THE NEUROTOXIN OF SHIGELLA DYSENTERIAE (SHIGA)[1]

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CONTENTS

The toxic properties of *Shigella dysenteriae* have been the subject of considerable controversy for nearly half a century. In particular, the neurotoxic factor has been the cause of discussion and dissension, as a study of textbooks by Topley and Wilson (106), Felsen (43) and Dubos (33) and special articles by Weil (114, 115, 116) and by Pillemer and Robbins (94) reveals.

This paper has as its purpose to review, from the time of discovery of the organism, the work on *Shigella dysenteriae* relative to the neurotoxic factor.

THE TOXINS OF S. DYSENTERIAE (SHIGA)

Although Chantemesse and Widal (29) and Grigorjeff (48) probably isolated the causative agent of bacillary dysentery before Shiga (98, 99), it was the latter who, in his classical report on the etiology of the disease, following isolation of a gram negative bacillary organism from a Japanese epidemic in 1896, gave the conclusive evidence. As a result, the genus name *Shigella* was derived from his name. Soon after Shiga's discovery, Kruse (59) in Germany, Flexner (44) in the Phillipines and a host of others during the following half century isolated the organism and related species in various parts of the world. This genus contains numerous species and types as shown by serological methods. Such methods have not demonstrated more than a single antigenic type of *Shigella dysenteriae* (Shiga) or *Shigella dysenteriae* Type I, hereafter referred to as *Shigella dysenteriae.*

Conradi (30), about five years following the discovery of the organism by Shiga, reported that autolysates of 18 hour agar grown cultures of *S. dysenteriae* would kill animals. His material was prepared by suspending the cells in saline at 37 C for 24–48 hours, diluting fivefold, filtering and concentrating at 35 C to $\frac{1}{10}$ to $\frac{1}{50}$ of the original volume. This product when injected intravenously

[1] This paper is based, in part, on work performed at the Biological Laboratories, Chemical Corps, Camp Detrick, Frederick, Maryland, and presented in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, University of Pennsylvania.

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into rabbits caused paralysis, diarrhea and collapse, with death in 48 hours. A lethal dose (LD) was 0.1 ml. When injected intraperitoneally into guinea pigs, the toxic material caused a fall in temperature and collapse. Again, the LD was of the order of 0.1 ml. Autopsy of these animals showed congestion and hemorrhage of the intestines. Conradi had apparently demonstrated a neurotoxic factor in his rabbits. The accompanying diarrhea in the rabbits and the pathology in guinea pigs indicate that the neurotoxic factor was contained in a crude mixture of growth products and obviously contained some of the somatic antigen having toxic properties similar to all other gram negative enteric microorganisms.

Todd (105), at about the same time, observed the same symptomatology in rabbits after injection of filtrate from broth cultures grown for a period of 4–6 weeks. He reported that the guinea pig, mouse and monkey were far less susceptible to the material than rabbits and horses. He made no attempt at separation of his material into fractions. The greater toxicity that he found, after long incubation, may be attributable to the alkalinity of the medium he used, which would prevent destruction by acid of the toxin and encourage autolysis of the cultures. Todd brought out the interesting and fundamental point that the organism of Flexner, *Shigella paradysenteriae* Flexner (*Shigella flexneri*), did not form any soluble toxin causing paralysis. This was the first indication that *S. dysenteriae* differed from the other organisms of the genus in this respect, a finding frequently corroborated since.

Vaillard and Dopter (109) and Dopter (31, 32) contributed early discussions of the pathology found in paralytic rabbits. After injection of 24 hour broth cultures, they found chromatolysis of the anterior horn cells, with small interstitial hemorrhages and focal necroses of the grey matter occurring frequently. These pathological findings provided additional strong evidence for the existence of a central nervous system toxin. Considering the crude nature of the materials used in the investigation of the toxin up to this point, the progress toward the biological identification of the material was encouraging.

Kraus and Dörr (58) took the next step by observing from their data that there were two toxins produced; a soluble toxin, fatal to rabbits but not to guinea pigs, and an insoluble toxin, which was fatal to both species. The former was found in 8–10 day broth or saline filtrates of 24 hour cultures and gave rise to specific neutralizing antibody. The latter, however, was present only in the cell body and was insoluble. This first statement of the "two toxin theory" started a controversy and a series of investigations which has continued to the present since the presence of an exo-neurotoxin has been disputed as recently as 1949 (25, 26).

The possibility that there were two toxins was also suggested by Flexner and Sweet (45), who specified that one was a neurotropic toxin and the other intestinal, although they did not separate the two. The material used by these authors was the product of agar-grown cells. After growth for 24 hours at 37 C, the cells were washed, heat killed, kept at 37 C for varying periods, and then filtered through a Berkfeld filter. This preparation kept its toxic properties even

up to 27 days at 37 C, a finding in agreement with Todd (105). These authors also contributed further information on the stability of the toxin. They injected the material intravenously into rabbits with resultant diarrhea, paralysis, convulsions and death. The paralysis appeared in the front legs first, then extended to the hind limbs. Some of these animals lived as long as 10 days after the onset of paralysis. Pathological findings included small hemorrhages in the brain and softening of the grey matter in the spinal cord. In the intestine there were congestion of the serosa, thickening of the walls, edema, pseudomembranes and hemorrhages, especially in the cecum.

A few years later, Pfeiffer and Ungermann (92) and, subsequently, Pfeiffer and Lubinski (91) further defined the two toxins as an exotoxin, causing neurotoxic symptoms in rabbits, and an endotoxin, which produced hypothermia and peritoneal exudation in guinea pigs.

Bessau (8) agreed with the previous theories on the two toxins noted above. He observed also that antitoxin produced in animals against the neurotoxin would neutralize the neurotoxic properties, but would not alleviate the intestinal symptoms produced in animals when the endotoxic material was injected. These findings are interesting in the light of later studies by other workers (74) indicating that antiserum produced against the somatic antigen endotoxin could protect animals against experimental infections with *S. dysenteriae*. This suggests that, similar to the typhoid organisms, a protective antigen is present in the somatic endotoxin complex. In further studies Bessau (8) found that the neurotoxin was without effect in guinea pigs while the "marasmic" toxin affected guinea pigs as well as rabbits.

There were some early workers, among them Horimi (55), who reported finding three toxins. In addition to those mentioned by others, they reported a third which produced intestinal hyperemia in both rabbits and guinea pigs and additionally affected the adrenal and mesenterial glands of the guinea pigs. More recent work has not supported these findings, the bulk of subsequent research leading more certainly to the conclusion that the toxins are two in number.

Olitsky and Kligler (81) performed classical experiments with Shiga toxicity, which have been referred to by many subsequent authors. Concluding that disagreements over the nature of the toxins were probably occasioned by differences in methods of preparation, these authors performed a series of careful studies on factors affecting the production of both of the toxins of *S. dysenteriae* and on the comparative effects of the toxins in rabbits. Though some of their protocols would have benefited from inclusion of an increased number of animals, various facts were ably and amply demonstrated. The exotoxic neurotoxin was found in the filtrate of 5 day broth cultures, making its appearance after the acid phase of growth was completed. Aeration greatly increased the yield of exotoxin, and presence or absence of sugar in the media was of no importance in toxin production and potency. A lethal dose was 0.5 ml whereas less could cause paralysis with recovery. The pathology in rabbits was confined to the central nervous system. There were no intestinal lesions. The endotoxin, on the

other hand, was found in broth cultures only after very prolonged incubation, that is, after autolysis. Agar cultures washed off the medium after 24 hours and incubated in saline for 2 days could be used for endotoxin preparation. In this case, the exotoxin had to be removed by heating at 80 C for 1 hour or by neutralization with anti-exotoxic serum, a serum that had no effect on the endotoxin. Pathological findings indicated that the endotoxin was a strict enterotoxin. The evidence in this report in favor of two toxins, one a neurotoxin, is more conclusive than any that preceded it.

