The combination of Clostridium perfringens epsilon antitoxin with toxin and toxoid
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A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology at Montana State College
Montana State University

Abstract:
Clostridium perfringens epsilon toxoid is used for the immunization of sheep against enterotoxemia. It is produced commercially in the form of formalinized broth cultures which may or may not be treated with trypsin before formalinization. Treatment with trypsin converts the almost non-toxic epsilon prototoxin, the form in which the bacteria produce this substance, into the highly toxic epsilon toxin. Toxoid prepared from the trypsin-activated toxin is more highly antigenic than is toxoid prepared from prototoxin. Unfortunately, treatment with trypsin reduces the ability of toxoid to combine with antitoxin, a property that is used to evaluate commercial preparations of toxoid. This inability to use an in vitro test for the evaluation of ah apparently superior commercial product has prevented the use of bacterins prepared with trypsin activation. The present investigation was designed to get some information about the combination of epsilon antitoxin and toxoid and to study the factors involved in present methods of testing.

Dried Clostridium perfringens Type D Standard Toxin, Toxoid and Antitoxin were obtained from the Animal Inspection and Quarantine Division, Agricultural Research Service, United States Department of Agriculture. Type D diagnostic serum, with a known amount of the various antitoxic fractions, was obtained from Burroughs Wellcome Co. Twelve Clostridium perfringens Type D strains were used to produce broth cultures in liter amounts. The cultures were divided into five fractions as follows: (1) whole culture, (2) whole culture formalinized, (3) whole culture trypsin-activated and formalinized, (4) Seitz filtered, and (5) Seitz filtered and trypsin-activated.

The various culture fractions were reacted with Wellcome serum using single diffusion and double diffusion in agar. A non-specific line of precipitation was obtained as well as a specific line of precipitation to the epsilon antitoxin when epsilon toxin was able to react with it at optimum proportions. The most discrete lines of precipitation were observed with 0.2 percent to 0.3 percent Wellcome serum in the agar, incubated at refrigerator temperature, and observed frequently after 60 hours incubation. Lines of precipitation did not form when materials which had been trypsin-activated and formalinized more than approximately two months were reacted with Wellcome serum nor when materials were reacted which had been trypsin-activated approximately six months. Materials formalinized for approximately six months produced only indistinct lines of precipitation. The loss of ability of toxin and antitoxin to form lines of precipitation correlated with the loss of ability of the toxin and antitoxin to combine, as measured by a combining power test in which mixtures were injected into white mice intravenously.

Avidity tests, which measure the firmness of combination of toxin and antitoxin, were carried out on Standard Toxin and Antitoxin (United States Department of Agriculture), Wellcome serum and two of the broth cultures which when trypsin-activated contained considerable amounts of epsilon toxin. All materials tested demonstrated a high degree of avidity.

Combining power tests were made In which combination of Standard Antitoxin and Standard Toxoid were compared when the concentration of reacting materials were at low levels and high levels. These

comparisons demonstrated that combination occurred much better when high concentrations of reactants were used. Since Standard Toxin and Standard Antitoxin were known to be avid it would appear that Standard Toxoid is not avid so that it dissociates from the Standard Antitoxin in higher dilutions.

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ABSTRACT

Clostridium perfringens epsilon toxoid is used for the immunization of sheep against enterotoxemia. It is produced commercially in the form of formalinized broth cultures which may or may not be treated with trypsin before formalinization. Treatment with trypsin converts the almost non-toxic epsilon prototoxin, the form in which the bacteria produce this substance, into the highly toxic epsilon toxin. Toxoid prepared from the trypsin-activated toxin is more highly antigenic than is toxoid prepared from prototoxin. Unfortunately, treatment with trypsin reduces the ability of toxoid to combine with antitoxin, a property that is used to evaluate commercial preparations of toxoid. This inability to use an in vitro test for the evaluation of an apparently superior commercial product has prevented the use of bacterins prepared with trypsin activation. The present investigation was designed to get some information about the combination of epsilon antitoxin and toxoid and to study the factors involved in present methods of testing.

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Avidity tests, which measure the firmness of combination of toxin and antitoxin, were carried out on Standard Toxin and Antitoxin (United

States Department of Agriculture), Wellcome serum and two of the broth cultures which when trypsin-activated contained considerable amounts of epsilon toxin. All materials tested demonstrated a high degree of avidity.

Combining power tests were made in which combination of Standard Antitoxin and Standard Toxoid were compared when the concentration of reacting materials were at low levels and high levels. These comparisons demonstrated that combination occurred much better when high concentrations of reactants were used. Since Standard Toxin and Standard Antitoxin were known to be avid it would appear that Standard Toxoid is not avid so that it dissociates from the Standard Antitoxin in higher dilutions.

CHAPTER I

INTRODUCTION

Clostridium perfringens Type D was first described by Wilsdon (1931, 1932) and Bennetts (1932). They found it to be the cause of enterotoxemia, a disease characterized by the production within the intestine of the infected animals of Clostridium perfringens epsilon toxin which is subsequently absorbed into the blood stream. Bosworth and Glover (1934-35) were puzzled by the fact that although the bacteria they recovered from the intestine in cases of this disease apparently produced this toxin in vivo, cultures in the laboratory were only slightly toxic. Further investigation demonstrated that the addition of intestinal contents to culture fluid increased the toxicity of the latter some forty times. Bosworth and Glover further demonstrated that this increase in toxicity was caused by the proteolytic action of the trypsin in the intestinal contents. Similar activation was carried out in vitro not only with trypsin, but also with pepsin, papain or other proteolytic enzymes. It was apparent, then, that the bacteria produced a relatively non-toxic substance designated "prototoxin" by Turner and Rodwell (1943) which could be converted to a relatively active toxin by the action of any of a number of proteolytic enzymes.

Epsilon prototoxin and epsilon toxin differ in a number of characteristics. Turner and Rodwell (1943) pointed out that the prototoxin was quite resistant to heating, being able to withstand 70° C. for 15 minutes. Epsilon toxin, on the other hand, was destroyed by being heated for 15

minutes at 70° C. Moreover the effect of formalin on the ability of prototoxin and toxin to combine with antitoxin is different for Schuchardt and Munoz (1957) found that trypsin-activated toxin, after treatment with formalin, was no longer able to combine with antitoxin whereas prototoxin after treatment with formalin still possessed this ability.

