PREPARATION AND PROPERTIES OF SHIGA TOXIN AND TOXOID*

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All strains of dysentery bacilli in the smooth (S) phase produce a toxic component—usually designated endotoxin—which is intimately associated with the specific somatic polysaccharide antigen (O antigen). In addition to this somatic toxin, many strains of *Shigella dysenteriae* (Shiga dysentery bacilli) produce under certain cultural conditions another toxic fraction which can be readily separated from the specific polysaccharide, and which is released in solution in the culture medium. This toxin is believed by most workers to be a protein comparable to the classical exotoxins and to exhibit a specific affinity for nervous tissue; it is often referred to as Shiga exoneurotoxin. The literature concerning the relationship between these two toxic fractions has been analyzed in several recent articles and will not be reviewed here (1, 3–5, 9–12, 14, 15, 18, 21, 22, 26–29). The present paper deals only with the preparation and properties of the so called Shiga exoneurotoxin, and of the toxoid derived from it.

Materials and Methods

Cultures.—The different strains of *Shigella dysenteriae* (Shiga) vary greatly in their toxicogenic power. On the other hand, it is known that the production of the neurotoxin is completely independent of the virulence of the culture and of the production by the latter of the specific polysaccharide present in the O antigen (characteristic of the smooth phase) (1, 4, 5, 14, 15, 21, 25, 29). Our preliminary experiments were carried out with virulent smooth strains of *Shigella dysenteriae.* It was soon found possible, however, to isolate from one of these strains a rough (R) avirulent variant which produces as much exoneurotoxin as the parent smooth culture; this R variant completely fails to elicit the production of type specific (O) antibody when injected into rabbits, and does not manifest the characteristic agglutination in the presence of specific Shiga antiserum. In addition to its lack of virulence, this R variant presents many other advantages for the study of the toxin. It grows abundantly in ordinary bacteriological media. It exhibits a tendency toward autoagglutination; because of this property the bacterial bodies (or bacterial debris after autolysis) can be readily separated from the soluble components of the cultures by centrifugation or filtration, a property which facilitates the different operations required for the concentration and purification of the

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toxin. Finally, it does not produce the somatic toxin associated with the O antigen, a fact which simplifies the titration of the exoneurotoxin in crude fractions and its separation in purified form. Most of the experiments to be reported in the present paper were therefore carried out with the R Shiga variant mentioned above, the purity and homogeneity of the culture being verified at frequent intervals by plating on MacConkey agar.

Media.—The R Shiga culture grows readily in media containing amino acids or hydrolysates of casein as a source of nitrogen. Unfortunately, toxin production is low under these conditions and it has been found preferable to employ, instead, media containing bacteriological peptone and meat extract in order to obtain adequate yields of toxin.

As will be shown in the following pages, the crude toxin can be conveniently separated from the culture filtrate by acidification at pH 4.0–4.2. It is therefore important that the uninoculated medium itself be freed of any material precipitable at acid reaction in order to minimize contamination of the toxin with broth constituents. To this end, the concentrated peptone-meat extract mixture is first treated with HCl (to pH 4.0) and any insoluble material is eliminated by filtration through paper. Removal of the acid-precipitable fraction does not decrease the nutritive value of the broth.

It has also been found (see below) that toxin production by our strains is much decreased when the culture medium contains inorganic iron. Removal of this metal is achieved by adding to the medium adequate amounts of calcium and phosphate ions at alkaline pH; the precipitate of calcium phosphate carries along the inorganic iron and can be removed by filtration through paper. Sodium pyrophosphate is subsequently added to the medium in order to bind any iron that might be introduced as a contaminant.

Toxin production is also associated with strict aerobic metabolism of the Shiga bacillus. This may account for the fact that fumaric acid increases appreciably the yield of toxin since this acid probably plays a rôle in oxidative reactions. Addition of glucose (0.2 per cent) increases the amount of bacterial growth and does not interfere with toxin production, provided adequate aerobic conditions are maintained during growth.