Preparation of exotoxin, free from endotoxin, apparently was not a difficult matter. Since it appeared in the medium before cellular autolysis (1–3 days), it could be harvested at that time as already described. To obtain endotoxin with no traces of exotoxin was a more troublesome matter. McCartney and Olitsky (66) succeeded in doing this by growing the cells anaerobically. This method produced an endotoxin which had no effect on the central nervous system of rabbits. This was further evidence of the dual nature of the toxic products of *S. dysenteriae*. These authors were not successful in separating the toxins by making use of differential diffusibility through a collodion membrane. They attributed their failure to the irregularity of membranes available to them. It was observed in the dialysis experiments, however, that the neurotoxin was found in the dialysate before the dissolution of the cells and before endotoxin could be demonstrated. Their experiments indicated that the organisms would grow and produce toxins of both kinds when inoculated into a collodion sac which was placed then intra-abdominally in rabbits. Antibodies were formed in the host against both exotoxin and endotoxin as shown by animal protection and serological tests.

The work of Okell and Blake (80) challenged the exotoxin theories of previous workers (66, 81). In their studies three differently prepared toxic materials (dry cells, filtrate and ammonium sulfate precipitated filtrates) had the same immunological properties and produced the same symptoms and lesions in animals. They failed to describe the properties further, but the results indicated that all three preparations contained the neurotoxic factor. It was their belief that this toxin was merely a substance of the cell released to the medium following autolysis of the cell. This toxic factor they called an endotoxin although they did admit that it resembled the classical exotoxins in all other respects. It would appear that subsequent workers (4, 112) have placed considerable emphasis on the meaning of this report. Okell and Blake merely found the neurotoxin within the cell. The fact that they omitted any discussion concerning the somatic intestinal toxin leads one to assume that they considered the organism of Shiga to produce only one toxic substance. This assumption may or may not be correct. It is of particular interest to note that they found higher antitoxic titers were engendered by the injection of culture filtrates than from whole dried cell preparations. This would imply that much of the antigen was present in the culture filtrate rather than in the cell or on the cell surface, and that the somatic antigen usually ascribed to being at the surface of the cell is probably not involved. It is our opinion that Okell and Blake ignored the question of one or

two toxins and grouped the toxic factors of the organism as "Shiga toxin" for their production of potent antitoxins.

Barg (4) also believed that there was only one toxin produced by the organism and that rabbits and guinea pigs reacted differently in symptoms and pathology to the toxin. In 1936 the position of Okell and Blake was somewhat strengthened by corroboration on the part of Waaler (112). This worker in an effort to repeat the work of Olitsky and Kligler (81) obtained results which entirely failed in their original purpose and indicated to him that the two toxins were one and the same. The differences reported by Olitsky and Kligler he supposed resulted from a difference in dosage used. The symptoms and pathology in rabbits caused by the filtrate of 3–7 day broth cultures Waaler found very like those following injection of the autolysate from 24 hour agar-grown cells. The rabbits showed both paralysis and diarrhea. Anatomical changes, like those described by previous authors, occurred in both the spinal cord and in the cecum. Antitoxin formed by injecting his endotoxin into rabbits was effective in protecting animals against the "exotoxin". These results, in absolute contradiction to those he started out to corroborate, he attributed to the fact that the earlier workers had not used a wide enough dosage range to illustrate the true state of affairs. Injection of small doses of Waaler's "exotoxin" into rabbits resulted in nervous system symptoms only. Rabbits receiving larger doses showed, in addition to these, diarrhea and marked intestinal pathology. Although not entirely satisfied with his explanation, Waaler concludes that a small dose of the toxin is neurotoxic, but larger doses show obvious enterotoxic manifestations.

In retrospect and in the light of later work, as well as earlier, it does seem likely that both of Waaler's materials were mixtures of bacterial products, the more highly toxic neurotoxin causing the symptoms when small amounts of a preparation were given and the intestinal manifestations appearing when the dosage reached the range of its lower toxicity.

A new phase of investigation was begun by Kurauchi (60) who employed chemical fractionation as a means of separating components of the organism. One of his accomplishments was the demonstration of a specific polysaccharide derived from the cells that was active in the agglutinin and precipitin reactions. Meyer and Morgan (67) found that this polysaccharide was capable of neutralizing hemolytic action of Shiga heterophile antibody.

Boivin and his associates (9–24) in prolific writings in the French literature have corroborated the two-toxin theory by means of chemical fractionation. Their method of separation consisted essentially of a trichloracetic acid precipitation of the neurotoxic protein. The somatic antigen, a complex molecule whose specificity was determined by a polysaccharide, remained in solution and could be purified by further chemical treatment. The polysaccharide could be split off by hydrolysis. Analysis showed that the polysaccharide contained 2–3 per cent nitrogen and represented 40–50 per cent of the complete antigen. Further studies indicated it was a hapten and that the complete antigen consisted of a polysaccharide–lipid–fatty acid–protein complex. The observation by these

workers indicated that the neurotoxin was found in young cells and old filtrates. The intestinal or endotoxin was found in the cells as well, and both toxins were released by autolysis. The neurotoxin was found in both rough (*R*) and smooth (*S*) variants while the endotoxin was found only in the smooth (*S*) variants and made up about 10 per cent of the organism by dry weight.

Haas (49–54), working independently in Germany, agreed with the results of Boivin and his group. He reported that the exotoxin was specific for the Shiga organism and could be obtained from both rough (*R*) and smooth (*S*) variants. The endotoxin (somatic antigen) was similar to that of the genus *Salmonella* although no cross-reactions were noted in serological or animal protection tests.

Olitzki and Leibowitz (82) and Olitzki, Benderesky, and Koch (83) also attempted fractionation of *Shigella* antigens. The results with several methods of fractionation, including boiling the organisms in saline with and without glycerol followed by ethyl alcohol precipitation of the filtrate, indicated several fractions varying in toxicity and antigenicity. However, Koch and Olitzki (57) corroborated the finding that the thermolabile neurotoxin is present in both *R* and *S* variants, whereas they found the thermostable factor only in *S* cells.

Morgan and his co-workers, Partridge and Schutze (69–74), working independently in England obtained data similar to the foregoing by using different methods of chemical fractionation and differential solubility with phenol, alkali, glycol and formamide. Although they were interested mainly in the somatic endotoxic antigen of the cell, their results are quite important in comparing the chemical and biological (physiological and antigenic) properties of their complete and degraded materials with the properties of the exo-neurotoxic factor, described by other investigators. Such a comparison throws much light upon the controversy of whether one or two toxins are produced by the Shiga organism.

In summary, their data showed that the somatic antigen consisted of a complex of carbohydrate, lipid and protein. The carbohydrate was a specific hapten containing phosphorus. On degradation, the phosphorus could be split off, and the carbohydrate solutions lost their viscosity and slight toxicity. The lipid was of the cephalin type, nontoxic and nonantigenic. The protein, in the undegraded form, contained a phosphorus prosthetic group and was antigenic. The removal of the prosthetic group removed antigenicity. The conjugated proteins of *Shigella dysenteriae* and *Salmonella typhosa* were found to be chemically and immunologically indistinguishable. The protein material was only slightly toxic and apparently unrelated to any neurotoxin described by other workers. The carbohydrate-phospholipid complex was not antigenic, but the carbohydrate-conjugated protein showed antigenicity. The undegraded carbohydrate could be recombined with the conjugated protein and then become antigenic. The undegraded carbohydrate could combine with the simple protein but did not become antigenic, indicating that the phosphorus prosthetic group linkage was essential in the antigenicity. When the carbohydrate was adsorbed onto collodion or to other proteins, it did not regain antigenic properties, but in the undegraded form it could combine with the conjugated protein of the typhoid organism and become antigenic and specific for the Shiga organism. The rough (*R*) organisms

contained no carbohydrate, and their somatic antigen was only slightly antigenic even though similar to the smooth (*S*) protein. The *R* and *S* cells contained at least 10% of other proteins. The degraded carbohydrate did not recombine with the conjugated protein. The Shiga carbohydrate induced typical passive anaphylaxis in guinea pigs and was related to the Shiga Forssman antigen which may be found in some strains of the organism. The Forssman antigen is found in no other *Shigella* species. Utilizing the complete somatic antigen, Morgan and his co-workers were able to produce antibodies in humans which would cause agglutination of Shiga cells and would protect mice against 50 million viable cells suspended in mucin.