Such inability of an antigen to combine with the antibody to which it gives rise is unique in the field of immunology and raises several problems of a theoretical nature. It raises some practical problems also, for it renders it impossible to use a combining power test in the evaluation of immunizing agents prepared for the production of epsilon antitoxin or for the prevention of Type D Clostridium perfringens enterotoxemia in sheep, cattle and goats. The use of an immunizing agent prepared from prototoxin activated by trypsin is desirable, for several workers (Batty and Glenny, 1948, and Smith and Matsuoka, 1954) have pointed out that the trypsin activation of prototoxin markedly increased the ability of the material to elicit antitoxin.

Combining power tests were first used for the evaluation of the probable immunizing potency of Clostridium perfringens Type D culture filtrates by Baldwin, Frederick and Ray (1948). They were working with toxoid preparations that had been prepared from non-activated cultures and concluded that such a method of evaluation was satisfactory.

The present studies were initiated to investigate some of the factors involved in the reaction between Clostridium perfringens epsilon antitoxin and toxoid or toxin in an effort to obtain some understanding of the apparently anomalous situation in which an antigen does not com-

bine with its antibody. Studies also were made in an effort to modify the combining power test so that it could be used with toxoid produced either from non-activated or activated toxin.

CHAPTER II

MATERIALS AND METHODS

A. DEFINITIONS ADOPTED

Throughout this paper a number of terms will be used. The definitions of the terms used are similar to those of Biological Products Sections (4), Animal Inspection and Quarantine Division, Agricultural Research Service.

1. Activated Standard Toxin: Standard dried toxin as issued by Animal Inspection and Quarantine Division reconstituted appropriately and treated with trypsin for 45 minutes at 37° C. or activated standard dried toxin appropriately reconstituted.

2. Minimum Lethal Dose (M.L.D.): The smallest quantity (weight) of Activated Standard Toxin which, when injected intravenously, kills 80 per cent or more Swiss albino mice weighing 16 to 20 grams each. The test period is 48 hours and a minimum of 5 mice are used in each test group.

3. Toxin Indicator Dose (T.I.D.): The smallest quantity of Activated Standard Toxin which, when mixed with any given amount of appropriately diluted and combined standard bacterin and standard antitoxin, will cause 80 per cent or more of test mice inoculated intravenously to die within 48 hours.

4. Combining Power Unit (C.P.U.): One-tenth of the smallest quantity of bacterin which when incubated for 1 hour at room temperature with 10 units of antitoxin will neutralize that antitoxin to the extent

that the addition of 10 T.I.D.'s of activated toxin results in a mixture lethal to at least 80 per cent of test mice within 48 hours following intravenous injection.

B. STANDARD MATERIALS

1. Standard lyophilized Clostridium perfringens Type D Toxin, Antitoxin and Bacterin were supplied by Animal Inspection and Quarantine Division in vials under vacuum. After opening and release of vacuum all dried standards were stored in vacuo over $CaSO_4$ except when used to prepare standard solutions.

During the course of these studies two series of Standard lyophilized materials were provided. Standard Toxin Series (a) as issued was not trypsin-activated; Series (b) was trypsin-activated.

a. Standard Toxin

Series (a) (not trypsin-activated) Lot A-4

M.L.D. (80%+)	0.013 mg.
T.I.D.	0.048 mg.

Series (b) trypsin-activated) Lot II-A S

L_0	0.0740 mg.
L+	0.1000 mg.
M.L.D.	0.003 mg.
T.I.D.	0.021 - 0.028 mg.

b. Standard Antitoxin

Series (a) Lot III

Contents contained 400 U.S. units per ml. when restored with 5 ml. distilled water.

Series (b) Lot IV-A

Contents contained 330 U.S. units or 36 International units when restored with 5 ml. distilled water.

c. Standard Toxoid

Series (a) Lot II-Af

1 C.P. unit - 0.0165 mg.

Series (b)

1 C.P. unit - 0.022 mg.

2. Diluent: All dilutions were carried out using diluent having the following composition:

Bacto-Peptone (Difco) - 1.0%

NaCl (C.P.) - 0.25%

Distilled Water, q.s. - 100.0%

pH 6.8 - 7.2

Autoclave 15 minutes at 15 pounds steam pressure (121° C.).

C. ACTIVATING TRYPSIN

From the beginning of these studies in September, 1960, until June 1, 1961, trypsin used was produced by Nutritional Biochemical Corporation (1-300; purchased in 1958). After this supply of trypsin was exhausted, from June 1, 1961, until the termination of these studies July 5, 1961, Difco Certified Trypsin (1-250 Control 448347) was used.

Activating trypsin 0.25 per cent was always prepared by adding 0.25 gram trypsin to 10 ml. Bacto-Peptone diluent in a screw-capped tube which

was placed in warm water and mixed until the trypsin was dissolved. This solution was added to the toxin to be activated in the proportion of 1:10.

D. STANDARD PREPARATIONS

1. Activated Standard Toxin Solution

After opening the vial of Standard dried toxin, or after removing from the vacuum storage dessicator, a minimum of 40-50 mg. was quickly but accurately weighed, transferred to a flask and diluted to volume with diluent. Diluent for Standard Toxin Series (a) contained 0.25 per cent Trypsin (Nutritional Biochemicals Corporation 1-300 purchased 1958) with toxin of this series being incubated at 37° C. for 45 minutes. The final concentration of dried toxin was 1.0 mg. per ml. Upon completion of activation of Series (a) or appropriate dilution of Series (b) the solution was divided among a number of small screw-capped tubes and stored at approximately -16° C. This frozen toxin was used up to four weeks after preparation. Once thawed it was not refrozen for future use.

2. Standard Antitoxin Solution

Contents were restored according to directions on the label using 5 ml. of distilled water. This restored antitoxin was further diluted to obtain a solution containing 10 antitoxin units per ml. This solution was distributed into screw-capped tubes and stored at approximately -16° C. This frozen antitoxin was used up to one year after preparation.

3. Standard Bacterin Solution

A minimum of 40-50 mg. was quickly but accurately weighed and diluted

to contain 10 C.P.U. per ml. This solution was distributed into screw-capped tubes and stored at approximately -16° C. This frozen bacterin was thawed and used up to one month after preparation. Once thawed it was not refrozen for future use.