In practice, the preparation of the medium involved the following steps:

1. Preparation of the concentrate.
   500 gm. peptone[1]
   125 gm. meat extract[1]
   2500 ml. H_2O
   Dissolve. Add HCl to pH 4.0 (approximately 40 ml. concentrated HCl). Filter twice. Keep concentrate in refrigerator
2. Preparation of broth (for 1 liter).
   50 ml. concentrate
   950 ml. H_2O
   10 gm. Na_2HPO_4, 12 H_2O
   1 ml. 50 per cent NaOH
   10 ml. 10 per cent $CaCl_2$

The resultant pH is 8.3–8.6. The abundant precipitate forming upon addition of the calcium chloride is removed by filtration through paper and the following substances are then added to the filtrate:

2 gm. glucose
1 gm. fumaric acid
5 gm. Na pyrophosphate

The medium should be clear with a final pH 6.8–7.2. It is distributed in pyrex containers and autoclaved at 20 pounds pressure for 15 minutes.

[1] Different brands of peptones and of meat extract were used and found equally satisfactory.

Toxicity and Immunity Tests.—On a weight basis rabbits are much more susceptible than white mice to the lethal effect of the Shiga toxin; in fact the LD_{50} is approximately the same for both animal species. Because of great variation in the susceptibility of individual animals, and the necessity of carrying out large numbers of assays for determining the toxicity and immunizing efficacy of different preparations, mice were selected as test animals in spite of their greater resistance. Mice 6 to 8 weeks of age were used since they were found to be more susceptible than younger animals, irrespective of breeds tested and of body weight. The toxin preparations were diluted in physiological saline (at neutrality) and injected in a volume of 0.2 ml. by the intravenous or intraperitoneal routes. Although the toxic activity is greater (approximately twice) when the material is injected intravenously, the intraperitoneal route was selected for reasons of convenience. The LD_{50} was determined by the Reed-Muench technique, 8 animals being used for each toxin dilution (24).

The Effect of Aerobiosis on the Amount of Growth and of Toxin Produced by Shigella dysenteriae R

It has been repeatedly shown that the production of toxin by *Shigella dysenteriae* is much greater under aerobic than under anaerobic conditions of growth (20, 29). Aerobiosis during incubation is usually provided by growing the organism in liquid media distributed in extremely shallow layers or on the surface of agar or silica gel media (7). We have found it more convenient, and more efficient, to secure adequate aerobic conditions in liquid media by agitating the culture on a shaking table or by aerating it with a stream of air bubbles finely divided with a powerful stirrer during the whole period of incubation. Fumaric acid was also added to the medium on the assumption that it might favor aerobic metabolism since it is known to participate in oxidative reactions. The following experiment illustrates the effect of agitation and of fumaric acid on the growth of, and toxin production by, *Shigella dysenteriae* R.

The medium was the peptone-meat extract broth described above, freed of inorganic iron and containing 0.2 per cent glucose. It was divided into two lots, one of which received 0.1 per cent fumaric acid. Both batches were adjusted to pH 7.0. The media were distributed in Erlenmeyer flasks of 500 ml. capacity and inoculated with an 18 hour culture of Shiga R (0.5 ml. inoculum per 100 ml. medium). Some of the flasks had received 400 ml. of medium and were left undisturbed during incubation, thus providing semianaerobic conditions of growth. Other flasks had received only 200 ml. of medium and were violently agitated during the whole period of incubation. All cultures were incubated (aerobically or anaerobically) for 48 hours at 34°C.; they were allowed to autolyze without agitation for an additional 3 days at the end of the incubation period.

The cultures were chilled in the refrigerator overnight and treated (in the cold) with enough HCl to bring them to pH 4.0. There is formed under these conditions a flocculent precipitate which contains the bacterial bodies and most of the soluble products released from the culture. This precipitate was washed with water, desiccated *in vacuo*, in the frozen state, over P_2O_5 and weighed. Toxin production was determined by the intraperitoneal injection into mice of graded amounts of the total culture or of the acid precipitate resuspended (before desiccation) in a dilute sodium carbonate solution at pH 8.0.

