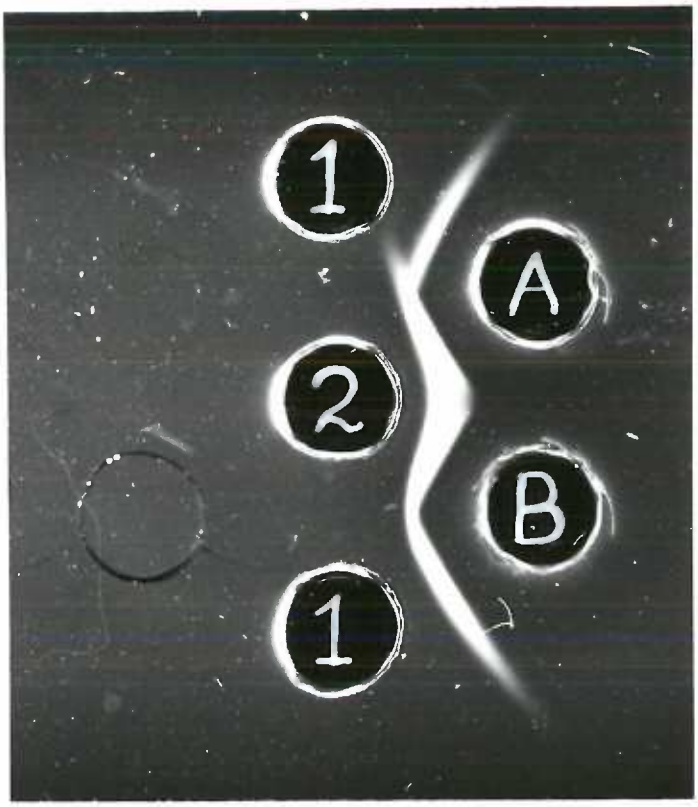
# RESULTS

## Mouse Serum

The first indication that immunization with toxin as opposed to toxoid would raise antisera of differing specificities can be seen in Figure 1. The antitoxoid antibodies form a line of identity between the toxin and toxoid antigens, however, the antitoxin antiserum recognizes only partial identity between the antigens. This spurring effect appears rather early during immunization in the pool of serum from all 20 mice in the antitoxin group. At forty one days after the initiation of the immunization the spurring was evident, and showed a marked increase in intensity after the intravenous boost. The direction of the spur seen in Figure 1 is toward the toxoid well suggesting that the toxoid antigen lacks antigenic determinant(s) detectable with antitoxin.

Passive hemagglutination results supported this interpretation, and are shown in Table I. The antitoxin and antitoxoid antisera had approximately the same end points when tested against toxin coated erythrocytes. However, when tested against toxoid coated erythrocytes, the antisera had somewhat different titers. In fact, the antitoxoid antiserum had an end point that was eight times higher than the antitoxin. These results held consistently when testing the two antisera in this system.

Figure 1 - Gel diffusion patterns comparing antitoxin (A) and antitoxoid (B) antisera against diphtheria toxoid (1) and diphtheria toxin (2). Toxoid concentration was 100 µg/ml; toxin concentration was 250 µg/ml.


1
A
2
B
1

Although the end points of each titration could vary by one well, in each individual assay the eight fold difference was consistently evident even though each test utilized different antigen-coated erythrocyte preparations. Table I also shows passive hemagglutination results using Fragment A coated erythrocytes. As can be seen, the antitoxin and antitoxoid contain approximately equal titers against Fragment A coated erythrocytes. These results were surprising considering later data regarding neutralizaiton of Fragment A enzymatic activity in vitro. Table I also shows the results of testing rabbit Anti-Fragment A antiserum tested against toxin, toxoid and Fragment A coated erythrocytes; Anti-Fragment A had a higher titer against Fragment A coated RBC's than either of the two mouse antisera.

To see if the above results were due to relative differences in the class of antibody present in the antitoxin or antitoxoid sera, passive hemagglutination tests were done after treatment with dithiothreitol, according to the method of Pirofsky and Rosner (66). In all cases, the maximum reduction of the end point was only one well (not shown) and no differences were observed between the respective antisera after dithiothreitol treatment. These results suggest that the differences in titers seen above could not be explained by the relative amounts of IgM contained in the sera.

Passive hemagglutination inhibition studies were then done with the three antigens used to coat the erythrocytes, to see if there existed any differences in the ability of the antigens to inhibit the agglutination of the antigen coated erythrocytes. The results are seen in Figures 2-4. Figure 2 shows that when toxin is

TABLE I

PASSIVE HEMAGGLUTINATION USING ANTITOXIN OR ANTITOXOID ANTISERUM

| Antiserum | Titer [a] | | | |
|---|---|---|---|---|
| | Protein Coated on Erythrocytes | | | |
| | Toxin | Toxoid | Fragment A | None |
| Antitoxin[b] | 25,600 | 1,600 | 1,280 | 0 |
| Antitoxoid[b] | 51,200 | 12,800 | 640 | 0 |
| Anti-Fragment A[c] | 10,240 | 640 | 5,120 | 0 |
| Normal Mouse Serum[d] | 0 | 0 | 0 | 0 |

a. Reciprocal of last dilution showing confluent agglutination across bottom of well. We do not consider a one tube difference in endpoint significant.

b. Starting dilution 1:100

c. Starting dilution 1:10

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

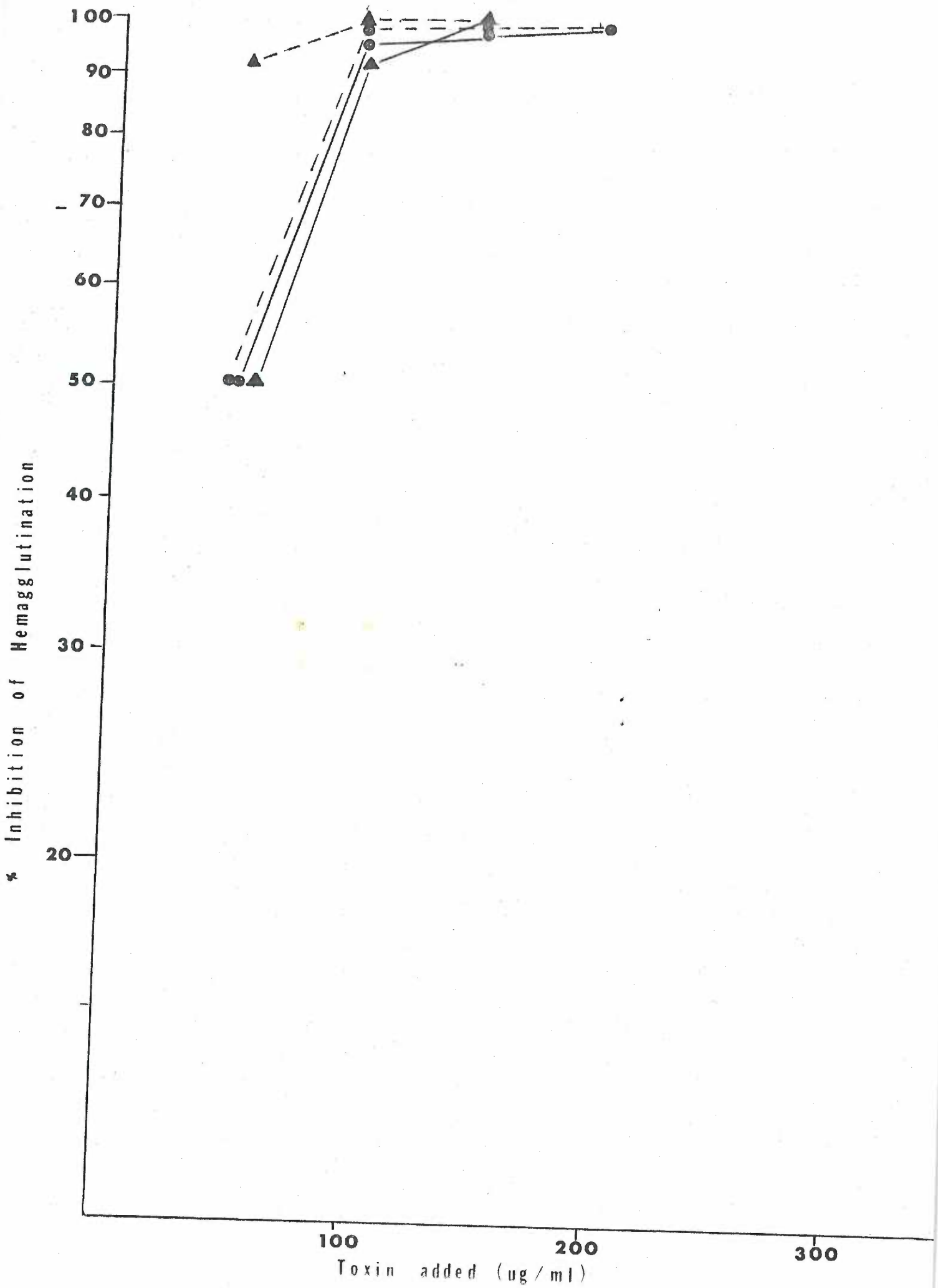
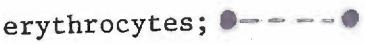
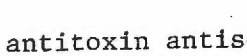

100
90
80
70
60
50
40
30
20
% Inhibition of Hemagglutination
100
200
300
Toxin added (ug/ml)

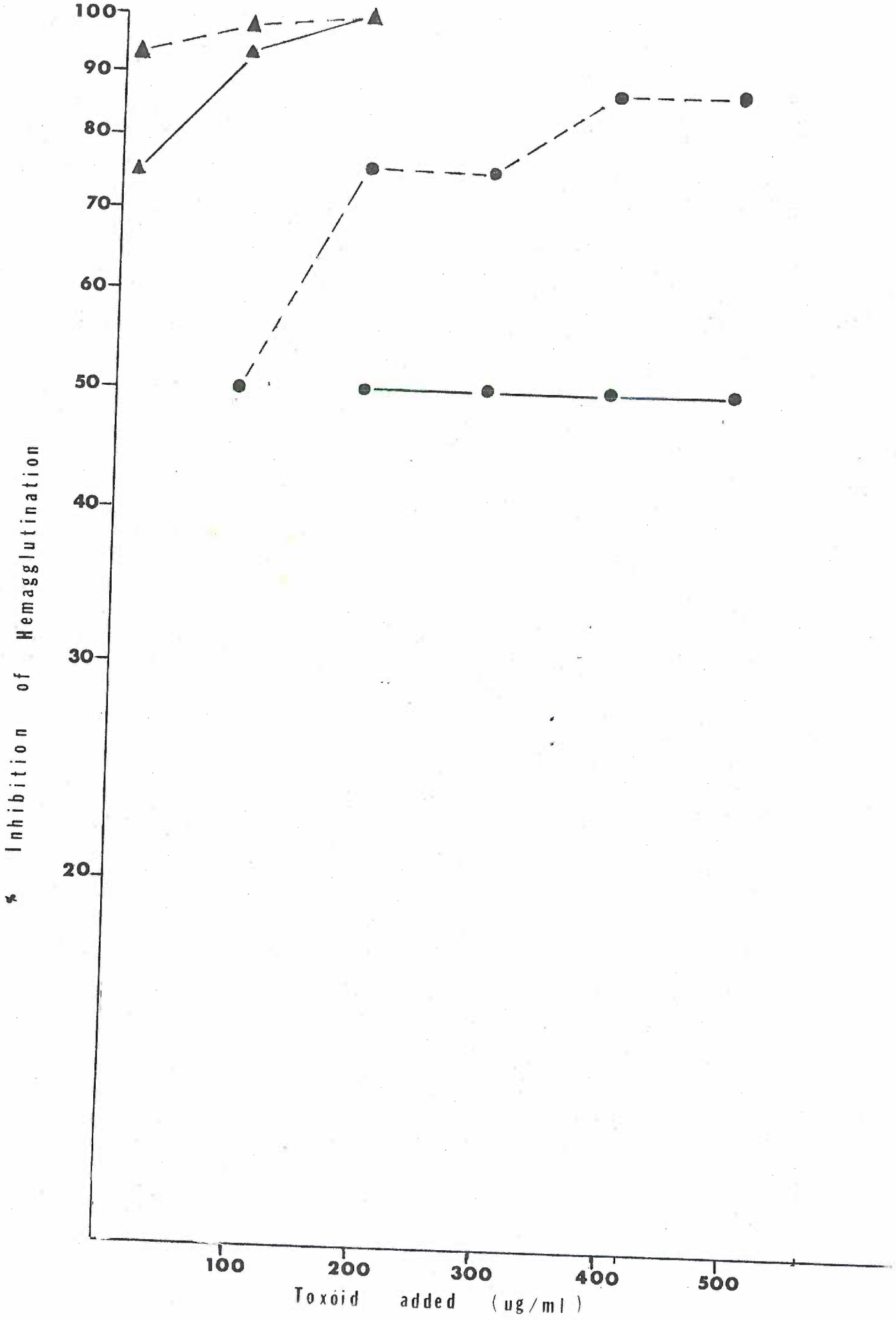
Figure 2 - Passive hemagglutination inhibition studies using toxin as the competing antigen. Symbols: ●———● antitoxin antiserum tested with toxin coated erythrocytes; ●– – –● antitoxoid antiserum tested with toxin coated erythrocytes; ▲———▲ antitoxin antiserum tested with toxoid coated erythrocytes; ▲– – – –▲ antitoxoid antiserum tested with toxoid coated erythrocytes.