E. CULTURE MEDIA

Toxin Run 1

The medium used in this experiment consisted of Bacto-tryptone 3 per cent, trypticase 1 per cent, yeast extract 1 per cent, and approximately 0.005 per cent magnesium sulfate. The pH was adjusted to 7.4 - 7.6 and the medium was sterilized by autoclaving for 25 minutes at approximately 121° C. One-half per cent glucose was added in the form of a sterile 10 per cent solution shortly before inoculation.

Toxin Run 2

The medium used in this experiment consisted of trypticase 3 per cent, yeast extract 1 per cent, small amounts of ground beef muscle, and approximately 0.005 per cent magnesium sulfate. The pH was adjusted to 7.4 - 7.6 and the medium was sterilized by autoclaving for 25 minutes at approximately 121° C. One-half per cent glucose was added in the form of a sterile 10 per cent solution shortly before inoculation.

F. CULTURES

Thirty stock cultures of Clostridium perfringens Type D were tested for toxin production. These stock cultures were furnished by Veterinary

Research Laboratory, Agricultural Experiment Station, Montana State College. They had been obtained from pathological specimens submitted for diagnosis and organisms from all selected stock cultures produced considerable epsilon toxin. With the exception of one stock culture obtained in May, 1960, they had not been transferred for two to four years. They had been maintained in a brain-egg-liver broth infusion stock culture medium.

The results of preliminary tests of toxin production of these cultures after trypsin activation indicated that strains 5431, 5639, 6778, 6779, 7070, 7071, 7072 and 7738 produced more than ten thousand mouse M.L.D.'s per ml. These strains were then used in further attempts to produce broth cultures which could be trypsin-activated with a resultant high level of epsilon toxin.

After testing and selection of the eight strains considered to be potentially most toxigenic serial transfers of each were made into tubes containing 10 ml. of fresh medium at intervals of a few hours and incubated anaerobically until the organisms appeared to be multiplying very rapidly. Apparent multiplication was based upon the development of turbidity and active gas production. Contents of tubes were then inoculated into 100 ml. amounts of fresh medium in 125 ml. Erlenmeyer flasks which in turn, after incubation and evidence of rapid growth, were used as inocula for liter amounts of medium.

Incubation was carried out in a water bath at 37° C. without anaerobic precautions. The pH of the cultures was checked as soon as active gas production was evident and readjusted to approximately 7.8 at 60-90

minute intervals using 1 N. NaOH which was added slowly with a minimum of stirring of the cultures. After 10 hours incubation when active gas evolution had nearly ceased, the pH was adjusted to 7.8 and the cultures were placed in the walk-in refrigerator at approximately 40° F. (app. 4° C.).

It was noted that any attempt to readjust the pH of actively growing cultures above 7.8 resulted in a rapid cessation of apparent growth with no recovery even after six to eight hours of additional incubation.

Testing of activated samples of material from each of the flasks of Toxin Run 1 by dilution and intravenous injections of 0.3 ml. amounts in mice revealed that only Strain 6778 produced more than 8000 mouse M.L.D.'s per ml.

Toxin Run 2 was initiated using essentially the same procedure as in Toxin Run 1 to obtain rapidly growing organisms using 10 per cent culture inocula. Incubation was carried out without anaerobic precautions in water baths at 35° C. Evidence of active gas evolution had nearly ceased after approximately 12 hours incubation. The pH of the cultures was checked and readjusted to 7.6 - 7.8. The cultures were left in the water bath overnight. The following morning the cultures were removed from the water bath and the pH rechecked and found to be still in the 7.6 - 7.8 range.

Trypsin activation of samples of Toxin Run 2 cultures along with appropriate dilution and intravenous injection in 0.3 ml. amounts in white mice revealed that toxins of Strain 5431 produced more than 6600 mouse M.L.D.'s per ml.; Strain 6778 more than 8250 mouse M.L.D.'s per ml.; and Strain 6779 more than 9900 mouse M.L.D.'s per ml.

In Toxin Run 1 produced September 23, 1960 liter amounts of whole culture materials were prepared from Strains 5639, 6778, 6779, 7071, 7072 and 7738; with Toxin Run 2 produced October 5, 1960 Strains 5431, 5639, 6778, 6779, 7070 and 7071 were used.

Samples of each culture fluid from these runs were treated five different ways, as follows:

Fraction I - approximately 400 ml. held as whole culture material at refrigerator temperature (40° F.).

Fraction II - approximately 200 ml. treated with formalin to give a final concentration of 0.6 per cent and stored at 35° C. for about two weeks; then placed in refrigerator.

Fraction III - approximately 200 ml. whole culture material treated with trypsin at 35° C. for approximately four hours, then formalin added to give a final concentration of 0.6 per cent, the mixture stored at 35° C. for approximately two weeks; then placed in refrigerator.

Fraction IV - approximately 100 ml. whole culture Seitz filtered at room temperature and then placed in the refrigerator.

Fraction V - approximately 100 ml. whole culture Seitz filtered at room temperature, trypsin-activated approximately 4 hours at 35° C. and then placed in the refrigerator.

Fractions II and III were checked after approximately two weeks incubation at 35° C. for completeness of toxoiding by injecting white mice with 0.3 ml. of trypsin-activated culture fluid intravenously. No deaths

resulted. Also, sterility was checked by inoculating tubes of Thiogel medium to which 0.5 per cent agar had been added and incubating anaerobically for two weeks. No growth resulted.

G. ANTITOXIN PREPARATIONS

1. Clostridium perfringens Type D diagnostic serum produced at the Wellcome Research Laboratories, Beckenham, England. This was of equine origin and contained 0.35 per cent cresol. In preliminary work antitoxin marked K2085 with an expiration date of May 1, 1962 was used. Antitoxin marked K2319, with an expiration date of October 1, 1962 and containing antitoxic values of 16 alpha units per ml., less than 0.5 beta and iota units per ml. and 1000 epsilon units per ml. was used.

2. Three different lots of Clostridium perfringens Type D antitoxin secured from commercial veterinary producers. These were of equine origin.

