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I hereby recommend that the thesis prepared under my supervision by Patricia J. Perkins

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The Toxin of Clostridium Oedematiens
Its Production and Some of its Properties

A Dissertation submitted in partial
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by

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General

Introduction

Purpose and Scope of Work

Because of the times, the importance of the study of the organisms which cause gas gangrene has increased many times. The organism, Clostridium oedematiens has been found in 20-30% of the cases of gas gangrene, which is usually a mixed infection(14).

In animal experimentation, the sulfa drugs have been found particularly ineffective against experimental infection with oedematiens cultures.(7) The drugs have also been found ineffectual in inhibiting in vitro growth. In this laboratory Cl. oedematiens has been grown in the presence of sulfadiazene.(36).The use of penicillin to protect animals against such experimental infections has proven effective as a therapeutic agent.(7). Because of its comparitively rapid disappearance from the blood stream, it does not serve in a prophylactic capacity. The development of a toxoid, or some similar immunizing agent, was deemed necessary for the protection of military personnel engaged in active warfare.

The first step in the development of such an agent is the consistent production of a toxin of relatively high potency on a medium with which there will be as little danger of reaction as possible, when the material is injected into animals or human beings. One of the phases of this problem was the study of the toxin production with Cl. oedematiens on such a medium.

For all bacterial toxins, with the exception of Cl. welchii, and Cl. septicum the only test for potency is the animal test employ-

ing mice, guinea pigs, or rabbits. In vivo tests are of long duration, usually requiring at least three days for observation of the animals after injection. In the event of the development of an in vitro test, such as the one which has been developed for Cl. welchii toxin(20)(22), the speed with which toxin production methods could be developed would increase. After some preliminary work on enzymic activities of the toxin of Cl. oedematiens, the hemolytic activity was singled out for the study of the possible development of an in vitro test. In the course of this work, some of the characteristics of the reaction of the hemolysin of Cl. oedematiens filtrates were investigated.

A few immunological experiments using mice, rabbits, guinea pigs and pigeons have been carried out with the toxins prepared on the media developed and toxoided with formalin.

History of Toxin Production with Cl. Oedematiens and the Study of the Enzymic Properties of the Culture Filtrates.

The organism was first isolated by Novy, in 1894, and was called Bacillus oedematis maligni II. This has been renamed Clostridium novyi. Some years later, Weinberg and Sequin (42) isolated from a case of gas gangrene an organism which they called Bacillus oedematiens, one of the characteristics of which was the production of a gelatinous edema in the muscle of animals injected with the organism. This organism, reclassified in the genus, Clostridium, is the one which has been used throughout this work. Weinberg believed that novyi was an atoxic variant of oedematiens, but most authors use the names interchangeably (3).

Weinberg (42) was the first to demonstrate the presence of a thermolabile toxin in the filtrates of cultures of Cl. oedematiens. By comparison with toxins obtained at present, his preparations, grown on 2% glucose broth, were weak, containing only four MLD/ml. (guinea pig). Preparations containing 100 MLD/ml. (guinea pig) were subsequently prepared by Weinberg and Seguin.(43). Since that time, 1917, toxin production has been studied on media containing 1.0% peptone, 0.2% glucose and sterile rabbit muscle,(24),on broth with meat at the bottom of the tube (33), liver broth (13), broth with cooked meat(40), with 20 in vitro passages on a variety of media the best of which was a digest of peas(17) fermented Reidel's peptone with no glucose(40)and on a medium containing 5% gelatin, 1% peptone, phosphate, magnesium sulfate and sodium chloride(30) . With all media used, toxin production was carried out under anaerobic conditions obtained with the use of parrafin, vaselin mixtures, mineral oil or a nitrogen-carbon dioxide atmosphere over the cultures, or evacuation of air. The potency of the toxins produced has increased noticeably, Walbum's filtrates containing 50,000 MLD/ml. (mice).

The enzymic activities of the toxin of Cl. oedematiens have been studied in a desultory fashion. Some of the filtrates contain a gelatin dissolving enzyme, but Walbum (40) did not find any such activity in his filtrates of high potency.

The toxic filtrates of Cl. oedematiens have an active fibrinolysin (31)(32) , which is thermolabile, withstanding heating at 60°C for up to thirty minutes. The fibrinolytic activity was not inhibited by oedematiens antitoxin.

The hemolytic activity of the filtrates has been investigated by Celarek(4) who compared recovery of hemolytic and lethal activities of toxic filtrates upon precipitation with ammonium sulfate and upon adsorption on benzoic acid in acetone. Walbum (34) has studied the importance of pH in the hemolytic reaction of filtrates, but has not made any attempt at correlation of hemolytic and lethal activity. Reed, Orr and Baker, (30) used hemolytic activity as an indication of lethal potency of some of their preparations, but have not published experimental justification of this practice.

The hemolytic activity of the toxin has been used to calculate the strength of the toxin or the antitoxin in the case of Cl. welchii (23), and, more recently, in the case of Cl. septicum(11)(16). The experiments of the last section of this work were designed to demonstrate whether or not the lethal and hemolytic activities of Cl. oedematiens were correlated.

- 11.

The Lethal Toxin, Testing and Production.

Method of Testing the Potency of Toxins.

For purposes of comparison of various toxic filtrates and toxin preparations, the minimum lethal dose for mice and the antitoxin combining power of the toxins was determined.

The minimum lethal dose, MLD or LD_{50} was measured in mice weighing 17-20 gms. Appropriate dilutions were made and each dilution injected into mice. In the early part of the work, the dilutions were made in saline, later, dilutions were made in 4% pancreatic digest of beef heart(5). A typical set of dilutions for a toxin of low potency is given in Table 1 with the value in MLD or LD_{50}/ml/ for each dilution. LD_{50} signifies that if, at any particular dilution, 1/2 the animals injected survive and 1/2 die, while all animals receiving mixtures containing more toxin die, the toxin tested contains the LD_{50}/ml. calculated for that dilution.

Table 1.

Dilution of Toxic Filtrate for Determination of MLD
0.2 ml. of each dilution injected per mouse

Dilution #	Ml. toxin to 50 ml. saline	MLD/ml.
1	0.5	500
2	0.25	1000
3	0.167	1500
4	0.125	2000

Where all the animals at one point died and all those at the next higher point survived, the results are reported as MLD

within the limits, e.g. 1000 +, 1500 -. Where one animal survived at each of two points and all animals at lower points died, or where anomalies occurred as when two animals at an intermediate point survived and both animals at the higher and lower levels died, the results are reported as MLD within limits, omitting the + and - signs, e.g. 1000-2000. Where only one animal died at any point and all animals below that point died, while all above survived, the results are reported as LD_{50}, end-point values, e.g. 1500.

Injections were made immediately after diluting the toxins. It has been found that intramuscular and subcutaneous injection give approximately the same results, while intraperitoneal injection gives potencies lower than intramuscular injection. Subcutaneous injection was adopted as the routine procedure. The mice were observed for 3 days after injection.

The combining power of toxins was determined by the official method outlined by Bengston(2). A dried toxin was prepared by precipitation with ammonium sulfate and standardized against official standard antitoxin.* This dried toxin was then used as a secondary standard to determine the strength of the commercial antitoxin** used in routine testing. This antitoxin was diluted suitably and toxic filtrates were tested by two methods, one with the toxin constant and one with the antitoxin constant.

* Kindly supplied by the National Institute of Health

** Kindly supplied by Lederle Laboratories, Inc.

In the latter method 140 TD , test doses, (1 test dose = 0.02 units of antitoxin) equivalents of antitoxin were mixed with various amounts of toxin, the mixtures were diluted to a final volume of 2 ml. with saline and incubated for 1 hr. at room temperature. After the incubation period 0.2 ml. of each mixture was injected into each of two mice. The animals were observed for 3 days after injection.

In the alternative method, 0.5 ml. portions of the toxin were mixed with varying amounts of antitoxin, the number of test doses differing by 10-50 TD in successive mixtures. A few confirmatory titrations were carried out at 5 TD intervals, but the routine procedure was to titrate at wider intervals.

Table 11 gives the results of an experiment in which combining power was determined by both methods concurrently.

Table 11.

Combining Power of Toxin with Antitoxin Varied and Constant

Conditions	Titre, TD/ml.
Antitoxin varied	130 - 140
Antitoxin constant	140 - 156

As may be seen from Table 11, indications are that the results with the two methods were comparable. Throughout this work, the test for combining power was carried out with the toxin constant and the antitoxin varied.

Strains of Clostridium oedematiens Used.

Two strains have been used throughout this work; N104, sent to us by the National Institute of Health and N21B, received from Lederle, Inc. In a preliminary study both strains were used, but N21B

seemed to produce a toxin of higher potency more consistently than N104, on the median tested. Six toxin batches were set up in duplicate, with B medium, solvent extracted beef kidney and dextrin. The inocula from cultures N104 and N21B were treated identically. One batch of each duplicate was inoculated with N104, one, with N21B. The results are presented in Table 111. The MLD of the filtrates were tested only on Lot # 6.

Table 111.

Comparison of Toxin Production by Two Strains N 104 and N21B.

Lot #	TD/ml	
	N104	N21B
1	10-	10
2	10+,20-	20+, 40-
3	30+,40-	50+ ,70-
4	50+	50+-,60-
5	30+,40-	80+ ,100-
6	0+,50-	50+ ,100-
	MLD/ml.	
6	2000 + , 5000- ,	5000

It may be seen from the table above, that under the conditions of toxin production used, N21B was a better toxin producing culture than N104. The former culture was used exclusively in later work.

Media and Materials Used for the Production of Toxic Filtrates.

Beef heart infusion broth was prepared according to Adams(1). Fresh beef heart was ground and 2 lbs. boiled with 2 l. of tap water for 20 minutes. The cooked meat mixture was filtered. To the filtrate was added the following:

Bacto-Peptone (Difco)	1.0 %
NaCl	0.5 %
$NaC_2H_3O_2$ H_2O	1.0 %

The pH was adjusted to 7.6-7.8 by the addition of 1/3 saturated NaOH and stored in the cold room under toluene.

Casein hydrolysate prepared by Mueller's (25) method was used as the source of the amino acids of casein in preliminary work. 200 gms. of commercial casein were hydrolyzed with 8 N HCl using a reflux condenser. Hydrolysis was continued for about 20 hours, at the end of which time, the hydrolysate was evaporated in vacuo to a gummy mass. The evaporated material was dissolved in 2 l. of distilled water. A paste of litharge (PbO) was slowly added to the dissolved hydrolysate until the pH was about 4. The lead treated mixture was filtered by suction. Lead was removed from the filtrate by the addition of a saturated solution of BaS. About 50 ml. was used for 2 l. of filtrate. Excess barium was removed by the addition of 10 N H_2SO_4 until the clear filtrate gave a slight precipitate upon addition of a saturated solution of $BaC_2H_3O_2$. The addition of BaS and H_2SO_4 was adjusted so that the filtrate contained a slight excess of sulfide and sulfate. The treated filtrate was filtered again and aerated to remove excess H_2S.

The iron was removed from the hydrolysate by adsorption on $Ca_3(PO_4)_2$. This method of deferration was used for removal of iron from all digests. The pH of the solution to be deferrated was adjusted to 7.8-8.0. 2 M Ca, as $CaCl_2$, was added and the mixture brought to boiling. The flame was removed from under the mixture and solid $Na2HPO_4 \cdot 12\ H_2O$ added until the clear solution showed no further precipitation upon addition of a saturated solution of the phosphate. The mixture was filtered while hot and the filtrate stored under toluene at 50 C.

The presence of iron in hydrolysates or digests was demonstrated by a modification of the Feigl (8) spot test for iron. 0.1-0.2

ml. of the material to be tested was mixed with a small amount of sodium hydrosulfate in the depression of a deep-depression spot plate. 0.02-0.05 ml. of a 0.2% solution of 3 3' dipyridyl was added and the whole mixed by the addition of water from a wash bottle and stirring with an applicator stick. A pink color developed immediately if iron were present in the solution tested. A blank test on the reagents was run at the same time as the test on the unknown to insure that no color developed with the reagents alone.

The test was used as a semi-quantitative test. Standard solutions of $FeSO_4 \cdot 7-_2O$ and $FeCl_3 \cdot 6H_2O$ were prepared containing 0.1 mg./ml. The standard solutions were distributed in amounts equivalent to 1,3,5, and 10 ugFe sodium hydrosulfite was added to the $FeCl_3$ solutions. 0.02 ml. of the dipyridyl solution was added to all standard test "spots" and water to a final volume of 1.02 ml. An accurately measured amount of the material to be tested was put in a depression adjacent to the standard test "spots". The color was developed and compared to that in the standards. From this comparison, the amount of iron per ml. of solution could be calculated.

Shortly after the work on Cl. oedematiens had been started, a Difco product, casamino acids (A Mueller casein hydrolysate) was tested in this laboratory for toxin production with Cl. welchii and found satisfactory. This product was finally adopted as the source of the amino acids of casein.

The medium most frequently used was a casamino acids medium developed in this laboratory for keeping the Cl. welchii culture(34). This medium will be referred to as B medium throughout this paper. B medium was usually prepared at four times the final concentration to

facilitate storage and the addition of supplements. In preparation of 4 l. of medium at four times the final concentration, the following procedure was used.

I 256 gms. casamino acids - dissolved with gentle heating in 1 l. of water. (1600 mgs. N/l)

II 92.2 gms. $Na_2HPO_4 \cdot 12H_2O$
7.68 " KH_2PO_4
320 mgs. $MgSO_4 \cdot 7H_2O$
320 mgs. tryptophane
320 mgs. cystine
Dissolved with gentle heating in
1 l. water

I and II were mixed and combined with
80 gms. sodium succinate.

The pH of the above mixture was adjusted to 7.8-8.0 by the addition of a saturated solution of Na_2CO_3.

After the solution was cooled, 80 ml. of the stock vitamin solution containing 1 mg. each of Ca-d-pantothenate, pimelic acid, nicotinic acid, pyridexine, thiamin and 0.1 mg. of riboflavin in 5 ml. was added.

The medium was diluted to volume, 4 l., and stored at 50°C, under toluene.

B medium was supplemented with solvent extracted beef heart, solvent extracted beef kidney, pancreatic digests of beef heart, beef kidney, and liver cake.[2]

The <u>solvent extracted beef heart</u> was prepared using fresh beef heart freed of gross fat and ground. 1 k. of the ground meat was thoroughly mixed with 2 l. of alcohol and kneaded with the alcohol for 1/2 --3/4 hr. The alcohol was removed as thoroughly as possible by filtering and pressing the tissue in a cloth bag. The tissue was dried by spreading in a thin layer in a current of air. After drying, the meat was reground in a coffee mill to particles of 2 mm. or less in diameter. The dried reground tissue was kneaded with 1-2 l. of an alcohol-ether mixture (1:1) for 1/2 -- 3/4 hr. The alcohol-ether was re-

moved by filtering and pressing the tissue in a cloth bag. The tissue was dried by spreading in a thin layer and exposing to air for about 24 hours.

Extraction was thorough enough that no fat droplets were present when the tissue was autoclaved with B medium. The necessity for this was clearly demonstrated. A lot of solvent extracted meat escaped thorough extraction. Toxin production dropped from 200 TD/ml. of filtrate to less than 50 TD/ml. When the meat which gave the poor yield was reground and extracted with alcohol-ether mixture, toxin production increased to the former level.

Solvent extracted beef kidney was prepared in the same manner as solvent extracted beef heart.

Pancreatic digest of beef kidney was prepared from fresh beef kidney freed of gross fat and ground. 1.5 kg. of kidney was washed with running tap water for about an hour. The washed kidney was mixed with an equal volume of tap water, heated to boiling and boiled about 5 minutes. The water was removed as thoroughly as possible by filtering and pressing the tissue in a cloth bag. 2.5 l. of distilled water and about 100 gms. freshly ground pancreas was added to the cooked meat. The pH of the mixture was adjusted to about 9.0 with saturated NaOh solution. The mixture was incubated at 45° for 24 hours. During the incubation period, the pH was adjusted to 9.0 at 8 hours and again at 18 hours.(20).

After digestion, the pH of the mixture was adjusted to about 4.0-4,5 with HCl and the digest filtered. Solid $Na_2HPO_4 \cdot 12\ H_2O$ was added to the filtrate until the pH was about 7.8.

A digest prepared in this manner contained 4.44% solids, (110° for 24 hrs.), 5.2 mg. N/ml. (Folin-Kjeldahl method) and 1.4 ug. Fe/ml.

Pancreatic digests of beef heart and liver cake* were prepared in the same way with a few modifications .

In addition to being used with B medium, the pancreatic digests were also tested as toxin production media with the addition of a salts supplement. This supplement, like B medium, was usually made up at four times the final concentrations. The supplement contained per liter at final concentration:

$Na_2HPO_4 \cdot 12\ H_2O$	5.76 gms.
KH_2PO_4	0.48 gms.
$MgSO_4.7\ H_2O$	0.02 gms.
$Na_2HPO_4 \cdot 12\ H_2O$	5.76 gms.
KH_2PO_4	0.48 gms.
$MgSO_4.7\ H_2O$	0.02 gms.

The salts were dissolved and stored at 50 C.

In later work a solution of cysteine·HCl and ascorbic acid was used in inoculum cultures. A solution was made containing 10% each of the crystalline solids of cysteine - HCl and ascorbic acid.

Toxin Production.

The experimental work on toxin production may be divided into two periods; the first, during which the culture was transferred daily and not stored and the second, wherein the culture was transferred only every week or two, and was stored for three weeks to two months before using as a seed for inocula. Only MLD titrations were performed in the first period, with titres ranging from 1500-4000 MLD/ml. and

* Liver cake is the residue from the commercial preparation of liver extract. It was supplied for this work by Lederle Company.

occasionally exceeding 5000 MLD/ml. During the later period the MLD titre ranged from 5000-20,000 MLD/ml., occasionally exceeding 20,000 MLD/ml. The Td titre ranged from 100-240 TD/ml.

Toxin Production - Early Work.

Anaerobic Conditions. When the cultures were received they were incubated on beef heart infusion broth with 0.3% glucose. The carbohydrate was sterilized separately in 25% solution and added to the medium after autoclaving. The infusion broth was distributed in 40 ml. amounts in pyrex tubes, 200 x 25 mm. To each 40 ml. portion was added 1 gm. of the dried meat residue from the infusion broth preparation. A duplicate set of tubes was inoculated deeply with 1 ml. of a 48 hour culture on the same residue grown under vaspar (vaseline:paraffin, 1:1). To one of the set of tubes, sterilized mineral oil was added to a thickness of about 1/2 inch. The cultures were incubated 48 hours at 37°. At 24 hours the tubes were removed from the incubator and growth estimated visually. At 48 hours portions of each culture were centrifuged and dilutions were made in saline and injected into mice to determine the MLD. The results are shown in Table IV.

Table IV.

The Effect of Mineral Oil on Growth and Toxin Production.

Mineral Oil	Growth in 24 hours.	MLD/ml. in 48 hours.
added	+++	1000-
not added	+++	1000

+++ = heavy growth

From Experiments such as the one reported indicated that mineral oil did not enhance growth and, perhaps, inhibited toxin production. The

practice of employing mineral oil to insure anaerobic conditions in the medium was discarded since it was felt that the presence of the meat or of substance in the medium itself afforded a sufficiently low oxidation-reduction potential for the growth of the organisms.

Comparison of B and Infusion Broth Media. Since toxin production on a medium less complex than an infusion broth medium was desired, the toxins formed by Cl. oedematiens grown on infusion broth and on B medium were compared. Infusion broth tubes were prepared as above. 40 ml. of B medium were distributed in pyrex tubes 200 x 25 mm. To each portion was added 1 gm. of solvent extracted beef heart. Both sets of tubes were inoculated deeply with 1 ml. of a 24 hr. culture and incubated 24-48 hours. At the end of this time, portions of the cultures were centrifuged. The centrifugate was diluted in saline and the dilutions were injected into mice to determine the MLD. The results are presented in Table V.

Table V.

Comparison of B and Infusion Broth Medium for Toxin Production.

Experiment #	Inoculum	MLD/ml.	
		BH	IB
1	IB	1500+	1000
2	BH	1000,1500	1000
3	BH	2000,2500	1000
4A	IB		500,1000
4B	BH	2500 +	

BH - B medium with solvent extracted beef heart
IB - Infusion broth with water extracted beef heart

B medium served as well as infusion broth for inocula, and better than infusion broth as a toxin production medium. Succeeding experiments in

the early portion of the experimental work were performed using B medium with solvent extracted beef heart.

Pigeon Passage. At first the culture was passed through pigeon regularly. The pigeon breast muscle was cleaned with alcoholic iodine solution. 0.1-0.2 ml. of the previous pigeon culture stored at 5°C was injected aseptically into the muscle. The pigeon died in 4-8 hours and remained at room temperature for 11-12 hours. At the end of this time, the entire breast region was cleared of feathers, the skin flooded with alcoholic iodine, and cut away using sterilized instruments. The skin was removed by making a longitudinal incision over the breast bone and horizontal incisions to the left and right above and below the site of injection. The exposed muscle was flooded with the iodine solution and the fascia and top muscle layer removed after making incisions in the same manner as for the removal of the skin. One or more small cubes of tissue were cut from the muscle so exposed and transferred immediately to B medium with solvent extracted beef heart and 0.6% glucose. The entire procedure was done as rapidly as possible and strict aseptic technique was used. The cultures were incubated for 24-48 hours after which they were stored at 5°C.

Despite the aseptic technique pigeon cultures often showed contamination. If a culture became contaminated, the contaminant was eliminated by heat shock. One ml. of the contaminated culture was transferred to B medium with solvent extracted beef heart and the culture was immediately heated in an 80° water bath for 1/2 hour. Heat shock did not lessen the production of toxin by any particular culture. Table VI.

Table VI.

Effect of Heat Shock on Toxin Production.

Conditions	MLD/ml.
Culture from inoculum before heating	3000
after heating	3500

Pigeon passage was finally eliminated as a routine procedure since during one of the periods of low toxin production, pigeon passage every other day did not restore the culture. It might be necessary to pass a culture through pigeons if it had been stored for a very long time, over three months, but some factor present in the medium seems more important to the maintenance of a high level of toxin production by any particular culture.

The basic procedure for toxin production in the early period of experimental work, may be summarized as follows. The culture was transferred every day or every other day. Forty ml. of B medium was placed in a pyrex tube. To this was added 0.75-1 gm. of solvent extracted beef heart. The tubes were autoclaved for 20 minutes at 12 pounds. If they were to be used immediately, the tubes were cooled to about 35°. If the tubes were to be used in succeeding days, they were stored at room temperature, heated 1/2 hour in a boiling water bath and cooled just before inoculation. After the tubes were cooled, 0.6% dextrin was added. The dextrin was a 25% suspension of Corn Products 150, sterilized for 20 minutes at 12 pounds. The 40 ml. tubes of B medium with solvent extracted beef heart and 0.6% dextrin were inoculated at the bottom with a minimum disturbance of the medium with 1.0 ml. of the previous day's culture. The cultures were incubated 24-48 hours at 37°C and used as inocula for toxin production, or tested themselves for potency of toxin in the culture.

Toxin was produced in 40 ml., and 3 l lots. The 40 ml. lots were prepared as above. The 1 and 3 l lots were made in Erlenmeyer or Florence flasks. For a one liter lot, 500 ml. portions of B medium were distributed in two one liter flasks. Twenty gms. of solvent extracted beef heart or kidney was added to one flask which would serve as the incubation vessel. These were autoclaved for twenty minutes at twelve pounds, cooled immediately in running water and poured together. The medium in the flask containing no meat was poured into that with meat while the mouths of both flasks were flamed. 0.6% of sterile dextrin was added and the flask inoculated at the bottom with 20 ml. of a twenty-four hour culture prepared as above.