Istrati (56), Steabben (102), and Olitzki *et al.* (83–85) agreed essentially with Haas and others, stating that the neurotoxin was specific to Shiga and could be found in both *R* and *S* cultures. Olitzki declared that autolysis must take place to liberate it. Other workers, such as Takita (103), had suggested autolysis as a means of getting maximum toxin in the "filtrate" of Shiga cultures. All of these observations stemmed from the original experiments of Conradi (30) and his observations on the presence of toxin in autolysates.

In two papers, summarizing his work on the toxins and antigens of *S. dysenteriae*, Boroff (25, 26) reports his inability to find evidence for the existence of an exo-neurotoxin. He considers that all the toxic properties are due to a single endotoxin, which is also the dominant antigen. Tal (104) points out, however, that Boroff's so-called somatic antigen differs markedly in toxicity and other characteristics from that found by others (57). It seems likely that his failure to find a separate and distinct exotoxin may be occasioned by incomplete separation of the toxic factors rather than by actual absence of a neurotoxin.

Our studies (39, 40) have tended to corroborate the two-toxin theory by producing according to a method somewhat similar to that of Dubos (35) a toxic material devoid of any enterotoxic characteristics. A typical paralysis in rabbits and mice, reproducible and fatal, regularly followed injection by any route of appropriate amounts of the toxin. The LD_{50} dose for 20 gram mice injected intraperitoneally was in the range of 0.1 microgram of material on a dry weight basis. The material was characterized as a protein which could be produced by young cells, destroyed at 75 C for 60 minutes, antigenic and with a marked specificity for the central nervous system. No diarrhea was produced and pathological examination failed to reveal any intestinal lesions. Injection of whole bacterial cells or impure material, on the other hand, resulted in a mixture of symptoms and lesions resembling those so often reported previously.

The endotoxin and somatic antigen of *S. dysenteriae* are synonymous. The somatic antigen is also the agglutinogen and the antigen causing formation of protective antibodies in experimental infections in animals. Investigations (9–24, 56, 57, 69–74, 82, 83, 104) have shown it to be a heat-stable chemical complex made up of polysaccharide–phospholipid–protein. It is similar to the endotoxins of the other gram negative enteric bacilli but is antigenically distinct, by reason of the polysaccharide hapten, from all the other members of this rather large, heterogeneous group. The toxic properties arise in considerable part from the

phosphorous groupings or linkages of the polysaccharide–protein (104). Besides the intestinal lesions and diarrhea caused by the antigen, numerous other pathological findings have been observed in mice, rats, guinea pigs and rabbits. Included are leucopenia, hypothermia, decrease in both liver glycogen and adrenal ascorbic acid, and adrenal hemorrhages (104).

NEUROTOXIN FORMATION

In the review just presented of the development of the two-toxin theory, the various authors used many different strains of the organism. Since few if any of these workers compared the toxin-producing abilities of several strains, it was thought possible that some variability in results might be caused by lack of constancy of toxigenicity of different strains.

Studies in our laboratories (39) suggested the role of strain toxicity from the following results. Eight Shiga strains were available to determine the differences in their ability to produce neurotoxin. A medium devised for Shiga toxin work by Dubos and Geiger (35) was used in this study. The cultures were grown at 34 C in 200 ml of medium contained in a 2,800 ml Fernbach flask on a shaking machine (100 opm) to provide optimum aeration. Samples were removed at 1, 3, 5, 7 and 14 day intervals to test for neurotoxin in the supernatant fluid. The cells were removed by centrifugation at 3,000 rpm over a 15–20 minute period. Mice were injected intraperitoneally with serial dilutions of the supernatant fluid. No deaths from infections or intestinal (diarrheal) symptoms in these mice were observed. Mice died with paralytic symptoms usually on the second through fourth days following injections of the test fluids.

After one day of incubation, 4 of 8 strains showed the presence of neurotoxin in the supernatant fluid. At 3 days of incubation, 7 of 8 strains had neurotoxin in the medium and with higher levels than at the first test period. After 5 days of incubation, several of the strains forming toxin early, had begun to lose some of their toxicity and the last strain to show toxin in the supernatant fluid had begun to do so. With 7 days of incubation, two of the slower strains continued to form toxin while the remainder lost slightly in total toxicity. At the 14 day incubation period, all strains showed a definite decrease in toxin present in the medium. This could mean that the aeration may have oxidized and destroyed the toxin or that the shaking procedure might have denatured the toxin. This point is discussed further in another section of this report on destruction of the toxin, including denaturation by shaking and oxidizing agents.

Not only was there a time variation in the neurotoxin formation, there was a difference in the amount of toxin formed by different strains. The 14-2, 14-4 and P52A strains in this and other experiments showed LD_{50} values above 1:40 dilutions. The others, except 14-5, approximated 1:40 as the LD_{50} value whereas the 14-5 strain never reached an LD_{50} value at 1:20. (The 14- series of strains was received from the Army Medical School, and the P-series was obtained from the University of Pennsylvania.) Obviously the strain and the incubation time of the organisms are directly responsible for the amount of toxin harvested.

Other studies (40) indicate a complete lack of correlation between ability of a strain to form toxin and its infectivity for mice. That is, powerfully toxigenic strains might be fatal for mice in small or great numbers when injected with 5 per cent mucin. The opposite would also hold. It was also observed that not only were the strain of organisms and time of incubation important, but factors involving nutrition of the organisms affected toxin production. In some media no neurotoxin formation was noted. This was particularly true in a synthetic medium where ammonium ions were used as a source of nitrogen. Again it was observed that where large amounts of fermentable carbohydrate (glucose) were present and high aeration was absent, no detectable neurotoxin was present. If cultures containing the glucose were aerated, then some neurotoxin was formed. The facultative metabolism of the organism apparently determines neurotoxin formation. When carbohydrate metabolism predominates and the pH is lowered, less neurotoxin is formed. If the medium is buffered or kept at pH levels above 7.0 and preferably near 8.0 by titration, more neurotoxin is found. The lower pH levels do not inactivate the neurotoxin, however, since stability studies indicate that the toxin is relatively stable for long periods at levels near pH 5.0. The cells produced under these conditions agglutinated with specific serum in a manner qualitatively and quantitatively similar to cells producing neurotoxin, indicating that the somatic antigen was present and reactive.

Numerous workers have suggested that the neurotoxin of *S. dysenteriae* is specific to the Shiga bacillus and that it may be formed by both the *R* (rough) and *S* (smooth) variants. Such reports have been made by Haas (49), Istrati (56), Steabben (102), and Dubos and Geiger (35). Morgan (70) reported that the *R* organisms contained no carbohydrate and that their somatic antigen was only slightly antigenic even though similar to the *S* protein. In experiments designed to confirm that neurotoxin is produced irrespective of phase (39, 40) we have compared the abilities of *R* and *S* variants to produce the toxin under different conditions. In summary, the variants responded similarly to changes in media, pH, temperature, time and oxidation-reduction potential. They produced or failed to produce neurotoxin under similar conditions. Such data indicate that the neurotoxin is unrelated to the somatic antigen of the *S* phase of the organism and, in fact, furnish additional evidence in favor of two toxins. It would appear that the neurotoxin is a metabolite of active cellular metabolism.