3. Fowl Sera: One hundred ml. amounts for immunization were withdrawn from bottles containing previously prepared whole culture from Run 1 of Strain 6778 and Run 2 of Strains 6778 and 6779. These materials were centrifuged, Seitz filtered, trypsin-activated at 35° C. for approximately two hours, formalinized to give a final concentration of 0.4 per cent and stored eight days at 35° C. Tests for toxicity were then performed on each of these materials using intravenous injection of 0.3 ml. amounts into white mice. No mice died.

A portion of these materials were mixed with equal parts of an adjuvant composed of approximately 80 per cent light mineral oil and 20 per

cent emulsifier (Arlacel A). These mixtures were then forced three times thru a sterilized homogenizer.

Each of these three toxoid-adjuvant mixtures was used to immunize White Leghorn hens weighing five to six pounds. The first injection consisted of 1 ml. intravenously of toxoid filtrate plus 1 ml. intramuscularly of toxoid-adjuvant. Eighteen days later a booster injection of 1 ml. of toxoid-adjuvant was given intramuscularly.

Twelve days following the booster injection the fowl were anesthetized with 0.75 to 0.8 ml. of Na pentobarbital and bled after reflecting the skin over the right jugular vein and severing the vein. Fifty to seventy five ml. of whole blood was collected by this method from each bird. One bird died before bleeding and blood from a second was rejected due to extensive lesions of undetermined origin being evident on post-mortem. The sera collected was preserved with Merthiolate (Thimerosal - N. F. Lilly) 1:10,000.

H. PRECIPITIN REACTIONS IN GELS

Both single and double diffusion precipitin reactions were carried out using Difco Purified Agar and Special Agar-Noble; Baltimore Biological Laboratories K-Agar and Consolidated Laboratories "Ionagar" No. 2.

Agar media were placed either in new 90 mm. X 15 mm. petri plates or new 90 mm. quadrant plates. Plexiglass dies were constructed with holes drilled in various sizes to accomodate various size cork borers at various distances from one another. The pattern of a central well surrounded

by six wells equidistant from one another as well as from the central cup was followed. In early work the bottoms of the cups cut by the cork borer were sealed with agar; in later work 1 per cent Formvar (Shawinigan Products Corporation, 350 Fifth Avenue, New York City) in ethylene dichloride was used. The plates were coated by flooding them and after being dried a few minutes were flamed to remove any excess. The Formvar solution was prepared freshly each day.

Merthiolate (Thiomersal - N. F. Lilly) 1:10,000 was incorporated into all agar preparations as otherwise bacterial growth from whole culture materials rapidly obscured lines of precipitation.

Incubation was carried out in an atmosphere of relatively high humidity. Preliminary tests for optimal conditions were carried out with identical cup charges at 35° C., room temperature and refrigerator temperature. It was determined that observations on reactions carried at 35° C. should be started at 18 hours; on reactions at room temperature at 36 hours; and on reactions at refrigerator temperature at 60 hours. Frequent observations after these periods of time allowed photographs to be taken when the lines of precipitation were most discrete. The results of several comparative runs indicated that the most discrete lines of precipitation occurred when the reaction proceeded at refrigerator temperature. This may be due to the fact that by this method both temperature and humidity may best be maintained quite uniformly throughout the reaction period.

E. IMMUNODIFFUSION ON CELLULOSE ACETATE DISCS

Immunodiffusion under oil with the antigens and antisera placed on

cellulose acetate filter membranes 5 centimeters in diameter was carried out essentially as outlined in the pamphlet "Electrophoresis With Cellulose Acetate Strips" supplied by Consolidated Laboratories, Inc., Chicago Heights, Illinois.

Upon completion of the diffusion the membranes were cleared and stained.

CHAPTER III

EXPERIMENTAL

A. PRECIPITIN REACTIONS IN GELS

Experimental analysis of single and double diffusion reactions in gels were used in an effort to evaluate the factors responsible for the inability of trypsin treated toxoids to neutralize antitoxin. This technique also was used to try to characterize the various antigenic fractions contained in the whole cultures and filtrates of culture Runs 1 and 2.

Gel diffusion studies were started approximately ten weeks after the production of cultures. Comparisons of the disappearance of the area of antigen-antibody reaction surrounding charged antigen cups, in experiments when antiserum was contained in the agar, are based upon the area of reaction seen when like materials were initially reacted.

Preliminary experiments using single diffusion in gels demonstrated that the optimal concentration of Wellcome Clostridium perfringens Type D antiserum in agar was 0.2 - 0.3 per cent (Figures 5 and 8). This concentration best elicited antigen-antibodies reactions when whole culture trypsin-activated antigens of Run 2 were placed in the cups. One well-defined line of precipitation appeared along with slight amounts of precipitate in the agar in the area between the line and the cup (Figures 5 and 8). Less defined lines of precipitation were noted when 0.1 per cent and 0.5 per cent (Figures 6 and 7) antiserum was incorporated, with the line of precipitate not being visible when 0.05 per cent Wellcome anti-

serum was used (Table I).

Using 0.3 per cent Wellcome antiserum in K-Agar correlation was evident between the toxin content of activated materials of Run 2 Strains 5431, 6778 and 6779 and the distance between the line of precipitation and the antigen charged cups.

Lines of precipitation were noted around cups charged with Run 2 trypsin-activated materials of Strains 5431, 6778 and 6779 from Fractions I, II, IV and V. Fraction V materials were Seitz filtered and trypsin-activated 25-26 days with lines of precipitation still evident. Fraction III was whole culture material both trypsin-activated and formalinized for 53-54 days with lines of precipitation not evident. Lines of precipitation produced by whole cultures of Strains 5431, 6778 and 6779 Toxin Run 2 reacted with 0.3 per cent Wellcome antiserum in agar disappeared as follows: (1) completely in approximately two months after being trypsin-activated and formalinized, (2) completely in approximately six months after being trypsin-activated but not formalinized, (3) after approximately six months lines of precipitation which were not discrete were still evident with formalinized materials, (4) after approximately six months slightly decreased lines of precipitation were evident with Seitz filtered materials, and (5) after approximately six months no apparently decreased lines of precipitation were evident with whole culture materials.

Pictures were taken of some plates showing the lines of precipitation. These lines were discrete but not prominent. However, it was found to be possible to demonstrate these lines by contact printing on photographic paper.