Toxin production on B medium with solvent extracted beef heart ranged from 500-2500 MLD/ml. routinely. On B medium with solvent extracted beef kidney, the potency was greater ranging from 2500 to 4000 MLD/ml.

These yields continued for about 4 months, after which, the culture regularly produced toxin of 500 MLD/ml. or less. Ten serial passages of the cultures through pigeon breast muscle failed to restore the ability of the culture to produce high potency toxins.

During the four months period before the potency of the toxins decreased, experiments were performed to determine the optimum conditions for toxin production.

The optimum concentration of glucose and dextrin was tested with culture $N_{21}B$ grown in 40 ml. lots in B medium with solvent extracted beef heart and varying amounts of glucose or dextrin added as sterile solutions to the medium after autoclaving. The optimum for both carbohydrates was found to be between 0.5 and 1.0% (Table VII). Also, dextrin was

established as a better carbohydrate source than glucose for toxin production.

Table VII.

Optimum Concentration of Glucose and Dextrin for Toxin Production on BH Medium.

Carbohydrate %	MLD/ml. with Glucose	Dextrin
0	500 - 500	1000
0.1	1500	1500-2500
0.5	2000	2500
1.0	1500-	2000-2500

Indication was obtained from one experiment with washed and unwashed dextrin that the soluble materials washed out of the dextrin were important for toxin production. Duplicate toxin batches were set up with B medium and solvent extracted beef kidney. To one batch was added 0.6% dextrin washed* with distilled water, to the other batch 0.6% dextrin unwashed. After 48 hours incubation, MLD titrations were run. The batch with unwashed dextrin gave 2500 MLD, that with washed dextrin, less than 500 MLD.

The final pH of toxin cultures at the end of the incubation on B medium with solvent extracted beef heart or kidney with dextrin as the carbohydrate source was 6.8-7.2. with glucose as the carbohydrate the pH was lower, 6.0-6.2. It was thought that the fall in pH might be detrimental to the toxin production. If the medium were buffered so that the pH did not fall when glucose was the carbohydrate source, toxin production might equal or exceed that using dextrin. A 0.4 M solution

* Supplied by A.A. Tytell, Department of Biological Chemistry, University of Cincinnati Medical School.

of $Na_2HPO_4 \cdot 12\ H_2O$ was prepared, the pH of the solution being adjusted to 7.6-7.8 before diluting to final volume. A similar solution of Na_2CO_3 and a 2% solution of sodium glycerophosphate were prepared. These were added to the medium in final concentrations of 0.02 and 0.04 molar before autoclaving. The media tested with added buffer were B medium with 2.0 solvent extracted beef heart, B medium with 2.0% solvent extracted beef kidney, and B medium with 3% pancreatic digest of liver cake (percent solids). After autoclaving, 0.6% glucose was added to all tubes with one exception. 1% dextrin was added to this tube. The tubes were inoculated with 1 ml. of a 24 hour culture and incubated 24-48 hours at 37°C. Experiment I was performed with 10 ml. of media in pyrex tubes 120 x 15 mm. while experiments II and III were carried out with the usual 40 ml. amounts. (Table VIII).

The pH of the cultures with additional buffer was between 6.8 and 7.2 at the end of the incubation period. It may be seen from Table VIII that the presence of additional buffer enhanced the potency of the toxin produced in all media tested. Phosphate proved to be better in this capacity than carbonate or glycerophosphate. The optimum concentration for the additional phosphate appeared to be 0.02-0.04 M.

The nitrogen content of B medium is 1600 mgs./l. A lower nitrogen content was tested for toxin production. B medium was prepared containing 1000 mgs. N/l. and distributed in 10 ml. amounts in pyrex tubes, 160 x 15 mm. Various supplements were added, the tubes were autoclaved for 20 minutes at 12 pounds and cooled. 0.6% glucose was added and the tubes were inoculated at the bottom with a minimum disturbance of the medium with 0.25 ml. of a 24 hour culture on B medium

Table VIII.

Toxin Production with Additional Buffers on B medium with Various Supplements.

Experiment #	Medium	Buffer	Conc.	MLD/ml.
1	BH	phos.	0.02 M	1500 + , 2500
	BH	phos.	0.04 M	2500
	BH	phos.	0.06 M	2500 - 3500
2	BPDLC	phos.	0.02 M	1500
	BPDLC	phos.	0.04 M	1500 + , 2500
	BH	phos.	0.02 M	1500 + , 2000
	BH	phos.	0.04 M	3500
3	BK	phos.	0.02 M	3000 - 4000
	BK	phos.	0.04 M	5000
	BK	phos.	0.02M	
		car.	0.02 M	4000
	BK	phos.	0.02 M	
		glyph.	0.5 M	3000 - 4000
	BH	phos.	0.02 M	1500
	BH	phos.	0.04 M	2500
(dextrin)	BH	phos.	0.02 M	2500

The phosphate concentration of B medium is 0.02 M.
BH - B medium with solvent extracted beef heart.
BK - B medium with solvent extracted beef kidney.
BPDLC - B medium with pancreatic digest of liver cake.
phos. - phosphate
car. - carbonate
glyph.- glycerophosphate

with solvent extracted beef heart and 0.6% glucose. The media tested with low nitrogen content medium were B medium with 2.5% solvent extracted beef heart, with 2.5% lipid free yolk* of egg and with 2 and 3% pancreatic digest of liver cake. Table IX.

It may be seen from the results presented in Table IX., that under the conditions of the experiment B medium with 1600 mgs. N/ml. gave a yield of slightly higher potency than B medium with 1000 mgs. N/ml. with the supplements tested.

* Kindly supplied by C.B. Giddings.

Table IX.

Toxin Production on Various Supplements in B Medium with 1600 and 1000 mgs. N/ml.

Experiment #	Medium	Mgs. N/l	MLD/ml.
1	BH	1000	1500-
		1600	1500-
	BE	1000	500+, 1000-
		1600	1500 , 2500-
2	BH	1000	1500-
		1600	1500+ ,2500-
	BPDLC	1000	1500-
		1600	1500

BH - B medium with solvent extracted beef heart.
BE - B medium with lipid free egg yolk
BPDLC - B medium with pancreatic digest of liver cake.

Although the iron requirements of Cl. oedematiens have not been thoroughly investigated, there are indications that there may be a concentration of iron which is optimum for toxin production(35) A pancreatic digest of liver cake added to B medium in a concentration of 3% solids gave a toxin containing 1500 MLD/ml. The same digest deferrated and mixed with B medium at the same concentration of solids gave a yield of over 2500 MLD. These two digests were tested in the same run which makes the comparison just. However, B medium which contains about 32 ug of iron per l. gives a toxin of no higher potency when it is deferrated.

Lipid free egg yolk proved to be an excellent supplement for B medium, Table XI. Since the toxins made were to be used in immunological experiments, it was felt that the introduction of a substance as complex and as harmful to the mammalian system as egg yolk was a partial defeat of one of the purposes of the work, this promising lead was dropped.

Table X.

Toxin Production on Lipid Free Egg Yolk in B Medium.

Experiment #	Medium	MLD/ml.
1	BH	500 +, 1000 -
	BE	1500 +, 2500 -
2	BH	1500 +, 2500 -
	BE	2500 +

Containers for Toxin Production Cultures. Toxin production was tested on B medium with solvent extracted kidney and 0.6% dextrin distributed in Erlenmeyer, Florence and Kjeldahl flasks. In the first two, were 1 l . lots, while the Kjeldahl flask contained 320 ml., sufficient to bring the medium up into the neck of the flask. Although the exposed portion of media in the liter Erlenmeyer and Florence flasks was small, (the medium came up into the narrow part of the flasks), the area was still larger than in the case of the Kjeldahl flask. It was thought that cutting down the exposure of the medium to oxygen might enhance toxin production. There was no increase in the potency of toxins produced in a Kjeldahl flask, over those produced in either an Erlenmeyer or a Florence flask.

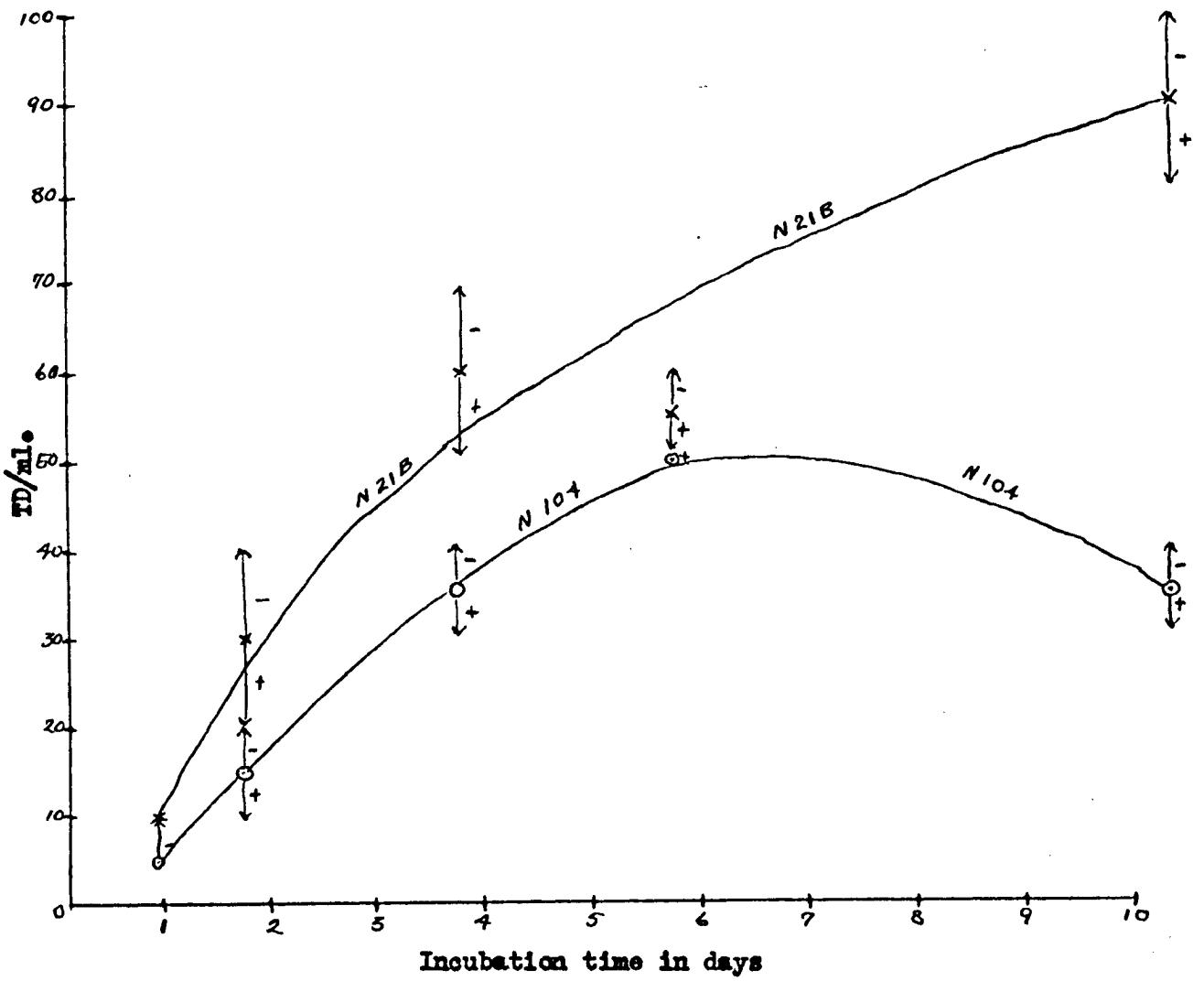
When the potency of the toxins produced decreased, an experiment to determine the optimum incubation time for toxin production was run. An experiment conducted at the beginning of the problem had indicated that 24-48 hours was the optimum incubation time for cultures grown on B medium with solvent extracted beef heart and 0.6% dextrin. After 4 months culture on this medium it was felt that the organism might have become adapted to the medium in such a way as to change the optimum time requirement.

Accordingly, B medium was distributed in 40 ml. portions. To each portion was added 1 gm. of solvent extracted beef kidney. The tubes were autoclaved for 20 minutes at 12 pounds, cooled, and inoculated at the bottom with 1 ml. of a 26 hour culture of $N_{21}B$ on B medium with solvent extracted beef heart and 0.6% dextrin. A similar set of tubes was prepared and inoculated with N104 carried on the same medium as N21B. The two sets of tubes were incubated at 37°C. At various intervals after the beginning of incubation, two tubes of each strain were removed. Portions of the four cultures were centrifuged and the test dose titre of each determined. Fig. 1. As may be seen on examination of the chart, culture N104 showed a definite optimum at 5 days incubation time, while N21B seemed to increase consistently up to 10 days incubation time. This is in contrast to reports of Dr. Danielson,(5) who finds that the optimum incubation time for toxin production with N21B is 5 days. The medium and procedure were different in the latter case which may account for the variation. Another factor in the experiment reported above was that the test on the tenth day was done on only one tube. In such a case there is possibility of error.

It was shown very definitely by the above work that the culture had changed during four months of sub-culturing on B medium with solvent extracted beef heart. Here-in lies a suggestion for future study and a possibility which must be reckoned with in practical production of consistently high toxic filtrates. What is the inadequacy of the medium which causes this drop in productive powers of the organism? If the factor in the medium or technique cannot be elaborated, would it be a good practice to keep a storage culture on infusion broth medium

Fig. I

THE EFFECT OF INCUBATION TIME ON TOXIN PRODUCTION
N104 N21B



with water extracted beef heart, transferred every 4-6 months and stored in the cold, to which recourse could be made in case of failure of the particular culture in use?

Toxin Production - Later Work.

During the second experimental period on the study of toxin production the procedure given on page 17 was altered. The culture was stored for a period before use,(41) the medium for inocula was changed, and the use of B medium with pancreatic digest of kidney as a medium for the production of toxin was investigated. In the following pages, the routine procedure used during this second period will be described first. Subsequently, the experimental details which led to the establishment of the routine procedure and others which were elaborated too late in the work to be incorporated in the routine method.will be discussed.

The medium for storage cultures was the usual Bmedium distributed in 40 ml. amounts with 2% solvent extracted beef heart, autoclaved 20 minutes at 12 pounds and cooled to about 35°C. As before, if the tubes were not used immediately after sterilizing, they were stored at room temperature and heated for 1/2 hour in a boiling water bath and immediately cooled just before inoculation.

Storage cultures were transferred every week or every two weeks on the above medium. 1 ml. of a sterile 25% solution of dextrin was added to 40 ml. of medium (final concentration of dextrin 0.6%). The tubes containing the carbohydrate were inoculated at the bottom with a minimum disturbance of the solution with 1 ml. of the previous week's culture. The cultures were incubated 24-48 hours at 37°C and stored at room temperature for at least three weeks. In case of contamination which

occurred frequently, the new storage culture was heat shocked as described on page16 and incubated the full 48 hours.

Storage cultures served as seed for inocula cultures which in turn served as seed for toxin production lots. Inocula cultures were prepared on B medium distributed in 40 ml. portions, each portion containing 2% solvent extracted beef kidney. To each tube, before autoclaving was added 0.4 ml. of the solution containing 10% each of cystéine, as the hydrochloride, and of ascorbic acid. The tubes were sterilized and cooled. Immediately after cooling, 0.4 ml. of a sterile 10% solution of glucose was added to each 40 ml. portion of medium (final concentration of glucose 0.1%). The tubes were then inoculated at the bottom with 1 ml. of a storage culture at least 3 weeks old, and incubated 24 hours at 37°C.

Such cultures were used at the end of the 24 hour incubation period as inocula for toxin production media. In this experimental period, the media tested for toxin production were B medium with solvent extracted beef kidney, B medium with pancreatic digest of beef kidny, B medium with pancreatic digest of liver cake, and salts supplement with pancreatic digest of liver cake. Only a few experiments were performed using the two latter media, consequently, only the kidney media will be discussed at present.

The preparation of the media containing B medium and solvent extracted beef kidney is the same as that with B medium and solvent extracted beef heart described in the study of the first experimental period on toxin production, page 15 . The medium for 250, 500, 1000, and 3000 ml. lots was divided into two portions for sterilization. One portion contained the kidney , added to a final concentration of 2%. Sterilization was at 12 pounds for 20 minutes, with the time increased to 30 minutes for 3 l lots. All batches were cooled immediately after auto-

claving to about 35°C. The portion containing no meat was poured into that with meat, and sterile 25% dextrin was added to a final concentration of 0.6%.

B medium with pancreatic digest of kidney was usually prepared in 250 ml. lots. To the B medium was added the pancreatic digest of beef kidney in an amount sufficient to give a final concentration of 0.5% solids of beef kidney digest. The medium was divided into two portions, autoclaved and cooled as above. After the cooled portions were poured together, sterile 10% glucose was added to a final concentration of 0.1%.

Inoculation and Incubation for toxin production lots was the same for both media. Lots were inoculated shallowly (inoculum poured or pipetted to top of medium) with a 24 hour inoculum culture, 1 ml. of culture to 100 ml. of medium. Incubation was from 4-5 days at 37°C.

The yield on B medium with solvent extracted beef kidney and dextrin was between 100 and 200 TD/ml. Of 14 filtrates selected at random from the experimental work of the second period, 5 filtrates contained over 200 TD/ml., 5 between 150 and 200 TD/ml. and 4 over 100 TD/ml. Two of the later cultures were tested at 100 and 200 TD/ml., while the other two were tested at 100 and 150 TD/ml. Very few MLD titrations were performed during this period, but the few values which were determined lay between 5 and 20, 000 MLD/ml. with most of the toxins falling in the lower range. The MLD value obtained on any particular filtrate is very dependent on the method of dilution.(5) The determination of MLD is of little value since great variation are encountered which are independent of the medium used. Little work was done in this experimental period on MLD titrations.

The yield on B medium with pancreatic digest of beef kidney was more variable than that on B medium-solvent extracted kidney, but often equaled and sometimes surpassed the latter. The purpose of the former medium was to develop a completely liquid medium which would lend itself more thoroughly to complete analysis and better control than the medium which contained some solid material not in solution or suspension. Such a medium has been developed employing pancreatic digest of liver cake.[41] The availability of the liver cake is limited, and it was thought desirable to develop a medium the constituents of which might be more attainable.

In the establishment of the routine described above, three factors in the storage of the culture were important, time of storage, temperature of storage and medium for storage.

The effect of storage time on toxin production may be seen by comparison of filtrates prepared on B medium with solvent extracted beef kidney and 0.6% dextrin. The data presented in Table XI were obtained on such filtrates. Storage cultures were prepared on 40 ml. portions of B medium with 2% solvent extracted beef heart and 0.6% dextrin. After 24 hours at 37°C, the cultures were stored at room temperature. After a certain storage time 1 ml. of these cultures was inoculated into a 40 ml. portion of B medium with 2% solvent extracted beef kidney, 0.1% cysteine. HCL, 0.1% ascorbic acid and 0.1% glucose. Inocula cultures so prepared were incubated about 24 hours at 37°, and used immediately for toxin production lots. Toxin was produced in 250 ml. lots, except for experiment #7 where production was on 40 mls. of medium, on B medium with 2% solvent extracted beef kidney and 0.6% dextrin. The toxin cultures were incubated 4-5 days at 37°C and tested immediately after harvesting.

Table XI.

Time of Storage of Culture and Toxin Production

Experiment #	Length of storage.	TD/ml.
1	2 mos.	240
2	3 wks.	240 +, 280-
3	4 wks.	100 +, 200-
4	4 wks.	150 +, 175-
5	10 wks.	50 *
6	9 wks.	100
	7 wks.	100 +, 200-
7	4 wks.	200 +
	2 wks.	200 +
	5 days	100 +, 150-
8	1 mo.	150 +, 160-
	1 mo.	150 +

* In this case incubation was at 52° for about 12 hours of the third day. This may account for the low titre obtained.

To determine the effect of the temperature at which the cultures were stored, cultures on B medium with solvent extracted beef heart and 0.6% dextrin, and on B medium with solvent extracted beef heart, cysteine, ascorbic acid and 0.1% glucose were stored at room temperature and in the cold room (5°C). After 10 weeks storage, these cultures were transferred to the usual inoculum culture medium, B, kidney, cysteine, ascorbic acid and glucose, and incubated for 24 hours at 37°C. After the incubation period, these inocula cultures were seeded into toxin production media, incubated 4-5 days and tested for potency, Table XII.

From Table XII it is apparent that there is little difference between toxin production with storage cultures stored at room temperaturesand with those stored at 5°C. The procedure of storing the

Table XII.

Temperature of Storage of Culture and Toxin Production.

Culture stored at	TD/ml.
rm. temp.	50
5°C	50 +, 100-
rm. temp.	50-
5°C	50-

cultures at room temperature was adopted as routine as a matter of convenience.

The medium for storage cultures used routinely was B medium with solvent extracted kidney and dextrin. This was compared with a medium consisting of B medium, solvent extracted kidney, cysteine, ascorbic acid and glucose,. Cultures which had been stored for the same length of time on these two media were transferred to the latter medium and incubated 24 hours at 37°C. These were inocula cultures for toxin production media. In any one experiment, the toxin batches were incubated the same length of time and tested concurrently.

From the data presented in Table XII, it may be seen that with one exception, BH medium with heart was a better storage medium than B, kidney, cysteine, ascorbic acid, i.e., toxins produced on either of the two toxin media from the former storage cultures were better than toxins so produced from the latter. The exception is in experiment #1 where toxin production from storage cultures on B-heart is only slightly better than those on B-kidney. In this case, the storage cultures were two months old, while in succeeding experiments the storage cultures were slightly younger. Whether this factor is important or not, it is still true that B medium with solvent extracted beef heart and dextrin

proved to be the best of the two media studied for storing the culture.

Table XIII.

Effect of Storage Culture Media on Toxin Production.

Storage Culture	TD/ml BH	BKCA
Experiment #		
1	240	200
2	50	50-
3	50 +, 100-	50-
4	100	50 +, 100-
5	100 +, 200-	50
6	100 +, 200-	50
7	200 +	50-
8	200 +	50-
9	100+ , 150-	50-

BH - B medium with 2% solvent extracted beef heart and 0.6% dextrin.
BKCA- B medium with 2% solvent extracted beef kidney, 0.1% cysteine, ascorbic acid, and glucose.

As inoculum medium, B, kidney, cysteine, ascorbic acid and glucose was used routinely. There were indications that the cysteine and ascorbic acid were not essential to production of high potency toxins, but experimental work on this point was too limited to define it accurately.

Toxin production on B medium with pancreatic digest of beef kidney compared favorably with that on B medium with solvent extracted kidney. The optimum concentration of solids, and the effect of the presence of dextrin, maltose, glucose, and glucose with additional phosphate were studied for one kidney digest, Table XIV. In some experiments reported, a toxin lot on B medium with solvent extracted kidney was run at the same time. This served as a control on the toxin producing ability of the culture, and as a means of comparison. In all

experiments reported in Table XIV, storage cultures were grown on B-heart-dextrin medium, inocula cultures on B-kidney-cysteine-ascorbic acid-glucose medium. Incubation, as usual, was at 37°C for 4-5 days, the average time being 116 hours.

Table XIV.

Toxin Production on B Medium with Pancreatic Digest of Kidney.