used as the competing antigen, there seems to be no difference in the inhibition curve of either the antitoxin or antitoxoid if toxin coated erythrocytes are used. If, however, toxoid coated erythrocytes are used, 50 µg of the toxin inhibits the antitoxoid serum much more effectively than the antitoxin (94% to 50% respectively). These results suggest that antitoxoid reacts more strongly (higher avidity) with toxin than with toxoid even though the antiserum was prepared to toxoid. These data are also consistent with later results presented below.

Figure 3 shows that when toxoid was used, the inhibition pattern is very unlike that obtained with toxin. Whereas 100 µg of toxin had been completely inhibitory to both antisera regardless of the antigen on the red cell (Figure 2), the toxoid was a less efficient competitor. When toxin coated erythrocytes were used, the toxoid was only able to reduce the titer of the antitoxin serum by 50% even at a toxoid concentration of 500 µg/ml. The antitoxoid serum titer on the other hand was reduced 87% by the addition of 400 µg/ml of toxoid and it appears that the titer might have been reduced further if more toxoid had been added. The same was not true with toxoid coated erythrocytes where the toxoid showed approximately equivalent abilities to inhibit the antitoxin or antitoxoid antisera. These results can be seen in the two upper curves on Figure 3.

When Fragment A was tested as a competing antigen (Figure 4), the results were also very interesting. The two lower curves in Figure 4 show that Fragment A is ineffective in competing with the toxin coated erythrocytes for either the antitoxin or the antitoxoid antibodies, reducing the titer of both by only 50%. Fragment A is,

Figure 3 - Passive hemagglutination inhibition studies using toxoid as the competing antigen. Symbols:  antitoxin antiserum tested with toxin coated erythrocytes;  antitoxoid antiserum tested with toxin coated erythrycytes;  antitoxin antiserum tested with toxoid coated erythrocytes;  antitoxoid antiserum tested with toxoid coated erythrocytes.


100
90
80
70
60
50
40
30
20
% Inhibition of Hemagglutination
100
200
300
400
500
Toxoid added (ug/ml)

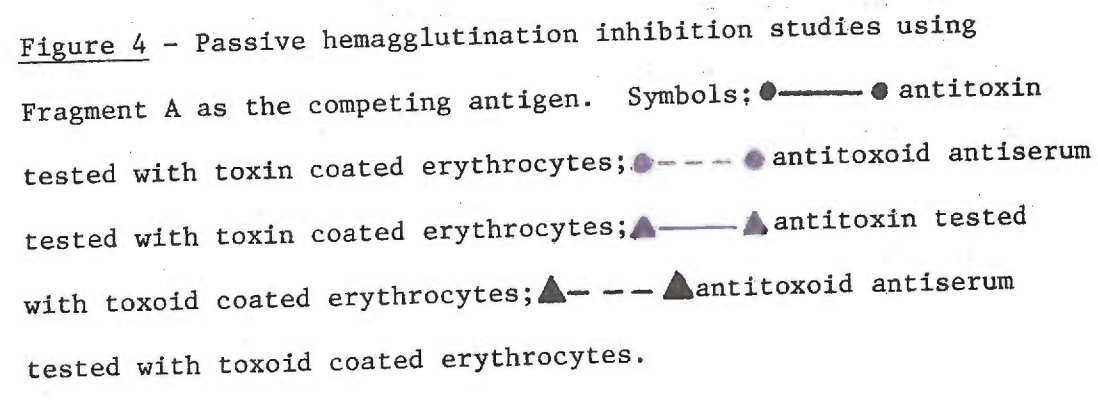
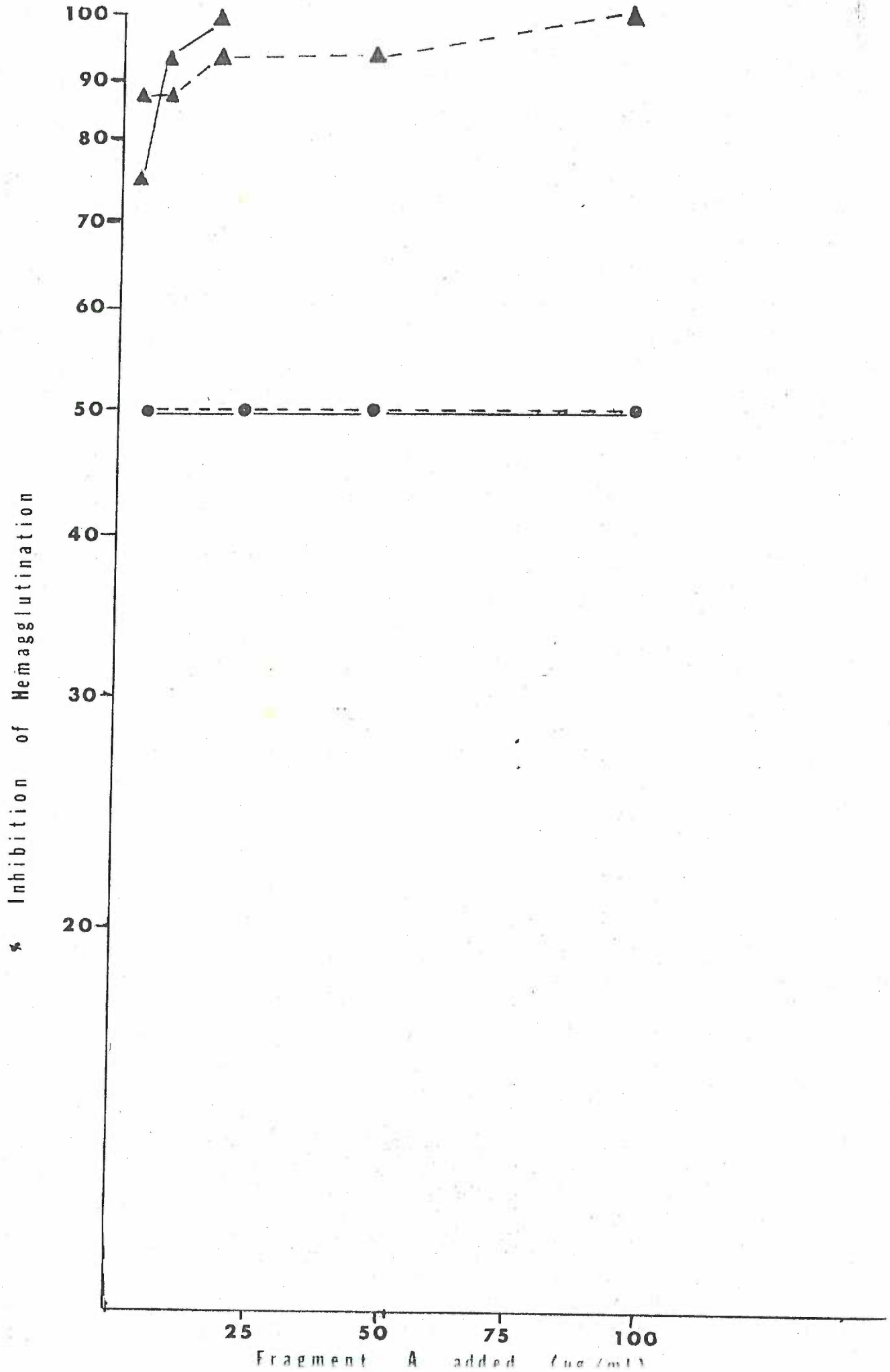


Figure 4 - Passive hemagglutination inhibition studies using
Fragment A as the competing antigen. Symbols: antitoxin
tested with toxin coated erythrocytes; antitoxoid antiserum
tested with toxin coated erythrocytes; antitoxin tested
with toxoid coated erythrocytes; antitoxoid antiserum
tested with toxoid coated erythrocytes.

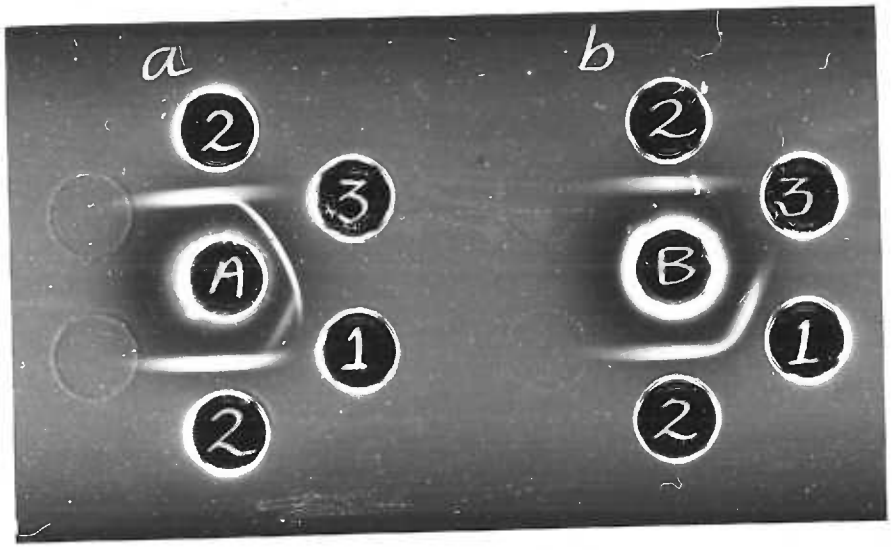

100
90
80
70
60
50
40
30
20
% Inhibition of Hemagglutination
25
50
75
100
Fragment A added (ug/ml)

however, effectively able to reduce both the antitoxin and anti-toxoid antibody titer using toxoid coated erythrocytes but the antitoxoid required a 10 fold higher concentration of free antigen than antitoxin to inhibit the agglutination 100%.

Based on these results we asked whether the antitoxin and anti-toxoid 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 (63). We assume this failure to be due to the relative insolubility and tendency of Fragment B to aggregate which has been reported by others (14, 13, 25). On the contrary there was good reactivity of Fragment A with antitoxin as shown in Figure 5a. 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 5b, as in Figure 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).