A search for factors contributing to greater toxin production by the Shiga organism has naturally been part of the study of the neurotoxin. As early as 1920 (81), it was observed that under aerobic conditions greater amounts of toxin were produced. This was reiterated by McCartney and Olitsky (66) when they found that increased aerobiosis also decreased the incubation time for neurotoxin production in stationary broth filtrates. These findings are in agreement with those of Dubos *et al.* (34) and of Olitzki and Bichowsky (85). Eaton (37) reviewed literature on bacterial toxins in general and stated that the lethal toxin of the staphylococcus (*Micrococcus pyogenes*) was not affected by anaerobic conditions. The toxins of tetanus, botulinus and clostridial gas gangrene are the

result of anaerobic conditions which are, of course, essential for the growth of the organisms producing them. Since the erythrogenic toxin of the streptococcus is likewise produced under conditions of reduced aeration, it would seem that only diphtheria and dysentery exotoxins require high aeration for their formation. Quantitative studies on the formation of neurotoxin under aerobic and reduced aerobic conditions will be reported elsewhere with an account of the effect of aeration by shaking.

In retrospect, it can be noted that the data of Conradi (30), Flexner and Sweet (45), and Okell and Blake (80) indicate high formation of toxin in 24–30 hours on solid media where maximum aeration could be obtained. McCartney and Olitsky (66) found toxin in 1–3 day broth cultures as did Dubos (35). Growth of the organism in liquid culture might require, under certain conditions, from one to three weeks for maximum toxin formation as compared to 18–30 hours on agar plate surfaces. Using a medium described by Dubos for *S. dysenteriae* (35) in Fernbach flasks for good aeration, we have found maximum toxin formation in three to four days at 34 C. Some toxin was formed as early as one day after inoculation, before autolysis had taken place.

Young agar cultures were found not to release toxin, but to hold it within themselves until autolysis caused its dispersion. In further work aimed at ascertaining optimum conditions for toxin production (39, 40), we determined that the pH 7.0 to 8.0 was most desirable for this purpose; when Dubos' medium was used with pH levels below 6.0 during the growth phase, little toxin was formed. Although toxin was produced at temperatures ranging from 20 C to 42 C, the higher the temperature, the more quickly the toxin was produced and the sooner this activity was terminated. For example, at 42 C, peak yield was at 3 days, and all toxicity was gone in 7 days; whereas at 20 C, toxicity was first noted at 7 days and at 28 days was still increasing.

The effect of iron on toxin formation has been a factor of significance with several of the exotoxins. Pappenheimer and Johnson (86) showed that iron in small quantities could inhibit the formation of diphtheria toxin. Mueller and Miller (75) and Mueller (76) investigated the effect of iron on the formation of both tetanus and diphtheria toxin and found that it caused a decrease in toxin production in both. Dubos and Geiger (35) removed the iron from liquid media since they considered that it would inhibit Shiga toxin formation. Their experimental data showed inhibition of neurotoxin formation in ranges where no effect on growth was observed. As little as one hundred thousandth per cent $FeCl_3$ showed this inhibitory property in all types of media tested. Our findings (39, 40) revealed that 0.01 M iron prevented toxin production and growth as well. Concentrations of 0.001 M to 0.000001 M decreased the amount of toxin formed without affecting growth. It was of interest that the cells grown in the presence of iron agglutinated as readily as cells grown in untreated media. This agglutination indicates that the formation of the type specific somatic antigen is not materially altered in such a medium although neurotoxin is not formed.

The low concentration of neurotoxin in the filtrate of liquid cultures, in comparison with other exotoxins, suggests that the toxic factor is an endotoxin and

that greater quantities of neurotoxin would be present within the cells. Our experiments with cells disrupted by sonic vibrations revealed that the cells contained no more exotoxin than did the surrounding medium. In fact, the supernatant fluids contained slightly greater amounts. Control toxin preparations exposed to sonic vibration did not show a loss in toxicity because of destruction by such treatment. It would seem that the neurotoxin is produced in much smaller quantities than are the toxins of tetanus, botulinus or diphtheria. That fact, plus the fact that the neurotoxin molecule is a less toxic substance per unit weight than the other exotoxins, caused some observers (25, 26, 80, 88, 112) to believe that it should be considered an endotoxin since some of them have been unable to separate the somatic antigen endotoxin and neurotoxin biologically or chemically.

PROPERTIES OF THE NEUROTOXIN

Biochemical properties. During the forty years following the first observations (30) that the Shiga bacillus has a neurotoxic principle, there was little interest in the chemical properties of the neurotoxin. Olitsky and Kligler (81) suggested that it appeared to be a globulin, the first indication, aside from its antigenic properties, that it might be protein. Boivin and co-workers (16 to 19) and others (50, 51, 52, 70, 72) agreed that the material could be precipitated by protein precipitants and that it had protein-like properties. Being mainly interested in the somatic antigen complex, however, they did not pursue the chemistry of the neurotoxin further.

Anderson (2) reported that the neurotoxin gave protein reactions with the biuret, Millon and xanthoprotein tests, as well as being nondialyzable and not coagulated by heat. It has been found (38) that nucleic acid is associated with the neurotoxic protein factor which gave positive Feulgen reaction and Dische diphenylamine reaction for desoxyribose.

Our studies with purified neurotoxin (39, 40) resulted in color reactions typical of protein and yielded precipitates with acids, metal salts, alkaloidal reagents and organic solvents in a manner similar to other proteins. Other tests indicated the presence of organic phosphorus and sulfur and possible traces of nucleic acid. The findings are summarized briefly in table 1.

Physical properties. Little information on the physical properties of the neurotoxin has been published. The presence of only relatively small quantities of the toxic material in filtrates might suggest that it is of large molecular size and so is prevented from passing the cell membrane. Our studies indicate that this is not so since we have found the neurotoxin in similar amounts within the cells and in the filtrates. It has been reported (2) that the toxin is nondialyzable and noncoagulable by heat: these properties indicate that it is neither a very small molecule nor an albumin.

The size of the molecule has been calculated in our studies (39, 40) through measurement of diffusion constants by a glass diffusion cell. The procedure at best reveals an approximate figure for particle size. In our hands accurate measurements were complicated by the fact that the toxic material could not be

obtained in high concentration per unit volume even with several procedures for purification and concentration. The resulting measurements of the diffusible material by mouse titration indicated that the neurotoxin approximated a molecular size of 73,000 with perhaps a range of 25–100 thousand.

Further studies on the physical characteristics revealed that the neurotoxin was extremely insoluble and formed viscous solutions when highly toxic solutions were attempted. The purified material was relatively heat labile being destroyed at 75 C in 60 minutes. Purification studies suggested that the neurotoxin was easily adsorbed onto various materials used as adsorption agents such as kaolin, charcoal and diatomaceous earth.

Immunological properties. The potent and specific antigenicity of the neurotoxin of *S. dysenteriae* was discovered early. As already noted, Bessau (8) observed that an antitoxin prepared against the paretic toxin could neutralize

TABLE 1

Biochemical properties of Shiga neurotoxin

TEST	GROUP TESTED FOR	RESULT
Millon	tyrosine (hydroxyphenol group)	+
Xanthoprotein	tyrosine, phenylalanine or tryptophan (phenyl group)	+
Hopkins-Cole	tryptophan	+
Biuret	protein (carbamyl groups)	+
Ninhydrin	amino acid nitrogen (free carboxyl and alpha amino group)	+
Sakaguchi	arginine or guanidine	+
Kjeldahl	total nitrogen	16 + %
Bial	pentose	+ (wk)

this material but not the intestinal toxin. Olitsky and Kligler (81) used this neutralization phenomenon for removing exotoxin from endotoxin preparations. Then in 1930, Okell and Blake (80) reported that both the bacillary bodies and filtrates gave the same antitoxin when injected into animals. As these antitoxins protected against each other in animal protection tests, they concluded that this meant a unity of antigens. Apparently, they did not consider the possibility that their preparations might contain mixed antigens which, of course, would give these cross-reactions.