Run #	Medium	% PDK	Carbohydrate	% con.	TD/ml.
1	BPDK	0.1	dextrin	0.6	50⁻
	BPDK	0.5	dextrin	0.6	100 +, 150⁻
	BPDK	1.0	dextrin	0.6	100 +, 150⁻
2	BPDK	0.5	glucose	0.1	150 +, 200⁻
			dextrin	0.6	100
			maltose	0.1	100 +, 150⁻
	BK	—	dextrin	0.6	150 +, 175⁻
3	BPDK	0.5	glucose	0.1	50 +, 100⁻
	BK	—	dextrin	0.6	50
4	BPDK	0.5	glucose	0.1	100 +, 200⁻
	BK	—	dextrin	0.6	100 +, 200⁻
5	BPDK	0.5	glucose	0.1	50
	BK	—	dextrin	0.6	50
6	BPDK	0.5	glucose	0.1	50 +, 100⁻
	BK	—	dextrin	0.6	100 +, 150⁻
7	BPDK	0.5	glucose	0.1	50⁻
			dextrin	0.6	50 +, 100⁻
	BPDK phos.	0.5 0.04 M*	glucose	0.5	200 +, 240⁻

* Phosphate added as 0.2M solution to a final concentration of 0.04 M additional phosphate before sterilizing.
BPDK - B medium with pancreatic digest of beef kidney.
BK - B medium with solvent extracted beef kidney.

The optimum concentration for the addition of pancreatic digest of beef kidney appeared to be between 0.5 and 1.0%, Run 1, Table XIV.

Glucose and maltose at 0.1% and dextrin at 0.6% served equally well as the carbohydrate source. The addition of 0.04 M phosphate to the medium as in the early work, page 19 , enhanced the production of toxin considerably.

Three toxin lots were produced with pancreatic digest of liver cake at 3-4% solids in the salts supplement, page 13 . The cultures were prepared with storage and inocula cultures prepared as above. The toxins produced contained 150-200 TD/ml.

Pancreatic digests of gluten, beef heart, liver and hog kidney, and a papain digest of casein,[19] mixed in concentrations of 2-4% with the salts supplement gave toxins of very low potency, less than 50 TD/ml.

111.

General Properties of Lethal Toxin Filtrates.

Among the properties of the lethal toxin which have been studied are the ratio of combining power, TD, to MLD, the stability with respect to MLD, the filtrability of toxic preparations and the reaction of toxic filtrates to precipitation with ammonium sulfate, and glycerol dialysis.

Ratio of MLD to TD.

As has been described in the previous section, the lethal powers of the toxin of Cl. oedematiens may be measured in two ways in vitro; by determination of the MLD and by determination of the number of test doses of antitoxin with which a definite portion of the toxin will react. The values of the latter determination are expressed in test doses per ml. of toxin. The ratio of MLD/TD of toxic filtrates is variable.

In Table XV are presented data on several toxic preparations for which both the MLD and TD values were obtained. The toxins listed were incubated for various times, from 1-6 days. The dilutions for the MLD determinations were made in saline, in 2% proteose-peptone in saline, and in 4% pancreatic digest of beef heart. However, even within a limited set of conditions there seems to be no constancy of the ratio, MLD/TD. It would seem, therefore, that the lack of correlation cannot be attributed to the variation either in length of time of incubation of the toxic preparations, or in the medium used for dilution of the toxins for determination of the minimum lethal dose.

Table XV.

Comparison of MLD and TD of Toxic Preparations.

Toxin #	Inc. Time	MLD/ml. x 10^{-3}	TD/ml.	MLD/TD
Dilution in saline				
13	2 days	1.5	40 +, 50-	33
10	1 day	2.5	20 +, 30-	100
41	5 days	5	50 +, 100-	50 - 100
100	4- 5 days	5	150 +, 200-	25 - 33
49	4- 5 days	5 +, 10-	240	21 - 42
50	4- 5 days	5 +, 10-	200	25 - 50
48	4- 5 days	10	200 +, 240-	45
51	4- 5 days	1	20 +, 60-	25
Dilution in proteose-peptone in saline				
102 A	1 day	2 - 3	50	40 - 60
77	4-5 days	5	100 +, 150-	33 - 50
79	4-5 days	5	50 +, 100-	50 -100
102 B	2 days	7.5	100 +, 120-	64
91	4-5 days	10	100 +, 200-	50 -100
74	4-5 days	10 +	240 +, 280-	39
76	4-5 days	10 +	100 +, 150-	73 -100
95 C	3 days	10 +	50 +, 100-	100 -200
Dilution in Pancreatic Digest of beef heart.				
110	4-5 days	5	50 +, 100-	50 -100
111	4-5 days	5	100	50
128	4-5 days	10	50 +, 100-	100 -200
130	4-5 days	10	100	100
101	5-6 days	20	150 +, 200-	25 - 33

Where the determination of MLD or TD did not give an end-point, i.e. where there were two values for any one determination, the ratio was calculated as lying within the range determined for either extreme. Where the test dose range was within 40TD , the average of the two values was used for calculating the ratio.

This ratio will be discussed further in connection with toxins precipitated with ammonium sulfate.

Stability of Toxic Filtrates.

The stability of the lethal toxin of Cl. oedematiens upon storage in the cold has been tested by comparison with the MLD titre

obtained immediately after harvesting with that obtained some time later. It has been found, Table XVl, that the MLD titre decreases rapidly during the first 3 - 6 days of storage to a relatively stable minimum value.

Table XVl.

Decrease in MLD Titre on Storage at 5°C.

Toxin #	Original MLD/ml.	Storage at 5°C	After Storage MLD/ml
116	2500 +	3 days	500
126	4000 +	2 days	1500
159	2500	9 days	1000
		16 days	1000
240	1500	6 days	500

A toxic filtrate was mixed with several reducing substances and with glycerol, to determine the effects of such materials on the stability of the toxin. 1 M solutions of ascorbic acid, cysteine • HCl, orcihol and sodium thioglycolate were prepared. The pH of each solution was adjusted to about 7.2 before the solutions were diluted to final volume. Hydroquinone was also tested, but because of the low solubility of this material, it was weighed and transferred to sterile tubes. Glycerol was added to the toxin. 25 ml. portions of a sterile toxic filtrate were transferred aseptically to labeled tubes. The reducing materials, not sterilized, were added using aseptic precautions, in various amounts to the portions of the filtrate. The toxin was tested immediately after filtering, and all solutions were tested after nine days storage in the cold room. Those solutions in which the MLD titre was still up at 9 days were retested at 16 days, Table XVll.

Table XVII.

Effect of Reducing Substances and Glycerol on the Stability of Toxic Filtrates

Materials added	Concentration Molar	MLD/ml. in 9 days	MLD/ml. in 16 days
—	—	1000	1000
ascorbic acid	.001	500 ≠ ,1500	
	.005	500 ≠ ,1500	
	.05	500-	
cysteine	.001	1500 ≠, 2500-	
	.005	2500	1500
	.05	500 ≠, 1500	
orcinal	.001	500	
	.005	1500	
	.05	500 ≠,1500-	
	.1	500-	
hydroquinone	.005	500	
	.05	500	
	.1	500-	
Na thiglycolate	.001	500	
	.005	1500	
	.05	2500	1500
glycerol	1.1	1500 ≠, 2500	

MLD/ml. immediately after filtering 2500

It may seem from Table XVII that only two of the substances tested maintained the MLD titre for a period of 9 days. Cysteine at .005 M and sodium thioglycolate at .05 M maintained toxicity of the filtrate for 9 days, but had little effect upon the toxicity over a period of 16 days. The remaining substances at the concentrations tested did not maintain the MLD titre and some showed inhibition of the toxic effect.

The pH at which the toxin was stored also affected the

stability. Walbum (40)found that with a toxin prepared on veal broth with peptone, the pH optimum for storage of 18 hours at 37?C was 6-7. In the present work, toxins have been adjusted to various pH and stored in the cold room. The toxins were tested after a period of 2-6 days, Table XVIII. Although only one of the filtrates (third filtrate in Run #2, Table XVII) retained the original MLD titre, the optimum pH was shown to be between 6.0 and 6.5.

Table XVIII

Effect of pH on Stability of Toxins Stored at 5 C

Run #	Original MLD/ml. x 10^{-3}	pH	MLD/ml. x 10^{-3} Value	After
1	3.0	6.9	2.5	6 days
		6.4	1. 5-2.5	
		6.1	1. 5-2.5	
		5.6	1.5	
2	5 + 10-	7.0	5	2 days
	10	6.7	5-	
	5 +.10-	6.3	5 +.7.5-	

Filtration of Toxin Preparations.

The toxin was found to be filtrable through Seitz filter pads, Jena glass filter and Berkfeld filter candles. The later method was adopted as the routine method of sterilizing toxic preparations. Preliminary clearing of cultures was first obtained by centrifuging the cultures in 500 ml. cups in a No. 2 centrifuge for 1 - 2 hours at 5 ^{0}C. Later, it was found that the toxin was not adsorbed by Super Cel (Johns Manville Corp). In addition, passage of

the culture through a Sharples centrifuge did not reduce the toxicity. Both these methods were used for preliminary clearing.

For final filtration, a single experiment was performed with a Jena glass filter. The culture was first cleared by centrifuging. 150 ml. of the centrifugate was filtered, the first 100 ml. being discarded. The remaining 50 ml. passed through the filter very slowly. A toxin so filtered showed no loss in toxicity as measured by the MLD value, Table XIX. Since the process was very slow, it was not investigated any further.

Two experiments were performed with Seitz filters. In both cases, the culture was first cleared by centrifuging. In the first trial, 150 ml. were filtered, the first 100 ml. being discarded. This filtrate showed no loss in toxicity. In the second trial, only 100 ml. were filtered and the filtrate showed complete loss of activity, Table XIX. Since the recovery of toxicity apparently depended on the amount filtered this procedure was also discarded.

With a Berkfeld N filter, loss was encountered in only three trials out of thirteen. Filtration is the same between pH 6.6 and 7.4. In many filtrations, the MLD titre of the filtered material was higher than that of the material before filtration, Table XIX. Determinations of the MLD before filtration were made on 10 ml. portions centrifuged 1 hour, In 15 ml. cups in a #2 centrifuge. This centrifugation was in addition to the preliminary clearing. There may have been some loss of activity during this period of centrifugation which would account for the apparent increase in toxicity after Berkfeld filtration. Centrifuging was the only method available for comparison of filtration results.

Table XIX.

Filtration of Toxins

Run #	Clearing		MLD/ml. x 10^{-3}
	Preliminary	Final	
1	centrifuge	centrifuge	2.5 +, 3.5 -
		Jena glass	2.5 +, 3.5 -
		Seitz	2.5 +, 3.5 -
2	centrifuge	centrifuge	2.0 +, 3.0 -
		Seitz	1.0 -
3	centrifuge	centrifuge	2.5 +, 3.5 -
		Berkfeld N	1.5
4	centrifuge	centrifuge	3.0
		Berkfeld N	1.0
5	centrifuge	centrifuge	2.0 -
		Berkfeld N	3.5 +
6	centrifuge	centrifuge	3.5 +
		Berkfeld N	2.5 -
7		centrifuge (2 hrs.)	2.0 +
	Sharples	centrifuge (1 hr.)	2.0 +
		Berkfeld N	2.0 +, 2.5 -
8		centrifuge (2 hrs.)	1.0 +, 2.0 -
	Sharples	centrifuge (1 hr.)	1.0 +, 2.0 -
		Berkfeld N	2.5
9		centrifuge (2 hrs.)	1.0
	Sharples	centrifuge (1 hr.)	2.0
		Berkfeld N	3.0
10 pH 6.8	centrifuge	centrifuge	0.5 +, 1.5 -
		Berkfeld N	1.5 +, 2.5 -
7.2	centrifuge	centrifuge	1.5
		Berkfeld N	2.5
	Super Cel	centrifuge	2.5 +
		Berkfeld N	2.5
11	centrifuge	centrifuge	1.0 +, 3.0 -
		Berkfeld N	2.0
	Super Cel	centrifuge	2.0 +, 3.0 -
		Berkfeld N	2.0
12		centrifuge (2 hrs.)	0.5 +, 1.5 -
	Super Cel	centrifuge (1 hr.)	1.5 +
		Berkfeld N	0.5 +, 1.5 -

Ammonium Sulfate Precipitation of Lethal Toxin.

The lethal toxin of Cl. oedematiens was precipitated with ammonium sulfate. Berkfeld filtered toxins were treated with ammonium sulfate, 750 gms./l(2). The ammonium sulfate was usually added to the filtrate immediately after filtration, i.e., with the filtrate at room temperature. The precipitate rose to the surface of the fluid and as the process was carried out in a container with a large diameter at the top, the precipitate was easily skimmed from the top of the fluid. Excess fluid was drawn from the precipitate by suction filtration, and the precipitate thoroughly dried in vacuo over phosphorous pentoxide. The dried toxin was stored in this manner at 5°C.

The percent recovery of the toxin by this precipitation process was determined by dissolving the toxin in a suitable medium, to be discussed below, page 42 , and titrating for MLD or LD_{50} and TD values, Table XX. For toxin #2 in Table XX the LD_{50} value was used for determination of percent recovery since this was the only value obtained on the original filtrate. Recovery is usually about 25% with toxins containing over 70 TD/ml., toxins # 22A, 22B, and 101.

Table XX.

Percent Recovery on Precipitation of Toxins with ammonium Sulfate

Toxin #	Original Titre	% Recovery
2	1500 LD_{50}/ml.	11.9
22 A	70 +, 80- TD/ml.	32
22 B		21
45	160 +, 180 - TD/ml.	71
101	150 TD/ml.	23

All toxins were prepared on B, kidney, dextrin.

The recovery on toxin #45 in the above table was unduly good. The reason for this cannot be discovered by studying the procedure for preparation of the precipitate, since the process did not differ from that used in preparation of the other toxins.

The solubility of the ammonium sulfate precipitated toxins was very poor in saline (0.85%). It was noted that on addition of a small amount of alkali to a saline solution of the toxin, which was quite acid, the solubility appeared to improve. For some of the experimental work described in Chapter V , it was desired to adjust the pH within rather narrow limits. This was very difficult in the saline solutions. Phosphate buffer was tested and found to be an excellent medium for dissolving the toxin. The buffer used was 0.158 M phosphate, prepared for hemolysin tests, page 87 . Toxins were readily soluble in this buffer mixture from pH6.5-7.4. A weighed quantity of the ammonium sulfate precipitated material was dissolved in 1/2 the final volume with 0.158 M phosphate buffer. When the solid material was dissolved, the volume was made up with saline. The final concentration of phosphate in the mixture was 0.076 molar.

Testing. Ammonium sulfate precipitated toxins with the exception of two preparations were usually dissolved at 10 mg/ml. Toxins #13 and 45 proved to be active enough to dissolve at 5 mg/ml., while a reasonable titration could be obtained with #45 at only 1 mg./ml. Solutions of the two toxins at the various concentrations were compared for MLD or LD_{50} and TD values.

It may be seen from Table XXI that there was about 50% loss in MLD when toxin #13 was diluted at 5 mg/ml. and compared with

Table XXI

Concentration of Solution of Ammonium Sulfate Precipitated Toxins Compared with LD and TD

Toxin #	Concentration mg./ml.	MLD or LD_{50}/ml. x 10^{-3}	TD/ml.
13	10	12 +, 13-	162 +
	5	3 +, 4-	100 +, 120-
45	10	20 +, 30-	1000 +, 1200-
	5	10	400 +
	1	2.5	100 +, 120-

the dilution at 10 mg./ml. There was no loss shown with toxin #45 at any dilution.

The ratio of LD/T_d of the ammonium sulfate precipitated toxins varys from 20 to 80. This range was slightly lower than that reported by Bengston (2) where the "number of minimal lethal doses per 'test dose'" varied from 25 to 100 on toxins received from various countries. As may be seen on examination of Table XXII, the ratio is highest for toxins prepared from cultures grown only 24 hours , toxins 1 and 2.

Table XXII.

The Ratio of LD-Td of Ammonium Sulfate Precipitated Toxins.

Toxin #	Hrs. Culture Incubation	LD/mg.	TD/mg.	LD/TD
1	24	150 +, 175 -	2	75-88
2	24	150	3+,3.5-.5	42-50
9	45	200	6.5+,7.0-	30
13	42	600 +, 800-	22	27-36
22A	98	400	18	19
22B	98	800 +,1000-	48	16-21
45	116	2500	100 +,120-	21-25

Glycerol Dialysis of Toxic Filtrates.

Preparation and Testing. Two toxic filtrates were dialyzed against glycerol after Berkfeld filtration. Toxin NG1 was prepared from a culture grown on B medium with solvent extracted beef kidney and dextrin. The culture was filtered with Super Cel on the fifth day of incubation. Three days later, the Super Cel filtrate which had been stored at 5°C was filtered through a Berkfeld N candle. The filtrate as tested after passage through the Berkfeld contained 150 +t 175- TD/ml. After Berkfeld filtration, 150 ml. of the toxin was placed in a dialysis bag prepared from sausage casing. The material was then placed in glycerol and dialyzed for 24 hours, at room temperature. At the end of this time the dialysis bag contained about 25 ml. of fluid of thick consistency. This material, NG1 was stored at 5°C and tested at various intervals for LD and Td titre, Table XXIII.

NG 3 was prepared in much the same way as NG1 except that Berkfeld filtration and dialysis were carried out immediately after the toxin was harvested, and dialysis was performed in the cold room, 5°C. The culture was grown on B-kidney-dextrin medium for 4-5 days. At the end of the incubation period, the culture was cleared with Super Cel and filtered through a Berkfeld N candle. 173 ml. of the filtrate was tied in a dialysis bag of sausage casing and the whole immersed in glycerine, previously cooled to 5°C, and placed at 5°C for 24 hours. At the end of this period, the 35 ml. of fairly viscous fluid contained in the dialysis bag was removed, tested for MLD or LD_{50} and T_d and stored at 5°C. The material, NG3, was retested at various intervals, Table XXIII.

Table XXIII

Glycerol Dialyzed Toxins

Toxin	Interval after dialysis	MLD/ml. x 10^{-3}	TD/ml.
NG1	1 day	10/	400/, 500-
	4 days	8/, 10-	400/, 420-
	51 days	6-	380
	57 days	4	
NG3	1 hr.	40/	450/, 600-
	2 days	15/, 20-	450, 500
	6 days	15	500/, 550-
	26 days	20	550
	31 days	20/, 30-	500

The toxin prepared at room temperature, NG1, was concentrated about 6 tiies. About 50% of the combining power measured as TD was lost in the process. The MLD value, as seen in Table XXIII, decreased from over 10,000/ ml. to 4000/ml. in 57 days with some decrease being shown between the first and the fourth day of storage. The TD value could not be siad to have decreased between the first and the fourth day of storage and decreased 20-4- TD only during 51 days of storage.

The glycerol dialyzed toxin prepared at 5°C was concentrated about 5 times and showed practically no loss either of toxicity, measured as MLD/ml. within the first hour after removal from the dialysis bag, or of combining power, measured as TD/ml. The LD value decreased sharply between the first hour and the second day, but decreased not at all after that up to 31 days of storage at 5°. The TD value did not decrease during 31 days storage (Table XXIII).

IV.

Immunological Properties of Cl. Oedematiens Toxin.

The toxin of Cl. oedematiens is antigenic. Toxic filtrates treated with formaldehyde lose toxicity for the animal body but remain antigenic. Such materials, called toxoids, have been used in the active immunization of sheep(26) mice, rabbits, guinea pigs(5), and humans(37). In this work, report is made of the uses of toxoids in active immunization of guinea pigs, mice, rabbits, pigeons, and dogs. The work on dogs was done in collaboration with Dr. Andrew H. Dowdy, Strong Memorial Hospital, University of Rochester Medical College.

Preparation of Toxoids.

Toxoids were prepared from filtered cultures grown on various media. Some of the cultures were filtered through a Berkfeld N candle after preliminary clearing with centrifugation or with Super Cel. It was found, however, that toxoids prepared from cultures merely cleared with Super Cel were non-lethal for the animals tested, so in many cases, Berkfeld filtration was omitted from the procedure.

The filtrates were toxoided by the addition of 0.3% by volume commercial formaldehyde (40% formalin). After a time of storage with formaldehyde, the filtrates were found to pass the "safety test". A "safety test" was made by injecting each of 5 mice subcutaneously with 1.0 ml. of the toxoid. If the mice lived for the observation period of 5 days, the toxoid was considered ready to

be used for immunological experiments. Preparations stored at 5°C were detoxified in 2-3 weeks, while those incubated at 37° for 24-48 hours passed the safety test within a week.

The toxoids prepared in this manner and used in the experimental work below are listed in Table XXiV for purposes of reference.

Table XXiV.

Toxoids of Cl. Oedematiens Used in Immunological Experiments

Toxoid #	TD/ml.*	Culture Medium
1	20*	BH with 0.6% dextrin
2	45 +, 60-	BK with 0.6% dextrin
3	40	BH with 0.6% dextrin
4	90	BK with 0.6% dextrin
5	100 +, 150-	PDLC, 5% with salts supplement, 0.1% agar and 0.1% glucose
6	80 +, 100-	PDLC, 5% with salts supplement, 0.1% agar and 0.1% glucose
7	60 +, 80-	PDLC, 5%, with salts supplement and 0.1% glucose
8	50 +, 75-	PDLC, 5%, with salts supplement, 0.1% agar and 0.1% glucose
9	50 +, 75-	PDBH, 3.7%, with salts supplement, 0.1% agar and 0.1% glucose
10	25 +, 50-	PDBH, 3.7%, with salts supplement and 0.1% glucose
11 F	120 +, 140-	B with 0.5% PDLC, 0.1% agar and 0.1% glucose
12	150	BK with 0.6% dextrin
13	100 +, 130-	PDLC, 3.5%, with salts supplement and 0.1% glucose
14	100	PDLC, 3.5%, with salts supplement and 0.1% glucose
L13E	250	(Obtained from Lederle Laboratories, Inc.)

* Only MLD determined. TD approximated by comparison with similar filtrates.
* TD determined on all filtrates except # 2,3,4 and 11F before toxoiding.

BH = B medium with 5% solvent extracted beef heart.
BK = B medium with 2% solvent extracted beef kidney
PDLC = Pancreatic digest of liver cake.
PDBH = pancreatic digest of beef heart.

Immunization - Monovalent

The toxoids listed above were employed in monovalent (against Cl. oedematiens alone) immunization of guinea pigs, mice, rabbits, pigeons, and dogs.

Guinea pig. Two groups of guinea pigs were immunized. The first group received toxoid #1 which contained 1000 MLD/ml., titrated before toxoiding. Two animals were injected subcutaneously with 3.0 ml. of fluid toxoid, and 3, with 2.0 ml. of toxoid precipitated with 1.0% alum. After 3 weeks, the guinea pigs received a second dose. Toxoid #2, 45 +, 60- T̄D/ml. was used. The pigs which had first received fluid toxoid, were again injected subcutaneously with 2.0 ml. of fluid material, while the animals whose first dose had been of alum precipitated toxoid, were injected with 1.5 ml. of toxoid precipitated with 1.0% alum.

Twenty days after the second dose, the guinea pigs were challenged by intracutaneous injection of 0.026 mg. of ammonium sulfate precipitated toxin contained in 0.2 ml. The reactions of the test and normal non-immunized pigs which are presented in Table XXV were observed for fifteen days after the challenge.

Thirty-five days after the second dose, the animals were bled and serum antitoxin determined by the usual NIH technique. At the same time, the circulating antitoxin titre of a normal guinea pig and a control skin test guinea pig were determined. Seventy-nine days after the second dose, the animals were again bled and the circulating antitoxin titres determined, Table XXV. Three of the animals died of laboratory infections during the course of the experiment. The two remaining guinea

pigs which had received 1.0 ml. of fluid toxoid # 4, 80 +, 90-TD/ml. All four animals were injected subcutaneously with 1 ml. of fluid toxoid # L13E, 250 TD/ml. This dose was given 16 weeks after the second dose in the case of the first two animals, and 25 days after the first dose in the case of the last two. Twenty-one days after this dose, all animals were bled and the serum antitoxin titres determined. Table XXV.