Since the mouse antitoxin and antitoxoid show differences in their relative abilities to detect Fragment A, we wondered if this

Figure 5 - Gel diffusion patterns showing activities of (a) antitoxin (A) and (b) antitoxoid (B) against toxoid (1), toxin (2) and Fragment A (3). Toxin concentration, 150 µg/ml; toxoid concentration, 300 µg/ml; Fragment A, 75 µg/ml.


a
2
3
A
1
2
b
2
3
B
1
2

difference would also be evident in neutralization of the in vitro NAD-transferase activity of free Fragment A. Figures 6 and 7 show two representative curves obtained when comparing the antitoxin and the antitoxoid in such a system. Figure 6 shows that for this bleeding date, the mouse antitoxoid was virtually ineffective in neutralizing the enzymatic activity of Fragment A, even at the highest concentration of antibody possible which was 1:2. Antitoxin on the other hand was able to inhibit the enzymatic activity extremely effectively and still retained approximately 30% of its neutralizing capacity at a 1:80 dilution. When testing a pool of two other bleedings (Figure 7) there was some deviation from the above results. The antitoxoid was able to show some neutralizing capacity, but it consistently had greater than one tenth or less activity than the antitoxin pool (Figure 7). Figure 6 also shows the ability of rabbit Anti-Fragment A to neutralize the enzymatic activity. As can be seen, the rabbit Anti-Fragment A is only effective out to a 1:4 dilution which represents a 10 fold lower ability to neutralize Fragment A than the mouse antitoxin antiserum. These results were somewhat surprising considering the fact that the mouse antitoxin and antitoxoid sera had approximately equal titers against Fragment A in the passive hemagglutination system and had at least a 4 fold lower titer against Fragment A than the rabbit Anti-Fragment A.

Figure 6 - Neutralization of the ADP-ribosyl transferase activity of Fragment A by various antisera. Symbols: (●) rabbit anti-A; (○) mouse antitoxoid; and (△) mouse antitoxin (see Materials and Methods).

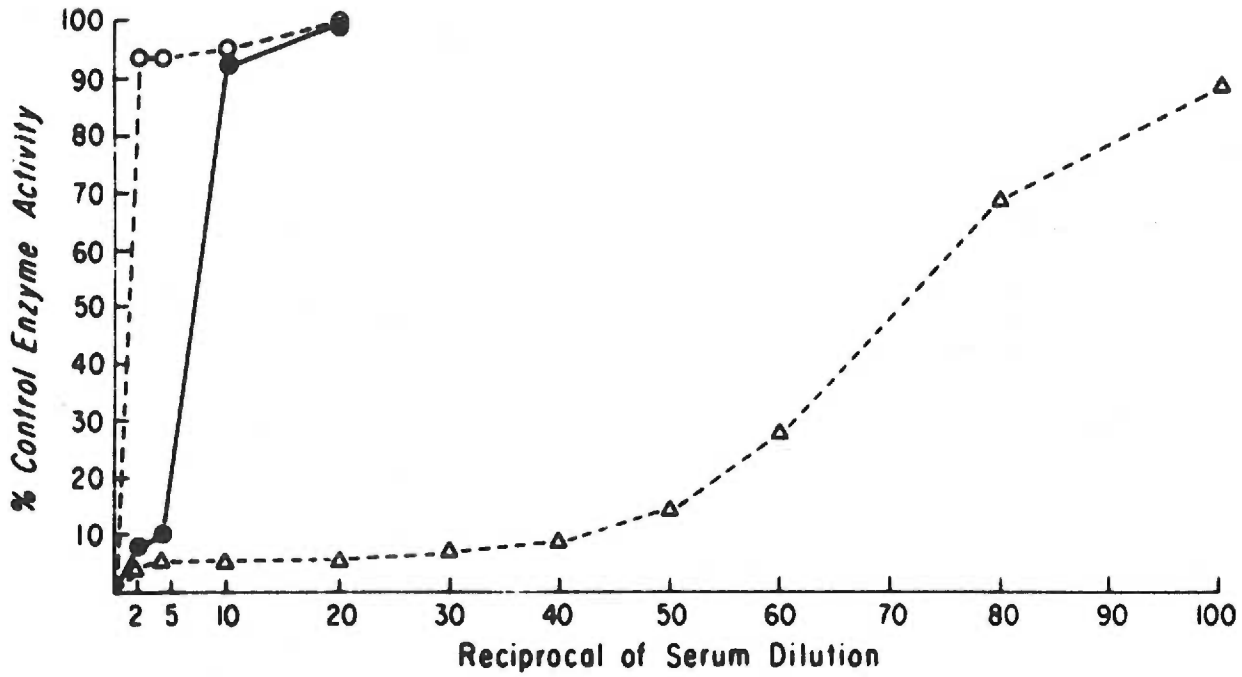
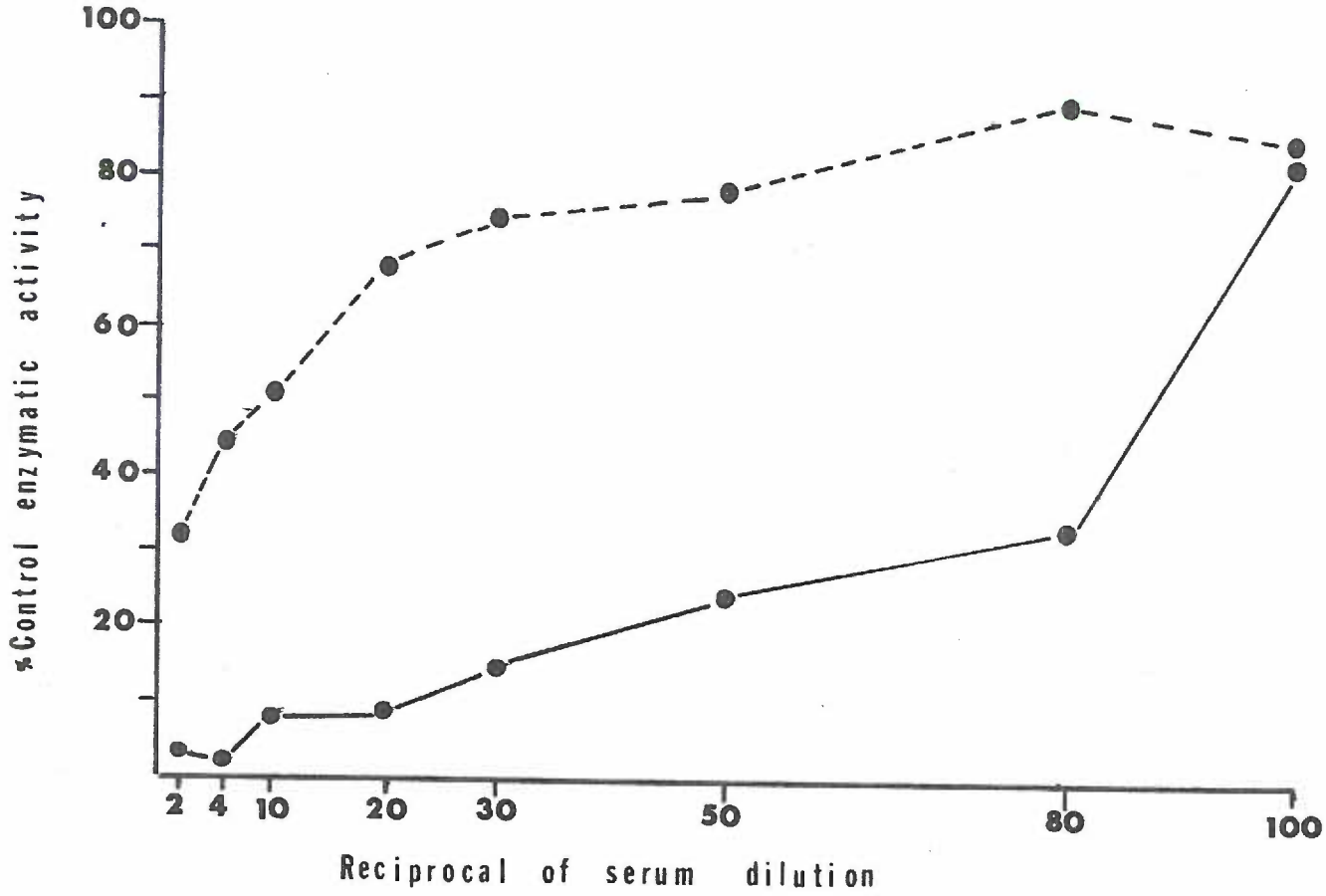

% Control Enzyme Activity
100
90
80
70
60
50
40
30
20
10
2
5
10
20
30
40
50
60
70
80
90
100
Reciprocal of Serum Dilution

Figure 7 - Neutralization of the ADP-ribosyl transferase activity of Fragment A by mouse antisera used for the immunoabsorbent column work. Symbols: (●——●) antitoxin; (●- - -●) antitoxoid. These antisera were pools of serum obtained on day 15 and 27 following the intravenous boost (see Materials and Methods).


100
80
60
40
20
% Control enzymatic activity
2
4
10
20
30
50
80
100
Reciprocal of serum dilution

To investigate further the differences in specificities between the antitoxin and antitoxoid antisera, immunoabsorbent columns were prepared coupling either toxin or toxoid to the matrix material (see Materials And Methods). One milliliter samples of either antitoxin or antitoxoid were passed over the toxin and toxoid columns, and the antibodies bound to the columns were eluted and collected. After column passage and elution, the samples were tested in three manners: 1) passive hemagglutination analysis, 2) ability to neutralize the in vitro NAD-transferase activity of Fragment A, and 3) gel diffusion.

The passive hemagglutination analysis of the samples can be seen in Table II. Passage of the antitoxin and antitoxoid antisera over the toxin column resulted in a significant reduction in the titer of both samples. The titer of the antitoxin was reduced 80 fold against toxin coated erythrocytes and 8 fold against toxoid coated erythrocytes. The same result did not occur after passage of the sera over the toxoid immunoabsorbent. The antitoxoid serum titer was significantly reduced (40 fold) against toxin coated RBC, but the antitoxin titer was only reduced 5 fold. Both antitoxin and antitoxoid antisera showed greater than 90% decrease when tested against toxoid coated erythrocytes. This means that the passage of the antitoxin serum over the toxoid column is approximately 1/16 as efficient at removing the antibodies compared to when the same serum is passed over the toxin column. The antitoxoid serum does not show this difference. These results again suggest that the specificities of the two antisera are different, supporting the results presented above.