The data presented in Table I summarize some of the results of the experiment. They reveal that the shaken cultures reached a higher pH and yielded

a more abundant growth than the cultures incubated under semianaerobic conditions. The effect of the fumaric acid is reflected especially in the toxicity of the cultures. The most toxic preparation was the one grown under aerobic conditions in the presence of fumaric acid; the least toxic was the semianaerobic culture without fumaric acid. Although this acid did not increase the total yield under anaerobic conditions, it increased markedly the toxicity of the culture. In all cases, the toxicity of the original culture was almost quantitatively recovered in the acid-precipitable material.

The Effect of Inorganic Iron on Toxin Production

Many authors have emphasized that production of Shiga toxin occurs most readily when the culture is incubated at alkaline reactions. For this reason,

TABLE I

The Effect of Agitation, with and without Added Fumaric Acid, on the Amount of Growth and of Toxin Produced by Shigella dysenteriae R

Fumaric acid	Aeration	Original pH	Final pH	Yield of bacterial autolysate	Toxicity for mice (LD_{50})	
					Original culture	Desiccated bacterial bodies
per cent				*gm./l.*	*ml.*	*mg.*
0	Shaken culture	7.0	6.9	0.61	0.08	0.042
0.1	Shaken culture	7.0	7.8	0.72	0.04	0.024
0	Still culture	7.0	6.5	0.32	0.9	0.25
0.1	Still culture	7.0	6.8	0.28	0.15	0.039

it is usually recommended that the medium be adjusted at pH 8.2 and that glucose be omitted to avoid the development of acid reaction during growth. Media prepared under these conditions are likely to be low in inorganic iron since this metal forms precipitates at alkaline reaction and is removed during filtration. It is known, on the other hand, that the production of toxin by *Corynebacterium diphtheriae* and *Clostridium tetani* is inhibited by the presence of excess inorganic iron in the culture medium (16, 17, 23). It appeared possible, therefore, that the favorable effect of alkaline reaction upon the production of Shiga toxin might be due in part at least to removal of iron. This is established in the following experiment.

The iron-free medium described in a preceding section was distributed in 200 ml. amounts in Erlenmeyer flasks (500 ml. capacity). $FeCl_3$ was added to some of the flasks in concentration of 0.001 per cent and 0.00001 per cent. Each flask was inoculated with 1 ml. of Shiga R culture (obtained from iron-free broth). The flasks were placed on a shaking table and agitated during the whole period of incubation (36 hours at 34°C.). The yield and toxicity of the cultures were determined by the methods described for the preceding experiment.

The results summarized in Table II reveal that the final pH of the medium and the total yield of bacterial growth are not significantly affected by the addition of 0.00001 per cent or even 0.001 per cent $FeCl_3$ to the medium. Yet even the lower concentration of inorganic iron is sufficient to decrease the production of toxin by the culture. In other experiments not reported here, it was established that inorganic iron always exerts the same inhibitory effect on toxin production, whatever the composition of the medium, the conditions of incubation, or the virulence of the culture used (R or S forms).

It is apparent that with the strains of *Shigella dysenteriae* used in the present experiments, satisfactory production of toxin is obtained by cultivating the organism under strict aerobic conditions in a meat extract-peptone medium free of inorganic iron. It is not at all necessary to grow the culture at alkaline reaction when inorganic iron has been removed from the medium, and glucose does not inhibit toxin production if aeration is adequate.

TABLE II

The Effect of Inorganic Iron on the Amount of Growth and of Toxin Produced by Shigella dysenteriae R

Amount of $FeCl_3$ added to medium	Original pH	Final pH	Yield of bacterial autolysate	LD_{50} per mouse (6 wks. old)	
				Culture	Bacterial autolysate
per cent			*gm./l.*	*ml.*	*mg.*
0	7.0	7.8	0.6	0.06	0.03
0.00001	7.0	7.7	0.5	0.15	0.07
0.001	7.0	8.0	0.72	>1.0	>1.0

Although the data reported in the present paper deal only with an avirulent R variant of *Shigella dysenteriae*, identical results have been obtained with two virulent S cultures of the same species. It must be emphasized, however, that even when grown in the presence of an excess of iron and under anaerobic conditions, the S cultures exhibit an irreducible toxicity which resides in the type specific 0 antigen produced by these organisms. In this respect, cultures of smooth Shiga bacilli do not differ from those of other members of the *Shigella* group, the Flexner, Boyd, and Sonne strains for example, and no experimental technique is as yet available to grow the smooth variants of these organisms in a non-toxic form.