It will be noticed on examination of Table XXV that both the fluid and the alum precipitated toxoids protected the guinea pigs in the skin test experiment. The animal receiving fluid was not as well protected 20 days after the second dose as the animals receiving alum precipitated toxoids.

In the case of determination of circulating antitoxin, the alum precipitated toxoid in Group 1, pigs #2 and 3 proved more antigenic after two doses than did the fluid pig #1. Even after 79 days, a pig which had been injected with alum precipitated toxoid, pig #2, had a circulating antitoxin titre over 0.5 units per ml. After 3 doses, the difference between the antigenicity of alum precipitated and fluid toxoids was not apparent, compare pigs # 1 and 3.

Where the potency of the toxoids used was higher, animals #4 and 5, two doses of fluid toxoid gave a good serum antitoxin titre.

Table XXVi.

Immunization of Guinea Pigs with Cl. Oedematiens Toxoids
Toxoid Dosage

Pig #	First Dose Tox. #	State	ml.	Second Dose Tox. #	State	ml.	Days after 1st.	Third Dose Tox. #	State	ml.	Days after 2nd
Group 1											
1	1	fl.	3.0	2	fl.	2.0	27	13E	fl.	1.0	114
2&3	1	ap.	2	2	ap.	1.5	27	13E	fl.	1	114
Group 11											
4&5	4B	fl.	1	13E	fl.	1	25				

Degree of Immunity

Pig #	Reaction to 1C Injection Days after	Dose #	Reaction	Antitoxin Titre Days after	Dose #	u/ml.
1	20	2	local edema, no lesion	35 79 21	2 2 3	0.5-1.0 0.04 1.0,-2.5
2	20	2	no edema or lesion	35 79 21	2 2 3	1.0-5.0 0.5-1.0 1.0-2.5
3	20	2	no edema, lesion 3 mm. on 8th day healed by 15th day	35	2	5.0 + died of laboratory infection
Controls 3 animals			gen. edema of abd. & thor,,lesions by 7th day, 1-2 cm., not healed by 15th day.	skin test normal		0.1- 0.1-
4				21	2	1.0-5.0
5				21	2	1.0-5.0

Fl. = fluid toxoid
ap. = toxoid precipitated with 1.0 % alum

Rabbits. Three groups of rabbits were treated by subcutaneous injection with 1% alum precipitated toxoids of varying strength. (except as otherwise designated). The animals of Group 1 were each injected with 1 ml. of toxoid #2, 45/, 65- TD/ml. This dose was followed in twelve days by a similar dose of the same toxoid. At intervals after the second dose, the rabbits were bled and the circulating antitoxin titre determined, Table XXVl.

The two rabbits in Group 11, were injected subcutaneously with 1 ml. of toxoid, #3, 45/ 55- TD/ml. At intervals after this single dose, blood was obtained from the animals and the serum antitoxin determined, Table XXvl.

Three animals remaining from Groups 1 and 11 (one from each group died of laboratory infection during the experimental period described above) received 1 ml. of fluid toxoid #11F, 120/, 140- TD/ml. This toxoid dose fell 129 days after the second dose for the rabbits of Group 1, and 113 days after the first dose for the rabbits of Group 11. Seven days after this last toxoid dose, the three animals were challenged with intramuscular injection of culture. The culture used for challenge was a 24 hour growth on B medium with solvent extracted beef heart and glucose, which was allowed to stand over night at room temperature following the 24 hour incubation period. 0.5 ml. of this culture was used for challenge. Two normal rabbits receiving a similar injection of culture died within 24 hours, while the treated rabbits lived throughout the observation period of ten days.

Group III* consisted of two rabbits, each of which were injected subcutaneously with 1 ml. of toxoid #11F, characterized above. A_t intervals after this dose, the animals were bled and the circulating antitoxin titre determined. Twenty-eight days after the first dose, both animals received a similar dose of the same toxoid. Twenty-one days after this second dose, the rabbits were again bled and anti-titre of the serum was determined, Table XXVI.

Table XXVI.

Immunization of Rabbits with Cl. Oedematiens Toxoids

Group #	Days between toxoid dose # 1&2	Days between toxoid dose # 2&3	Days after	Dose #	Antitoxin Titre Units/ml. serum Rabbit # 1	2	3
1	12	129	11	2	2/,5-		1/,2-
			28	2	1/,2-	.04/,10	1
			120	2	died	0.5-	.1,.25-
					4	5	
11	113	no #3 dose	14	1	(pooled)	.5/,2-	
			104	1	.1/,.25-	died	
					6	7	
111	28	no #3 dose	7	1	.04-	.04-	
			12	1	2/,4-	4/	
			21	2	5/,10-	10/	

Cl. oedematiens toxoids have been shown to be antigenic for rabbits. A single dose of toxoid, provoked a serum antitoxin titre

*Immunization of rabbits and antitoxin titration of sera were performed by M.L.Freese and C. Hoffling under direction of P.J. Perkins.

of 0.5 to 2 units in twelve to fourteen days, Groups 11 and 111, Table XXV1. The antitoxin content of the serum of rabbits was higher when the animals received a more potent (higher TD value) toxoid; compare the titres of the rabbits in Group 111, injected with 130 TD/ml. toxoid with those in Group 11, injected with 50 TD/ml. toxoid.

It was indicated that the serum antitoxin rises sharply between the 7th and 12th days after a single toxoid dose, Group 111, Table XXV1. With two doses or one dose of toxoid, Groups 1 and 11, the serum antititre diminished over a period of thirteen to fourteen weeks, but even at the end of that time was higher than 0.1 unit of antitoxin.

From the results of culture challenge of three immune rabbits, page 52 , it was evident that if the rabbits showed at least 0.1 unit of circulating antitoxin, and then received a dose of fluid toxoid, consisting of about 130 test doses, seven days before challenge, they would resist killing doses of oedematiens culture.

Mice. In the immunization of mice with monovalent toxoids a preliminary experiment was set up to determine the efficacy of fluid as compared with alum precipitated toxoids. Portions of toxoids #1, 20 TD/ml. and #2, 45, 65- TD/ml. were precipitated with 1% alum. The mice were divided into four groups of three mice each. The mice in Group 1 were injected subcutaneously with 0.25 ml. of toxoid #1, fluid; in Group 11, with 0.25 ml. of toxoid #1, alum precipitated; in Group 111, with 0.25 ml. of toxoid #2, fluid; and in Group 1V, with 0.25 ml. of toxoid #2, alum precipitated.

Three weeks after the toxoid dose, the mice were all challenged with 4 MLD of ammonium sulfate precipitated toxin contained in 0.5 ml. The challenge injection was intramuscular. The survivals during the observation period of five days are presented in Table XXVll.

At first glance, it would seem that from the data presented in Table XXVll that alum precipitated toxoid was more antigenic than fluid toxoid, however, it is possible that the conditions of the experiment were not optimum for the action of fluid toxoid, and that had conditions been so the fluid toxoid might have proved as good or better than the alum precipitated toxoid. For further comparisons note: the antitoxin titre of guinea pigs immunized with fluid and alum precipitated toxoid, Table XXV., page 51 . The resistance of mice to toxin challenge after two doses of alum precipitated and after two doses of fluid toxoid, Table XXX, page 61 ; and the immunization of mice with divalent oedematiens - welchii toxoid, Table XXXlll, page 66 . Under the conditions of these experiments, the alum precipitated toxoid appeared to give a better immunity than the fluid.

Table XXVll
Comparison of Fluid and Alum Precipitated Toxoid in Immunization of Mice against Oedematiens Toxin.

Group #	Toxoid #	State	Challenge Survived/Tested
1	1	fl.	1/3
11	1	ap.	3/3 *
111	2	fl.	1/2
1V	2	ap.	3/3

* One of the animals in this group died before the challenge was given.
fl. = fluid toxoid
ap. = toxoid precipitated with 1% alum.

Toxoid was treated with various concentrations of alum to determine the optimum concentration for antigenicity of alum precipitated toxoids. Alum treated toxoids differed from alum precipitated toxoids in that in the former, the precipitate formed on addition of alum was separated from the original fluid by centrifugation and resuspended in a less antigenic medium, while in the later, the precipitate remained suspended in the original medium.

Alum treated toxoid was prepared in the following manner. An aliquot of toxoid #L13E was precipitated with 0.5% alum. The precipitate was centrifuged down and the supernatant decanted. The precipitate was then resuspended in toxoid #10, 25/, 50- TD/ml. to a volume equal to that of the fluid before precipitation. Similar aliquots were treated in the same way with 1.0,1.5 and 2.0% alum.

Mice were injected subcutaneously with a single dose of 0.05 (0.5 ml. of a 1:10 dilution of the alum treated toxoid in saline), 0.1 and 0.2 ml. of the toxoid containing 0.5% alum. Similar groups of mice were injected in like manner with the toxoids containing 1.0, 1.5 and 2% alum. The mice which received the 0.05 and 0.1 ml. doses were challenged after three weeks with 30 MLD of ammonium sulfate precipitated toxin contained in 0.5 ml. Those receiving 0.2 ml. toxoid doses were challenged with the same amount of toxin in five weeks. Table XXVIII.

The optimum concentration of alum as far as antigenicity is concerned was between 1.0 and 1.5%. There was little difference in response to doses of 0.1 and 0.2 ml. at any concentration of alum tested except at 2.0% in which case the poor condition of the animals

Table XXVIII.

Alum Concentration and Immunization.

% alum	0.5	1.0	1.5	2.0
Toxoid dose, ml.	Survived/Tested			
0.05	1/7	4/7	3/5	5/7
0.1	4/10	5/10	10/10	0/10*
0.2	4/8	6/7	4/8	8/8

* The mice injected with 2% alum treated toxoid, 0.1 and 0.2 ml. were in very poor condition, showing loss of weight and hair, at the time of the challenge.

at the time of challengeing unquestionably influenced the results in an adverse manner.

The comparative antigenicity of toxoids of various combining powers was determined by immunization of mice. The animals received subcutaneously a single dose of toxoid precipitated with 1.0% alum. After a period of time, the mice were challenged with ammonium sulfate precipitated toxin in phosphate buffer-saline. Challenge doses were administered subcutaneously. The challenge dose was contained in 0.2-0.6 and occasionally 1.0 ml. of fluid. Mice were observed for three days after challenge and the results recorded in Table XXIX.

The following preliminary conclusions may be drawn from the experimental data presented in Table XXIX. With a dose of 0.1 ml. of 1.0% alum precipitated toxoid, and a period of three weeks between toxoid injected and challenge toxin injection, there was no correlation between combining power and antigenicity of toxoids. The conditions of the experimental work were limited. Under a different set of con-

ditions it is believed that some correlation might be found.

The immunity conferred by any toxoid increased with increasing length of time between the toxoid and challenge toxin injections, up to twenty-one days, compare the results obtained with toxoids #4B at 0.1 ml., #5 at 0.1 ml. and #11F at 0.1 ml.

The immunity conferred by any toxoid is greater with 0.1 ml. than with 0.05 ml. when the toxoids are precipitated with 1.0% alum. This conclusion is corroborated by the evidence presented in Table XXVIII, page 57 . The increased efficacy of larger toxoid doses in immunization is also shown in immunization of pigeons, Table XXXI, page 63 .

Mice were protected with two doses of oedematiens toxoid against a challenge of up to 250 MLD of toxin. Mice were injected subcutaneously with two doses of 0.1 ml. each of toxoid #11F, 120 #, 140- TD/ml. at the intervals noted in Table XXX. The toxoid was precipitated with 1.0% alum previous to injection. After the second dose, at the times noted in Table XXX these animals were challenged with ammonium sulfate precipitated toxin, the challenge dose being contained in 0.25-1.0 ml. The animals were observed for five days after the challenge dose. Unfortunately for comparative purposes, allanimals challenged survived except the controls, although the challenge dose was increased on successive challenges in the hope of reaching an end point. The data are presented for the interest that the high challenge level holds.

Table # XXX also includes data obtained on the resistance of mice to two doses of toxoid #2, 454, 60- TD/ml. injected as fluid and precipitated with 1.5% alum. Mice were injected subcutaneously with 0.25 ml. each of fluid and alum precipitated toxoid.

Table XXIX.

Immunization of Mice with a Single Dose of Alum Precipitated Oedematiens Toxoid

#	Toxoid TD/ml.	ml.inj.	Challenge after -days	MLD	Survived Tested	% Survived*
10**	25 +,50-	0.1	21	5	7/8	88
				15	6/8	75
9**	50 +,75-	0.1	21	5	6/8	75
				30	4/8	50
8**	50 +,75-	0.1	26	5	3/8	38
				10	3/8	38
7**	60 +, 80-	0.1	26	5	6/6	100
				10	5/6	83
4B	80 +,90-	0.1	16	5	2/6	30
				10	1/6	17
		0.1	21	5	14/21	67
				10	8/18	44
6	80 +,100-	0.1	26	5	3/6	50
				10	4/6	67
4A	90 +,100-	0.1	21	5	3/7	43
				10	4/7	57
5	100 +,150-	0.05	16	2	0/6	0
				10	0/6	0
		0.1	16	10	2/6	30
				25	2/6	30
		0.1	21	5	12/13	92
				10	6/9	67
13	100 +,130-	0.1	20	5	7/8	88
				15	6/7	86
14	100	0.1	20	5	7/7	100
				15	7/7	100
*11F	120 +,140-	0.1	7	5	1/6	17
			14	8	36/36	100
12	160 +,180-	0.1	20	5	9/9	100
				15	8/8	100
L13E	250	0.1	20	5	20/22	100
				15	20/20	100

Controls run with all tests at the lowest LD used for challenge, survived/tested = 0/5.

* Because of the small number of animals used in most of the tests, percent survived is not accurate, it is included in the table to facilitate reading.

** Student experiments conducted by F. Matthews, J. Franklin, B. DeSalvo, C. Hoffling, M.L. Freese, B. Curtis, J. Hagans under the direction of P.J.P.

At various intervals, the dose was repeated. At ten days after the second dose in each group, the mice were challenged subcutaneously with four or eight MLD of ammonium sulfate precipitated toxin contained in 0.25 or 0.5 ml. respectively. The challenged mice were observed for five days after the toxin injection.

It may be seen from Table XXX, that the lower potency toxoid, #2 was a poorer antigen after two doses, than the more potent toxoid, 11F. It was also apparent, Table XXX, that two doses of

Table XXX

Immunization of Mice with Two Doses of Toxoid.

Toxoid #	Toxoid ml. 1st & 2nd doses	Time Between doses -days	Challenge Days after 2nd dose LD		Survived / Tested
11F	0.1	10	7	4	4/4
				10	4/4
				20	4/4
			15	12	4/4
				35	4/4
				75	4/4
		21	7	25	3/3
				100	4/4
				200	4/4
			15	65	3/3
				130	3/3
				250	4/4
2,ap	0.25	10	10	4	5/6
				8	7/11
		42	10	8	3/3
2,fl.	0.25	10	10	4	0/6
				8	1/5
		42	10	8	0/2

fluid toxoid #2 spaced at 11/2 or at six weeks did not give the protection, measured by survivals, that the same doses at the same intervals of the same toxoid precipitated with 1.0% alum gave.

A group of nine mice were treated with alum precipitated and fluid toxoid and challenged with culture. The mice were injected subcutaneously with 0.25 ml. of toxoid #3, 40 TD/ml., precipitated with 1.0% alum. Sixteen weeks later, these animals received 0.1 ml. of fluid toxoid #11F, 120 +, 140- TD/ml. one week after the second dose, the mice were challenged with oedematiens culture, injected intramuscularly. The culture was grown on B medium with solvent extracted beef heart and 0.5% glucose. Incubation of the culture was for twenty-four hours at 37°C. After the incubation period the culture was allowed to stand over night at room temperature before it was used as challenge. The challenge dose was 0.25 ml.

Seven out of the nine treated mice survived the challenge for the observation period of five days, while three untreated controls injected with the same amount of culture died within twenty-four hours.

Pigeons. Pigeons were protected against death by toxin injection by treatment with 1.0% alum precipitated toxoid #3, 40 TD/ml. Ten pigeons were divided into two groups of five birds each. Group 1 was injected in the breast muscle with 0.5 ml., Group 11 with 1.0 ml. of the toxoid. Ten days later the dose was repeated. Ten days after the second toxoid dose, all pigeons were challenged with 20 MLD (pigeon titre) of ammonium sulfate precipitated toxin contained in 0.5 ml. Table XXXI.

Previous to the toxin challenge, and eight days after

the second toxoid dose, the pigeons were bled from the heart. The antitoxin titre of the pooled sera was 0.5 +, 2- units of antitoxin per ml.

Table XXXl.

Immunization of Pigeons with Two Doses of Alum Precipitated Oedematiens Toxoid.

ml. of toxoid	Toxin Challenge Survived/Tested
0.5	3/5
1.0	5/5
Total	8/10
Controls	0/4

Alum precipitated oedematiens toxoid has proved antigenic in pigeons, as measured by the circulating antitoxin titre obtained, and the resistance of treated birds to lethal dose of oedematiens toxin, after two toxoid doses.

Dogs. Dogs have been treated with oedematiens toxoid with a resultant demonstrable antitoxin titre and increased resistance to infection induced by injection of Cl. oedematiens culture. The immunizing and challenging work was done by Dr. Andrew H. Dowdy, Strong Memorial Hospital, University of Rochester Medical College. Six of the dogs, Tx-1n to Tx-6n, Table XXXll had been previously immunized with Cl. welchii toxoid, challenged with welchii culture and recovered. The recovery of these dogs had been moderate to very rapid and all were in good condition throughout the experiment with Cl. oedematiens.

Six of the dogs had received no treatment previous to the experiment with Cl. oedematiens toxoid, Tn-1 to Tn-6. During the course of the experiment, all of the dogs in the Tn group with the exception of Tn-2 developed distemper. The recovery from distemper was complete in all dogs except Tn-6 which died and Tn-1 in which the condition had improved to fair by the time of the third bleeding.

All dogs were injected intramuscularly with 1 ml. of oedematiens toxoid #4, 80/, 90- TD/ml. precipitated with 1.0% alum. The antitoxin titre of all sera before immunization was less than 0.04 units per ml. Two weeks after the first toxoid dose, the dogs were bled and circulating antitoxin titre determined on the sera, Table XXXll A and B. One month after the first toxoid dose, the animals received a second dose of the same toxoid, and two weeks later were bled. The antitoxin titre of the sera were determined, Table XXXll A and B.

Three weeks after the second dose of toxoid, the dogs were challenged by intramuscular injection of 0.5 ml. of Cl. oedematiens culture.

From examination of Table XXXll, it may be seen that the animals previously immunized with welchii toxoid gave a better response to the toxoid of Cl. oedematiens, Table XXXll A , than did the dogs which had had no previous treatment, Table XXXll B. The antitoxin titres of the former set of dogs were generally higher than those of the latter as tested after the first toxoid dose. As only one end point was reached in each set of dogs in the determination of antitoxin titresafter the second toxoid dose, it is not possible

Table XXXII A

Immunization of Dogs with Alum Precipitated Cl. Oedematiens Toxoid.

Dog No.	Weight Age Sex Kind	Source and Previous Treatment	Toxoid Treatment	1st Blood Sample 1st Toxoid Dose	2nd Blood Sample 2nd Toxoid Dose	Units AT/mf. on 2nd Sample	3rd Blood Sample	Units AT/ml. on 3rd Sample	Holding Record of Dog	Condition at Time of Challenging Infection	Challenging Infection - Local Sepsis: Date	Degree	Rate of Recovery
Tx - 1n	14.5 K. 3 yrs. M. Elk-Hound	Pa. Welchii toxoid Immunization. Challenged. Very rapid recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	4.6+	1-/25/43 - Blood	20.0 +	Remained in Excellent Condition	Very Good	11/4/43	+ +	Rapid
Tx - 2n	8.2 K. 4 yrs. F. Airdale	Pa. Welchii toxoid Immunization. Challenged. Medium recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.04+ 0.25-	10/25/43 - Blood	5.0 +	Condition Improved. Increased Weight.	Very Good.	11/4/43	+	Rapid
Tx - 3n	15.7 K. 3 yrs. Otter-Hound	Pa. Welchii toxoid Immunization. Challenged. Rapid recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	2.6+ 4/6-	10/25/43 - Blood	5.0 + 10.0 -	Continued Good Condition.	Very Good.	11/4/43	+ +	Rapid
Tx - 4n	10.2 K. 3 yrs. M. Shepperd	Pa. Welchii toxoid Immunization. Challenged. Rapid recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	2.6+ 4.6-	10/25/43 - Blood	10.0 +	Improvement. Increased Weight.	Good.	11/4/43	+++	Rapid
Tx - 5n	15.7 K. 5 yrs. F. Hound	Pa. Welchii toxoid Immunization. Challenged. Very rapid recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	4.6+	10/25/43 - Blood	20.0+	Remained in Exdellent Condition.	Very Good	11/4/43	+++	Medium
Tx - 6n	8.2 K. 2 yrs. M. Wire-haired Terrier	Pa. Welchii toxoid Immunization. Challenged. Medium recovery.	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.25+ 0.7-	10/25/43 - Blood	5.0+	Improvement from Good to Excellent Condition	Very Good	11/4/43	+++	Rapid

Cl. novyi - .5 c.c.		Degree and Duration of Immunity	Autopsy Reports
Toxicity			
Degree	Rate of Recovery		
∄	Rapid	Moderate. Not complete Chall. 3 weeks after 2nd toxoid	—
0	—	Practically complete. Chall. 3 weeks after 2nd toxoid	—
∤	Rapid	Moderate. Not complete Chall. 3 weeks after 2nd toxoid	—
∤	Rapid	Moderate. Not complete Chall. 3 weeks after 2nd toxoid	—
∤	Rapid	Moderate. Not complete Chall. 3 weeks after 2nd toxoid	—
∤	Rapid	Moderate. Not complete Chall. 3 weeks after 2nd toxoid	—

Table XXXII B

Immunization of Dogs with Alum Precipitated Cl. Oedematiens Toxoid.

Dog No.	Weight Age Sex Kind	Source and Previous Treatment	Toxoid Treatment	1st Blood Sample 1st Toxoid Dose	2nd Blood Sample 2nd Toxoid Dose	Units At./ml/ on 2nd Sample	3rd. Blood Sample	Units At./ml. on 3rd. Sample	Holding Record of Dog	Condition at Time of Challenging Infection	Challenging Infection - Cl. Oedematiens - .5 cc.					Degree and Duration of Immunity	Autopsy Reports
											Date	Local Sepsis Degree	Local Sepsis Rate of Recovery	Toxicity Degree	Toxicity Rate of Recovery		
Tn - 1	10.5 K. 2 yrs. F. Setter	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.04 -	10/25/43 - Blood	5.0 +	Probable Distemper. 5 doses Oral Sulfadiazine 9/21 - 10/18. Condition Now Fair	Fair.	11/4/43	+++	Rapid	++	Rapid	Moderate. Not Complete Challenge 3 weeks after 2nd Toxoid	--
Tn - 2	12.7 K. 2 yrs. F. Hound	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.04 -	10/25/43 - Blood	2.0 + 5.0 -	Condition Good. Slight Decrease in Weight.	Good	11/4/43	+++	Rapid	+	Rapid	Moderate. Not Complete Challenge 3 weeks after 2nd Toxoid	--
Tn - 3	10.5 K. 4 yrs. F. Schnauser	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.04 -	10/25/43 - Blood	5.0 +	Probable Mild Distemper. 3 Doses Oral Sulfadiazine 9/25 and 9/28. Complete Recovery.	Good	11/4/43	++	Rapid	+	Rapid	Moderate. Not Complete Challenge 3 weeks after 2nd Toxoid	--
Tn - 4	10.9 K. 3 yrs. F. Setter	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.25 + 0.7-	10/25/43- Blood	5.0 +	Probable Distemper. 6 Doses Oral Sulfadiazine. 9/21 - 9/28 Complete Recovery.	Good	11/4/43	+++	Rapid	++	Rapid	Moderate. Not Complete Challenge 3 weeks after 2nd Toxoid	--
Tn - 5	10.0 K. 2 yrs. M. Setter	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	9/27/43 - Blood 10/11/43 - Toxoid	0.25 + 0.7 -	10/25/43 - Blood	5.0 +	Probable Mild Distemper. 1 Dose Oral Sulfadiazine 9/28. Complete Recovery	Good	11/4/43	+++	Rapid	+	Rapid	Moderate. Not Complete Challenge 3 weeks after 2nd Toxoid	--
Tn - 6	9.1 K. 1-1/2 yrs. M. Setter	Pa. none	Alum ppt. 1 c.c.	9/14/43 - Blood 9/14/43 - Toxoid	Died 9/27/43	--	--	--	Probable Distemper. 3 Doses Oral Sulfadiazine. Died 9/27/43.	----	----						Distemper

Table XXXII C

Controls

Dog No.	Wgt.K.	Date of Inoc. 1943	Amount and Type of Inoculation (20 hr. culture)	Local Sepsis		Toxicity		Survival Time
				Degree	Rate of Recovery	Degree	Rate of Recovery	
Tn C-7	7	11/4	Cl. novyi .5 cc. IM	+++	0	+++	0	38 hours
Tn C-8	8	11/4	Cl. novyi .5 cc. IM	+++	0	+++	0	32 hours
Tn C-9	8	11/4	Cl. novyi .5 cc. IM	+++	0	+++	0	50 hours
Tn C-10	10	11/4	Cl. novyi .5 cc. IM	+++	0	+++	0	65 hours

to make a fair comparison in this case. The distemper infection in the Tn group possibly influenced the results.