TABLE II

PASSIVE HEMAGGLUTINATION OF ANTITOXIN AND ANTITOXOID AFTER PASSAGE OR ELUTION FROM IMMUNOABSORBENT COLUMNS.

| Antiserum | I.A. Column | TITER [a] Protein coated on RBC | |
|---|---|---|---|
| | | Toxin | Toxoid |
| Antitoxin | Before Passage[b] | 20,480 | 2,580 |
| | Passed over Toxin[c] absorbent | 256 | 256 |
| | Passed over Toxoid[c] absorbent | 4,096 | 512 |
| | Eluted from Toxin[c] immunoabsorbent | 2,048 | 2,048 |
| | Eluted from Toxoid[c] immunoabsorbent | 2,048 | 2,048 |
| Antitoxoid | Before Passage[b] | 20,480 | 10,240 |
| | Passed over Toxin[c] immunoabsorbent | 256 | 256 |
| | Passed over Toxoid[c] immunoabsorbent | 512 | 512 |
| | Eluted from Toxin[c] immunoabsorbent | 4,096 | 4,096 |
| | Eluted from Toxoid[c] immunoabsorbent | 4,096 | 4,096 |

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

b. Starting dilution 1:100

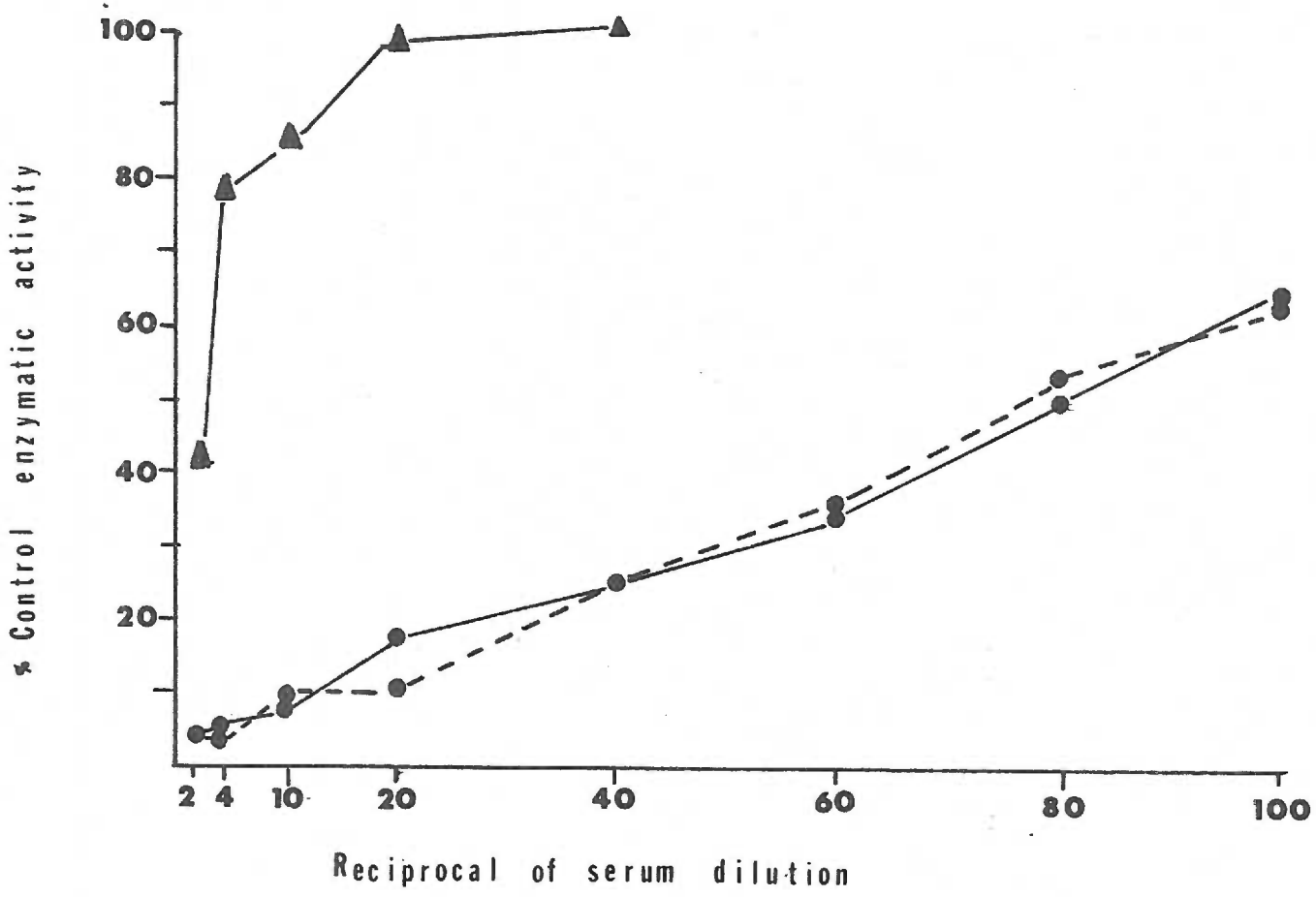
c. Starting with undiluted serum.

To investigate further the differences in the serum samples after passage over and elution from the immunoabsorbent columns, the samples were tested for their ability to neutralize the enzymatic activity of Fragment A. The results are presented in Figure 8 and 9. The column passed serum reacted differently after the toxin immunoabsorbent than the same serum passed over the toxoid immunoabsorbent (Figure 8). The antitoxin serum passed over the toxin column lost virtually all of its ability to neutralize the *in vitro* enzymatic activity of Fragment A, whereas the toxoid column passed antitoxin serum retained virtually all of its ability to neutralize the activity.

The antitoxoid serum passed over the toxin column similarly lost all of its limited neutralization capacity, but retained all of its limited Fragment A neutralizing antibodies when passed over the toxoid immunoabsorbent (not shown).

The antibodies eluted from the respective columns showed the expected result with respect to neutralization of Fragment A NAD-transferase activity (Figure 9). The antibodies eluted from the toxin column reflected the appropriate neutralizing abilities of the original serum samples; however, the amount of neutralizing antibodies contained in the eluted sample were less than what the original sample contained, again suggesting some loss due to the elution conditions.

Figure 8 - Neutralization of the ADP-ribosyl transferase activity of Fragment A by mouse antitoxin passed over the immunoabsorbent columns. Symbols: (●– – –●) antitoxin activity before column passage; (●——●) antitoxin activity after passage over a toxoid immunoabsorbent; (▲——▲) antitoxin activity after passage over the toxin immunoabsorbent.


100
80
60
40
20
% Control enzymatic activity
2
4
10
20
40
60
80
100
Reciprocal of serum dilution

Gel diffusion analysis was also done on the appropriate column passed serum or eluted antibodies. After passage over either the toxin or toxoid immunoabsorbent, neither the antitoxin nor antitoxoid contained detectable precipitating antibody. Testing the eluted antibodies on gel diffusion, however, also met with little success. At no time were we able to demonstrate precipitating antibody in the eluted samples despite concentrating the samples to ¼ of the original volume of serum. This I attribute to the 3 M NaSCN used for elution, which has been reported to denature antibody proteins (76). This idea is supported by passive hemagglutination results (Table II) which shows that the titers of the passed serum and eluted samples are not additive to the original titer contained in the appropriate samples.

We then asked whether there would be any differences in the ability of the antitoxin or antitoxoid to neutralize the in vitro toxicity of diphtheria toxin on tissue culture cells. The results are seen in Table III. The mouse antitoxin and the mouse antitoxoid showed relatively little difference in their abilities to neutralize the in vitro toxicity when tested at two different doses of toxin (see Materials And Methods). The rabbit Anti-Fragment A was also tested and showed virtually no ability to neutralize toxicity as has been reported (63).

Since the above results suggested that the two antisera differed little in toxin neutralization capacity in vitro, we asked whether the avidity of the two antisera for toxin would differ, since the in vitro results suggested that there may be little difference. To test this we used the rabbit skin test method of detecting free

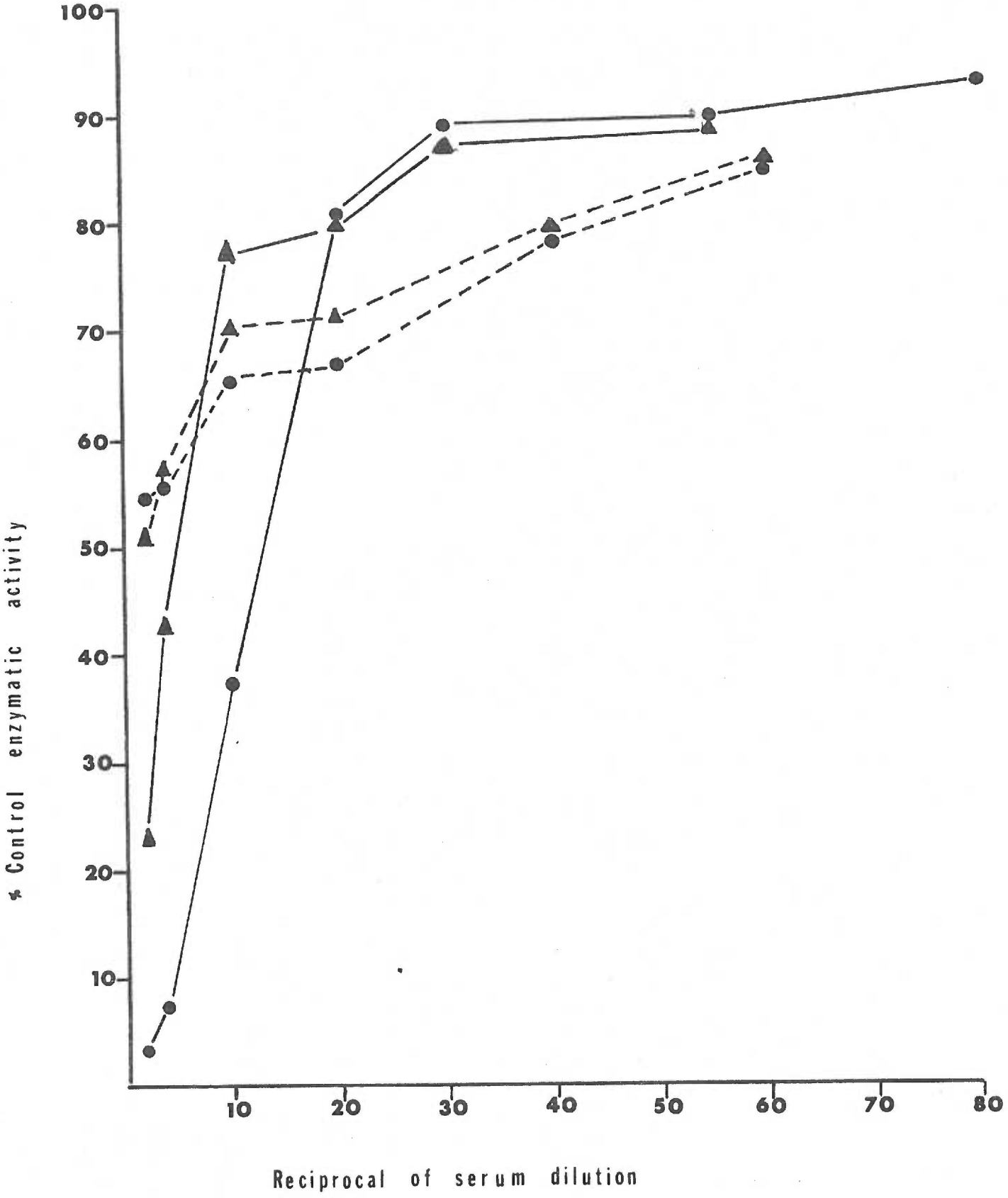

100
90
80
70
60
50
40
30
20
10
% Control enzymatic activity
10
20
30
40
50
60
70
80
Reciprocal of serum dilution

Figure 9 - Neutralization of the ADP-ribosyl transferase activity of various antisera eluted from the immunoabsorbent columns. Symbols: (●——●) antitoxin eluted from a toxin immunoabsorbent column; (▲——▲) antitoxin eluted from a toxoid immunoabsorbent column; (●- - -●) antitoxoid eluted from the toxin immunoabsorbent column; (▲- -▲) antitoxoid eluted from the toxoid immunoabsorbent column.

TABLE III

NEUTRALIZATION OF CYTOPATHOGENIC EFFECT OF DIPHTHERIA TOXIN IN HEP-2 CELL CULTURES.