Boivin and Mesrobeanu (14, 15) studied antisera prepared against chemically separated exotoxin and endotoxin from both *R* and *S* variants. In protection tests against the exotoxin, they found that anti-exotoxic sera derived from *R* or *S* variants would give protection against 10 lethal doses of toxin. Anti-endotoxic sera of both variants were inactive against exotoxins as were controls of normal sera. Farrell and Ferguson (41), using autolysates detoxified with formalin, protected mice against 50 MLD of toxin. By passive immunization, they were able to protect mice with rabbit antiserum against 50 MLD of toxin. However, no differentiation was made between exotoxin and endotoxin in these studies.

The roles of various antigens of the organism in producing protective antibodies were again investigated by Steabben (102), her results agreeing with those of others (14, 15). Antitoxic sera protected against the toxin but did not prevent infection by the whole cells. Antiserum against the *R* variant protected against *R* organisms but not against infection by *S* cells. An ultraviolet irradiated toxoid prepared from an autolysate of *S* cells by Branham and Habel (28) protected mice against both toxemia and infections.

According to Farrell *et al.* (41, 42), who studied the antitoxic content of human sera, this is of a very low level. The significance of this finding depends on one's views as to the importance of the neurotoxin in human infections. Branham and Carlin (27) suggested that in theory a serum both antibacterial and with a high antitoxin content would be more practical and would have higher use in clinical work. Their work with commercial antisera showed that antibacterial sera contained very low levels of antitoxic protection whereas strong antitoxic sera contained low levels of anti-infection protection for mice.

This last statement is in direct contradiction to assertions by Boroff (26) that "immunization of rabbits with heat-killed organisms of smooth and rough variants of *Shigella dysenteriae* or some of its fractions affords protection against both of the variants and their toxic products." A series of experiments wherein groups of rabbits were immunized respectively with *R* and *S* whole organisms, sonic lysates of these, partially purified toxin from both variants and somatic antigen prepared according to the method of Morgan (70) is said to support the hypothesis that there is "only one dominant toxin in *S. dysenteriae*" and that "the injection of any antigenic fraction of this organism, capable of inducing protective antibodies, will afford protection against the toxic effects of the whole organism as well as the purified toxin." If true this would greatly simplify the whole problem of the toxic properties of the organism—in fact, it would be solved. However, examination of this author's tables suggests the matter may not be quite so simple as the text would lead one to suppose. For example, immunization of rabbits with somatic antigen from *S* cells did not protect them against subsequent challenge with whole cells. Since the same animals had already survived a challenge with homologous somatic antigen, it seems that the whole cells contain a toxic factor not present in the somatic antigen. Rabbits immunized with toxin were found resistant to a challenge of whole organisms. This toxin, as the author states, was only partially purified and, therefore, cannot be said to be free of somatic antigen which would, of course, induce antibodies completing the protection of the animal against whole organisms. Not only is it difficult to correlate the data with the conclusions and theory presented by this author, but it is hard to accept his conclusions in the light of other evidence presented in this review.

Table 2 summarizes mouse protective tests (39, 40) which indicate that the antitoxic antibody and the antibacterial antibody are separate entities engendered by different antigens of *S. dysenteriae*. Several antisera were available for these protection tests. Rabbit antisera were produced against the *R* and *S* cells and against crude and purified neurotoxin preparations. In addition, a

commercial anti-*S* whole culture antitoxic serum from horses was also used (courtesy of Parke-Davis).

The rabbit antisera were prepared by injecting washed *R* and *S* cells that had been killed by 1 per cent formalin at 37 C and preserved by 0.5 per cent formalin. The crude toxin consisted of acid precipitated (pH 4.0) whole *R* broth culture resuspended in phosphate buffer at pH 7.0. It was detoxified by 0.5 per cent formalin at pH 8.5 and 37 C for 14 days. The purified toxin was a fractional acid precipitate of crude *R* toxin, having an LD_{50} of less than 1 microgram of protein material as based on nitrogen determinations. It was detoxified in the same manner as the crude toxin.

Mice were injected subcutaneously with 0.5 ml of diluted serum approximately 24 hours previous to receiving intraperitoneally a challenge dose of

TABLE 2

*Mouse protection tests**

SERUM	SERUM DILUTION PROTECTING 50% OF MICE AGAINST	
	10 LD_{50} neurotoxin	100 LD_{50} infectious units
Normal rabbit	—	—
Anti-*S* cell	—	1/400
Anti-*R* cell	—	—
Anti-crude toxin	1/80	—
Anti-purified toxin	1/160	—
Commercial antitoxin†	>1/640	1/150

* Six mice per dilution; 0.5 ml serum given subcutaneously 18–24 hours before intraperitoneal challenge.

— = no protection from undiluted serum.

† Parke-Davis.

either toxin or viable cells. Various routes were used for the sera and challenge doses to prevent localized immunity from interfering with the results.

In the tests, where approximately 10 LD_{50} of neurotoxin were given as the test dose, the anti-*R* and -*S* cell sera showed negligible protection. All of the antitoxic sera showed protection. The antitoxin against the purified toxin developed greater protective qualities even though less material was used in immunization of the rabbits. The commercial antitoxin, which was produced in horses with whole *S* cultures and which was concentrated and purified, yielded great protection, being active through a 1:640 dilution. When approximately 100 LD_{50} of neurotoxin were used as the test dose in mice, only the commercial antitoxin gave significant protection. Here it protected mice when used at less than a 1:200 dilution.

When the protected mice were challenged with living cells, the results were quite different. Approximately 100 LD_{50} of viable cells in gastric mucin were injected intraperitoneally in 1 ml quantities 24 hours following the injection of serum by the subcutaneous route. The data indicate that anti-*S* cell serum shows considerable protection, preventing death in 50 per cent of the mice at

1:200 and 1:400 dilutions. No other antiserum produced in rabbits against the toxins and the *R* cells showed protection. The commercial antitoxin protected mice against infection at the lower dilutions.

These results are evidence of the existence of two distinct antigens in the Shiga organism. Further evidence is provided serologically in table 3, which gives agglutination titers of *R* and *S* cells with *R*, *S* or antitoxic sera. The data reveal little cross agglutination between the *R* and *S* sera and cells. It is also shown that the antitoxin (commercial) from whole *S* culture autolysates has agglutinins for *S*, but not *R* cells. These data correlate well with the animal infection and toxin protection tests just described.

The practicality of antitoxic sera in therapeutic use has been questioned by Weil (115), in contrast to the cited statements by Branham and Carlin (27).

TABLE 3

Agglutinin titers of sera of rabbits immunized with R and S cell vaccines

SERUM	ANTIGEN	DILUTIONS OF SERUM								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560
R	+ *R*	4+	4+	4+	4+	4+	4+	3+	2+	2+
R	+ *S*	3+	2+	1+	—	—	—	—	—	—
S	+ *S*	4+	4+	4+	4+	4+	4+	2+	1+	—
S	+ *R*	4+	4+	1+	—	—	—	—	—	—
Antitoxin*	+ *R*	—	—	—	—	—	—	—	—	—
Antitoxin*	+ *S*	—	1+	4+	4+	4+	4+	4+	1+	—
Normal serum	+ *R*	—	—	—	—	—	—	—	—	—
Normal serum	+ *S*	—	—	—	—	—	—	—	—	—

* Parke-Davis.