In the case of the challenging infection, the immunity conferred was not complete, except in dog #Tx-2n which showed only a slight degree of local sepsis and no toxic reaction. However, as shown in Table XXXll, the recovery of all treated dogs from the infection with oedematiens was rapid except in the case of dog #Tx-5n where the recovery from local sepsis was only moderately rapid.

On comparison of the reaction of the two groups of dogs, those previously recovered from welchii challenge and those not previously treated, to the challenge infection, it may be said that the former group generally had a slightly weaker reaction than the latter. In other words, the Tx-n dogs showed slightly less local sepsis and toxic reaction than the Tn dogs. This did not affect the rate of recovery of the dogs from the challenge infection which, as has been mentioned above, was rapid in all treated animals. The challenge was lethal to all untreated controls, Table XXXlllC.

Immunization-Divalent.

One group of each of mice, rabbits and pigeons was treated with mixtures of oedematiens and welchii toxoids and challenged after a period of time with oedematiens and welchii toxins. In the case of rabbits, circulating antitoxin was determined at intervals before challenging.

Mice. The toxoids used for mice were Cl. oedematiens toxoid #2, 45 +, 60- TD/ml., and Cl. welchii toxoid #PF14A, Lb 5+. These toxoids were mixed before injection, one volume of oedematiens and one volume of welchii, and precipitated with 1% alum.

Table XXX 111.

Immunization of Mice with Divalent Oedematiens-Welchii Alum Precipitated Toxoid

Group #	Toxoid		Challenge		Survived Tested.
	ml.	#	LD	Toxin	
1	0.25	2, fl.	4	oedematiens	0/6
			8	"	1/6
2	0.25	2, ap.	4	"	5/6
			8	"	4/6
3	0.25	PF,fl.	7	welchii	0/6
			14	"	0/6
4	0.25	PF,ap.	7	"	5/6
			14	"	3/5
5A	0.25	2-PF,fl.	4	oedematiens	0/6
5B			7	welchii	0/3
			14	"	0/2
6A	0.5	2-PF,fl.	4	oedematiens	0/5
6B			7	welchii	0/3
			14	"	0/3
7A	0.75	2-PF,fl.	4	oedematiens	2/6
7B			7	welchii	0/3
			14	"	0/3
8A	0.25	2-PF,ap.	4	oedematiens	2/3
			8	"	2/3
8B			7	welchii	1/3
			14	"	1/3
9A	0.5	2-PF,ap.	4	oedematiens	2/3
			8	"	3/3
9B			7	welchii	2/3
			14	"	2/3
10A	0.75	2-PF,ap.	4	pedematiens	3/3
			8	"	2/3
10B			7	welchii	1/3
			14	"	2/3
11A	controls		4	oedematiens	0/3
			8	"	0/2
11B			7	welchii	0/3
			14	"	0/2

2 = Cl. oedematiens toxoid #2, 45 ℔. 60-
PF = Cl. welchii toxoid #PF14A, Lb 5 ℔
ap = precipitated with 1.0% alum
fl = fluid

The mice were divided into two groups which received toxoids in the amounts found in Table XXXIII injected subcutaneously. After eleven days, all animals received the same toxoids at similar doses. Ten days after the second dose, the animals were challenged by intramuscular injection of toxin. The animals previously injected with monovalent toxoid were challenged with the respective toxins. Those which had received the divalent material were divided into two groups one of which was challenged with oedematiens toxin, the other, with welchii toxin. The challenge dose in all cases was contained in 0.1 or 0.2 ml.

It has been shown that mice are protected by a 1:1 mixture of oedematiens-welchii toxoids, precipitated with 1% alum against challenging infections of both toxins. In the work here presented, there is no indication that the divalent mixture is less effective in protecting the mice than either of the monovalent toxoids.

The data given shows that alum precipitated toxoid under the conditions of the experiment, i.e. two toxoid doses ten days apart, challenge ten days after the second toxoid dose, was more effective in protecting mice against toxin challenge than fluid toxoid.

Rabbits.* Rabbits were injected subcutaneously with 1 ml. of a 1:1 mixture of oedematiens toxoid #11F, 120 +, 140- Td/ml. and welchii toxoid #PF11, 3 Lb/ml. The mixture was precipitated with 1.0% alum before injection. After twenty-four days the rabbits received a similar dose of the same toxoid mixture. The rabbits were bled and circulating antitoxin titres were determined before and after the second toxoid dose at the intervals noted in Table XXXIV.

*Student experiment conducted by M.L. Freese, and C. Hoffing under the direction of P.J.P.

Table XXXIII A

Summary of Table XXXIII

Toxoid #	Challenge	Survived Tested	% Survived
2 fl	oedematiens	1/11	9.1
2 ap	oedematiens	9/12	75
PF,fl	welchii	0/12	0
PF,ap.	welchii	8/11	73
2 PF, fl.	oedematiens	2/17	12
	welchii	0/17	0
2-PF, ap.	oedematiens	14/18	78
	welchii	9/18	50

Forty-three days after the second toxoid dose, these animals were challenged by intramuscular injection of culture mixture. An oedematiens and a welchii culture were mixed in the ratio 1:1. 0.5 ml. of this mixture was used as the challenge dose. Both animals survived the challenge for the observation period of five days, while three untreated control rabbits injected intramuscularly with 0.5 ml. of the same culture mixture died within twenty-four hours.

Table XXIV.

Immunization of Rabbits with Divalent Oedematiens-Welchii Toxoid, Alum Precipitated.

Bleeding		Antitoxin		
			Units/ml. serum rabbit #	
Days after	Toxoid dose #	Type determined	8	9
17	1	oedematiens	(pooled sera) 0.5	
21	2	oedematiens	1.0+, 2.5 -	1.0
		welchii*	0.2+, 0.5-	0.5+,1.0-
35	2	oedematiens	0.25+,0.5-	0.25+,0.5-
		welchii *	0, 2+,0.5-	0.2+2 0.5-

* Welchii antitoxin titre was determined by the lecithovitellin, acid soluble phosphorous methos (21).

A demonstrable titre of oedematiens antitoxin has been developed in rabbits after one dose of toxoid (see also page 53). Oedematiens and welchii antitoxin have been found in rabbit sera three and five weeks following two injections of divalent, oedematiens - welchii, toxoid.

Rabbits with a serum antitoxin of about 0.3 units per ml. oedematiens and 0.2 units per ml. welchii were protected against a lethal dose of a mixed culture of oedematiens and welchii.

Pigeons. Pigeons were treated with monovalent oedematiens and welchii toxoids, and with divalent oedematiens-welchii toxoid. The oedematiens toxoid was #3, 30/, 40- TD/ml, the welchii, PF14A, Lb 5 / . The divalent toxoid was a mixture of these, one volume of oedematiens with one volume of welchii. All the toxoids were precipitated with 1.0% alum.

The birds were injected in the breast muscle with the amounts of the toxoids shown in Table XXXV. Ten days later, they received a similar dose of the same toxoids. Ten days after the second toxoid dose, all birds were challenged with ammonium sulfate precipitated toxins which had been titrated for LD in pigeons. The pigeons were observed for five days after the challenging dose was administered. Survivals were noted and reported in Table XXXV.

Table XXXV.

Immunization of Pigeons with Divalent Oedematiens-Welchii Toxoid, Alum Precipitated.

Toxoid		Challenge		Survived
#	ml.	Toxin	Pigeon LD	Tested.
3	0.5	oedematiens	20	3/5*
	1.0	"	20	5/5*
PF	0.75	welchii	15	2/2
3-PF	1.0	oedematiens	20	4/5
		welchii	15	2/2
	1.5	oedematiens	20	4/5
		welchii	15	1/1
Controls		oedematiens	20	0/4
		welchii	15	0/3

* Reported also in Table XXXI, page 63 .

Pigeons were protected against lethal doses of both oedematiens and welchii toxins by two injections of divalent, oedematiens-welchii toxoid given at ten day intervals.

V.

Hemolytic Activity of Toxic Filtrates.

A hemolysin is present in filtrates from cultures of oedematiens(30)(39) This hemolysin is not as strong as that produced by streptococci (10), tetani , or welchii(23). (In welchii filtrates there are two hemolysins, that of α toxin and that of β toxin). The hemolytic activity of oed oedmatiens filtrates is comparable in potency to that of filtrates of Cl. septicum cultures(30)(39). Experiments were designed to determine whether oedematiens filtrates could be correlated with the lethal activity. If such were the case, determination of the lytic activity could be substituted for in vivo determination of lethal activity which is both expensive and time consuming.

Turbidemtric Method for Determination of Hemolytic Activity.

One of the simplest and most oftenused methods for the assay of hemolysins is visual estimation of 100% hemolysis caused by a certain amount of the lysin. In this method, serial dilutions of the lysin are prepared. The cell suspension is added to the different dilutions and the tubes observed to determine the highest dilution, i.e. the lowest amount of toxin, which will cause 100% hemolysis in a given time. The method may also be used to determine the time required for a given amount of lysin to effect 100% hemolysis. This method is, at best, qualitative, for if the dilutions are widely separated in concentration there may be a large error, and if smaller variations in concentration of the lysin are used, accurate determination of the end point becomes

difficult.

More accurate methods for the determination of hemolytic activity have been developed based on one of two facts: (1) as hemolysis of a particular suspension increases, the suspension becomes more translucent; and (2) as hemolysis increased, the amount of hemoglobin in the suspension medium becomes greater. Several methods based on the former principle have been used.

Percentage hemolysis may be determined with a simple opacimeter (12) in which an object, such as the filaments of a lamp, becomes visible through the suspension when the latter reaches a certain degree of translucency. Other methods involve the use of thermopiles or radiometers, (27), photoelectric cells, (15), and photometers, (28).

In the method based on the second principle stated above, the hemoglobin liberated into the suspension on hemolysis is estimated coloremetrically. Cells and lysin are mixed and after a certain length of time, the cells are centrifuged out of the mixtures and the hemoglobin present in the supernatant is measured. Vles(38) determined the hemoglobin concentration by the extinction coefficient of the adsorption band at 542 mm. in a visual spectrophotometer. Herbert (9) compared the solutions containing hemoglobin in a Leitz colorimeter, fitted with a green filter in the eyepiece, with a neutral grey filter. There are many variations of methods based on either of the two principles stated above.

The method used in this work is a turbidimatric method depending on the reflection of light from the cells of the suspension, less light being reflected when fewer cells are present. The method is independent of the observer's judgement of the appearance of an object when viewed through the suspension. It does not require any complicated apparatus and it eliminates the necessity of centrifuging the suspensions, steps which are necessary in one or the other of the methods described above.

The instrument used was the turbidity comparator,(18) in which an electronically regulated power source supplies the current for both amplifier and light source. Light entering two slits, one on either side of the suspension, wasreflected by the particles in the suspension on to a photoelectric cell placed at right angles to the beam of light. The current produced by the excited photocell was amplified and read on a microammeter. The amount of light reaching the photocell depended on the number, size and shape of the red blood cells in suspension.

A standard curve was sst up with 0.1 to 1.0 ml. of the cell suspension to be used. One ml. of the suspension was added to decreasing amounts of a filtrate, Both sets of tubes, the standard and thedetermination set, were made up to the incubation volume, and incubated for an hour at the specified temperature. At the end of the incubation period, all mixtures were made up to 10 ml. final volume, shaken thoroughly by inversion of the tubes and read on the turbidimeter. A standard curve graph was prepared from readings with the standard set plotted against ml. of cells used. The 100%

hemolysis point was set by adjusting the instrument. The mixture used was 1.0 ml. of the suspension with 9.0 ml..0.4% (NH_4) OH added. With this mixture, the instrument was adjusted to give a reading of zero on the microammeter. It was assumed in this standard curve that if 90% of 1.0 ml. of a certain suspension of cells were hemolyzed there would be present the same number of cells as would occur in 0.1 ml. of the original suspension; if 80% were hemolyzed, there would be present the number of cells in 0.2 ml. and so on. Under this assumption, ml. of cells was transposed to percent hemolysis. With the standard curve so established, it was possible to read the percent hemolysis in the tubes of the determination set directly. The above is an outline of the method which will be discussed in detail, with the experimental work which led to the establishment of the method, in the following sections.

Red Blood Cell Suspension.

Throughout this work rabbit red blood cells were used. The possibility of other animal red blood cells being less resistant has not been investigated in this work. According to Celarek, (4), "the toxins of the gas gangrene anaerobes hemolyze the red blood cells of man and rabbit equally well, do not hemolyze the red cells of the horse, and act on those of the sheep to only a small extent." In view of the relatively small activity of filtrates of Cl. oedematiens and the resistance series for red blood cells of different types to various lysins(6)(29) , It would be interesting to investigate this point.

Rabbit's red blood corpuscles were washed three times in 0.9% saline solution and suspended in saline at a concentration of about 20 to 30 percent cells. This concentrated suspension was stored at 5°C and diluted just before a test was to be made. The dilution was prepared to give a cell suspension with a concentration of 1.4-1.6% cells by comparison of TR with a concentration-reading curve prepared as in Fig.11, Run #1 and #2 before incubation. TR, top reading, was the reading in microammeters obtained when 1 ml. of any particular cell suspension was diluted to 10 ml. final volume and read on the turbidimeter. Since 1 ml. of cell suspension is used in testing for hemolytic activity, this reading, TR, corresponds to the reading at zero hemolysis.

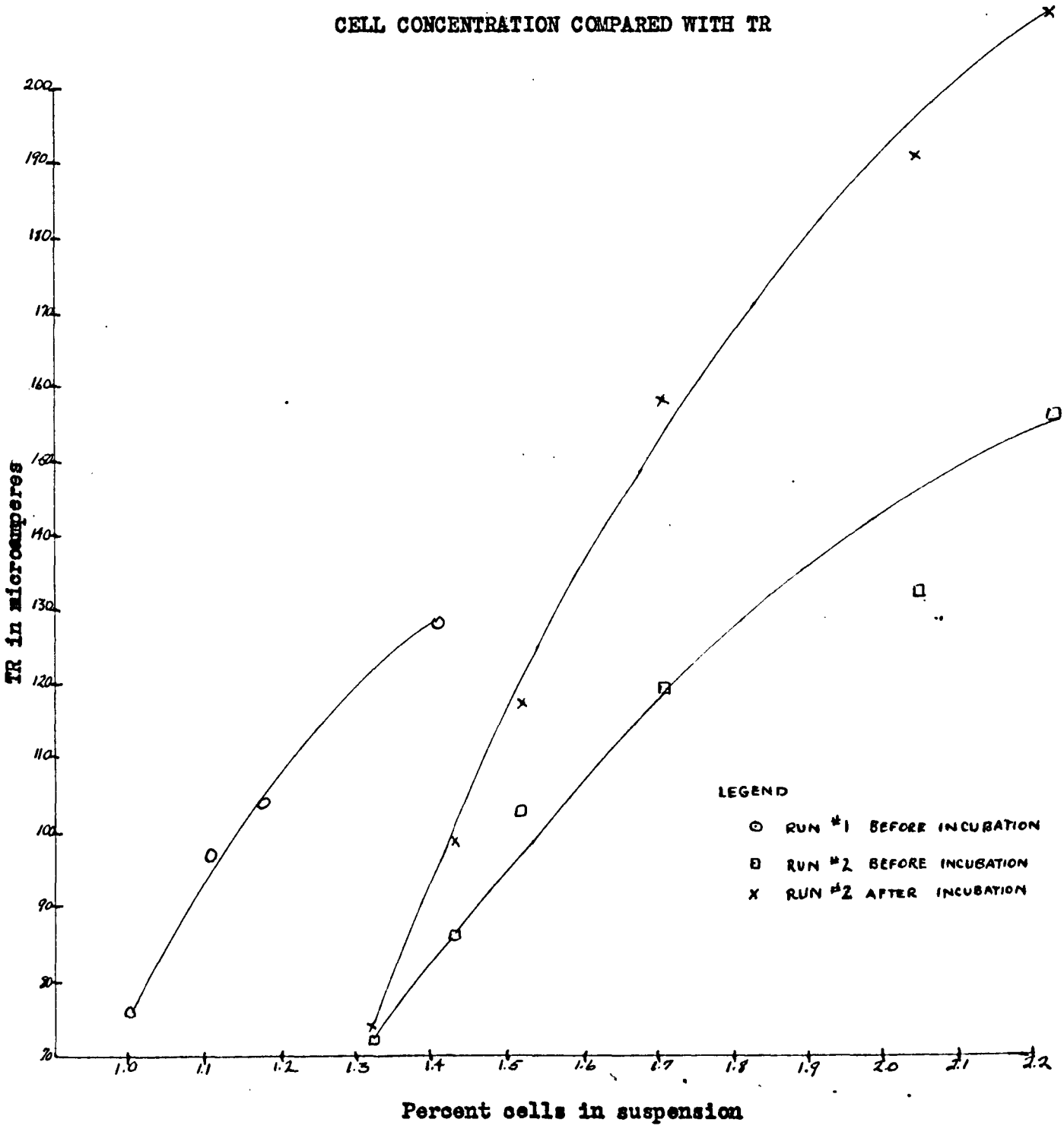
The shift in position of the curves for Run #1 and 2, Fig. 11, occurred when the lights on the turbidimeter were replaced. From this experience, it will be seen that if one or both of the 1 lamps on the instrument should burn out, a calibration curve of TR against concentration would have to be run before TR could be used as an estimation of the concentration of a cell suspension. The concentration of the dilute cell suspension was determined by centrifuging 2.5-3.0 ml. of the suspension at 3100 RPM for fifteen minutes in a Bauer and Schenck centrifuge tube. This tube designed for the determination of precipitable proteins in serum, is graduated from 0.1 - 0.4 ml. in 0.004 ml. divisions.

Standard Curve.

All turbidimetric determinations were made in pyrex test tubes, 120 x 15 mm., selected so that, with distilled water the

Fig. II

CELL CONCENTRATION COMPARED WITH TR



tubes of any one set gave readings which agreed within ± 1.5 micro - amperes. Two sets matched at different readings could be used for one run provided that a 100% hemolysis tube (see explanation above) was used for the zero setting of the instrument for each matching set of tubes.

The cells were distributed in amounts from 0.1-1.0 ml. at 0.1 or 0.2 ml. intervals. As has been mentioned above, it was assumed that 0.1 ml. of the original suspension contained the same manner of cells as 1.0 ml. 90% hemolyzed. Immediately the question of hemoglobin present in the latter mixture arises. It was found that addition of a hemolyzed suspension to the standard curve did not change the readings. A portion of the dilute suspension to be used in the test was hemolyzed by freezing (in dry ice, acetone mixture) and thawing (in hot water) as rapidly as feasible, 15-20 times, or until hemolysis appeared complete. Cells and hemolyzed suspension were distributed in the following manner:

tube #	0	1	2	3	4	5
ml. cells	0,	0.2	0.4	0.6,	0.8,	1.0
ml. hemolyzed suspension	1.0	0.8	0.6	0.4	0.2	0
corresponding % hemolysis	100	80	60	40	20	0

One ml. of saline was added to each mixture. A corresponding set was prepared with the hemolyzed suspension omitted and the volume made up to 2 ml. with saline. The 100% hemolysis tube for this set contained 1.0 ml. of cells. The volume was made to 10 ml. with 0.04% N_aOH. Both sets were incubated for one hour at 40 C, diluted to 10 ml. with 0.9% saline except for the 100% hemolysis mixture of the second set, and mixed thoroughly by inverting the tubes. The readings in microamperes of both sets of tubes were similar.

The possible presence of stroma in the mixtures hemolyzed by toxic filtrates also raises a question as to the acdeptance of the assumption that a mixture containing 0.1 ml. of the original cell suspension would give the same reading as a mixture containing 1.0 ml. of the original suspension with 90% of the cells hemolyzed. This possibility was eliminated by checking the turbidimetric measurement against a colorimetric determination run on the supernatants of mixtures cleared of cells by centrifuging. This comparison will be described in detail in a later section.

When a standard curve was set up with the cell suspension distributed in amounts from 0.1-1.0 ml. by 0.1 or 0.2 ml. intervals, each suspension made up to a volume of 2.0 ml. with 0.9% saline, the suspensions incubated one hour at the temperature of the run, diluted to 10 ml. with saline and read on the turbidimeter, the curves obtained by plotting readings on the ordinate and percent hemolysis (ml. of cell suspension) along the abscissa were similar to the saline curves in Fig. III and Fig. IV.