Preparation of Soluble Shiga Toxin

As already mentioned, the soluble toxin, which is the object of the present report, is often designated under the name of "exotoxin," to contrast it with the "endotoxin" associated with the somatic 0 antigen. Although the former toxin (exotoxin) appears early in solution in the medium, this constitutes no

proof that it is excreted into the medium by the living bacterium. Indeed, there exists experimental evidence that the toxin is released in a soluble form only after the death of the cells (4–6, 19). In agreement with this fact, we have observed that in the case of very young cultures (grown for 24 to 36 hours at 34°C.) the toxin sediments with the cells when the culture is centrifuged at 3500 R.P.M. for 10 minutes; separation of the toxin in a soluble form can then be obtained by extracting the bacterial sediment with 1 per cent sodium carbonate. The toxin is also released in solution when the whole culture, or the sedimented bacterial bodies, are allowed to undergo partial autolysis for a few days (2 to 4) at 37°C., at neutral or slightly alkaline reaction. Advantage has been taken of these observations in the following method of preparation of the toxin.

The medium, free of inorganic iron and of acid-precipitable material, is prepared as described in an earlier section. It is inoculated with a young culture of *Shigella dysenteriae* R (5 ml. of inoculum per 1000 ml. of medium) and incubated for 36 hours at 33 to 34°C. with constant agitation or other form of forced aeration. The fully grown culture is allowed to stand without agitation at 37°C. for 3 days. The pH of the culture at the end of this period should be 7.6-8.0 if aeration has been adequate during growth. Microscopic examination usually reveals that a very large percentage of the cells stain poorly and have undergone partial autolysis. The culture can then be stored in the refrigerator before extraction of the toxin; 0.5 per cent phenol may be added if desired.

Hydrochloric acid (10 per cent) is added to the chilled culture to bring it to pH 4.2. The precipitate is allowed to settle, separated by decantation, filtration, or centrifugation, and washed with 1 per cent NaCl solution. The toxic component is released into solution by extracting the washed precipitate at pH 7.5 with dilute sodium carbonate or with salt solution buffered with phosphate. Complete extraction may demand several hours and can be conveniently obtained with a volume of extracting fluid corresponding to 1/50 of that of the original culture. Separation of the soluble toxin from the insoluble inert bacterial debris is obtained by filtration over filter paper in the presence of a small amount of filter aid. The toxin is thus recovered in the form of a perfectly clear filtrate. It can be sterilized by passage through any of the classical bacteriological filters; it can be reprecipitated with acetic acid (at pH 4.2–4.4) and the precipitate, washed free of excess acid with water, can be desiccated *in vacuo* over P_2O_5 or by lyophilization.

The soluble extract—which is designated in the present paper as crude soluble toxin—exhibits an LD_{50} for rabbits and mice of approximately 10μg. It is fairly stable. Solutions at slightly acid or neutral reactions have been kept in the refrigerator for 2 years without significant loss of activity. The toxic principle becomes more unstable as the solution becomes more alkaline; at pH 9.0, heating at 60°C. for 5 minutes causes a complete and irreversible loss of activity. In contrast, only little inactivation follows heating at 90°C. for 15 minutes when the preparation is acidified to pH 6.0–6.5.

In agreement with others, we have found that the toxin is resistant to digestion with pepsin and papain, but is slowly inactivated when treated with trypsin at pH 8.0 (1, 4, 5, 11).

Purification of the Toxin

Shiga toxin is only slowly inactivated by trypsin. It appeared possible, therefore, that rapid treatment with this enzyme might destroy selectively some more readily digested protein contaminant and achieve some purification of the crude material. Crude toxin digested with commercial trypsin no longer precipitates with acetic acid at pH 4.2. However, the toxic principle still present in the digested material can be precipitated by adding to the solution 4 volumes of alcohol at pH 5.0. The toxin recovered under these conditions exhibits much greater biological activity (LD_{50} for rabbits and mice approximately 1 to 3 μg.). It contains less than 0.1 per cent phosphorus while its nitrogen content is increased from 10 per cent to 14 per cent or more. Unfortunately, it has not been possible to standardize this method of purification by trypsin treatment on account of the fact that the recovery of toxin is often very low due to some uncontrolled inactivation during enzyme digestion and alcohol precipitation.