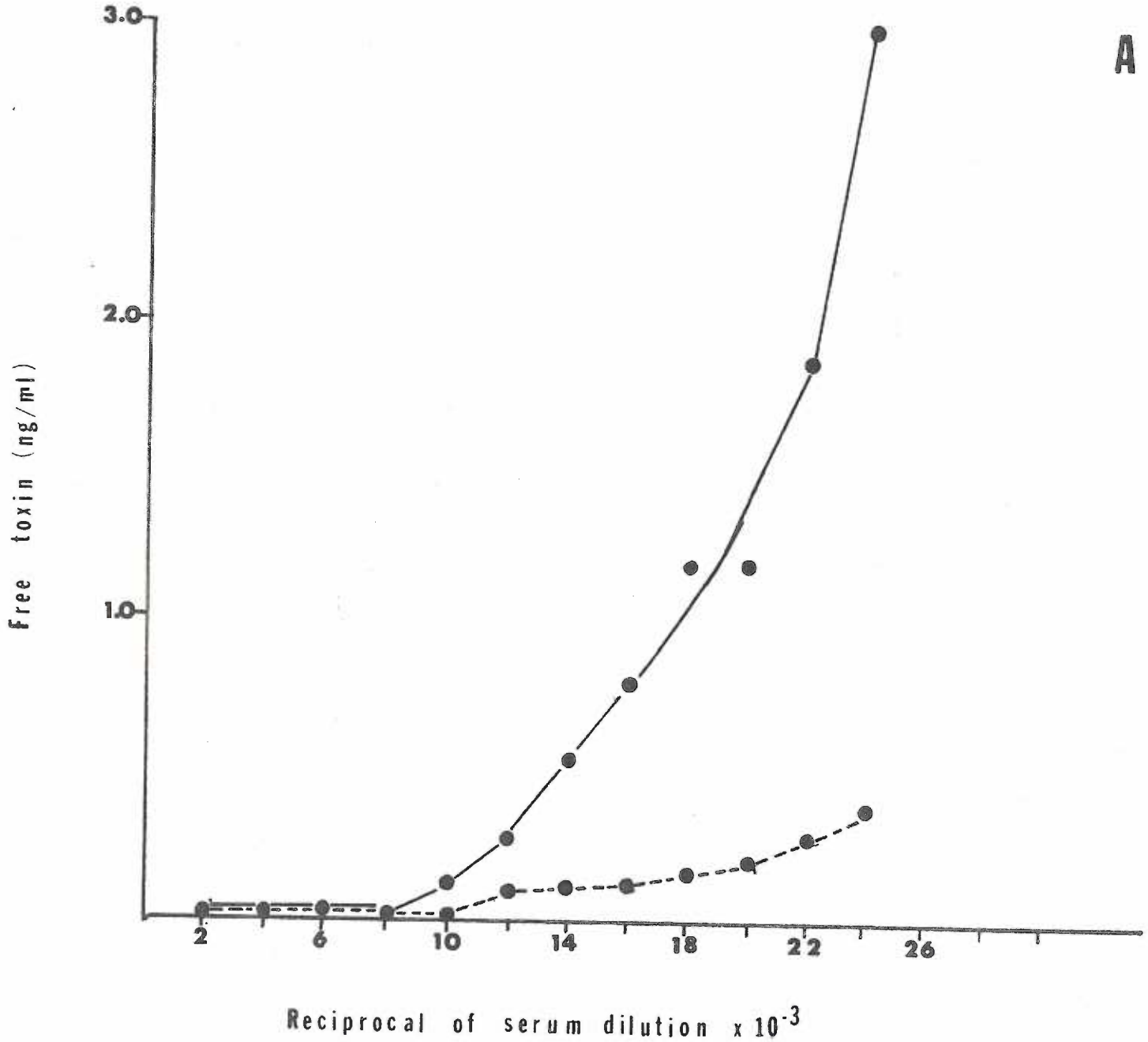
| Number of Minimal Cytotoxicity Doses | Neutralization Titer[a] | | |
|---|---|---|---|
| | Antitoxin | Antitoxoid | Anti Fragment A |
| Experiment 1 | | | |
| 2 m.c.d. (0.04 µgm) | 4,000 | 2,000 | 20 |
| Experiment 2 | | | |
| 10 m.c.d. (0.2 µgm) | 200 | 200 | <10 |

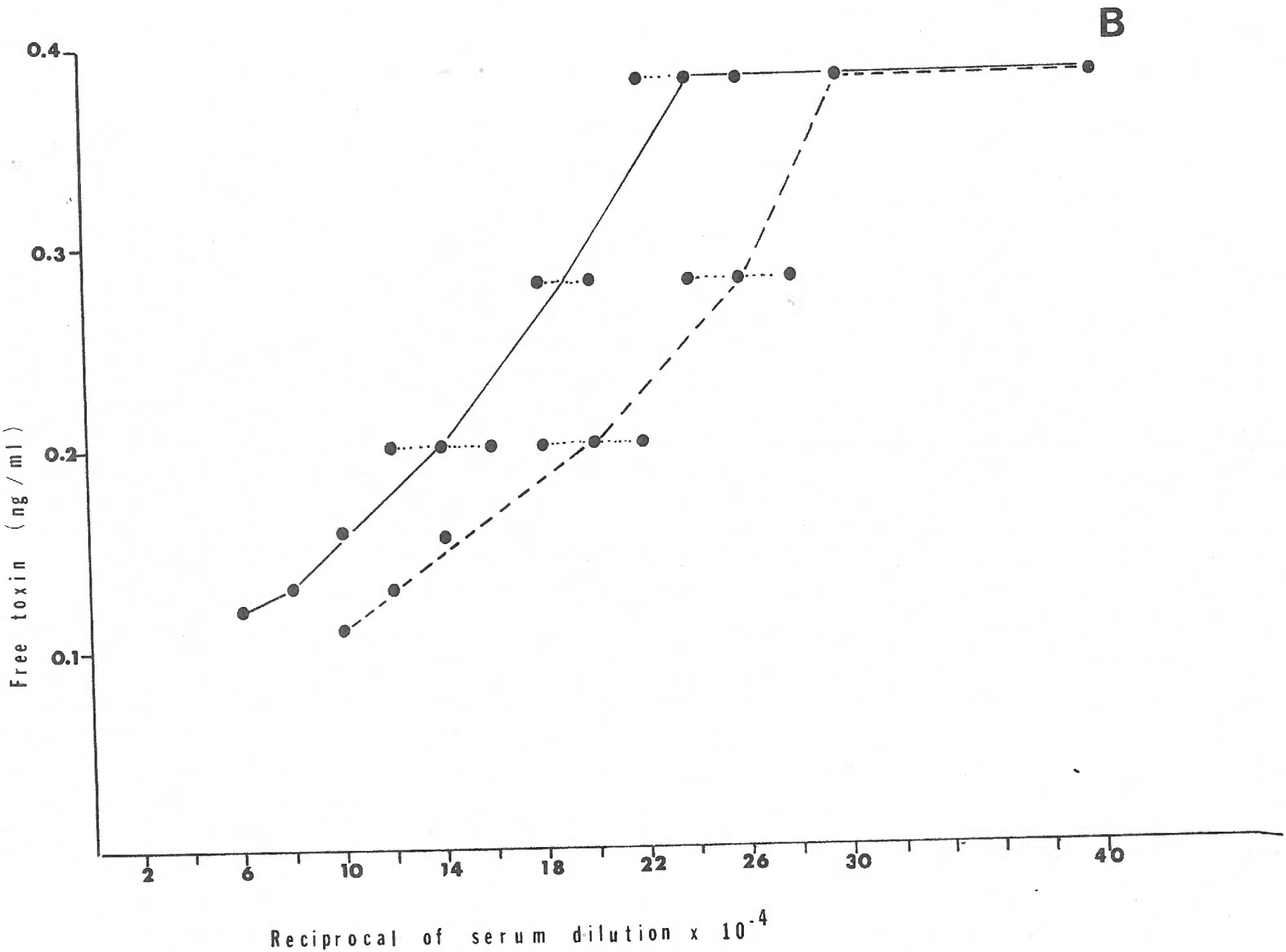
a. The titer represents the reciprocal of the highest dilution of serum giving complete cell protection after 48 hour incubation.

toxin as described by Fraser (22), and followed the in vivo method of assaying avidity described by Jerne (43). The results can be seen in Figure 10. The ability of antitoxin and antitoxoid to neutralize toxin seemed to differ only slightly when tested at the lower antigen dose (Figure 10b), but showed a significant difference when tested at a ten fold higher dose. The antitoxoid showed a much higher avidity for the toxin than did the antitoxin (Figure 10a).

All of the above data lead one to believe that there are significant alterations in the toxin molecule during the toxoiding procedure. These differences might be important in results reported previously regarding the relationship of diphtheria toxin to the β-phage. In 1954, Barksdale and Pappenheimer (4) reported the failure of antitoxoid antiserum to neutralize the infectivity of the β-phage concluding that the toxin is not structurally related to the β-phage particle. Recent evidence suggests that such a structural relationship between Fragment B of diphtheria toxin and the phage may exist (20,21). Considering the apparent alterations in the toxin molecule during toxoiding as evidenced by the differing specificities of the antitoxin versus the antitoxoid antisera, we asked whether such differences in recognition would be manifested in the non-recognition of a phage structural protein by the antitoxoid serum; can the antitoxin recognize the "native" Fragment B in the phage and therefore neutralize its infectivity? The results of the experiment can be seen in Figure 11. Neither the antitoxin antiserum nor the antitoxoid was able to neutralize the infectivity of the β-phage when compared to rabbit anti-β-phage antiserum, which showed greater than a 3 log reduction in phage titer. Even

Figure 10 - Measurement of free toxin in an antigen-antibody mixture using the rabbit skin test method of Fraser (22). The toxin doses used in the neutralization mixtures were (A) 5.0 ng/ml, and (B) 0.5 ng/ml. Symbols: (●——●) mouse antitoxin; (●- - -●) mouse antitoxoid.


A
3.0
2.0
1.0
Free toxin (ng/ml)
2
6
10
14
18
22
26
Reciprocal of serum dilution x $10^{-3}$


B
0.4
0.3
0.2
0.1
Free toxin (ng/ml)
2
6
10
14
18
22
26
30
40
Reciprocal of serum dilution x $10^{-4}$