He reports that apparently antitoxic immunity did not alter the course of infections in clinical trials. Observations of this type raise the question of the presence and/or the role of the neurotoxic factor in human cases of Shiga dysentery. It might be that the neurotoxin could be a laboratory phenomenon and perhaps it is not produced in significant enough quantities in the human body to play an important role in the course of the disease. Such a suggestion is not likely to be accepted readily by those who have read of, or have observed, some of the severe dysentery outbreaks caused by *S. dysenteriae*. It is true that numerous closely related organisms not forming a demonstrable neurotoxic factor can produce bacillary dysentery or dysentery-like syndromes sometimes as severe as true Shiga dysentery, the intestinal toxic manifestations with these other organisms being produced by the somatic antigen, namely, the endotoxin.

Stability of the neurotoxin. Bacterial exotoxins have been noted for their sensitivity to heat. Eaton (37) and Pappenheimer (88) have summarized data on the heat lability of several toxins. Todd (105) first reported that Shiga

neurotoxin was destroyed at 80 C in 60 minutes, but not at 70 C in the same period. Flexner and Sweet (45) confirmed Todd as did Olitsky and Kligler (81) and Waaler (112). Dubos *et al.* (34) and Anderson (2) pointed out that the neurotoxin was more sensitive to heat under alkaline or acid conditions than at neutrality. Others, such as Egami *et al.* (38), reported that higher temperatures were necessary for total detoxification. It must be remembered that the endotoxic somatic antigen had been found by Olitsky and Kligler (81) to be heat resistant. The author (39, 40) found almost complete destruction of both crude and purified toxin at 75 C for one hour. The crude material was slightly more resistant. Increasing temperatures decreased the detoxification time and at 100 C both crude and purified toxin were destroyed in 1 to 2 minutes. In preparations where the neurotoxin appeared to be heat stable, endotoxin might have been present in small amounts confusing the results and their interpretation.

In general, bacterial toxins have been found sensitive to acid and alkaline environments. Eaton (37), in a review article, indicated that this was true of diphtheria and tetanus toxins. In the succeeding years, Petermann and Pappenheimer (89) reported denaturation of diphtheria toxin at pH below 5.6 and above 10.8. Pappenheimer (88) quoted other investigators whose findings were similar. Putnam and his co-workers (95), summarizing work on botulinus toxin, reported lability of the toxin to pH changes in the narrow range of 6–8 and cited evidence of other bacterial toxins responding somewhat similarly.

The toxins of Shiga's bacillus appear to be more resistant to pH changes. Flexner and Sweet (45) reported that 0.5 per cent HCl did not destroy the toxin although Olitzki and Bichowsky (85) revealed that below pH 5 some toxicity was lost. Sensitivity to pH has been reported (2, 34) to be only associated with the resulting increased loss by heat. The former described experiments in which the effects of heat and pH changes were followed by flocculation techniques. Stability was greatest at pH 7–7.5. Most rapid loss of activity appeared in the alkaline range while pH levels to 5.5 showed some loss with increasing heat. Dubos and his collaborators (34) found that the toxin was slowly inactivated by heat at neutral and acid pH while rapid inactivation took place under alkaline conditions.

The resistance of the neurotoxic factor to acid and alkaline conditions is of practical importance in the procedures of precipitation by acids and various basic salts. Our observations (39, 40), made by following loss of toxicity for mice, corroborated the findings of earlier authors by demonstrating Shiga neurotoxin to be more resistant to detoxification by mild acid or alkali than other bacterial toxins. Between pH 6 and 8 no toxicity loss was noted in 7 days. At pH 4 and pH 10, slight loss was noted. Greater departures in either direction resulted in more rapid detoxification.

Further work (39, 40) showed that the toxin was not destroyed by drying from the frozen state or by desiccation under partial vacuum over drying agents such as P_2O_5 or H_2SO_4 at room temperature. The toxicity was not affected by sonic treatment of culture filtrates nor by shaking for 18 hours on a Kahn shaker. It was found (39, 40) that storage of the toxin in solution at 5 C was

not detrimental to the toxicity of either crude or purified preparations over periods of 6 months to a year. Lyophilized samples were kept at dry ice temperature and 5 C for periods of 1 to 2 years with no loss of toxicity. These results agree with those of Todd (105), who maintained toxin samples at room temperature (25 C) without loss for 4–5 months. Okell and Blake (80) reported stability of their preparation for 4 years in the dried state at cold storage temperatures. The neurotoxin was also found to withstand shaking with chloroform with no apparent loss of toxicity.

DETOXIFICATION OF THE NEUROTOXIN

The effectivity of various agents and procedures in detoxification is of positive importance in toxoid formation and negative in toxin production. Dumas, Ramon, and Bilal (36) described the detoxification of Shiga toxins by formalin and cited similar modifications in the toxicity of other bacterial toxins by formalin, namely diphtheria and tetanus toxins. Numerous investigators, including Anderson (2), Dubos and Geiger (35), Branham and Habel (28), and Farrell and Ferguson (41), showed that formalin detoxification was practical on numerous types of toxic preparations from Shiga cultures. This was more recently confirmed (39, 40) with crude and purified neurotoxin preparations. The purified material was detoxified in about 7 days at 37 C when a final concentration of 0.5 per cent formalin was used; the less pure toxin required about fourteen days. Results with phenol, used in the same concentration, were similar.

Ultraviolet radiation has been used by Branham and Habel (28) for the detoxification of whole Shiga culture autolysates. Detoxification of thin films was complete in 15 or 20 seconds depending on the pressure of the lamp used. Neurotoxin, free of somatic antigen, was detoxified in our studies in 64 minutes, the thickness of the film and the lamp used being at least partly responsible for the increased time requirement.

The effect of surface active agents on bacterial toxins has been under investigation for some time. As early as 1924, Larson and his associates (61, 62) reported that diphtheria and tetanus toxins were destroyed by soap and bile salts. Vincent (111) confirmed their work using tetanus toxin. Schmidt (97) also reported that salts of fatty acids would detoxify the diphtheria toxin. Again in 1935–1936, the destruction of diphtheria toxin by soaps of differing chemical constitutions was studied by Bayliss and Halvorson (6, 7). Inactivation of the lecithinase of *Clostridium welchii* was reported by Macfarlane and Knight (65) to occur upon treatment with sodium dodecyl sulfate. Neter (78), investigating the effects of antimicrobial substances, demonstrated that zephiran, a detergent of the dimethyl benzyl ammonium chloride structure, could inactivate tetanus toxin. The effects of surface active agents on biological materials, including bacterial toxins, as published up to 1948 have been reviewed by Glassman (47). In our hands (39, 40), the results with other toxins already mentioned have been found to be true also of Shiga neurotoxin. Ionic surface active agents destroyed the toxin in low concentration (final concentration 1 per cent), while a nonionic agent caused only partial loss of toxicity in the same test period of seven days.

Resistance or susceptibility of Shiga neurotoxin to destruction by enzymes has been a source of much disagreement. Flexner and Sweet (45) reported destruction by peptic digestion but resistance to trypsin after 18 hours of exposure. However, Boivin and Mesrobeanu reported destruction of toxin by trypsin. Dubos and his associates, after reporting complete resistance of the toxin to proteolytic enzymes (34), later (35) found slight detoxification by trypsin. Again, complete destruction by trypsin was reported by Anderson (2), who found less activity by pepsin and still less by papain. Egami (38) observed destruction by pepsin and pyloric enzymes, but none by papain, trypsin or takadiastase. Our studies (39, 40) indicated no detoxifying activity on the Shiga neurotoxin by pepsin, papain, pancreatin, pangestin, rennin or trypsin during 20 hours of exposure at the activating pH. The remarkable lack of correlation in these findings most probably can be attributed to the variable conditions of the tests, sensitivity of the methods used for toxicity determinations, and the exact nature of the toxic material under study.