Preliminary work using the double diffusion gel technique attempted to evaluate the merits of three types of agar (Ionagar, Noble and Purified) upon incubation at 35° C., room temperature and refrigerator temperature. Fraction I of Run 2 antigens 5431, 6778 and 6779, undiluted and diluted 1:2 and 1:4, were reacted against Wellcome serum, undiluted and diluted 1:2 and 1:4. The majority of the plates, after appropriate incubation periods, showed one rather heavy line of precipitate closely surrounding the central sera cups. The pattern was the same at all antigen dilutions, but the line of precipitation was weak in those plates in which the serum cup was charged with Wellcome serum in 1:4 dilution.

Further work was initiated to determine if the line of precipitate seen was non-specific due to some factor in the serum. Control plates with cups charged with undiluted Wellcome serum, the three commercial sera, and the fowl sera showed one line of precipitate which was non-specific since no antigens were reacted with the sera. It was found that with Wellcome serum the non-specific line of precipitate was considerably diminished at a dilution of 1:10 but was still evident at a dilution of 1:1000 (Figure 2). Control plates charged with activated, undiluted materials from all five fractions of Strains 5431, 6778 and 6779 showed no lines of precipitate.

Agar was poured into petri plates divided into quadrants. Cups were cut in the agar of each quarter so that the central cup was surrounded by six outer cups, all of which were equal distance from one another. Quadrants were designated in clockwise order, as Number 1 to 4 respectively. Cups in each quadrant were designated, in clockwise order, as Number 1 to 6

respectively (Figure 1 - Sketch). The central cup of two quadrant plates were charged with Wellcome serum, in a similar manner, so that Quadrant Number 1 contained Wellcome serum undiluted; Quadrant Number 2 Wellcome serum diluted 1:10 with normal saline; Quadrant Number 3 Wellcome serum diluted 1:100 and Quadrant Number 4 Wellcome serum diluted 1:1000. Using Run 2 of Strain 6778 the outer cups of each quadrant of one plate were charged Numbers 1 to 5 respectively with undiluted Fractions I to V. Number 6 cup was left empty in each quadrant. The outer cups of each quadrant of the second plate were charged Numbers 1 to 5 respectively with a 1:10 dilution of Fractions I to V. Two additional series of two plates each were charged in similar manner except that in one series Run 1 of Strain 6778 was used; in the other series Run 2 of Strain 6779. This experiment as outlined was then repeated with fowl sera being used in place of Wellcome serum to charge the central cups of each quadrant.

In plates in which quadrants A to D contained respectively undiluted antigens and antigens diluted 1:10, 1:100 and 1:1000 and Wellcome serum in the respective central cups was undiluted and diluted 1:10, 1:100 and 1:1000 quadrant C showed quite discrete double lines of precipitation. It would thus appear that the ratio of antigen to antibody in quadrant C was optimum. Quadrants B and D also contained double lines of precipitation, but they are not discrete (Figure 2). Quadrant A exhibits only a non-specific line of precipitation. In plates with fowl sera in the central cups quadrant B of Figure 4 showed heavy double lines of precipitation, which could not be demonstrated, however, in the photograph. Figure 3 shows heavy non-specific lines of precipitation. All plates were incubated

in the refrigerator under similar conditions of temperature and humidity. Wellcome serum was titrated and known to contain 1000 epsilon units per ml. and only 16 alpha units per ml. Double lines of precipitate were evident only in those quadrants containing an apparent optimum ratio of antigen to antibody with discrete lines being evident in different quadrants as the ratio of antigen to antibody was changed. It was believed that the second line of precipitate was due to the epsilon fraction, in the activated antigen materials, reacting with the high epsilon antitoxic fraction in the Wellcome serum.

Confirmation that only one line of precipitate would form, due to the reaction of the epsilon antigen and the epsilon antitoxin in Wellcome serum, was sought by use of the immunodiffusion technique. Undiluted Wellcome serum was reacted with undiluted activated Fraction I of Run 2 of Strains 6778 and 6779 by diffusion under oil. After diffusion was considered complete the oil was removed from the cellulose acetate discs with petroleum ether. The discs were dried, stained in 0.2 per cent Ponceau S and cleared in Whitemore oil Number 120. Observation of the cleared discs revealed only one distinct line of precipitate. No lines of precipitate were evident on control discs, on which only Wellcome serum had been placed and diffused, or on discs on which all toxin fractions of all Strains in both Runs 1 and 2 had been placed and diffused.

B. COMBINING POWER TESTS

Combining power tests were set up in an attempt to determine if the gradual disappearance of precipitability, previously noted when all Frac-

tions of Run 2 of Strains 6778 and 6779 were reacted against Wellcome antiserum, was correlated with the loss of ability of epsilon toxin to combine with antitoxin.

Fractions I, II and III of Run 2 of Strain 6779 were diluted 1:8. One ml. amounts of this dilution, from each fraction, were placed in each of a series of four tubes along with 1 ml. of Standard Toxoid, containing 10 C.P.U. per ml., in each of an additional series of four tubes. To each of the sixteen tubes 1 ml. of Standard Antitoxin containing 10 A.U. per ml. was added. Contents of all tubes were thoroughly mixed and they were incubated one hour at room temperature. Then in one tube of each series of four, 1 ml. of Standard Toxin, containing 5 mg., was added. In the additional tubes of each series Standard Toxin was added, containing respectively 3 mg. per ml., 1 mg. per ml. and 0.05 mg. per ml. Considerable differences in the amounts of indicator toxin added were made, as wide variations were expected in combining power of the various toxin fractions. After the addition of Standard Toxin the contents of all tubes were again thoroughly mixed and incubated 1 hour at room temperature. Each of five white mice were injected intravenously with 0.3 ml. of each mixture.

Mouse deaths occurred within one hour after injection at all levels of Standard Toxin indicator added to Fraction I (Table IIa). Mouse deaths occurred overnight at all levels of Standard Toxin indicator except the 0.05 mg. per ml., added to Fraction II (Table IIb). Only the 5 mg. per ml. amount of Standard Toxin added to Fraction III killed mice overnight (Table IIb). Mouse deaths occurred overnight at the 5 mg. per ml. and 3 mg.

per ml. Standard Toxin indicator levels when added to Standard Toxoid.