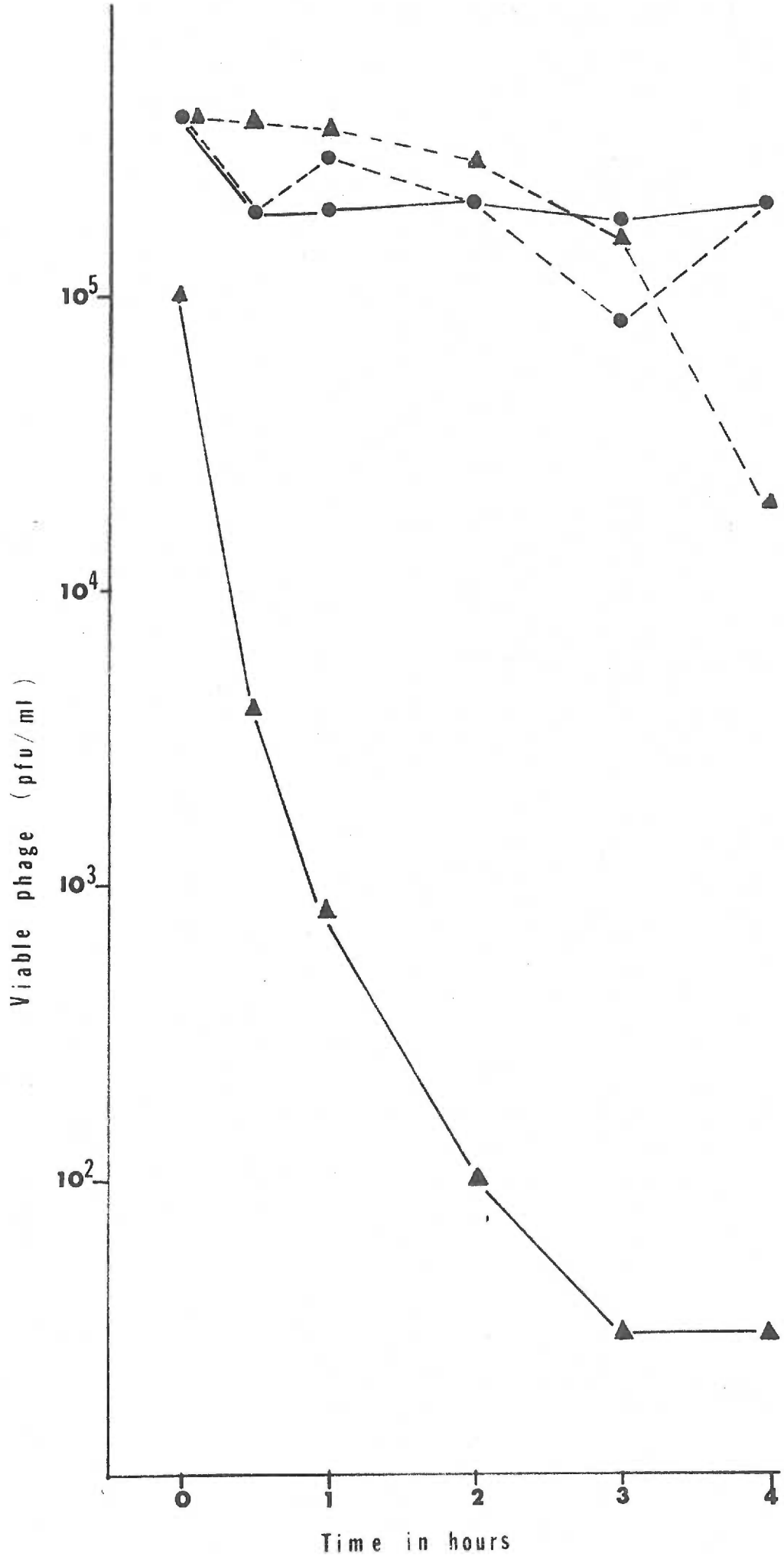

$10^5$
$10^4$
$10^3$
$10^2$
Viable phage (pfu/ml)
0
1
2
3
4
Time in hours

Figure 11 - Neutralization of Corynebacteriophage β infectivity of an indicator strain of C. diphtheria ($C_7$-). Symbols: (●——●) mouse antitoxin; (●- - -●) mouse antitoxoid; (▲- - -▲) normal mouse serum; (▲——▲) rabbit anti-phage.

facilitation of the reaction with rabbit anti-mouse gamma globulin was ineffective, reducing the titer only ½ log (data not shown).

One other question of importance is whether the quantity of antibody differed in either antiserum. To answer this question, reverse radial immunodiffusion tests were set up using a sheep anti-toxoid antiserum of known antibody content as the standard. The titers of the respective antisera can be seen in Table IV. The content of antibody of both the antitoxin and antitoxoid was approximately equal against diphtheria toxin, which supports the qualitative determination seen in the passive hemagglutination results (Table I). When tested against the toxoid antigen, the two sera had significantly different titers. In fact, the antitoxin had approximately 64% of the antitoxoid titer against the toxoid. These results are in accord with all of the other comparative data obtained with these two antisera.

TABLE IV

DETERMINATION OF ANTIBODY TITER BY REVERSE RADIAL IMMUNODIFFUSION

| | Titer[a] | |
|---|---|---|
| Antiserum | Toxin[c] | Toxoid[c] |
| Anti-toxin | 1.01 ± 0.26[b] | 0.87 ± 0.16[b] |
| Anti-toxoid | 1.10 ± 0.21[b] | 1.35 ± 0.13[b] |

a. Titer expressed in mg/ml.

b. Values expressed ± standard error.

c. Indicates the antigen incorporated into agar.

## ASCITES ANTIBODIES

One of the problems associated with obtaining antiserum from mice is the limitation in the quantity of antiserum. The total serum pool of the antibodies used for the majority of this work was 4 ml each of antitoxin and antitoxoid antisera. In an effort to gain larger amounts of antibodies, ascites were induced into mice in two different manners: 1) injecting the Erlich Lettre Ascites tumor into the mice used for the above work and 2) induction of an adjuvant-induced ascites fluid in mice by the method of Tung *et al.* (81- see Material and Methods).

The injection of Erlich Lettre Ascites tumor cells into the high responding mice used above gave mixed success. Large amounts of fluid were gathered, not all of which had detectable antibody as judged by gel diffusion analysis. After a number of tappings, the antibodies were precipitated out of the ascites fluid by the method of Harris (35) and concentrated. The samples obtained from both the antitoxin and antitoxoid mice were tested as above in gel diffusion, passive hemagglutination and *in vitro* neutralization of the NAD-transferase activity of Fragment A.

The antitoxin antibodies obtained showed the same partial identity between toxin and toxoid while the antitoxoid antibodies showed complete identity (see Figure 1). In passive hemagglutination tests there was still a difference (5 fold rather than 8 fold) between the antitoxin and the antitoxoid antibodies when tested against toxoid SRBC (data not shown). The same antibody sample had approximately equal titers when tested against toxin coated erythrocytes.

The above results show that the same types of differences present in the serum obtained from these mice could be demonstrated in the ascites antibodies isolated. I then asked if the differences would also be manifested in the ability to neutralize the enzymatic activity of Fragment A. Figure 12 shows that similar to the gel diffusion and passive hemagglutination systems, the differing specificities can also be demonstrated using neutralization of Fragment A. The antitoxin antibodies have significant neutralizing capacity out to a 1:20 dilution, and maintained significantly lower enzyme neutralizing capacity than the antitoxoid antibodies until higher dilutions were reached.

The second method of obtaining larger amounts of antibodies was a recently published method of Tung et al. (81). This method involves a series of intraperitoneal injections of the antigen in an emulsion of Freunds complete adjuvant (FCA), mixed at a ratio of 9 parts adjuvant to 1 part antigen. The ascites antibodies obtained in this manner were also able to show results similar to those seen in the other groups of immunized mice. Again, the antibodies obtained were tested by gel diffusion analysis, passive hemagglutination and neutralization of enzymatic activity. Figure 13 shows that immunization by this method was able to raise antibodies that reacted the same in gel diffusion analysis as does the other method of immunization. The pattern demonstrates that only partial identity between toxin and toxoid is recognized by the antitoxin antibodies whereas complete identity was seen using antitoxoid ascites antibodies. These results obtained are not only consistent with the previously presented data, but indicate that the reaction in the gel diffusion analysis is not a peculiarity of the particular method of

Figure 12 - Neutralization of the ADP-ribosyl transferase activity of Fragment A by ascites fluid obtained by ELA tumor injection (see Materials and Methods). Symbols: (●——● ) antitoxin ascites; (○——○) antitoxoid ascites.

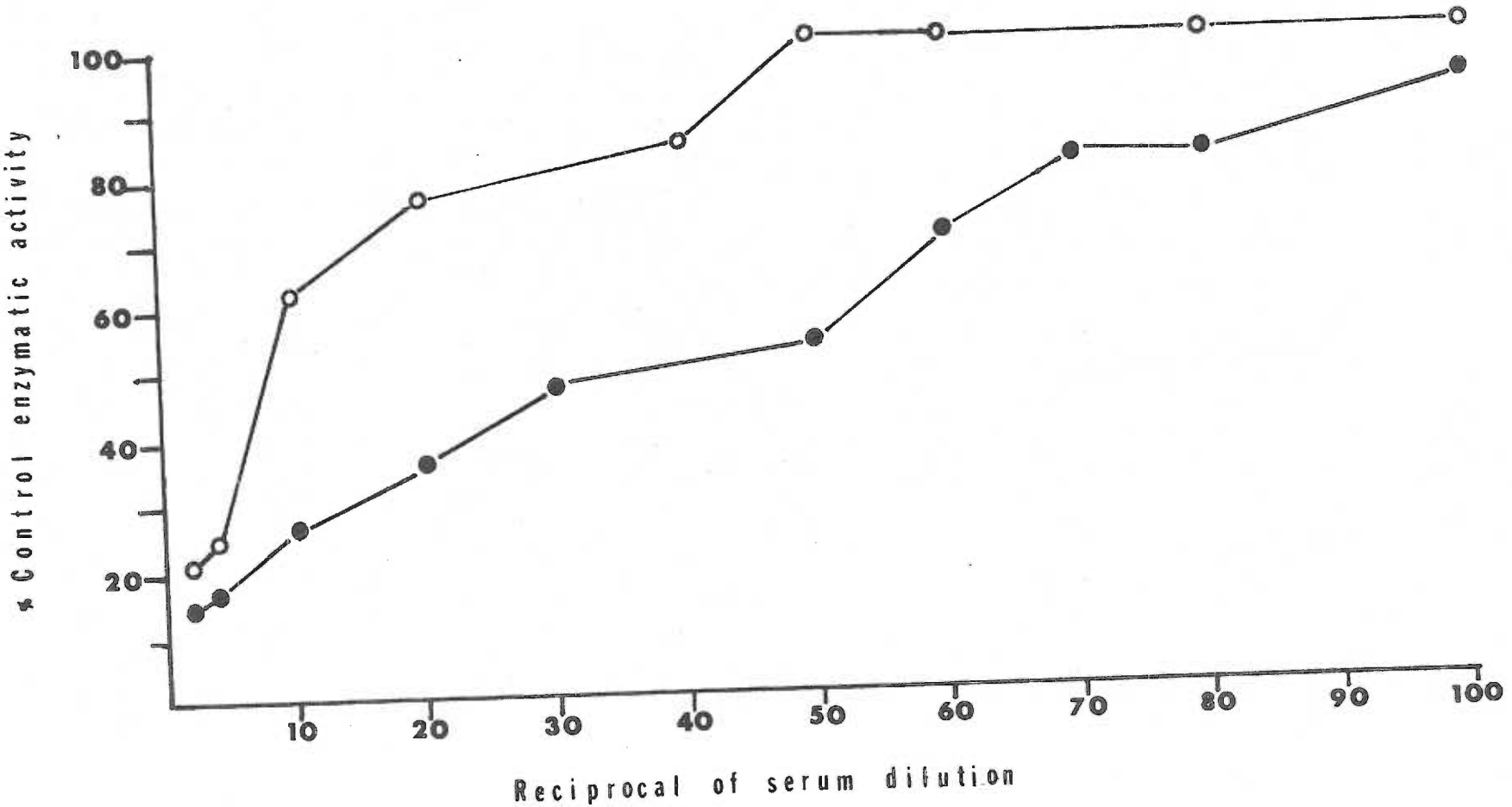
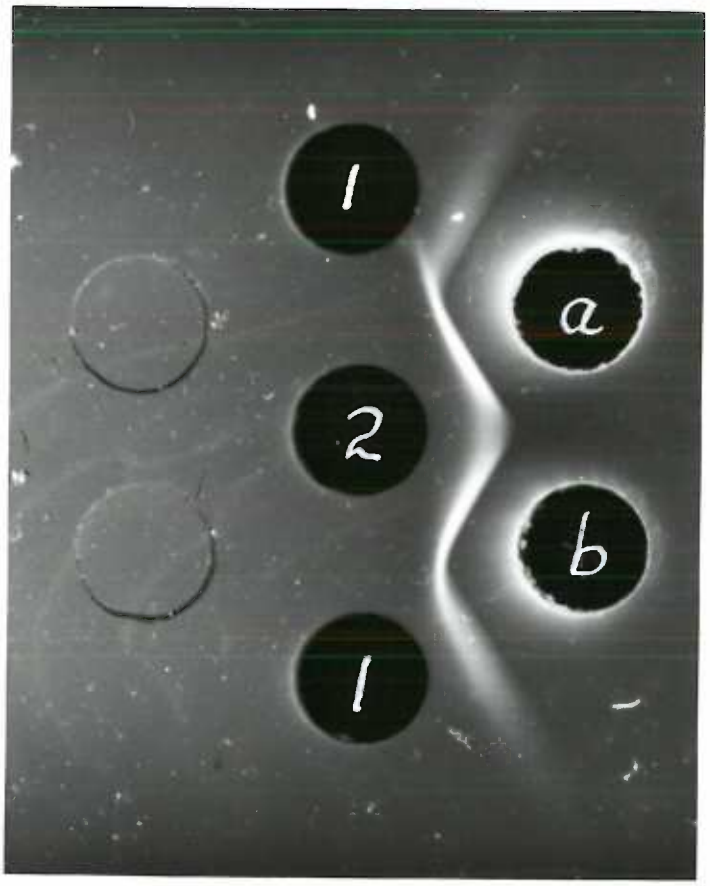

% Control enzymatic activity
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
Reciprocal of serum dilution

Figure 13 - Gel diffusion patterns comparing ascites antitoxin (a) and ascites antitoxoid (b) against diphtheria toxoid (1) and diphtheria toxin (2). Toxin concentration is 100 µg/ml; toxoid concentration is 100 µg/ml.