Other means of detoxifying toxins, including Shiga neurotoxin, have been reported. For example, Treffers (107, 108) demonstrated reduction of toxicity in the somatic antigen of *S. dysenteriae* by acetylation. Egami (38) found that Shiga neurotoxin could be destroyed by ketene, a method of acetylation. Ketene gas was also used by Boroff (25), who found that *S. dysenteriae* and sonically disintegrated materials prepared from it were detoxified by this method. Diazomethane or alcohol-HCl mixtures were also used by Egami (38) to lower toxicity of the neurotoxin.

Recently sulfur containing compounds have been tested frequently for their ability to reduce toxicity. Levaditi and Vaisman (63, 64) tested aromatic sulfur-containing compounds of the sulfonamide, sulfone and sulfoxid groups. The latter two showed considerable activity in detoxifying the endotoxins of coliform and typhoid organisms in mice. Wagner-Jauregg (113) has reviewed some of these results with *S*-containing compounds and a discussion of their roles in detoxification. Murthum and Glenk (77) described the lowering of toxicity of Shiga endo- and exotoxins as well as diphtheria toxin *in vivo* in mice by 2–4 mg/kg of sulfonamides. A prophylactic effect was also noted. Giving sulfonamides orally, Zahl and his co-workers (118) found protection against the endotoxins of *Salmonella* and *Shigella*. An interesting contrast to these reports is one by Wildführ (117) which points out that para-aminobenzoic acid, an analogue of the sulfonamides, increases toxin formation by the gas gangrene producing clostridia and by *Corynebacterium diphtheriae*. Moore and Marmorston (68) published data indicating that sulfonamides had no detoxification effect on *Shigella paradysenteriae* Flexner filtrates in mice even when used in growth inhibiting concentrations. Our own findings (39, 40) revealed that sulfapyridine and sulfamerazine were without activity in lowering the toxicity of the purified neurotoxin after 7 days in concentrations of 0.5 per cent. Para-aminobenzoic acid was also lacking in activity.

Other chemicals which have been studied include those which have an effect on the OR potential, that is, are active as oxidizing or reducing compounds.

Souto and Lima (101) found that ascorbic acid (a reducing substance) destroyed gas gangrene toxin; this was confirmed by Petherick and Singer (90) on diphtheria toxin. On the other hand, numerous reports indicate detoxifying activity by oxidizing agents. Velluz (110) reported that oxidation inactivates toxins *in vitro*. Ribeiro and Guimaraes (96) found that chlorophyll would neutralize by its oxidative properties both tetanus toxin and snake venom. These neutralized toxins could then be used for immunization. Hydrogen peroxide was used for detoxification purposes by Petherick and Singer (90), and subsequently Singer (100) reported that intracellular oxidases were important in such processes. They observed experimentally that oxidase granules contained lipid which they said combined with the toxin. The destruction of tetanus toxin by oxidative

TABLE 4

Effect of various factors on the toxicity of Shiga neurotoxin

DETOXIFICATION	NO EFFECT
Heat	Sulfonamides
Ultraviolet radiation	Antibiotics
Phenol	Penicillin
Formalin	Streptomycin
Surface-active agents	Gliotoxin
Cationic	Fumigacin
Anionic	Chloromycetin
Urea	Mercurials
Guanidine	Enzymes
Oxidizing agents	Proteolytic
H_2O_2	Amylolytic
$KMnO_4$	Lipolytic
	Surface-active agents
	Nonionic
	Desiccation
	Freeze drying
	Sonic treatment
	Shaking

conditions was reported by Pillemer (93). Barnes *et al.* (5), while developing antidysentery vaccines, observed loss of toxicity following oxidation with peroxide combined with ultraviolet radiation in the presence of caprylate ions. Peroxide and peroxidase, used together, could detoxify diphtheria or tetanus toxins (1); neither had any effect on the toxins when used alone.

Ascorbic acid was less effective against Shiga neurotoxin, causing only 50 per cent destruction in 7 days. Sodium thioglycolate had even less effect than this on the toxin. The Shiga neurotoxin was completely susceptible to the action of peroxide in 24 hours. Likewise, potassium permanganate detoxified the material in this space of time (39, 40).

Urea and guanidine, as would be expected of protein denaturants, were destructive of the neurotoxin (39, 40), urea more rapidly so than guanidine. Acetylsalicylic acid detoxified 75 per cent of the toxin in 7 days. Other studies

conducted on the neurotoxin (39, 40) indicate that it resists detoxification or neutralization by penicillin, streptomycin, gliotoxin, fumigacin and chloromycetin. Various reports, starting with Gaté and Papacostas (46), had stated that bacterial toxins might be affected by antibacterial substances. Neter (79), however, reported that, while tetanus toxin could be detoxified by clavacin and pyocyanase, penicillin and streptomycin did not have this effect. Mercurials were found to be ineffective as detoxifying agents, in concentrations in which they are used as antiseptics (39, 40). Table 4 is a summary of the effectiveness of some of the agents mentioned in detoxification of the neurotoxin.

CONCLUSIONS

Abundant experimental evidence, accumulated by many researchers using various approaches, indicates that *Shigella dysenteriae* (Shiga) produces, in addition to the endotoxin similar to that of other gram negative enteric bacilli, a neurotoxic exotoxin which can be isolated free of endotoxin by chemical means. It is formed by both *R* and *S* variants, the yield varying with strain, media constituents, incubation temperature and time and degree of aeration. Biochemical properties classify it as a protein noncoagulable by heat, nondialyzable and of low solubility. Heat lability is a property it shares with other exotoxins. Its molecular size is in the range of diphtheria toxin but much smaller than the botulinus toxins (87, 95). The material is antigenic, immunologically specific and active in eliciting production of neutralizing antibodies. It has been found stable to drying, sonic vibration, shaking with chloroform and to have a greater tolerance for pH changes than have other exotoxins. Detoxification can be accomplished with formalin, phenol, ultraviolet irradiation, ionic surface-active agents and various other physical and chemical protein denaturing factors. Injection of the neurotoxin into mice and rabbits results in paralysis and death after 48–72 hours with no intestinal symptoms. The LD_{50} for mice and rabbits is in the range of 0.1 microgram.

Relatively low yields of neurotoxin, the involved purification procedures necessary to free it from endotoxin and media proteins, and its low solubility have complicated studies on the neurotoxin for numerous workers who have contributed to our knowledge of it. Much of the confusion concerning the neurotoxin has resulted from the presence in cultures of the Shiga organisms of two toxic substances having different toxic manifestations in animals. Some of the misunderstanding over these toxic substances has been caused by various workers using the terms exotoxin, neurotoxin, endotoxin, somatic antigen too loosely, too rigidly or interchangeably in such a manner as to cause confusion.

Comprehensive studies are needed on purification and on elucidation of the nature of the neurotoxin by chemical analyses and physical means including electrophoresis, ultracentrifugation and spectroscopy. Another problem which still calls for clarification is the role of the neurotoxin in human dysentery infections. This question is of considerable clinical significance since the basis of both active and passive prophylaxis and therapy depends on the answer.