Addition of salmine and other protamines to solutions of the crude toxin causes the formation of an abundant precipitate which contains most of the phosphorus originally present in the toxin solution. Ribo- and desoxyribonucleic acid have been identified in this precipitate (13). The material which remains in solution still exhibits unimpaired the characteristic toxicity of the original material. In other words, toxicity does not depend upon the presence of nucleic acid, and it is probable that precipitability of the toxic material with acetic or hydrochloric acid in the crude material results from the formation of a nucleinate insoluble at pH 4.0–4.4

Removal of phosphorus without loss of toxic activity can also be accomplished by treating concentrated solutions of toxin with calcium chloride at alkaline reaction. Under these conditions, the toxic material remains in solution, whereas the precipitate carries along 75 per cent of the total tyrosine, 50 per cent of the total nitrogen, and 95 per cent of the total phosphorus (consisting chiefly of nucleic acids and nucleotides). The following examples illustrate this method of purification.

To four batches of 50 ml. each of crude toxin solution ST 26 at pH 9.0 there were slowly added 8, 6, or 3 ml. of 10 per cent $CaCl_2$ with simultaneous addition of NaOH to maintain the pH between 8.8 and 9.0. The three batches, as well as the fourth one which was kept as control, were made up to 60 ml. with distilled water. The precipitates were allowed to settle in the refrigerator overnight. The preparations were then brought back to room temperature and filtered through filter paper. The filtrates were assayed for nitrogen, phosphorus, and tyrosine as well as for toxicity in mice.

The results presented in Table III show that, despite equivalent LD_{50} values in all flasks, the amount of extraneous material was considerably less where the greatest amount of $CaCl_2$ had been added.

In another experiment 130 ml. of 10 per cent $CaCl_2$ were added to 325 ml. of crude toxin preparation ST 28, the reaction being maintained at pH 9.2 The clear filtrate was found to contain 0.068 mg. P and 0.45 mg. N per ml. and, in this case again, there was no significant loss of toxic activity.

Attempts were made to obtain further purification of the toxin by filtration over ionic exchange resins. The toxin solution filtered readily through an anionic resin (Ionac A) and the filtrate was found to contain as much nitrogen and exhibit as much toxic activity as the original material. When the same solution was added to a cationic resin (Ionac B) on the contrary, a heavy precipitate immediately formed over the resin particles, clogging up the system and preventing filtration of the concentrated toxin solution. Filtration over the cationic resin was possible only when very dilute solutions of the toxin were used and the filtrate obtained under these conditions was found to be free of

TABLE III

The Purification of Shiga Toxin by $CaCl_2$ at Alkaline pH (9.2)

Amount of 10 per cent $CaCl_2$ added per 50 ml. toxin*	Analysis of the filtrate			LD_{50} per mouse (6 wks. old)
	Nitrogen	Phosphorus	Tyrosine	
ml.	*mg./ml.*	*mg./ml.*	*mg./ml.*	*ml.*
0	1.6	0.42‡	1.20	0.0020
3	1.23	0.26	0.99	0.0020
6	1.25	0.26	1.05	0.0023
8	0.67	0.09	0.60	0.0025

* Volume made up to 60 ml. with NaOH and H_2O.

‡ Of which 0.1 mg. was free phosphorus.

nitrogen and to have lost its toxic activity. It appears, therefore, that our preparations of Shiga toxin are inert toward the negative charge of the anionic resin but exhibit marked affinity for the positive charges of the cationic resin. It will be remembered also that the toxin is fairly resistant to heat at slightly acidic reactions, but unstable at pH 9.0. These facts suggest that the toxin is closely associated with a protein of basic character which is obtained as a salt of nucleic acid in the first phases of the purification procedure.