1
a
2
b
1

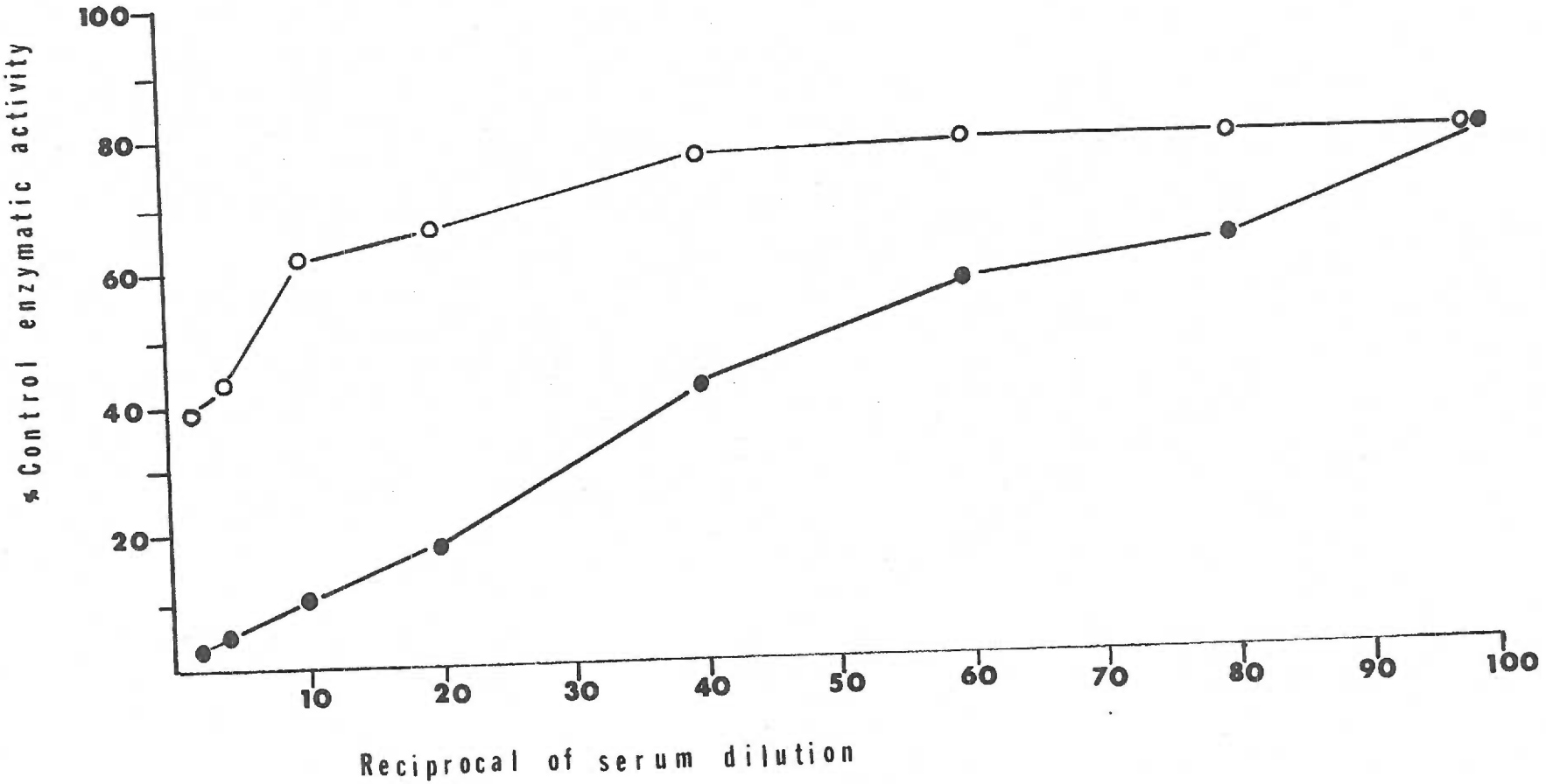
immunization used in the previous studies.

To further support this idea, Fragment A neutralization was tested and the results are presented in Figure 14. The capacities of the respective antibodies to neutralize the enzymatic activity of Fragment A are different, even though at the lower dilutions there appears to be relatively little difference. As the dilutions get higher, a marked divergence of the curves is evident. In fact, by a 1:50 dilution of the antisera, there is no neutralizing ability remaining in the antitoxoid preparation, while the antitoxin has approximately 50% of its neutralizing capacity remaining. In fact, the enzymatic activity never returns to 100% of control values with the antitoxin serum.

If the two methods of obtaining ascites antibody are compared, the method of Tung et al. (81) seems preferable. The time required to obtain the antibodies by this method is approximately 5 weeks. Each animal yields between 6 to 12 ml of ascites fluid at each tap, and each animal can be tapped 4-6 times. If ELA is used to obtain the ascites (37), 6-12 ml of ascites fluid can also be obtained per animal, but the fluid contains a great deal of tumor cells, which obviously reduces the total amount of clear fluid that is ultimately recovered.

The Tung et al. (81) method also has the advantage of obtaining higher titer of antibodies in the ascites. Equivalence of the antibodies in this way was generally between 75-250 µg/ml as judged by gel diffusion analysis. The ELA ascites was not even close to this amount, generally varying between 10 and 40 µg/ml for equivalence.

Figure 14 - Neutralization of the ADP-ribosyl transferase activity of Fragment A by ascites fluid raised by the method of Tung (84) (see Materials and Methods). Symbols: (●——●) antitoxin ascites; (o——o) antitoxoid ascites.


% Control enzymatic activity
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
Reciprocal of serum dilution

Lastly, the induction of the ascites with ELA resulted in a higher mortality over the period of time the animals are tapped and a higher amount of nonspecific protein that is precipitated with the antibodies during the clarification procedure (see Materials and Methods).

# DISCUSSION

The action of formaldehyde on the toxin molecule during the toxoiding process is not well understood. Formalin treatment is known to destroy the toxicity of the molecule (28, 29, 70), block ε-amino groups, and form cross-linkages between lysine and tyrosine or histidine via methylene bridges (9). It is clear from the data presented that the toxoiding process also alters the immunogenicity of the toxin. There is a variety of evidence supporting this contention which is very consistent. Gel diffusion analysis of both serum and ascites antibodies of the toxin immunized mice, show a reaction of only partial identity between the toxin and the toxoid (Figures 1 and 13). The direction of the spur suggests that antigenic determinants are lost during toxoiding. The antitoxoid antibodies were unable to distinguish between the two antigens and complete identity was seen in all cases. If new antigenic regions are formed during toxoiding, they are not detectable in gel diffusion.

Passive hemagglutination data support this idea. In the initial test, both antitoxin and antitoxoid antisera had approximately equal titers with toxin coated erythrocytes (Table I). However, when tested with toxoid coated erythrocytes, the antitoxin titer was significantly lower than the antitoxoid titer (1600 vs. 12,800). The higher antitoxoid titer could indicate a recognition of new antigenic regions on the toxoid even though they weren't detectable by gel diffusion analysis.

The antitoxin and antitoxoid preparations had equivalent anti-

body levels (1.02 mg/ml vs. 1.1 mg/ml) as determined by reverse radial immunodiffusion using toxin as the antigen; however, when the same sera were tested with toxoid as the antigen, the antitoxin had only 64% as much antibody as the antitoxoid antiserum (0.87 mg/ml vs. 1.35 mg/ml). A loss of antigenic determinants, or alterations of these determinants on the toxoid could explain the difference. It is also possible that the differences in titer could be due to differing affinities of the antisera for the toxoid. The antisera show approximately equal antibody levels against toxin despite the fact that antitoxoid has a much higher avidity. However, we were unable to make a comparison of the avidity of the two sera for toxoid which might account for the differences of titer observed.

Alterations in immunogenicity by toxoiding are reflected in the relative abilities of the antisera to neutralize the ADP-ribosyl transferase activity of Fragment A. Antitoxin has a much greater ability to neutralize the enzymatic activity than the anti-toxoid, which supports our gel diffusion results (Figures 6 and 7). The spur seen in Figure 1 could represent at least in part the differences in enzyme neutralizing capacity between the antitoxin and antitoxoid, especially since the antitoxoid was unable to re-cognize Fragment A by gel diffusion analysis (Figure 5b). In view of these results, we were surprised to find that both the antitoxin and antitoxoid had approximately equal titers when tested in passive hemagglutination with Fragment A coated erythrocytes (Table I). Passive hemagglutination inhibition data confirm this since these anti-A antibodies also interact sufficiently with the Fragment A antigen to inhibit hemagglutination. Thus, the antitoxoid antibodies

do recognize Fragment A determinants, and the differences observed between the antisera cannot be explained solely on the basis of anti-A activity.

Antitoxin and antitoxoid show obvious differences in their ability to recognize the enzymatic active site of Fragment A. There are several explanations for this difference. First, the two antisera could differ greatly in their concentration of antibody. This possibility, however, is not likely since reverse radial immunodiffusion data (Table IV) as well as passive hemagglutination results show that both have approximately equal antibody titers. The antibody present in both antisera are also resistant to dithiothreitol treatment indicating that neither had a significant IgM component (66). Secondly, the antitoxin and antitoxoid could differ in the avidity of the anti- Fragment A antibodies, the latter being of lower avidity and dissociating during the enzyme assay, and would explain the failure to precipitate in gel diffusion analysis. This possibility is also unlikely since the antitoxoid serum agglutinates Fragment A coated erythrocytes, has sufficient avidity to be inhibited by Fragment A in the hemagglutination assay (Figure 4) and has a much higher avidity than antitoxin when tested by the rabbit skin test method (22 - Figure 10). Thirdly, that the antibodies formed represent the recognition of Fragment A antigenic determinants that are presented during immunization in their native relationship in the toxin or toxoid molecule. The high neutralizing capacity of the antitoxin appears to be due to a recognition of the active site determinants on Fragment A present during immunization. The low enzymatic neutralizing capacity of the antitoxoid serum indicates either that

the active site regions of the toxin are masked during toxoiding or that the change in immunogenicity leads to the preferential recognition of non-active site determinants.