With non-hemolytic amounts of toxin added to 1.0 ml. of the cell suspension and incubated for one hour, it was noticed that the reading exceeded TR (zero hemolysis of the standard curve). This was especially noticeable when the cells were fresh or not more than three days old. It was thought that the increase in reading was due to some change in the size or shape of the cells brought about by the medium of the toxin. The effect was not apparently due to the toxin itself since it persisted after heating the toxin and after addition of an excess of antitoxin which inhibits the

Fig. III

COMPARISON OF STANDARD CURVE WITH SALINE AND WITH BUFFER

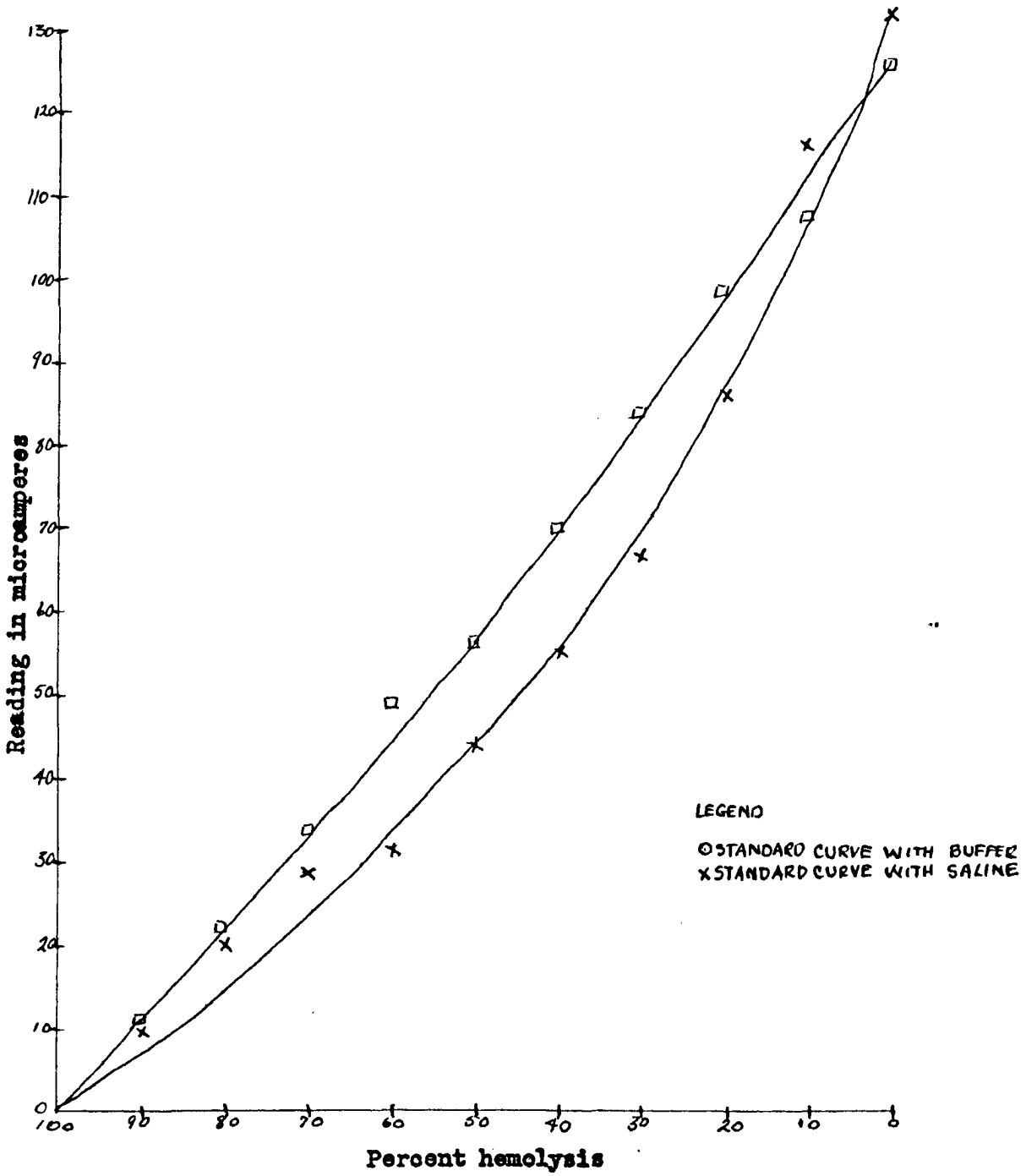
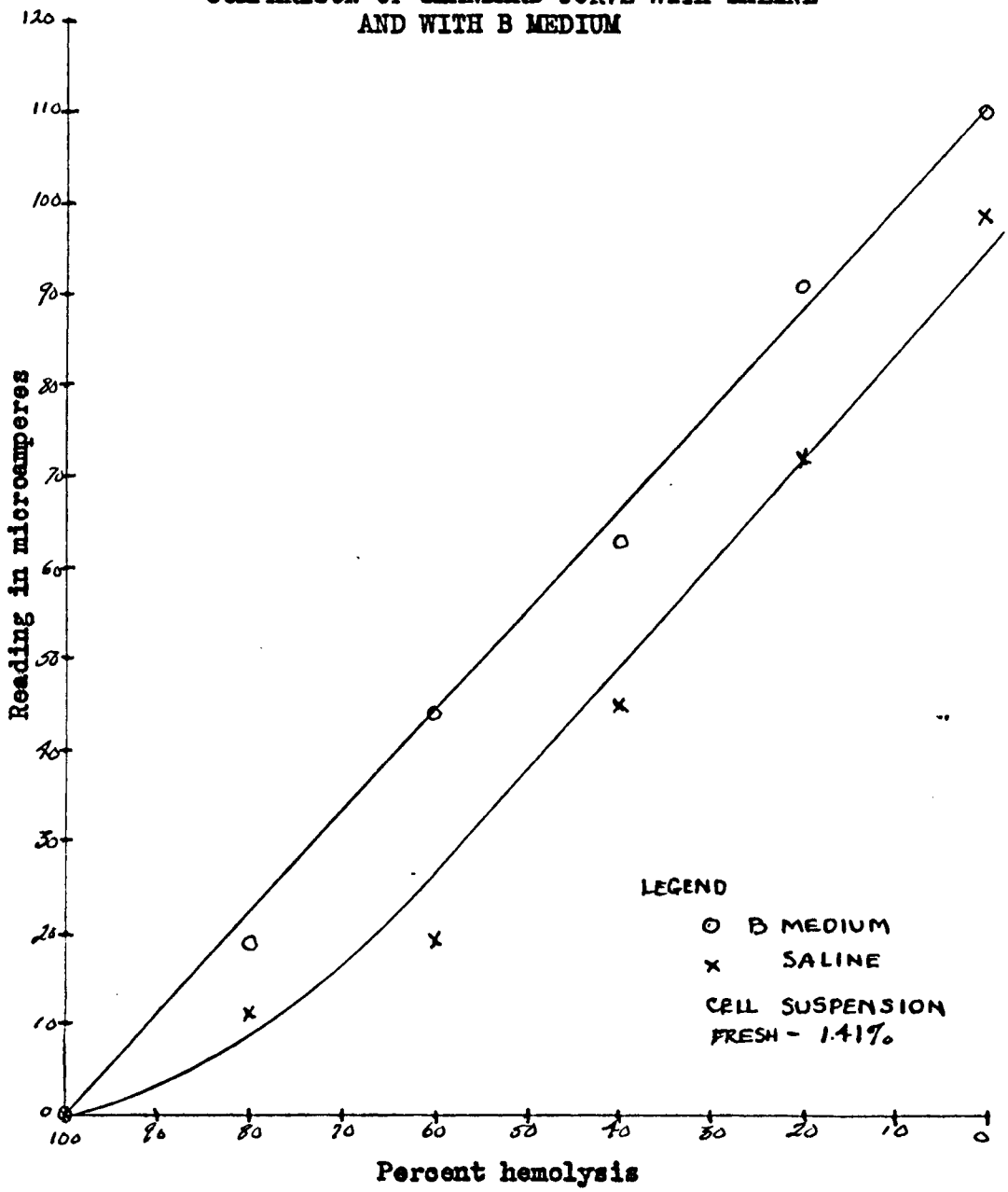


Fig. IV

COMPARISON OF STANDARD CURVE WITH SALINE AND WITH B MEDIUM



hemolytic activity. Something of the same effect may be noticed in Fig. 1, Run #2, after incubation, where it is seen that at concentrations above 1.4% cells, some change occurs in the suspension during incubation which increases the readings given by any particular concentration of cells. If some substance could be found which when added to the standard test would increase the readings to a maximum, the phenomenon of increase over TK with sub-hemolytic amounts of toxin would be eliminated.

Accordingly, different amounts of isotonic phosphate buffer 0.158 M (9) , at various pH, and different amounts of B medium were added to 1.0 ml. portions of the cell suspension. The mixtures were made up to 2 ml. volume with 0.9% saline and incubated for one hour at 40° C. One ml. of isotonic phosphate buffer pH 6.5 on 0.2 ml. of B medium were found to give the highest reading upon dilution of the incubated mixtures to 10 ml. The readings with Be medium showed more of an increase than did those with phosphate buffer. Standard curves with 1.0 ml. of isotonic phosphate buffer, pH 6.5, with 0.2 ml. of B medium, pH 6.5, and with saline were set up. The incubation volume in all cases was 2.0 ml. made up with 0.9% saline. The mixtures were incubated one hour at 40° C, diluted to 10 ml. and read on the turbidimeter, Fig. 111 and 1V. On comparison of turbidimetric with colorimetric determinations, it was found that readings taken on the B medium standard curve gave the best results. (These experiments will be discussed in detail in a later section). In the routine procedure finally adopted, 0.2 ml. of B medium, pH 6.5, was added to each tube in the standard run.

Titration of Hemolysin.

With each titration run a standard run was set up. In the titration run, 1 ml. of a 1.4-1.6% red blood cell suspension was added to decreasing amounts of the toxin or toxic filtrate. The mixtures were made up to volume with 0.9% saline and incubated in a constant temperature water bath. At the end of the incubation period, saline was added to all tubes to a final volume of 10 ml. The contents of the tubes were thoroughly mixed, and readings were taken on the turbidimeter. By comparison of the readings with the % hemolysis of the standard curve, hemolysis in any particular mixture could be determined. As illustration: if the standard curve were that given in Fig. 111 and a mixture in the titration curve gave a reading of 70, the percentage hemolysis would be 40 as read by the buffer curve, 30 as read by the saline curve.

An increase in incubation volume was found to decrease the hemolytic activity of a toxin. An ammonium sulfate precipitated toxin, dissolved in phosphate buffer saline at a concentration of 1 mg./ml. was distributed as shown in Table XXXV11 in two sets of tubes. One ml. of a cell suspension was added to each tube. The volume in one set was made up to 2 ml., in the other to 3 ml. with 0.9% saline. The mixtures were incubated for one hour at 37°C, diluted to 10 ml. final volume, thoroughly mixed, and read on the turbidimeter. Percent hemolysis was determined by comparison of the readings obtained with those of the standard curve, Table XXXV11.

Table XXXVII.

Effect of Incubation Volume on Hemolysin Determination.

Ml. Lysin	Inc. vol.	% Hemolysis 2 ml.	3 ml.
0.4		41	37
0.6		57	51
0.8		74	--
1.0		81	75
0.51*		50	--
0.59		--	50

* Value for 50% hemolysis calculated.

An increase in the temperature at which the lysin-cell mixtures were incubated increased the hemolytic activity of toxic filtrates. Mixtures of cells and lysin were prepared and incubated one hour at various temperatures. At the end of the incubation period, the mixtures were made up to ten ml. final volume, mixed and read on the turbidimeter. For each run a standard set was prepared to be incubated at each temperature. The percent hemolysis in the mixtures incubated at any one temperature was determined by comparison of the readings obtained with the mixtures, with the standard curve obtained for the particular temperature.

TABLE XXXVIII

Effect of Incubation Temperature on Percent Hemolysis in Lysin-Cell Mixtures.

Run #	Mixture #	% Hemolysis 35°C	40°C	45°C
I	a	0	--	25
II	b	9	--	40
	c	20	--	62
	d	48	--	93
III	e	--	22	35
	f	--	58	74
	g	--	87	93

The data presented in the above table indicated that the increase in hemolytic activity with increasing temperature was greater between 35 and 40 degrees than between 40 and 45 degrees. The temperature of 40°C was chosen as the routine incubation temperature since lysis of the cells by constituents of the mixtures other than the lysin would be less at 40° than at 45°.

The length of the period of incubation of cells-lysin mixture affected the percentage hemolysis obtained. Five sets of tubes were prepared, each set containing tubes for standard curve and tubes for titration curve. Standards were prepared with graded amounts of a 1.6% cell suspension, with 0.2 ml. B medium adjusted to pH 6.5, and saline to a final volume of 2.0 ml. added to each tube. Varying amounts of a culture filtrate were added to the tubes for the titration curve. 1.0 ml. of the cell suspension was added to each portion of toxin, and the volume of all mixtures was made up to 2 ml. Each set was incubated for a different length of time at 40°C; set #1 for one half hour, #2 for one hour, #3 for two hours, #4 for three hours, and #5 for five hours. At the end of each incubation period, the mixtures were made up to 10 ml. volume by the addition of 8 ml. of 0.9% saline. Thoroughly mixed and read on the turbidimeter. Percent hemolysis in the titration mixtures incubated for any one length of time was determined by comparing the readings obtained with the cell-lysin mixtures with those obtained with the standard curve mixtures incubated for the same length of time.

In this way, hemolysis with 0.1 to 0.5 ml. of filtrate, by 0.05 ml. increments, was determined for the various incubation periods. From this data, a curve was drawn for hemolytic

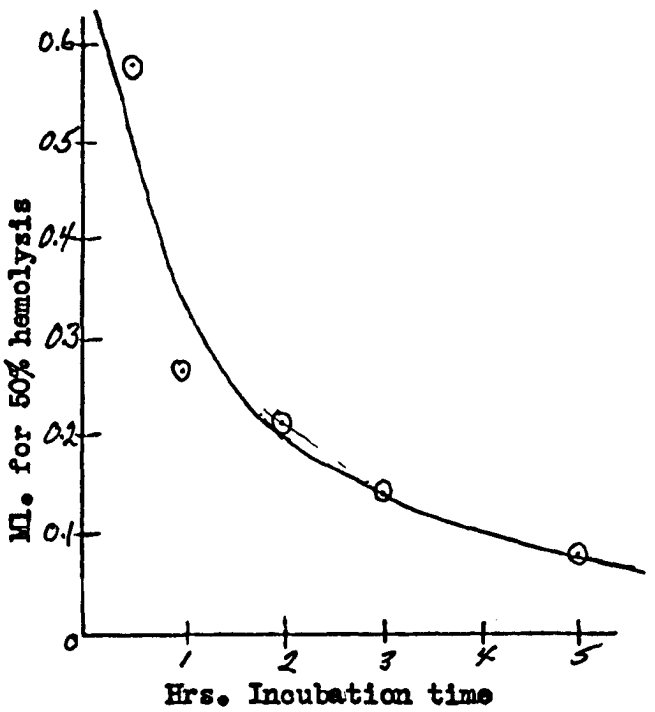
activity for each incubation period, with ml. of toxin plotted on the abscissa and percent hemolysis plotted along the ordinate. The curves obtained were similar to those shown in Fig. VI. From the curves so drawn, the end point for eachincubation period could be determined . The end point in all determinations of hemolytic activity was the number of ml. which would cause 50% hemolysis of the cell suspension under the conditions of the experiment.

The results are presented graphically in Fig. V in which the end point (ml. for 50% hemolysis) is plotted against the incubation time. On inspection of Fig. V, it may be seen that as the incubation period was increased, the ml. necessary for 50% hemolysis decreased, i.e., the hemolytic activity increased. The curve is very sharp between one-half and one hour and falls off more gradually from one to five hours. Despite the increased activity shown with longer incubation, one hour was chosen as the routine incubation time. During this period of time, it was felt that the cell suspension would show less autohemolysis than during a longer incubation. Also, one hour rather than a longer period was chosen as the routine incubation time in the interests of shortening the test.

The accuracy of the test method may be seen by comparison of the end point values determined at different times for dried, stable toxin preparations. Ammonium sulfate precipitated toxins were dissolved in isotonic phosphate, 0.158 M (PA) - 0.9% saline, 1:1 at a concentration of 10 mg./ml. (For a complete discussion of the method see page). With freshly prepared toxin solutions and cell suspensions not older than four days, results were obtained at various times during

Fig. V

EFFECT OF INCUBATION TIME ON HEMOLYTIC ACTIVITY OF A TOXIC FILTRATE



the experimental work. Table XXXVIII shows end points obtained on two dried toxins at different times.

On examination of Table XXXVIII it will be seen that the comparison of the end points obtained for any one toxin, titrated at different times is good.

TABLE XXXVIII.

Repitition of Determination of Hemolytic Activity of Ammonium Sulfate Precipitated Toxins.

Toxin #	Run #	Ml. for 50% Hem.
3*	1	0.060
	2	0.060
	3	0.061
	4	0.062
22A*	1	0.52
	2	0.53
	3	0.53

* Toxins dissolved in phosphate buffer saline, 10 mgs./ml.

To check further on the reliability of the method, colorimetric determinations were compared with turbidimetric assays. It was such comparison which lead to the adoption of the procedure of adding 0.2 ml. of B medium, pH 6.5, to the standard curve.

A turbidimetric assay was prepared. Two standard curves were set up, one containing graded amounts of cells mixed with 1.0 ml. of isotonic phosphate buffer, pH 6.5, and saline to 2 ml. final volume, the other containing graded amounts of cells and saline to 2 ml. final volume. Culture filtrate was added in increasing amounts to 1 ml. of the cell suspension, and these mixtures were made to 2 ml. volume with saline. The standard and titration sets were incubated one hour

at 40°C. After the incubation period, 8 ml. of saline was added to each mixture. The suspensions were thoroughly mixed and read on the turbidimeter. The percent hemolysis in the mixtures of the titration set was determined by comparison of the readings with those of the standard set containing buffer and saline, and with those of the standard set containing saline alone Fig. 111. It may be seen from Fig. V1 A that percent hemolysis in mixtures containing less than about 95% hemolyzed cells was higher when read on the buffer curve than when read on the saline curve.

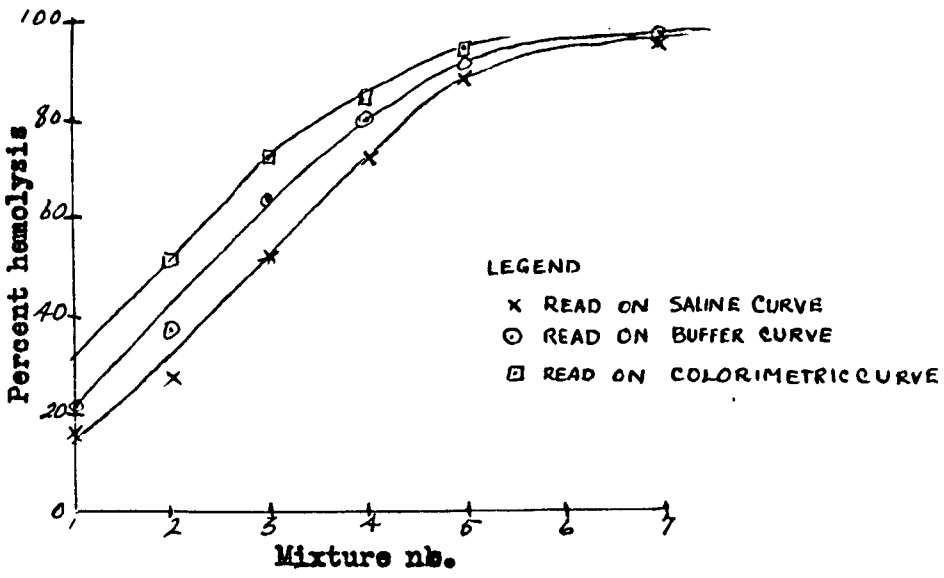
Immediately after reading, the cells were centrifuged from the mixtures. The supernatants were carefully separated from the cells and read on an Evelyn colorimeter. A standard curve was prepared for the colorimeteric determination with a portion of the cell suspension used for the turbidimetric assay hemolyzed by the addition of a small amount of saponin. This hemolyzed material was distributed in increasing amounts from 0.1-1.0 ml., corresponding to ten to 100% hemolysis of 1.0 ml. of the original suspension. Each amount was made up to 10 ml. final volume. Standard readings were taken on the Evelyn colorimeter with a filter with a maximum absorption of 540 mm. and a center setting with distilled water. From the readings obtained with the standard mixtures, a curve was prepared with readings plotted against percent hemolysis. Hemolysis in the titration mixtures was determined by comparing the colorimeter readings of these mixtures with the standard curve.

The results are plotted in Fig. V1 A, and it may be seen that percent hemolysis determined colorimetrically was higher

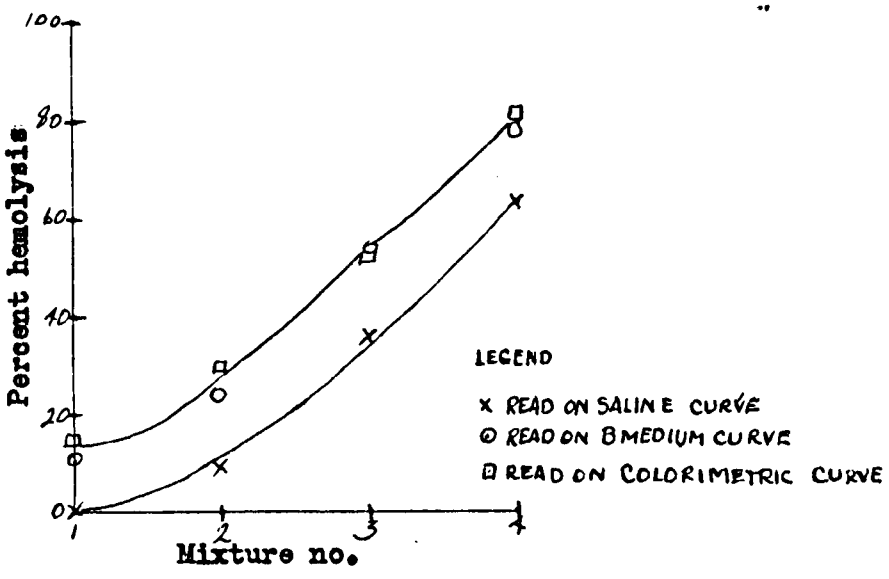
Fig. VI

PERCENT HEMOLYSIS DETERMINED COLORIMETRICALLY AND TURBIDIMETRICALLY

A



B.



than either of the two turbidimetric determinations, that with the buffer-saline standard, and that with the saline standard curve.

A similar determination was made with turbidimetric standards prepared with 0.2 ml. of B medium, pH 6.5 and saline to volume, and with saline alone. The titration mixtures were prepared as above. Both standards and titration mixtures were incubated for one hour at 40° C, diluted to 10 ml., the cells suspended by thorough shaking, and read on the turbidimeter. Hemolysis in the titration mixtures was read on both standard curves, Fig IV, and was higher as read on the curve in which B medium was present than as read on the curve in which B medium was absent, Fig. VI B.

Hemolysis was determined colorimetrically by the method outlined above. The results plotted in Fig. VIB were higher than those determined turbidimetrically using a saline standard curve but were in excellent agreement with those determined turbidimetrically using a B medium standard curve. The last results reported were taken as an indication of the reliability of the turbidimetric method and of the advisability of adding B medium to the standard curve.

Characteristics of the Hemolytic Reaction and the Hemolysin.

The activity of bacterial hemolysins is influenced greatly by the pH at which the lytic reaction is carried out, (10)(39) Observations of Walbum (39)on the effect of pH on the hemolytic activity of Cl. oedematiens filtrates have been confirmed. Portions of a filtrate were adjusted to various hydrogen ion concentrations by the addition of HCl or NaOH. The pH was measured with

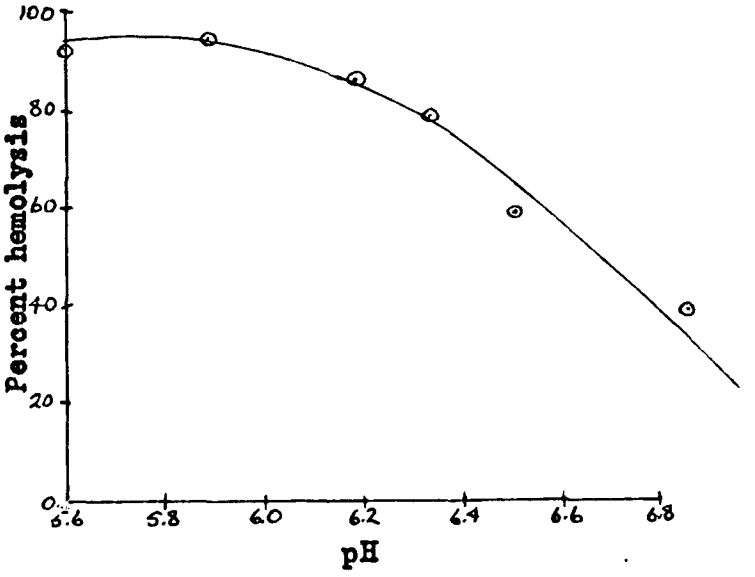
a glass electrode. The filtrate was from a culture grown on B medium which contained 0.02 M phosphate. The cells-lysin mixtures were assumed to be buffered by the amount of phosphate added with the filtrate. The percent hemolysis obtained with 0.4 ml. of each of the portions of filtrate was determined by the method described above. Controls were run with 0.4 ml. of toxin, incubated with a sufficient quantity of antitoxin to t stop the specific hemolysin, for one hour, at room temperature. As may be seen from Fig VII, in which percent hemolysis was plotted against pH, hemolytic activity decreased uniformly from pH 6.0 - 6.8. Walbum's data carried the curve farther to the alkaline side and showed a continuing decrease in hemolytic activity at higher pH. Below pH of about 5.8, the non-specific hemolysin, i.e, the hemolysin not neutralized by antitoxin, increased.