Susceptibility of Different animal Species to the Toxin

Albino mice can be used for assaying toxic activity although they are less susceptible than rabbits to the lethal effect of the toxin. It must be emphasized, however, that mice exhibit marked individual variation in their susceptibility (1, 2, 15, 30). In the course of the present work, several thousand mice, varying from 3 to 9 weeks in age and from 12 to 40 gm. in body weight, were obtained from four different breeders and tested with different prepara-

tions of toxin in a number of independent experiments. Analysis of the results suggests the following generalizations:–

There occurred between the 5th and 6th weeks of age a sudden and marked increase in the susceptibility of the mice used in our tests; the lethal dose (expressed as LD_{50} per animal) appeared to be essentially independent of the weight of the animal.[2] For example, mice of The Rockefeller Institute strain, 20 gm. in weight, exhibit high resistance to the toxin; they reach this or higher weight when 4 weeks old. On the other hand mice obtained from commercial breeders reached this weight considerably later (6 to 7 weeks) and were found to be very susceptible. When tested between the 3rd and 5th weeks of age, at a time when their weight ranged from 12 to 18 gm., they exhibited the same resistance as the mice of The Rockefeller strain weighing 18 to 25 gm. What-

TABLE IV

Effect of Age on Susceptibility of Mice (Rockefeller Institute Breed) to Crude Shiga Toxin

Age	Average weight (96 mice per group)	LD_{50}	
		Toxin preparation ST 6a (desiccated bacterial bodies)	Toxin preparation ST_1TA_5
wks.	*gm.*	*mg.*	*mg.*
3	17.7	0.7	0.08
4	19.9	0.6	—
5	21.0	0.5	0.05
6*	23.5	0.2	0.03
7*	26.6	0.25	0.03
8*	29.3	0.2	0.035

* Varying numbers of necrotic foci were often found on the surface of the liver of control mice 6, 7, and 8 weeks old. Microscopic examination and cultural studies did not reveal any convincing evidence of bacterial infection (see text).

ever the strain used, the susceptibility of mice to Shiga toxin was found to depend upon the age of the animal and to be independent of its weight. The effect of age on the susceptibility of mice of The Rockefeller Institute breed is illustrated in Table IV.

Although it is tempting to assume that this age-susceptibility relationship is the expression of some fundamental physiological process, this need not be the case. It is possible that the older animals have become carriers of some latent infectious agent (a virus for example) which becomes activated and causes fatal outcome when the physiological equilibrium is disturbed by injection of the toxin. It may be worth mentioning in this respect that there

[2] It has been shown that mice varying from 12 to 35 gm. in weight are killed by the same minimal amounts of specific antigen prepared from dysentery bacilli. In other words, the susceptibility of mice to this endotoxin is also independent of the weight of the animal (30).

were found on the surface of the liver of many of the older animals numerous necrotic foci from which it was not possible to obtain evidence of bacterial infection. These necrotic foci were rare or absent in mice 3 and 4 weeks old and very common in animals 6, 7, and 8 weeks old. A number of workers in other laboratories in different parts of the country have confirmed this observation; it appears, therefore, that the occurrence of liver pathology in mice over 5 weeks of age was very common in the continental United States during the years 1944–1945 (20*a*).

Rabbits also exhibit considerable individual variation in susceptibility to the toxin, and nothing is known of the factors affecting this variability. In general, the lethal dose (by the intravenous route) varied from 2 to 20 μg. of crude soluble toxin per animal of 1.5 to 3 kg. body weight. As already stated, therefore, rabbits are much more susceptible per unit weight than mice, since the lethal dose is of the same order of magnitude for both animal species (15). Guinea pigs on the contrary, are extremely resistant even when the toxin is injected by the intracardiac route. No attempt has been made to determine the M.L.D. or LD_{50} in dogs. It has been observed, however, that injection of 1 to 5 mg. of toxin by the intravenous route usually results in immediate circulatory disturbances, followed by generalized hemorrhages and death within a few hours.

Although our preparations of Shiga toxin have not been injected into man, some accidental observations bear witness to the potential toxicity of the material. In two different laboratories, four workers developed fever, headache, nausea, diarrhea, and other ill defined symptoms after having been engaged in grinding or weighing desiccated preparations of bacillary bodies or of crude soluble toxin. The symptoms subsided after 18 to 36 hours and there was no evidence of bacterial infection. In all cases, the association of events pointed to an intoxication resulting from inhalation of fine particles of the desiccated toxic material.