C. AVIDITY OF ANTITOXINS AND TOXINS

Glenny, Barr, Ross and Stevens (1932) found that mixtures of toxin with loosely combining antitoxin still dissociate immediately on dilution, even after standing combined for two months. Samples of non-avid antitoxin have been tested by various in vivo methods resulting in an apparent value under one set of conditions which was less than one-third of the apparent value under other conditions (9). They found that with non-avid sera, it was possible to prepare mixtures of toxin and antitoxin such that rabbits will survive the intravenous injection of a 10 ml. amount but not of amounts varying from 0.001 ml. to 0.5 ml. The greater dilution of the small inocula in the rabbits' circulating blood apparently results in the dissociation of a lethal dose of toxin (20).

Glenny and his colleagues found that differences in avidity seriously affect the standardization of certain antitoxins, because in titrating many of these the level of testing is not definitely fixed but depends upon the potency of the toxin available at the time, and is therefore subject to continual change. The relative values of non-avid antitoxins were found to depend not only upon the dilution at which these tests are conducted, but also upon the endpoint chosen, because with non-avid sera there is a greater difference between the amount of antitoxin necessary to afford complete protection and that delaying death for some definite period. These workers believed that differences in avidity affected the relative neutralizing power and therefore the apparent antitoxic value of

the serum according to (a) the amount of toxin used in the test dose, (b) the endpoint accepted as a measure of degree of neutralization, (c) the route of administration, and (d) the species of animal used.

Glenny devised a method for estimating the avidity of antitoxic serum in terms of a dilution ratio, which was defined as the ratio of the amount of antitoxin necessary to form a neutral mixture with one lethal rabbit (L_r) dose of toxin in a total volume of 2 ml. (L_r 10) divided by the amount necessary to form a neutral mixture with the same amount of toxin in a total volume of 200 ml. (L_r 1000) (20).

Batty and Glenny (1947) found that the difference in firmness of combination between antitoxin and toxin and that of antitoxin and toxoid is far greater with a non-avid antitoxin. In titrations of avid antitoxin against activated toxin by the mouse intravenous method, a certain number of mice die after the first 24 hours, and less after 48 hours, but the number dying after 48 hours increases when titrations are made against unactivated toxin, and still further increases when non-avid antitoxin is titrated. Combining power tests are of special importance in assessing the total amount of antigen present, whether it be in the form of toxin or toxoid, and in the case of epsilon toxin or prototoxin as well. An arbitrarily chosen (Standard) antitoxin must be used (3).

The combining power potency test for Clostridium perfringens Type D bacterins as outlined in (4), while considered to be the best test available for evaluating this biological product, was beset with some difficulties. This was demonstrated by the inability of trained and very competent personnel of various veterinary biological producers to get

comparable results with repeated tests of the same bacterin. Serious efforts have been made by representatives of Animal Inspection and Quarantine Division to determine how improvements might be made in this test.

Sterne (1959) of Wellcome Laboratories, Beckenham, Kent, England, compared the combining power test, as carried out there, with the level of reagents and the method being used in the United States. He suggested that possibly Standard Antisera as issued for use in this test in the United States might be non-avid.

The author noted that with toxin-antitoxin neutralization tests using Fraction I of Run 2 of Strain 6779 and Wellcome serum that injected mice became markedly depressed and respired rapidly starting about thirty minutes after intravenous injection of a sublethal dose and lasting for several hours, with subsequent recovery. These symptoms and the fact that the level of lethality was not clearcut, due to scattered deaths in high dilutions which obviously should not have been lethal, indicated that this fraction of this Strain contained a toxic factor not neutralized by Clostridium perfringens Type D Wellcome antisera. Consequently, work with this Strain was dropped.

Repeated toxin dilutions of activated Fraction I of Runs 1 and 2 of Strain 6778 approximately six months after production, and subsequent intravenous injections in 0.3 ml. amounts into white mice indicated that 1 ml. of Run 1 contained 4000 mouse M.L.D.'s; and 1 ml. of Run 2 contained more than 6325 mouse M.L.D.'s (Table III). Fraction I toxin-antitoxin neutralization tests showed that 1 Wellcome epsilon unit would neutralize more than 400 mouse M.L.D.'s of Run 1 of Strain 6778 and approximately 380

mouse M.L.D.'s of Run 2 of Strain 6778 (Table IV). This would indicate that these materials could be well activated so that very little apparent prototoxin remains.

Toxin-antitoxin neutralization tests of activated Fraction I of Run 2 of Strain 6778 were set up at undiluted and 1:100 dilutions and reacted with Wellcome serum at appropriate dilutions of each toxin level. Trypsin activation when performed repeatedly on the same materials results in slightly different degrees of activation and thus slightly different lethal dilution levels for mice should be expected (15). Against undiluted toxin Wellcome serum protected white mice injected intravenously in 0.3 ml. amounts in a dilution range of 1:50 to 1:65; against 1:100 dilution of toxin, Wellcome sera protected in a dilution range of 1:10,000 to 1:12,000 (Table V). Standard Antitoxin, when reacted against Fraction I of Run 2 of Strain 6778 protected repeatedly at 165 antitoxin units per ml. but would not protect at 124 antitoxin units per ml. at the undiluted toxin level and protected at 1.0 antitoxin unit per ml. but not at 0.5 antitoxin unit per ml. level against the 1:100 toxin dilution (Table VI).

Repeated avidity tests with activated Fraction I of Run 1 of Strain 6778 undiluted and diluted 1:100 and Wellcome serum revealed that the serum would protect against equivalent (i.e. 1 ml. undiluted toxin and 1 ml. antiserum dilution 1:100) amounts of undiluted toxin at 1:100 dilution but not at 1:120 dilution; against 1:100 dilution of toxin the serum would protect in dilutions greater than 1:18,000 (Table VII).

Avidity tests of Standard Toxin and Standard Antitoxin were set up at twenty times difference in level of dilutions rather than one hundred

times difference in order not to rapidly deplete the stock of Standard Antitoxin. Standard Toxin levels used were 1 mg. per ml. and 0.05 mg. per ml. It was found that approximately 7.5 units of Standard Antitoxin per ml. were required to neutralize equivalent amounts of Standard Toxin at 1 mg. per ml. While 0.25 units of Standard Antitoxin per ml. were required to neutralize Standard Toxin at 0.05 mg. per ml. Standard Antitoxin, 0.125 units, would not neutralize Standard Toxin at the 0.05 mg. per ml. level (Table VIII).