Gill and Dinius (25) and later Pappenheimer et al. (63) suggest that the anti-A activity of an antitoxoid serum is due to free Fragment A in the immunizing preparation. The same free Fragment A in the toxin used for immunization could account for the high neutralization activity of the antitoxin serum. However, the data suggest that this may not be the case. Rabbit Anti-Fragment A has approximately a 5 fold higher titer in passive hemagglutination than either of the mouse antisera, yet has a 10 fold lower enzyme neutralizing capacity than antitoxin. Secondly, the amount of free Fragment A in an immunizing preparation required to produce the anti-A activity seen in the antitoxin should be detectable in gel diffusion. At no time were multiple lines of precipitation seen with either the toxin or toxoid antigens.

Immunoabsorbent column results support this contention since they indicate that toxoid lacks the ability to remove the enzyme neutralizing activity of the antitoxin, in keeping with the suggestion that this activity was developed due to antigenic regions present on the toxin molecule but not on the toxoid. Passage of antitoxin over a toxin immunoabsorbent (Figure 8) resulted in a complete loss of enzyme neutralizing activity. When the same serum was passed over the toxoid column the passive hemagglutination capacity was reduced significantly but the enzyme neutralization capacity was unchanged. If the anti-A activity (or neutralization activity) of either antiserum were due to

free Fragment A in the immunizing preparation, then passage of the antitoxin over either of the immunoabsorbents should have had an equivalent effect on this anti-A activity. Such was not the case, and in fact, the toxoid column had slightly more protein coupled to the matrix than the toxin column (3.5 mg vs. 2.7 mg) and should have depleted that activity to a greater extent. The antitoxoid antiserum passed over either column showed an equal reduction of titer and anti-A activity. It is also possible that the toxoiding process may only affect the active site of free fragment A that might be present in the toxoid preparation. However, we did not have sufficient Fragment A to test this hypothesis so are unable to distinguish between these two possibilities.

One other possibility that could explain the differential amounts of anti-A activity is that some in vivo nicking of the toxin could occur exposing more antigenic regions of the Fragment A. Toxoiding is known to stabilize the toxin molecule (9) and may in fact be the reason for the loss of toxicity (5). The toxin preparation used for the immunization was less than 5% nicked as assessed by SDS gel electrophoresis staining patterns; however, the fate of the antigen in vivo is difficult to determine. There is a possibility that the toxin is nicked after injection, but the release of free Fragment A is unlikely considering the rabbit anti-A data above.

Again, the data imply the existence of at least two antigenic regions on Fragment A; one active site related and one or more not related to the active site. However, one must also explain why the toxin elicits a high amount of antibodies directed to active site related determinants and yet is itself enzymatically inactive. The

fact that the native toxin is not enzymatically active, cannot be explained by steric hindrance by Fragment B. Even nicked toxin is enzymatically inactive, despite the relaxing known to occur after nicking (13). The enzymatic activity involves a reaction with both $NAD^+$ and elongation factor II, both of which have a rather high MW. Therefore, it seems probable that free fragment A is necessary for enzymatic activity.

The data presented allow some generalizations regarding the types of antibodies raised to the B-portion of the toxin. Tissue culture neutralization data (Table III) show that the two antisera contain approximately equal amounts of anti-B activity, since it has been shown that neutralization of toxicity of the toxin depends on the antibodies directed to the fragment (14,84). However, the avidity data show that the antitoxoid anti-B antibodies bind more efficiently to the B fragment and thus are of higher avidity than the antitoxin anti-B antibodies.

One other interesting specificity that the antitoxin possesses is shown in Figure 5a. There is only partial identity recognized between the toxin and Fragment A, a result not unexpected. There is also partial identity between toxin and toxoid as seen before (Figure 1). However, antitoxin also recognized partial identity between the toxoid and Fragment A. The direction of the spurring suggests a recognition of A determinants on the toxoid. Inhibition of passive hemagglutination by Fragment A (Figure 4) support this contention, since relatively small amounts of the inhibiting antigen were needed to reduce the ability of antitoxin to agglutinate toxoid sensitized erythrocytes to zero. In fact, a ten fold lower concen-

tration of free Fragment A is required to inhibit agglutination by the antitoxin antiserum than by the antitoxoid serum.

The differences in immunogenicity between the antigens is also reflected in the comparative avidity of the antisera. The results in Table III suggested that little difference existed between the antitoxin and antitoxoid in their ability to neutralize the ability of toxin to affect tissue culture cells. Whether these data were also representative of the relative avidity of the antisera or of the sensitivity of the tissue culture assay in detecting free toxin was questionable. These same antisera were tested using the rabbit skin test method of Fraser (22), a more sensitive method of detecting free toxin. In fact, nanogram amounts of free toxin can be measured accurately by this method. Testing the antisera at a low dose of toxin (0.5 ng/ml) the neutralizing abilities of the antisera appeared similar to the tissue culture results differing only slightly (Figure 10b). However, if 10 times the initial dose of toxin (5 ng/ml) was tested with 10 times the antibody levels, the two antisera differed significantly in their neutralizing capacity. Since avidity is a measure of how strongly the antiserum interacts with the toxin, the antitoxoid antiserum had a much higher avidity than the antitoxin antiserum as judged by this in vivo determination (43). Therefore, the toxoid is able to elicit an antiserum that has a much higher avidity for the toxin, even though both antitoxin and antitoxoid antibody titers were approximately the same against toxin. Pons (67) previously demonstrated that a standard antiserum prepared against toxoid, had a higher affinity for toxin than toxoid. Additionally, the results indicate that the avidity of these antisera seems

to be independent of the enzyme-neutralizing antibody titer against Fragment A. Since we only have one set of mouse antisera, the data, while in accord with the hypothesis concerning the inverse correlation between avidity and amounts of anti-Fragment A antibodies (63), do not prove it.

Alterations are known to occur in the toxin during toxoiding, but the specific changes are not known. That such alterations do occur is supported by a variety of data. First, the passive hemagglutination inhibition studies demonstrate a vast difference exists between the ability of toxin and toxoid to inhibit the agglutination of antigen sensitized erythrocytes, using either the antitoxin or antitoxoid. If toxin is used to inhibit agglutination, relatively little difference is noted between the inhibition curves of the toxin or toxoid coated erythrocytes, or the antitoxin or antitoxoid antisera (Figure 2). Thus, the free antigen is able to compete effectively for antibodies in this system. The toxoid antigen, on the other hand, has a large variation in its ability to inhibit agglutination (Figure 3). The toxoid shows limited ability in reducing the titer of either antiserum using toxin sensitized erythrocytes. The antitoxin serum titer is reduced only one well despite using concentrations up to 500 µg/ml. The antitoxoid serum titer is reduced 8 fold but the high amounts of toxoid required (300 - 500 µg/ml) suggest that relatively few determinants are present on the toxoid that are recognized as antigenically similar to the native toxin. When toxoid sensitized

erythrocytes are used, the toxoid is effectively able to inhibit the titer of both antisera, with nearly identical inhibition curves.

Formalin treatment of the dialyzed toxin is a random process (49). Raymond showed that the formalin acts primarily with ε-amino groups of the lysine, resulting in crosslinkages (9). The cross-linkage could either mask or create antigenic regions, depending on the degree of crosslinking. If new antigenic regions are formed on the toxoid, they are not detectable in gel diffusion analysis (Figures 1 and 5) nor are they evident in the passive hemagglutination inhibition results (Figure 4). Although the cross-linking does occur, the reactions are probably unpredictable on the toxin molecule and could affect different antigenic regions on different molecules at random. The resultant preparation should be extremely heterogeneous with respect to exposed antigenic regions. This type of heterogeneity in the immunizing preparation would be consistent with the experimental observations. Passive hemagglutination inhibition by toxoid is relatively ineffective with the toxin coated erythrocytes. The small but significant neutralizing capacity of some antitoxoid sera (Figure 7) and the immunoabsorbant data showing virtually no loss of the enzyme neutralizing capacity of the antitoxin serum after passage over a toxoid column (Figure 8) are also consistent with this idea.

If the toxoid preparations are so heterogeneous, they would have limited ability to compete with the toxin for binding sites on susceptible cells as has been noted previously (3). The inability of antitoxoid to precipitate Fragment A is also consistent with this

concept. The population of antibodies formed to any one antigenic region of Fragment A may be very small; small enough to account for the lack of precipitation in gel diffusion, yet large enough to agglutinate Fragment A coated erythrocytes. Such a possibility is feasible since the greater sensitivity of the passive hemaggluination is because of the greater ease in forming a lattice with the antigen attached to erythrocytes. Additionally, rabbit anti-Fragment A is unable to precipitate toxoid in gel diffusion (not shown), yet has a low but significant titer against toxoid coated erythrocytes in passive hemagglutination (Table I). This is analogous to the antitoxoid-Fragment A results. An alternative explanation for the above data is that Fragment A possesses only two antigenic regions (active site and non-active site). The antitoxoid antiserum only has antibodies to one region of Fragment A -- the non-active site region (Figures 6 and 7) -- and the toxoid antigen only has one of the Fragment A antigenic regions exposed. Precipitation requires at least two antigenic regions be recognized by the antibodies (60).

Elwell and Iglewski (20) and later Elwell (21) suggest that the B portion of the toxin is related to a structural protein of the Corynebacteriophage β. The failure of antitoxin to neutralize phage infectivity agrees with earlier studies using an antitoxoid antiserum (4). These results might have been predicted since the antitoxoid antisera have higher avidity for the toxin than for the toxoid. However, the data do not disprove a possible relationship between the toxin and the phage. The failure of the antitoxin to neutralize may be a result of the attachment of the antibody to the head of the phage.

Secondly, it is possible that the antibody is attaching to sites that will neutralize infectivity but it either does not have a high enough titer or does not recognize enough determinants to neutralize the phage. Since the neutralization reaction was not facilitated with rabbit anti-mouse IgG, it appears that there was little if any attachment of any antibodies to the phage. It is also possible that the B fragment is in fact a protein of the phage, yet none of the antigenic determinants are exposed.

The fact that two different methods of immunization were able to elicit antitoxin antibodies displaying similar reactivities, indicates that such specificities are probably not a result of some peculiar factor of the immunization procedure. The ascites antibodies demonstrated similar reactivities in gel diffusion, passive hemagglutination and Fragment A neutralization. Thus, a more likely conclusion is that the results demonstrate real differences existing between the toxin and toxoid. Species variations in the immunologic recognition of this foreign antigen also can not be discounted at this time, especially the possibility that the rabbit anti-A results represent a difference in species recognition of Fragment A. The mouse is relatively resistant to diphtheria toxin (74) and may also differ in its response to certain antigenic determinants. Since similar types of antibody specificities can be shown in other animals using the CRM-197, a molecule antigenically similar to the toxin, such a conclusion while possible is unlikely.

## SUMMARY AND CONCLUSIONS

Immunizing mice with toxin and toxoid leads to antisera of quite different reactivities. Antitoxin is able to recognize antigenic alterations in the toxoid, most of which seem to be loss of determinants present on the toxin. The pieces of data supporting this conclusion are the direction of the spur in gel diffusion analysis, detectable by antitoxin, the significantly lower titer of the two antisera in passive hemagglutination analysis with toxoid RBC, as well as the lower titer of the antitoxin as judged in reverse radial quantitation of antibody levels. Antitoxin and antitoxoid also differ significantly in their capacity to neutralize the enzymatic activity of Fragment A. Also the antisera differ significantly in their avidity for toxin. All of these differences were detectable despite relatively equal titers of the antisera when tested against the toxin antigen.

The results suggest that toxoiding results predominantly in the alteration of antigenic regions on Fragment A. This is shown by the greater affinity (avidity) of the antitoxoid for toxin, the inability to precipitate Fragment A in gel diffusion, despite a significant anti-A titer in passive hemagglutination, and the lower enzymatic neutralizing capacity of the antitoxoid antiserum. Fragment A of the toxin does not appear to be masked antigenically by Fragment B in the toxin's native configuration. There also exist at least two antigenic regions on Fragment A, one active site related and the others not associated with the active site.

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