The Preparation and Immunizing Efficacy of Shiga Toxoid

Like most other species of Gram-negative bacilli, *Shigella dysenteriae* (Shiga) growing in the smooth (S) phase, produces a toxin which is closely associated or identical with the somatic 0 polysaccharide antigen and which is extremely resistant to detoxification. The toxic activity of the substance described in the present report can, on the contrary, be destroyed by a number of different treatments; prolonged digestion with trypsin, heating to 60°C. at slightly alkaline reactions, precipitation with acetone at room temperature (which renders the material insoluble), etc. All these treatments, however, result at the same time in the complete loss of specific antigenicity of the toxin.

It is also possible to deprive Shiga toxin of most of its toxic properties by treatment with low concentrations of formaldehyde (1, 8–11, 29). Detoxification with formalin is most rapid and most complete at alkaline reactions (pH

9.0 and above) but, in agreement with other workers, we have found that there also occurs a partial or complete loss of specific antigenicity under these conditions (9, 10). Detoxification remains incomplete if treatment with formalin is carried out at pH 7.0, 7.5, or even 8.0 for several months at 37°C. Satisfactory conversion of our toxin preparations into toxoid has been obtained under the following conditions.

The solutions of crude soluble Shiga R toxin were prepared in concentrations corresponding to 0.4 to 0.6 mg. nitrogen per ml.; these preparations exhibit an activity of 700 to 1000 LD_{50} for white mice (6 weeks of age) by intravenous injection. They were treated with 0.5 per cent commercial formalin for 2 to 3 weeks at 37°C., the pH being maintained between 8.4 and 8.6 during the whole period of treatment.

Toxoid prepared under these conditions could be injected by the intravenous route in 5 ml. amounts into rabbits without significant reactions. Most mice receiving 0.2 ml. of the material survived, although 0.5 ml. usually caused death within 1 to 7 days. Intraperitoneal injection of 5 ml. of the toxoid into guinea pigs causes death by a shock-like condition within a few hours. Other guinea pigs which had received 1 ml. of the material by the subcutaneous route exhibited little or no reaction. It is clear, therefore, that treatment of Shiga toxin with 0.5 per cent formalin at pH 8.5 results in an appreciable degree of detoxification of the material (300- to 500-fold). It is still uncertain whether the residual toxicity is due to unmodified toxin or to unrelated toxic material present in the preparation.

The antigenicity of the toxoid was tested in white mice by injecting the soluble material or alum-precipitated toxoid, and challenging the immunized animals with active soluble toxin or desiccated bacillary bodies. The results are illustrated by two representative experiments carried out in two different laboratories (The Rockefeller Institute and Biological Laboratories of E. R. Squibb and Sons).

Two preparations of toxoid were used—toxoid S 146 and alum-precipitated toxoid S 153—both prepared from Shiga R toxin of approximately 900 LD_{50}/ml. initial potency.

In one experiment groups of 30 mice[3] 20 to 24 gm. of weight and over 7 weeks of age received by the subcutaneous route different amounts of physiological saline or toxoid in a total volume of 0.2 ml. On the 33rd day after the initial injection they were challenged with different amounts of toxin S 210 (in solution in 0.4 per cent Na_2HPO_4 $12H_2O$ and 0.85 per cent NaCl) given intravenously. The results are presented in Table V.

The second immunization experiment was carried out according to a similar plan. The mice were of The Rockefeller Institute stock; they were 3 weeks old at the beginning of the immunization schedule and weighed approximately 18 to 20 gm; 50 animals were used for each amount of toxoid. They were challenged with amounts of crude soluble toxin (ST 14) ranging from 0.005 mg. to 0.04 mg. for the controls which had received physiological saline instead of toxoid, and from 0.02 to 0.16 mg. for the immunized animals. The LD_{50} (intraperitoneal route) for each group of immunized and control animals is presented in Table VI.

[3] Mosher's Mouse Colony, Riverside, New Jersey.