Avidity tests were then set up using both Runs of Fraction I of Strain 6778 and Wellcome serum at one hundred times difference in level of dilutions as well as Standard Toxin and Standard Antitoxin at twenty times difference in level of dilutions. Mixtures were made in great enough amounts so that duplicate tests could be done. With one test run toxin-antitoxin mixtures were incubated at room temperature for only twenty minutes; with the other test run the mixtures were incubated for three hours.

Mouse deaths occurred at essentially the same level of dilution of antiserum when injected with toxin-antitoxin mixtures incubated either for only twenty minutes or for three hours (Tables IXa and IXb). However, the three hour incubation at room temperature apparently destroyed the toxin at the high levels of dilution. Mouse deaths occurred later, after injection with materials incubated three hours when compared to the time of death in mice injected with the same materials incubated only twenty minutes. This, too, may have been due to some destruction of activated toxin with prolonged incubation.

D. COMBINING POWER TESTS AT LOW AND HIGH REAGENT LEVELS

Sterne (15) of Wellcome Laboratories in England suggested that since English workers obtained satisfactory combining power test results, using a level of reagents approximately twelve and a half times more concentrated than those used in the United States, that low concentration of reagents might be responsible for failure to obtain satisfactory results upon repeated testing of the same materials.

Preliminary combining power tests had shown that using Series (b) Standard Toxin at the lowest indicated T.I.D. level, (0.021 mg. per ml.) with white mice obtained from Veterinary Research Laboratory and under our conditions of testing, that mice were killed even at the 10 T.I.D. minus 10 M.L.D. level.

The combining power test procedure as outlined (4) uses two levels of Standard Toxin indicator, to be added to the control mixtures of 10 A.U. of Standard Antitoxin and 10 C.P.U. of Standard Toxoid. These levels are 10 T.I.D. and 10 T.I.D. minus 10 M.L.D. Mice injected at the 10 T.I.D. level should die; those injected at the 10 T.I.D. minus 10 M.L.D. level should live. The test was devised in this manner as a control measure to prevent excessive challenge by adding too great amounts of Standard Toxin Indicator.

A combining power test series of seven tubes was set up. To each tube 1 ml. of Standard Antitoxin and 1 ml. of Standard Toxoid were added, mixed well and allowed to incubate one hour at room temperature. Standard Toxin made up to 0.22 mg. per ml. was then added to the first tube of the

series; Standard Toxin concentration was reduced to 0.20 mg. per ml. which was added to the second tube; with toxin concentration being reduced 0.02 mg. per ml. in each of the subsequent tubes of the series until the last tube contained Standard Toxin made up to 0.10 mg. per ml. Contents of the tubes were again thoroughly mixed and allowed to incubate an additional hour at room temperature. White mice, weighing 22-26 grams, were each injected with 0.3 ml. intravenously of the mixtures containing the various levels of Standard Toxin indicator. Three mice were injected with each mixture containing a different level of toxin indicator. Injections were started at 11:15 A.M. and completed about noon June 16, 1961. Results are shown in Table X.

Investigation was made on how the weight or age of mice affected their sensitivity to Clostridium perfringens Type D Standard Toxin. Mice for testing were available from the colonies of Veterinary Research Laboratory, Agricultural Experiment Station, Montana State College. Mice were weaned at three to four weeks of age weighing 16-20 grams. Mice at approximately eight weeks of age weighed 26-30 grams. Since it was not always possible to obtain weanling mice, it was necessary to determine how the use of 26-30 gram mice might affect test results. Series (a) Standard Toxin was trypsin-activated and diluted to contain amounts ranging from 0.10 mg. per 0.3 ml. to 0.16 mg. per 0.3 ml. as outlined in Table XI. White mice were injected with 0.3 ml. amounts intravenously. This test was repeated using only the 16-20 gram and 26-30 gram mice with toxin dilutions ranging from 0.10 mg. per 0.3 ml. to 0.13 mg. per 0.3 ml. with similar test results being obtained. Results would indicate that the

26-30 gram mice are at least 10 per cent more susceptible to the toxin. Susceptibility may be correlated more with the age of the mice than with the weight.

Further confirmation of the T.I.D. value was sought by setting up a series of combining power tests as outlined in Table XIIa using a 10 T.I.D. level of 0.22 mg. and a 10 T.I.D. minus 10 M.L.D. level of 0.19 mg. Mice used weighed 25-30 grams. Results indicated that again the T.I.D. level of Standard Toxin was excessive. Whereas all mice were killed at both toxin indicator levels at 10 A.U.-C.P.U. of Standard Materials no deaths occurred at 80 A.U.-C.P.U. With Toxin Run 2 of Strain 6778, where only the undiluted toxin killed all mice at the 80 A.U. level, mice were killed with a toxin dilution of 1:15 at the 10 A.U. level (Table XIIb). A valid comparison could not be made with Toxin Run 1 of Strain 6778 since mice were not killed with undiluted toxin (Table XIIc).

Adjustments were made in reagent levels as outlined in Tables XIIa, XIIb, and XIIc and then combining power tests were set up as outlined in Tables XIIIa, XIIIb, and XIIIc using a 10 T.I.D. level of 0.20 mg. and a 10 T.I.D. minus 10 M.L.D. level of 0.17 mg. Again all mice, weighing 25-30 grams, were killed at both toxin indicator levels at 10 A.U.-C.P.U. (Table XIIIa).

With Toxin Run 2 of Strain 6778 differences in dilution levels at which mouse deaths occurred, as compared to results shown in Table XIIIb could have been due to the fact that the material was trypsin-activated for 1 hour and 40 minutes instead of approximately 1 hour. This was done since difficulty had been experienced in getting the Difco lot of trypsin

being used to go into solution readily. However, what was considered to be even more plausible is the fact that on this day toxin dilutions inadvertantly were made in Bacto-Peptone diluent which was at room temperature. Toxin dilutions previously had been made in Bacto-Peptone diluent which was still ice-cold.