Effect of Dilution. Many of the toxins had to be diluted for accurate determination of hemolytic activity. The largest dilution made was one volume of toxin with nine volumes of diluent)1:10). When the diluent was saline, there was no decrease noted in the hemolytic activity of any particular filtrate . In some cases there was, rather, an apparent increase in hemolytic activity on dilution of a filtrate in saline, while in most cases, no difference was noted.

In view of the importance of pH to the determination of hemolytic activity, buffer diluents were tested. These were a phosphate buffer and B medium, which on testing proved to be

Fig. VII

EFFECT OF pH ON HEMOLYTIC ACTIVITY OF A TOXIC FILTRATE



non-hemolytic. The phosphate buffer used was 0.158 M which at pH 6.5 is isotonic with, has a vapor pressure equal to, 0.9% saline.(9). It was found that dilution in this buffer mixed with saline in the ratio 1:1 gave greater hemolytic activity than dilution in either saline or B medium. For example, one filtrate diluted 1:10 in saline gave an end-point of 0.037 ml. (calculated as ml. of the original filtrate), in B medium, an end-point of 0.022 ml. and in buffer-saline, an end-point of 0.016 ml. The low result (high ml. of toxin indicated low hemolytic activity) with the saline dilution may have been due to the poor buffering capacity of this dilution.

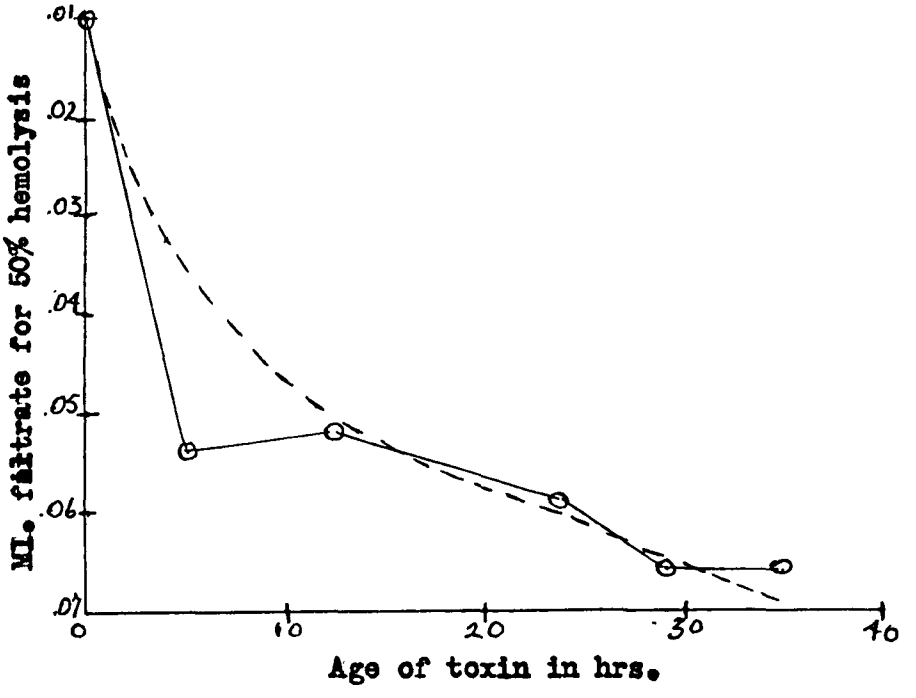
From experience such as the one described above, isotonic phosphate buffer was adopted as the diluent.

Stability of the hemolysin. Hemolytic activity of the filtrates was destroyed by heating in an 85°C water bath for ten minutes. A portion of a filtrate, of which 0.05 ml. (0.5 ml. of a 1:10 dilution in buffer-saline) gave 75% hemolysis, was heated for ten minutes, in an 85° water bath. 0.5 ml. of a 1:10 dilution of the heated material in buffer-saline gave zero hemolysis when tested with the same suspension under the same conditions of incubation volume, time and temperature.

The hemolytic activity of filtrates decreased on standing at room temperature. A toxic filtrate stored at room temperature was tested at intervals over a period of two days. The end-point results (ml. for 50% hemolysis), obtained by titration of the hemolysin by the method outlined above, at the various intervals were plotted against the age of the filtrate in hours, Fig.VIII. The zero point

Fig. VIII

DECREASE IN HEMOLYTIC ACTIVITY OF A TOXIC FILTRATE ON STORAGE AT ROOM TEMPERATURE



was obtained by titration of the filtrate with 0.005 M thioacetic acid. (This titration gave the optimum hemolytic activity of the filtrate). Justification of the use of the end-point so determined as the activity of the filtrate at zero hours age is found under the discussion of the effect of thioacetic acid on hemolytic activity of toxic filtrates. In this latter work, it was shown that filtrates titrated, at various intervals after harvesting, with the optimum concentration of thioacetic acid gave the same end-point even after considerable aging) .

According to Fig. VIII the hemolytic activity of the filtrate tested decreased (increasing ml. for 50% hemolysis denoted decreasing hemolytic activity) over a thirty-five hour period. The results of this experiment were corroborated by comparison of the activity of filtrates stored at 50° C at various intervals. The decrease in activity noted in the latter cases was slower to occur than in the case shown graphically in Fig. VIII.

Effect of Cysteine on the Hemolytic Activity of Filtrates.

It was thought that thedecrease inactivity of the hemolysin might be an oxidative reaction comparable to that found with streptolysin-O(10).With this in view, the action of cysteine on the hemolytic activity of toxins was investigated. A solution of cysteine .HCl was prepared in saline such that the concentration was 1.0 M with respect to cysteine. From the 1.0 M solution, a dilution in saline was prepared such that the concentration was 0. 01 M cysteine and the pH was 6.5 Various amounts of the 0.01 M solution were added to a definite quantity of the hemolytic filtrate.

For instance, toxin #1, Fig. 1X was distributed in 0.5 ml. quantities. To each 0.5 ml. portion, cysteine was added in increasing amounts so that the concentration of cysteine in successive mixtures of toxin and cysteine was 0, 0.5, 0.9, 1.3, etd., x 10^{-3} molar. The percent hemolysis caused by each mixture acting on 1 ml. of a 1.6% cell suspension was determined by reading percent hemolysis from a standard curve set up with each assay.

Cysteine itself under the conditions of the experiments was non-hemolytic at the highest concentrations tested. This may be seen by examination of Fig. 1X. The last point in the assay of toxin #2 contained the highest amount of cysteine tested. The concentration of cysteine in the final incubation mixture, cells-lysin-cysteine-saline, 2 ml., at this point was 0.002 molar. The cysteine had completely inhibited the lysin and showed no lytic effect itself. This was confirmed by addition of cysteine alone, in amounts corresponding to those added to the lysin, to 1.0 ml. of the cell suspension.

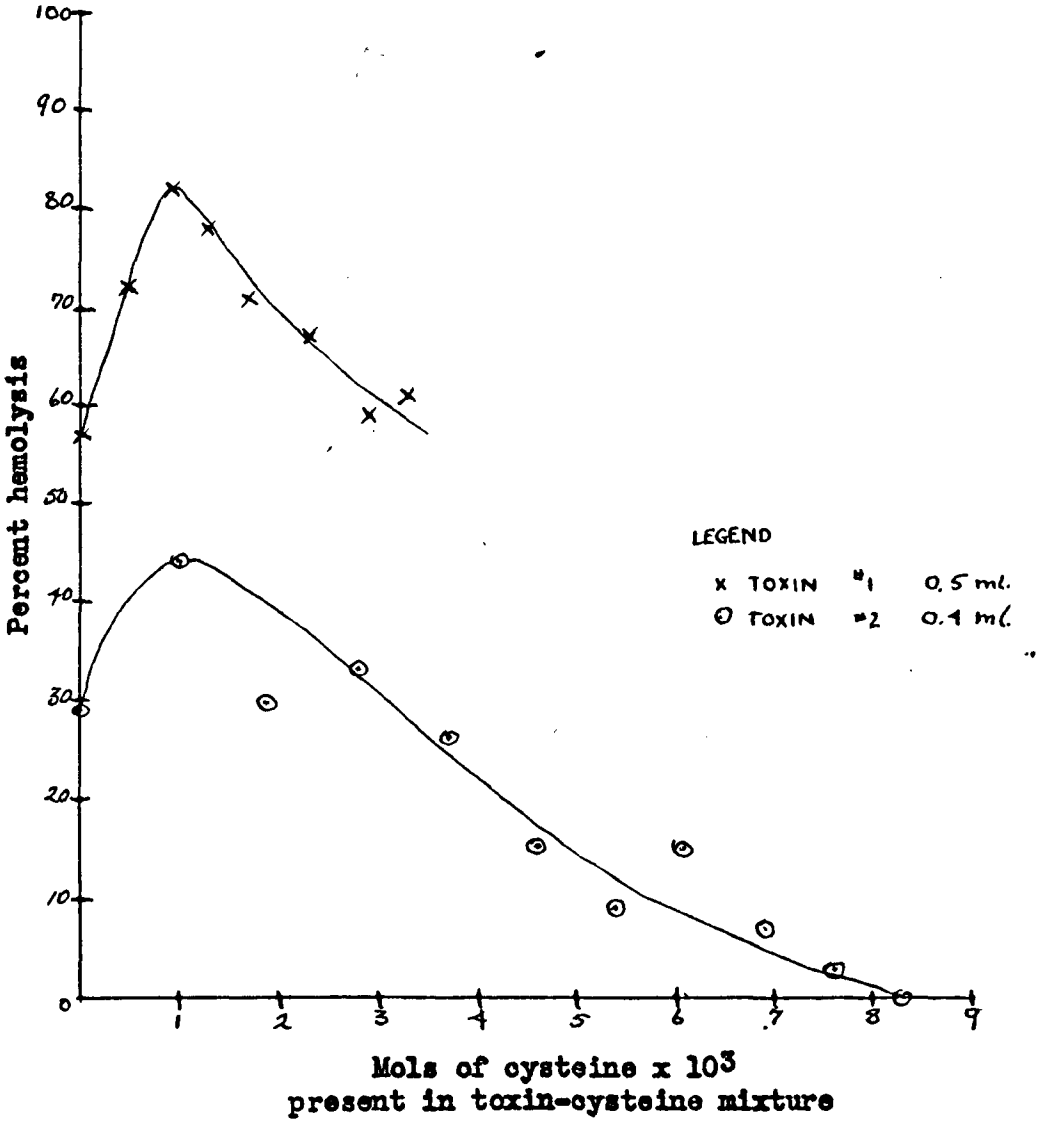
Repitition of experiments such as the one described above, and the ones presented graphically in Fig. 1X showed that cysteine increased the activity of the hemolysin of Cl. oedematiens filtrates about 1-1/2 times. The optimum concentration of cysteine in lysin-cysteine mixtures for this increase in activity was seen to be rather sharp and to have a value of about 0.001 molar, for all toxins tested.

The Effect of Thioacetic Acid on the Hemolysin of Filtrates.

Reducing agents, such as cysteine, increased the

Fig. IX

OPTIMUM CONCENTRATION OF CYSTEINE IN ACTIVATION OF HEMOLYSIN OF TOXIC FILTRATES



hemolytic activity of streptolysin-O about ten times.(10). Since the activity of cysteine for the hemolysin of Cl. oedematiens filtrates was only 1/10 of that for the streptolysin, it was hoped that some other compound containing labile SH groups would be more active. The effect of thioacetic acid was investigated, and found to be an activation effect.

The optimum concentration of thioacetic acid for activation of the hemolysin was determined by a method similar to that used in the determination of the optimum concentration of cysteine. A 1 M suspension of thioacetic acid in saline was prepared. From this a dilution was made in buffer, 0.158 M phosphate, pH 6.5, mixed with saline 1:1. The concentration of the dilute solution used was 0.01 M. The pH was adjusted to 6.5 by the addition of about 0.005 ml. of dilute NaOH.

Increasing amounts of the 0.01 M thioacetic acid solution were added to quantities of the filtrates which without thioacetic acid would cause al small amount of hemolysis. Thioacetic acid was added to give the concentration shown in Fig. X in the toxin-acid mixture. After 15-30 minutes 1.0 ml. of the cell suspension was added. Saline was added to each mixture to a final volume of 2 ml. and themixtures were incubated for one hour at 40°C. Percent hemolysis in each mixture was read from the standard curve set up with each determination.

Hemolysis by the thioacetic acid alone was tested by adding amounts of the 0.01 M acid solution to 1. 0 ml. of cells. The amounts added were equal to those used in the assay of optimum concentration. With 0.0025 M thioacetic acid in the final

incubation mixture, there was only about 5% hemolysis. This concentration was the highest used in any test.

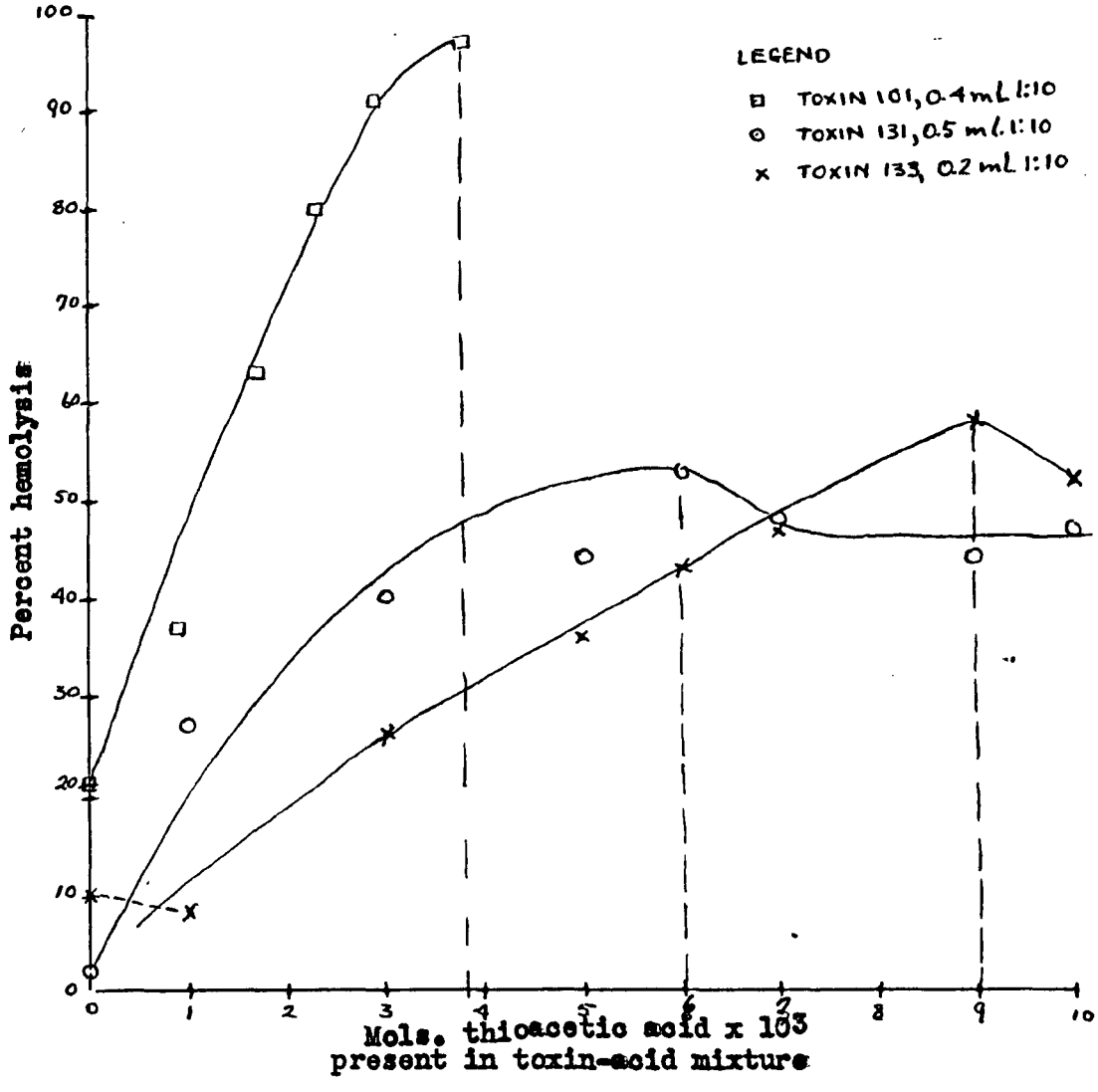
From Fig. X, in which are plotted determinations for the optimum concentration of thioacetic acid for three filtrates, it may be seen that thioacetic acid increased the hemolytic activity of Cl. oedematiens filtrates about five times. The optimum concentration for toxin #101, Fig. X appeared to be about 0.004 molar, for toxin # 131, 0.006 molar, and for toxin #133 about 0.009 molar. The low value for the optimum for toxin # 10 may be incorrect. The amount of toxin used gave 20% hemolysis without thioacetic acid and the curve for added thioacetic acid above 0.003 molar fell above 90% hemolysis, a portion of the graph which it is felt does not give an accurate picture of the effect.

As the 1.0 M thioacetic acid suspension aged, it was noticed a white precipitate formed on the walls of the container. Suspensions of various ages were compared for potency in increasing hemolytic activity, Table XXXIX. In Run #1, 1.0 M thioacetic acid suspensions were added to a 1:10 dilution of a filtrate in buffer-saline to a concentration of 0.005 M thioacetic acid. An end-point (ml. for 50% hemolysis) titration was run on each mixture. In Run #2 1.0 M thioacetic acid suspensions of various ages were added to a 1:10 dilution of a filtrate in buffer-saline to a concentration of 0.008 M thioacetic acid.

From the Table XXXIX, it may be seen that over a period of a month, the efficiacy of the thioacetic acid suspension decreased markedly, Run #1. If the thioacetic acid suspension

Fig. X

OPTIMUM CONCENTRATION OF THIOACETIC ACID IN ACTIVATION OF HEMOLYSIC ACTIVITY OF TOXINS



were stored four days a slight decrease in the potency of the suspension was noticed, Run #2. The decrease in potency of the thioacetic acid on four days storage was so slight as to fall almost within the limits of error of the test. However, for comparative purposes, a thioacetic acid solution was prepared each day that a determination was run.

Table XXXIX.

Activity of Thioacetic Acid Suspensions Various Ages.

Run #	Storage Time	% Hemolysis	Ml. Toxin
1	0*	50**	0.016
	2 weeks	50	0.025
	4 weeks	50	0.037
11	0*	65	0.02
	2 days	63	0.02
	4 days	60	0.02

* Thioacetic acid suspension prepared immediately before testing.
** Results for Run #1 are from end-point titrations.

The Effect of Antitoxin on the Hemolytic Activity of Filtrates.

Cl. oedematiens antitoxin completely inhibited the hemolytic activity of culture filtrates of Cl. oedematiens, with different filtrates showing different antihemolytic combining power. The antitoxin used throughout the hemolysin work was a concentrated preparation* in glycerol containing 750 units Cl. oedematiens antitoxin and 1600 units Cl. sordelli antitoxin per ml.

*Obtained from Lederle Laboratories, Inc.

The antihemolytic unit was arbitrarily chosen as equivalent to the antitoxin test dose, i.e. that amount of the above antitoxin which contained one oedematiens antitoxin test dose, was said to contain one antihemolytic unit, AHU.

The antihemolysin combining power was determined by mixing toxin and antitoxin in various proportions and incubating themixtures at room temperatures. After the incubation period, the mixtures of hemolysin and antihemolysin were treated as mixtures in a hemolysin assay. 1.0 ml. of a 1.4-1.6% suspension of rabbit red blood cells and saline to 2 ml. final volume were added to each mixture. The mixtures containing added cells and saline were incubated for one hour at 40°C, diluted to 10 ml. with saline, and read on the turbidimeter. Percent hemolysis in the various mixtures was read from the standard curve. The AHU end-point selected dépended on the method used for titration. When the titration was run with hemolysin constant, the end-point was that point at which the percent hemolysis showed no further degrease. When the antihemolysin was constant in the lysin-antilysin mixtures, the end-point was calculated as lying between the values for the mixture in which there was no hemolysis and the next succeeding mixture in which hemolysis was present. (Further details of the two methods are given in the division on comparison of the end-point determinations obtained using both methods.

The effect of the time of incubation of the hemolysin-antihemolysin mixtures before the addition of cells. Triplicate sets of tubes were prepared with constant hemolysin content and varying amounts of antihemolysin. One of the sets

was incubated for one-half hour, one for one hour, and the third for two hours at room temperature. At the end of the room temperature incubation period, cells and saline were added and percent hemolysis was determined with one hour incubation at 40°C. With the filtrate tested, the same AHU titre was obtained for each period of hemolysin-antihemolysin incubation, Table XL.

Table XL.

Determination of Antihemolytic Combining Power with Increasing Lysin-antilysin Incubation Periods.

Time of Incubation of Lysin with antilysin	AHU/ml.
30 minutes	140 ≠ , 160-
1 hour	140 ≠ , 160-
2 hours	140 ≠ , 160-

For incubation of hemolysin and antihemolysin, one hour was chosen as the routine length of time. This it was felt would be nearly optimum in cases in which the lysin and antilysin might combine more slowly than in the example presented in Table XL, and, at the same time, would keep the method of reasonably short duration for routine analyses.

Comparison of AHU end-point determination with hemolysin constant and with antihemolysin constant. The description of the method of determining the combining power with hemolysin constant follows. To a constant amount of filtrate which would cause at least 30% hemolysis were added increasing amounts of the antihemolysin. In some assays, the antihemolysin prepara-

tion was diluted in saline to contain 100 AHU/ml. This was the case in the determination of the antihemolytic combining power of toxin #L, Fig. XIA. In the experiment presented graphically in Fig. XI A, 0.5 ml. portions of filtrate were mixed with 0,0.05 , 0.1, 0.15, 0.2,---ml. of antihemolysin, corresponding to 0, 10, 20, 30, 40, ---AHU/ml. of filtrate. The mixtures were incubated at room temperature for one hour, cells and saline were added and the percent hemolysis was determined after one hour incubation at 40° C.

It may be seen from Fig. XI A, that hemolysis decreased regularly to a minimum of 20%. (This latter hemolytic activity was seldom higher than 20% in the filtrates tested and usually ranged between 0 and 5%. The hemolysis not inhibited by antihemolysin was called the non-specific hemolysis). The end-point in the determination shown in Fig. XI A, taken at the intersection of the decreasing hemolysis and non-specific, "basic" hemolysis curves was 45 AHU/ml. shown at X.

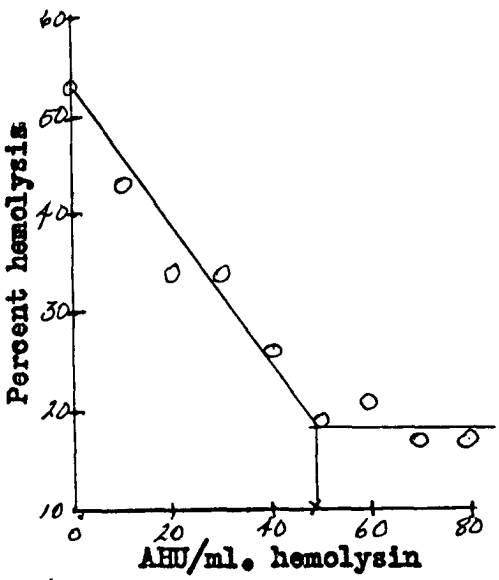
In other assays, the antihemolysin was diluted to contain 200, 20, or 10 AHU/ml., depending on the strength of the hemolysin and the amount of the hemolysin used for testing the combining power.