The results presented in Tables V and VI establish that mice immunized with Shiga toxoid develop an appreciable active immunity against the toxin. It is worthy of note that one single injection of alum-precipitated toxoid proved to be a satisfactory method of immunization.

The results presented in this paper deal only with the resistance of mice immunized with Shiga toxoid to the homologous toxin. It may be mentioned, however, that immunization with toxoid also resulted in the development in

TABLE V

Immunization of Mice, 7 Weeks Old, from Mosher's Mouse Colony, with Shiga Toxoid

Antigen (subcutaneously) (in 0.2 ml. volume)	Amount of toxoid injected on following days			Death-survival ratios of mice challenged on 33rd day with following amounts of toxin			
	1st	6th	12th	0.2 mg.	0.4 mg.	1.0 mg.	2.0 mg.
	ml.	*ml.*	*ml.*				
Saline control	—	—	—	6/15	8/14	—	—
Fluid toxoid S 146	0.033	0.033	0.033	—	0/10	2/10	0/8
Alum-precipitated toxoid S 153	0.033	0.033	0.033	—	0/10	1/10	1/7
Alum-precipitated toxoid S 153	0.1	—	—	—	0/10	0/10	0/8

TABLE VI

Immunization of Mice (3 Weeks Old, Rockefeller Institute Breed) with Shiga Toxin

Antigen (subcutaneously) (in 0.2 ml. volume)	Amount of toxoid injected on following days			LD_{50} of toxin ST 14 injected (i.p.) on 33rd day
	1st	6th	12th	
	ml.	*ml.*	*ml.*	*mg.*
Saline control	—	—	—	0.015
Fluid toxoid S 146	0.033	0.033	0.033	0.06
Alum-precipitated toxoid S 153	0.033	0.033	0.033	0.06
Alum-precipitated toxoid S 153	0.066	—	—	0.09

these animals of an appreciable degree of resistance against infection with the virulent Shiga bacilli (injected by the intracerebral route) and with Flexner or Sonne bacilli (injected by the intraperitoneal route). The serum of rabbits and mice immunized with Shiga toxoid was also found to possess a greatly enhanced bactericidal activity *in vitro* (in the presence of guinea pig complement), not only for R cultures of Shiga bacilli but also for the S variants of the species, as well as of other members of the *Shigella* group.

SUMMARY AND CONCLUSIONS

1. *Shigella dysenteriae* (Shiga) can, under the proper cultural conditions, produce a soluble toxin which is independent of the specific somatic polysac-

charide antigen. A method is described for the rapid production of this toxin by an avirulent R variant of this organism.

2. The amount of bacterial protoplasm synthesized, and the yield of toxin produced, are very much increased when the culture is grown under conditions which favor aerobic metabolism and which do not permit accumulation of organic acids.

3. Although addition of an excess of inorganic iron to the medium does not interfere with the synthesis of bacterial protoplasm, maximal toxin production is obtained only in media which have been freed of the metal.

4. R Shiga bacilli grown under conditions of extreme aerobiosis at pH 7.0 in a simple medium free of inorganic iron and containing fumaric acid, glucose, meat extract, and peptone, give rise within 24 to 36 hours to cultures exhibiting a high degree of toxicity. The toxic principle rapidly becomes soluble as the cells die; it can be concentrated, purified, and freed of phosphorus by selective precipitation methods.

5. The toxin is fairly resistant to heat at pH 6.0 but rapidly denatured at pH 9.0. It is only slowly inactivated by trypsin. It filters readily and without loss of activity through anionic exchange resins but is precipitated and adsorbed by cationic resins which retain the nitrogen and toxic activity of the preparation.

6. The most active preparations of toxin available possess an LD_{50} of 1 to 10 μg. for mice and rabbits. Young mice (up to 5 weeks of age) are more resistant than older animals.

7. The soluble toxin can be detoxified by treatment with 0.5 per cent formalin at pH 8.5; detoxification does not take place at lower pH whereas treatment at higher pH destroys specific antigenicity. Mice immunized with Shiga toxoid (in solution, or in the form of an alum precipitate) develop active immunity to the toxin.

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