With Toxin Run 1 of Strain 6778 mice died at a 1:7 toxin dilution and survived at a 1:8 toxin dilution at the low level of reagents; mice died at a 1:1.5 and survived at 1:1.75 toxin dilution at the high level of reagents with a toxin indicator dose of 40 T.I.D. (Table XIIIc).

At this time it was felt that the correct levels of toxin dilutions necessary to run a series of combining power tests had been closely determined. Test series were set up using low and high levels of reagent materials at various toxin indicator levels. Results of tests using Standard materials and Toxin Run 2 of Strain 6778 are outlined in Tables XIVa and XIVb; results of Toxin Run 1 of Strain 6778 in Tables XVa and XVb. Mice used weighed 25-30 grams.

Results outlined in Tables XIV and XV indicated that a proper range of toxin dilutions had been established. Combining power tests were set up using materials from Fractions I-V inclusive of Toxin Run 2 of Strain 6778. These were trypsin-activated if they already had not been so treated. After activation was complete five toxin dilutions of each fraction were made, these being 1:7.5, 1:10, 1:12.5, 1:15 and 1:17.5. Standard Antitoxin was used at the 10 A.U. level; Standard Toxin at the 10 T.I.D. level. Standard materials control was set up at 10 A.U.-C.P.U. with Standard Toxin being added at 10 T.I.D. (0.17 mg.) and 10 T.I.D. minus 10 M.L.D. (0.14 mg.)

levels.

Mice weighing 25-30 grams, injected at both toxin indicator levels died at approximately the same times indicating a slight over-challenge. With Fraction I mice were dead within eight hours after injection at toxin dilutions of 1:7.5 and 1:10. At the 1:12.5 dilution at approximately eighteen hours post-injection all three injected mice exhibited central nervous system disturbances shown by symptoms of twisting and rolling and two were comatose. At sixty six hours post-injection one of these affected mice was dead. One mouse died within eight hours post-injection at the 1:17.5 toxin level (Table XVIa).

With Fraction II at eighteen hours one mouse was dead and two mice exhibited symptoms of twisting and rolling at a dilution of 1:7.5. All mice injected with the remaining dilutions of Fraction II survived. No deaths occurred in Fractions III, IV or V (Tables XVIb and XVIc).

The experiments in this section show that combination of toxoid and antitoxin occurs more firmly when the reactions are carried out using reagents concentrated eight times more than those reagents previously used in the outlined test (4).

CHAPTER IV

DISCUSSION

In this study the Clostridium perfringens Type D diagnostic serum used was titrated and known to contain 1000 epsilon units per ml. Run 1 trypsin-activated broth culture of Strain 6778 contained approximately 4000 mouse M.L.D.'s per ml.; Run 2 broth culture contained approximately 6325 mouse M.L.D.'s per ml. Using either Run 1 or Run 2 trypsin-activated broth culture material it was demonstrated that one epsilon unit would neutralize approximately 400 mouse M.L.D.'s. Calculations thus reveal that the epsilon concentration in Wellcome serum was approximately 62 times greater than the epsilon toxin contained in Run 2 of Strain 6778 and approximately 100 times greater than the epsilon toxin contained in Run 1.

When work was first begun with the agar gel precipitin reactions the author believed that since the trypsin-activated broth cultures were not concentrated that considerable differences existed in their content of epsilon toxin and the concentration of epsilon fraction in the Wellcome serum.

This was confirmed by preliminary testing of Run 2 of Strain 6778 activated toxin reacted with Wellcome serum in neutralizations tests. Initial work, using the single diffusion technique, demonstrated that the concentration of Wellcome serum to be mixed into the agar must be within the range of 0.2 per cent to 0.3 per cent in order for lines of precipitation to appear discretely. Inclusion of somewhat lesser or greater

amounts of serum in the agar prevented the development of optimum proportions of reacting antigen with antibody and resulted in the formation of non-discrete lines of precipitation. The time, for which the reactions were carried out, was important because the most discrete lines of precipitation persisted for only a few hours after which discreteness was gradually lost. The temperature at which agar gel precipitin reactions were carried out might be varied depending upon what is desired. During preliminary work, in which economy of time is important, reactions might be carried out at 35° C. Later if it were desired to take photographs of precipitation patterns then reactions might be carried out at refrigerator temperature to obtain the most discrete lines of precipitation.

The ability of a Clostridium perfringens Type D whole culture to combine with antitoxin after trypsin activation alone would appear to be influenced by the length of time elapsing between activation and testing. Agar gel precipitin reactions, which correlated with the ability of toxin to combine with antitoxin, showed that lines of precipitation appeared in broth cultures of several strains of organisms reacted 25-26 days after trypsin activation but failed to appear in materials reacted approximately six months after activation.

Using a single diffusion serum agar technique the author confirmed the finding of Schuchardt and Munoz (1957) that formalin produced a loss of the ability of trypsinized toxin to give a specific precipitate with antitoxin with complete loss occurring in less than fifty four days. This disappearance of precipitability correlated with the loss of ability to combine with antitoxin.

The loss of the ability of a non-activated, formalinized broth culture to combine with antitoxin, while it proceeds relatively slowly, is of practical importance. Commercial Clostridium perfringens Type D bacterins may carry an expiration date, after which they are not to be used, ranging from eighteen months to three years. It would seem that this loss of combining power would preclude the use of the combining power test, should the need arise, for some months before this biological product would reach its expiration date.

Avidity studies indicated that Standard materials along with other toxins and Wellcome antitoxin were avid. In all cases except when Standard Toxin was reacted with Standard Antitoxin the Activation Ratio was less than unity. This has been interpreted as indicating a greater dissociation in more concentrated solutions (9). The Activation Ratio of Standard Toxin with Standard Antitoxin was approximately unity.

Mouse deaths occurred at a clear cut level of toxin-antitoxin dilution. The majority of mouse deaths occurred within eight to ten hours after injection. An occasional death occurred within twenty four hours post-injection with only a rare death occurring thereafter. Mice injected with near-lethal dilutions, when observed eighteen hours after injection, exhibited symptoms of a central nervous system disturbance as evidenced by twisting and rolling movements. These same mice even though comatose generally survived throughout the ninety six hour observation period.

Observed time of mouse deaths with Standard Toxin as well as Toxin Runs 1 and 2 of Strain 6778 during both toxin-antitoxin neutralization tests and combining power tests indicated an unusual pattern of mouse