In all assays, the volume of the lysin-antilysin-cells-saline mixture did not exceed two ml.

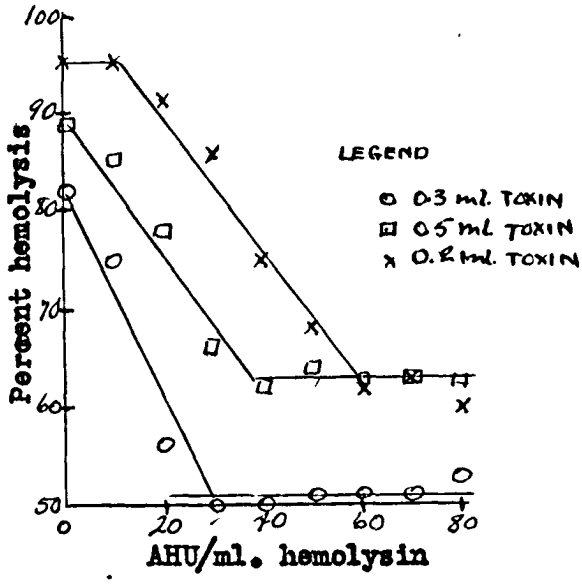
The end-point obtained by this method was not independent of the amount of filtrate used. In Fig. XI B, data are presented graphically which demonstrate this effect. For the

Fig. XI

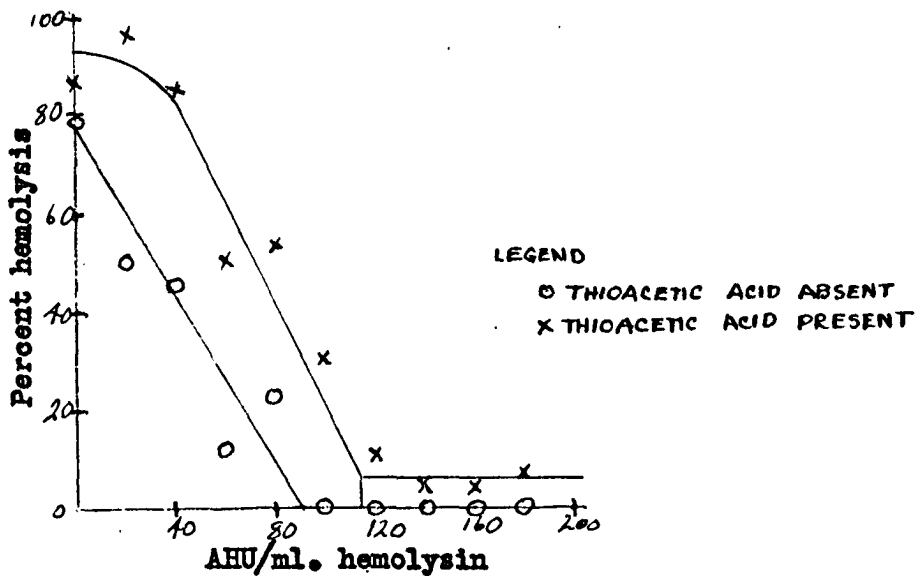
EFFECT OF ANTITOXIN ON HEMOLYTIC ACTIVITY-
TOXIN CONSTANT



A. Toxin L constant at 0.5 ml. antihemolysin increasing



B. Toxin 254 constant at 0.3, 0.5, 0.7 ml.- antihemolysin increaseing



C. Effect of thioacetic acid on antitre of toxin filtrate 101-4 days old

data given, three sets of tubes were prepared. The first contained 0.3 ml., the second, 0.5 ml., and the third, 0.7 ml. of filtrate # 254. Aliquots of antihemolysin equivalent to 0, 10, 20, 30, 40,---AHU/ml. of filtrate were added to each set and the mixtures were incubated for one hour at room temperature. Cells and saline were added at the end of the hour, and the mixtures were incubated a second hour at 40° C. After the 40 incubation period, the mixtures were diluted to 10 ml. with saline, and the percent hemolysis was determined turbidimetrically.

From Fig. X1 B, it will be seen that the end-point in AHU/ml. increased with increasing amounts of filtrate. It will be noticed that filtrate #254 had a very high non-specific hemolysis which may have had some effect on the values. The data is collected in Table XL1, which also contains corroborative data obtained on three other filtrates.

Table XL1.

Determination of AHU/ml. with Increasing Amounts of Hemolysin-Lysin Constant.

Run #	Ml. Filtrate	% Hemolysis with no antihemolysin	AHU/ml.
1	0.3	82	25
	0.5	90	38
	0.7	95	60
11	0.1	-- *	25
	0.5	--	50
111	0.1	--	65
	0.5	--	120
1V	0.1	--	75
	0.5	--	120 +

* Percent hemolysis not determined accurately, but over 80% with 0.1 ml. of the filtrates tested in Run #11, 111, and 1V

In the method in which antihemolysin was constant, ten or 20 AHU of antihemolysin were placed in each of a set of tubes Increasing amounts of filtrate were added and the mixtures were incubated for one hour at room temperature. Following this, cells and saline were added, the mixtures were incubated for one hour, at 40°C, diluted and read on the turbidimeter. The end-point was determined by examination of the readings obtained. As an example: to 10 AHU of antihemolysin, a filtrate, diluted 1:10 in buffer-saline, was added as shown in Table XLll. The mixtures were treated as described above, and the readings listed in Table XLll were obtained.

In this case, there was sufficient hemolysin in 0.08 ml. (0.8 ml. of the 1:10 dilution) to neutralize 10 AHU of antihemolysin and give close to 80% hemolysis, while in 0.05 ml. there was apparently not enough hemolysin to neutralize the antihemolysin present. As the end-point was taken as lying between the point where no hemolysis was shown and the succeeding point in which hemolysis was demonstrated, the filtrate tabulated in Table XLll contained between 63 and 100 AHU/ml.

Table XLll

Determination of AHU/ml. of Filtrate Antihemolysin Constant.

Ml. of filtrate diluted 1:10	Reading Microamperes	End-Point AHU/ml.
0.25	86	
0.3	87	
0.4	90	
0.5	88	100
	12	63

Table XLlll shows the comparison of AHU/ml. of filtrate determined with hemolysin constant to that determined with antihemolysin constant.

Table XLlll

Determination of AHU/ml. of Filtrate Hemolysin and Antihemolysin Constant

Run #	AHU/ml.	
	Hemolysin Constant	Antihemolysin Constant
1	75 $\frac{+}{140}$, 100-*	63 $\frac{+}{143}$, 100-
11		

* Hemolysin constant at 0.1 ml. (0.5 ml. of 1:5 dilution)- 90% hemolysis with no antihemolysin.
* Hemolysin constant at 0.1 ml. - 100% hemolysis with no antihemolysin.

The effect of the presence of thioacetic acid on the determination of AHU/ml. of toxic filtrates. If thioacetic acid were added to filtrates in concentrations of 0.005-0.01 molar before the determination of the AHU content of the filtrate were made, the values obtained were slightly higher than those obtained in the absence of thioacetic acid, Fig. Xl C and Table XLiV.

Table XLIV.

Effect of Thioacetic Acid on Determination of AHU/ml.

Toxin #	Ml. of Toxin	Concentration of Thioacetic Acid	% Hemolysis	AHU/ml.
101	0.1	0	79	80 ≠ ,100-
	0.1	0.005 M	87	120 ≠ ,140-
110	0.5	0.	100	50 -
	0.5	0.01 M	100	50
112	0.5	0.	100	50 ≠ , 100-
		0.01 M	100	120

Comparison of Hemolytic and Lethal Activity.

Toxic Filtrates. The antihemolysin combining power of toxic filtrates prepared on various media was determined with the hemolysin content constant and the antihemolysin content varied. The results, tabulated in Tables XLV A - XLV D, were compared with the in vivo determination of antitoxin.combining power, reported as. TD/ml.

The sections, A-D, of Table XLV, each contain the results obtained on one medium. Within the sections, the values are arranged in order of increasing TD/ml. for purposes of comparison.

The filtrates in section A, Table XLV, were received from Lederle Laboratories, Inc. The media on which the filtrates were prepared is not known. At the time of determining the antihemolysin comgining power, the in vivo antitoxin combining power was also determined. The medium used in preparation of the filtrates reported in section B, Table XLV, was pancreatic digest of liver cake, 3.0-4.0% with salts supplement and glucose.

Table XLV-A

Comparison on Antitoxin and Antihemolysin Combining Power of Filtrates

A

Medium Unknown

Toxin #	TD/ml.*	Antihemolysin Determination		
		ml. filtrate tested	% hemolysis no antilysin	AHU/ml.
NS 14	10 +, 20-	0.5	26	10 +, 20-
NS 16	10 +, 20-	0.7	34	10 +, 20-
255	10 +, 20-	0.7	30	10
254	20 +, 30-	0.3	82	30
NS 15	40 +, 50-	0.7	45	50
2	50 +	0.5	16	20
8	70 +	0.7	54	80

B

Medium - Pancreatic Digest of Liver Cake with Salts Supplement

L	10-	0.5	53	43
37	63-	0.1	100	80
38	63-	0.1	92	55
42	150 +, 200-	0.5	73	63-
43	200	0.5	100	63-
44	200 +	0.5	90	63-

C

Medium - Pancreatic Digest of Beef Kidney with B Medium.

		**		
135	50	---	--	63 +, 100-
104	50	0.5	57	50-
116	50	0.5	98	50 +, 100-
106	50 +, 100-	0.5**	89	50-
134	50 +, 100-	-- -	--	63 +, 100-
114	100 +, 200-	0.5**	100	100 +, 200-
136	200 +, 240-	--	--	63 +# 100-

with 0.008 M thioacetic acid

* Determined in mice

** Determination performed with antihemolysin constant.

Table XLV

D

Medium - B medium with Solvent Extracted Beef Kidney

Toxin #	T^D/ml.*	Antihemolysin Determination		
		ml. filtrate tested	% hemolysis no hemolysin	AHU/ml.
13	40 +, 50-	0.5	95	70
108	50-	0.5	24	50-
109	50-	0.5	83	100 +, 200-
105	50	0.5	98	50 +, 100-
115	50	0.5	100	50 +, 100-
39	63-	0.1	87	60
107	50 +, 100-	0.5	84	200
110	50 +, 100-	0.5	100	50-
112	50 +, 100-	0.5	100	50 +, 100-
22	70 +, 80-	1.0	23	60
111	100	0.5	100	50-
102 B	100 +, 120-	0.1	97	100-+
101	150	0.1	79	80 +, 100-
101	with 0.005 M thioacetic acid	0.1**	87	120 +, 140-
131	150	--**	--	63 +, 100-
133	150	--**	--	125 +, 167-
132	150 +, 160-	--	--	63 +, 100-
45	160 +, 180-	0.5	100	60 +
50	200	0.5	35	160 +, 200-

** Determination performed with antihemolysin constant.
* Determinediin Mice.

The filtrates in section C were prepared on B medium with 0.5% pancreatic digest of beef kidney and 0.1% glucose with the exception of toxin #136, in which the phosphate concentration was 0.06 molar (the phosphate concentration of B medium is 0.02molar) and the glucose concentration was 0.5%. The medium used in the preparation tabulated in section D was B medium with 2% solvent extracted beef kidney and 0.6% dextrin.

With six of the seven filtrates tested in section A, Table XLV, there was good correlation between the in vivo test dose determination and the in vivo antihemolytic unit determination. These tests were run before the method was refined. The rabbit red blood cell suspension used was about 5% cells, and the readings were taken on a turbidimeter less sensitive than the one used for the rest of the experimental work. It is possible that this fact may explain the high degree of correlation obtained between in vivo and in vitro results with this group of filtrates.

Of the six filtrates grown on pancreatic digest of liver cake medium, tested as shown in section B, only one, filtrate #38, showed any agreement between the in vivo and the in vitro determinations.

Filtrates, tested as shown in section C, gave a slightly better correlation of the antitoxin and antihemolysin results than did the filtrates of section B . Of the seven filtrates tested, three , filtrates #116, 134, and 114 , showed fair correlation.

Of the preparations on B-kidney-dextrin medium, nine out of nineteen, or almost 50% showed fair agreement between the in vivo and the in vitro tests.

The degree of correspondence between the antitoxin and the antihemolysin results in all filtrates except those prepared on pancreatic digest of liver cake medium makes further investigation of this reaction necessary. It has been found in the lecitho-vitellin test for determination of Cl. welchii toxicity, that it is necessary to add an indicator toxin, that is, a toxin which has a high MLD titre

as compared with the Lb, to the toxin-lecitho-vitellin system before good agreement can be assured between the in vitro, and the in vivo determinations(21). A similar necessity maybbe indicated in the case of the attempt to titrate the antitoxin combining power of Cl. oedematiens filtrates by determination of the antihemolytic combining power. A standard dry preparation should be made which would have a high hemolysin titration end-point (ml. for 50% hemolysis) and a relatively low AHU content per mg. This would be used in the following manner. The filtrate to be tested would be incubated with an excess of antihemolysin for one hour at room temperature. The indicator hemolysin would then be added to the mixtures to determine the excess of antihemolysin present. Such a procedure would eliminate the possibility of a filtrate containing such a small hemolytic activity in relation to the antihemolytic combining power, that the AHU end-point would be false.

Summary

1. Lethal activity of Cl. oedematiens filtrates was determined by testing for MLD or LD_{50} by subcutaneous injection, and for combining power by incubation of toxin with antitoxin and injection of portions of the mixtures into mice. The ratio of MLD to TD (test dose, where 1 test dose is equivalent to 0.02 units of antitoxin) in filtrates was found to vary from 25 to over 100, in ammonium sulfate precipitated toxins, from 20 to 80.

First Period of Toxin Production

2. With cultures N104, from the National Institute of Health, and N21B, from Lederle Laboratories, Inc., toxin was produced on casein acid hydrolysate medium, B medium, containing the hydrolysate (1600 mgs.N/l.) with 0.02 M phosphate ($Na_2HPO_4.12H_2O$, 5.76 gms./l.; KH_2PO_4, 0.48 gm./l), $MgSO_4.7\ H_2O$, l-tryptophane and l-cystine (20 mgs./l. each), sodium succinate (5 gms./l.) and Ca-d-pantothenate, pimelic acid, pyridoxin, thiamine-HCl (1 mg./l. each) and riboflavin (1 mg./l.). The medium, pH 7.6 - 7.8, was supplemented with extracted meats and various digests.

3. The cultures were grown without strict anaerobic conditions. Pigeon passage was found unnecessary for maintenance of virulence. Contaminants were eliminated by heat shock, which did not decrease the ability of the culture to produce toxin.

4. With seed cultures transferred daily on B medium with solvent extracted beef heart and dextrin, toxin obtained after 24 - 48 hrs.

incubation at 37° C. on B medium with solvent extracted beef heart ranged in potency from 500 to 2500 MLD/ml., while that obtained on B medium with solvent extracted beef kidney ranged from 2500 - 4000 MLD/ml.

5. Dextrin was shown to be better as a source of carbohydrate for toxin production than glucose when the medium was B-heart. The optimum concentration for both carbohydrates on the above medium with 24 - 48 hrs. incubation at 37° C. was about 0.5%. It was indicated that washed dextrin was unsatisfactory for toxin production. When 0.02 - 0.04 M additional phosphate was added to B-kidney or B-pancreatic digest of liver cake medium with 0.6% glucose, the toxin production was found to increase about 2000 MLD/ml.

6. B medium with a nitrogen concentration of 1600 mgs./l and with supplements of solvent extracted beef heart, pancreatic digest or liver cake and lipid free egg yolk was found to yield a toxin of slightly greater potency than B medium 1000 mgs.N/l. with the same supplements.

7. Experiments conducted at the beginning of this experimental period showed that the optimum time of incubation of toxin production cultures, as tested by MLD for mice, was 24 - 48 hrs. After 4 months daily subculture on B-heart medium with dextrin, the optimum time of incubation for toxin production with culture N104 was indicated to be 5 days, as tested by combining power of the toxin. Toxin production with N21B was higher and appeared to increase up to 10 days.

Second Experimental Period-Culture N21B

8. The culture was transferred every week or every other week and stored for three weeks to two months at room temperature. Under the experimental conditions tested, no difference in toxin production was indicated when inocula were prepared from cultures stored at room temperature or from cultures stored at 5° C. B-kidney-dextrin medium served better as storage culture medium than B-kidney-cysteine-ascorbic acid-glucose medium when inocula cultures were prepared on B-kidney-cysteine-ascorbic acid-glucose medium and toxin production was on B-kidney-dextrin or on B-pancreatic digest of kidney-glucose medium.

9. Inocula cultures were prepared from storage cultures, and incubated 24 - 48 hrs. at 37° C. The medium used for inoculum cultures was B-kidney-cysteine-ascorbic acid-glucose. There were indications that the cysteine and ascorbic acid were not essential for inocula cultures.

10. Toxin production in 250 - 3000 ml. lots was as good on B-pancreatic digest of kidney-glucose medium as on B-kidney-dextrin medium. The potency of the toxins produced was 100 to over 200 TD/ml. and 5000 to 20,000 MLD/ml. The optimum solids concentration for toxin production with pancreatic digest of kidney was between 0.5 and 1.0% solids of pancreatic digest.

11. Pancreatic digest of liver cake at a concentration of 3 - 4% solids in salts supplement yielded toxins containing 150 - 200 TD/ml.

12. As carbohydrate source, on B-pancreatic digest of beef kidney medium, 0.1% glucose was better than 0.1% maltose (one experiment only) which was better than 0.6% dextrin. Addition of 0.02 M phosphate increased toxin production on this medium with 0.5% glucose from less than 50 TD/ml. to over 200 TD/ml. (one experiment performed.)

Properties of Toxic Filtrates.

13. The MLD titre of toxic filtrates decreased on storage at 5° C. to a minimum level in about a week. The original MLD titre was maintained for 9 days by the addition of 0.005 M cysteine, or 0.05 M sodium thioglycollate to the filtrate. The titre of the filtrate in the presence of these substances had fallen after 16 days to the minimum level. Ascorbic acid, orcinol, hydroquinone, and glycerol did not maintain the MLD titre over the 9 day period. The optimum pH for the storage of the filtrates at 5° C. appeared to be between 6.0 and 7.0 .

14. Preliminary clearing of cultures was obtained by centrifugation in a #2 or a Sharples centrifuge, or by filtration with Super Cel. Final clearing was accomplished by filtration through a Berkfeld N. There was no loss of toxin potency.

15. The toxin was precipitated with ammonium sulfate, with recovery of 20 - 30% of the original activity with the exception of one preparation in which the recovery was 70%. Ammonium sulfate precipitated toxins dissolved in 0.158 M phosphate buffer at pH 6.5, gave 2-110 TD/mg, and 150-2500 MLD/mg. The ratio of MLD to TD was from 20 - 80.

16. The toxin was dialyzed against glycerol with 50% loss of potency when dialysis was at room temperature and no loss of potency when dialysis was at 5° C. The material prepared in the cold, showed 50% loss of potency as measured by MLD in the first two days, and no further loss of MLD titre up to 31 days. This preparation showed no decrease in combining power up to 26 days and a slight decrease in 31 days.

17. Toxoids were prepared by formalization and guinea pigs were immunized with 2 and 3 doses of fluid or alum precipitated toxoid. The pigs showed a serum antitoxin titre and were protected against intracutaneous infection with toxin.

18. Rabbits were treated with alum precipitated toxoids. They showed appreciable antitoxin titres (over 0.5 units) after the first and second doses of toxoid. Treated animals resisted lethal injections of oedematiens culture.

19. Mice treated with a single dose of toxoid showed better resistance to toxin challenge when the toxoid was alum precipitated than when it was fluid. The optimum concentration of alum for precipitation of toxoid in protection of mice against toxin challenge appeared to be between 1.0 and 1.5% when the mice were treated with a single dose of 0.05 - 0.2 ml. (one experiment only). No correlation was demonstrated between the combining power of toxoid and protection of mice challenged with toxin (only about 8 animals per toxoid.) Mice treated with 2 doses of 1 1 TD each of alum precipitated toxoid were protected against injection of up to 250 MLD of toxin. Mice treated with 2 doses of alum precipitated

toxoid, withstood culture challenge.

20. With divalent, oedematiens-welchii, alum precipitated toxoid, 2 doses, mice were protected against toxin challenge with oedematiens and with welchii toxins. Rabbits treated with 2 doses showed a titre of over 0.2 units of antitoxin of both oedematiens and welchii per ml. of serum. The rabbits survived the injection of a killing dose of mixed oedematiens welchii culture. Pigeons treated with 2 doses survived a toxin challenge of both oedematiens and welchii.

21. The hemolysin of filtrates of Cl. oedematiens cultures was titrated by a turbidimetric method. A standard curve was prepared with a suspension of the red blood cells of the rabbit in 0.9% saline B medium and 0.9% saline. The addition of hemolyzed cell suspension to the mixtures of the standard curve was found unnecessary for the assay. Hemolysin was titrated by addition of graded amounts of filtrate to 1.0 ml. of a 1.4 - 1.6% suspension of rabbit red blood cells in 0.9% saline. The mixtures were incubated at 2.0 ml. volume for 1 hr. at 40° C., diluted to 10 ml., and mixed and read on the turbidimeter. Per cent hemolysis was determined by reference to a graph of the standard curve. The end point adopted was the number of mls. necessary for 50% hemolysis of 1.0 ml. of a 1.4 - 1.6% suspension of rabbit red blood cells. Titres of filtrates and ammonium sulfate precipitated toxins dissolved at 10 mg./ml. in 0.15% M phosphate buffer and saline 1:1, ranged from 0.01 to 0.5/ml.

22. Increasing the incubation volume of the cells-lysin mixture decreased the hemolytic activity. Increasing the incubation

temperature of the cells-lysin mixture from 35 - 40° C. increased the hemolytic activity.

23. The accuracy of the method was determined by comparison of the titration value of ammonium sulfate precipitated toxins on different days. The colorimetric and turbidimetric assays showed good agreement.

24. Hemolytic activity of the filtrates was found to decrease regularly with increasing pH from 5.8 to 6.8 .

25. The hemolysin could be diluted 1:10 in saline without decrease in activity. Dilution of 1:10 in isotonic phosphate buffer, 0.158 M, and 0.9% saline, 1:1, increased the hemolytic activity.

26. Hemolytic activity was destroyed by heating filtrates at 85° C. for 10 minutes. Activity decreased on storage at room temperature or at 5° C.

27. The hemolytic activity has increased 1 1/2 times by the addition of 0.001 M cysteine. Above the optimum concentration, 0.001 M, cysteine inhibited hemolytic activity. Thioacetic acid at a concentration of 0.006 to 0.009 M increased hemolytic activity about 5 times. No decrease in activity with concentrations of thioacetic acid over the optimum was demonstrated.

28. Hemolytic activity of most filtrates was completely inhibited by antitoxin. The hemolysin-antihemolysin mixtures were incubated for 1 hr. at room temperature and then treated as mixtures in a hemolysin titration. Increasing the amount of filtrate present in the hemolysin-antihemolysin mixture,

increased the combining power, AHU, end-point. Addition of thioacetic acid to the lysin-antilysin mixtures increased the AHU end-point.

29. When antitoxin combining power, determined as TD in mice, and antihemolytic combining power of filtrates was compared, about 50% correlation was found.

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