REFERENCES

1. AGNER, K. 1947 Detoxicating effect of verdoperoxidase on toxins. Nature, **159**, 271–272.
2. ANDERSON, C. G., BROWN, A. M., AND MACSWEEN, J. C. 1945 The production, purification and titration of the neurotoxin of *Shigella dysenteriae*. Brit. J. Exptl. Path., **26**, 197–208.
3. ANDO, K. 1929 A simple method of obtaining soluble specific substances from various bacteria (*Streptococcus viridans*, *B. dysenteriae* and *B. mallei*) (preliminary report). J. Immunol., **17**, 555–557.
4. BARG, G. S. 1932 Zur Charakteristik der Wirkung des Dysenterie-toxins. Z. Immunitätsforsch., **74**, 372–379.
5. BARNES, F. W., DEWEY, M. M., HENRY, S. S., AND LUPFER, M. H. 1946 Chemical detoxification of dysentery antigen. J. Bact., **51**, 621.
6. BAYLISS, M., AND HALVORSON, H. O. 1935 Germicidal and detoxifying properties of soaps. J. Bact., **29**, 9–10.
7. BAYLISS, M. 1936 Effect of the chemical constitution of soaps upon their action on diphtheria toxin. J. Infectious Diseases, **59**, 131–136.
8. BESSAU, G. 1911 Ueber die Dysenteriegifte und ihre Antikörper. Zentr. Bakt. Parasitenk., Abt. I., Orig., **57**, 27–56.
9. BOIVIN, A. 1936 Sur le comportement comparé des endotoxines et des exotoxines vis-à-vis de l'acide trichloroacetique. Compt. rend., **203**, 284–286.
10. BOIVIN, A., AND IZARD, Y. 1937 Methode pour la purification à l'acide trichloracétique, des toxines et anatoxines diphtériques, tétaniques et staphylococciques. Compt. rend. soc. biol., **124**, 25–28.
11. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur la nature chimique et sur les propriétés biologiques, des antigénes somatiques et des toxines des bactéries. 6th Congré de Chimie Biologique, 401–413.
12. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines des bacilles dysenteriques. Sur la nature et sur les propriétés biologiques, des principes toxiques susceptibles de se recontrer dans les filtrats des cultures sur bouillon du bacille de Shiga. Compt. rend. soc. biol., **124**, 442–444.
13. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines des bacilles dysentériques. Sur les principes toxiques du bacille de Flexner. Compt. rend. soc. biol., **124**, 1078–1081.
14. BOIVIN, A., AND MESROBEANU, L. 1937 Sur le pouvoir protecteur antitoxique des serums obtenus en injectant à l'animal des endotoxines glucido-lipidiques. Compt. rend. soc. biol., **124**, 1092–1094.
15. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines des bacilles dysentériques. Sur le pouvoir protecteur antitoxique des serums obtenus en injectant à l'animal l'endotoxine-antigene O du bacille de Shiga et du bacille de Flexner. Compt. rend. soc. biol., **125**, 796–799.
16. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines des bacilles dysentériques. Sur l'existence d'un principe toxique thermolabile et neurotrope dans les corps bactériens du bacille de Shiga. Compt. rend. soc. biol., **126**, 222–224.
17. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines des bacilles dysentériques; sur l'identité entre la toxine thermolabile et neurotrope des corps bactériens du bacille de Shiga et l'exotoxine présente dans les filtrats des cultures sur bouillon de la mème bacterie. Compt. rend. soc. biol., **126**, 323–325.
18. BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines du bacille dysentérique. Sur la signification des toxines produites par le bacille de Shiga et par le bacille de Flexner. Compt. rend. soc. biol., **126**, 652–655.

19. BOIVIN, A., AND MESROBEANU, L. 1937 Sur l'existence de toxine thermolabile et neurotrope (exotoxine) dans les corps bactériens du bacille de Shiga. Compt. rend., **204**, 1759–1761.
20. BOIVIN, A., MESROBEANU, L., AND IZARD, Y. 1938 Sur la separation, par voie chimique, des endotoxines glucidolipidiques et des exotoxines protéiques. Compt. rend. soc. biol., **127**, 651–654.
21. BOIVIN, A., AND MESROBEANU, L. 1938 Sur la spécificité qui se manifeste dans l'action immunisante anti-infectieuse des antigènes glucido-lipidiques. Compt. rend. soc. biol., **128**, 358–360.
22. BOIVIN, A., AND MESROBEANU, L. 1938 Recherches sur les toxines des bacilles dysentériques. Sur les rapports qui existent entre la presence d'antigène glucido-lipidique chez le bacille de Shiga et la virulence et le pouvoir vaccinant de cette bacterie. Compt. rend. soc. biol., **128**, 446–448.
23. BOIVIN, A. 1940 Les deux toxines du bacille de Shiga et leur place dans la classification général des toxines bacteriennes. Rev. Immunol., **6**, 86–115.
24. BOIVIN, A., DELAUNAY, A., AND SARCIRON, R. 1940 Sur la liberation de l'antigène glucido-lipidique et de la neurotoxine par les cellules mortes du bacille de Shiga. Compt. rend. soc. biol., **134**, 361–363.
25. BOROFF, D. A. 1949 Study on toxins and antigens of *Shigella dysenteriae*. I. Toxicity and antigenicity of whole organisms and various fractions of *Shigella dysenteriae*. J. Bact., **57**, 617–632.
26. BOROFF, D. A., AND MACRI, B. P. 1949 Study on toxins and antigens of *Shigella dysenteriae*. II. Active protection of rabbits with whole organisms and fractions of *Shigella dysenteriae*. J. Bact., **58**, 387–394.
27. BRANHAM, S. E., AND CARLIN, S. A. 1948 Studies with *Shigella dysenteriae* (Shiga). II. The effect of serums on experimental infection and intoxication in mice. J. Infectious Diseases, **83**, 66–77.
28. BRANHAM, S. E., AND HABEL, K. 1946 Preparation and evaluation of an irradiated toxoid from the toxin of *Shigella dysenteriae*. J. Immunol., **54**, 305–314.
29. CHANTEMESSE, A., AND WIDAL, F. 1888 Le microbe de la dysenterie epidémique. La semaine Méd., **8**, 153.
30. CONRADI, H. 1903 Ueber lösliche, durch aseptische Autolyse erhaltene Giftstoffe von Ruhr- und Typhus-bazillen. Deut. Med. Wochschr., **29**, 26–28.
31. DOPTER, C. 1905 Effets experimentaux de la toxine dysentérique sur le système nerveux. Ann. inst. Pasteur, **19**, 353–366.
32. DOPTER, C. 1905 Effets experimentaux de la toxine dysentérique sur le système nerveux central. Compt. rend. soc. biol., **58**, 400–402.
33. DUBOS, R. J. 1945 *The bacterial cell.* Harvard University Press, Cambridge, Mass.
34. DUBOS, R. J., HOBERMAN, H. D., AND PIERCE, C. 1942 Some factors affecting the toxicity of cultures of *Shigella dysenteriae*. Proc. Natl. Acad. Sci. U. S., **28**, 453–458.
35. DUBOS, R. J., AND GEIGER, J. W. 1946 Preparation and properties of Shiga toxin and toxoid. J. Exptl. Med., **84**, 143–156.
36. DUMAS, J., RAMON, G., AND BILAL, S. 1926 Anatoxine dysentérique. Ann. inst. Pasteur, **40**, 134–140.
37. EATON, M. D. 1938 Recent chemical investigations of bacterial toxins. Bact. Revs., **2**, 3–45.
38. EGAMI, F., NISHIMIYA, K., HOYSOYA, S., AND YAGI, Y. 1947 Chemical studies on the exotoxin of *Shigella dysenteriae*. Chem. Abst., **41**, 3504. (Published in J. Chem. Soc. Japan, **64**, 309–311, 654–658, 1943; **65**, 564–568, 1944.)
39. ENGLEY, F. B., JR. 1949 Some characteristics of the neurotoxin of *Shigella dysenteriae* (Shiga). Bact. Proc., 97.
40. ENGLEY, F. B., JR. (To be published.)
41. FARRELL, L., AND FERGUSON, H. 1943 Shiga toxoid. Can. J. Pub. Health, **34**, 130–139.

42. FARRELL, L., FRASER, D. T., AND FERGUSON, H. 1944 Trial of dysentery toxoid (Shiga) in human volunteers. Can. J. Pub. Health, **35**, 311–316.
43. FELSEN, J. 1945 *Bacillary dysentery, colitis and enteritis*. W. B. Saunders Co., Philadelphia, Pa.
44. FLEXNER, S. 1900 On the etiology of tropical dysentery. Bull. Johns Hopkins Hosp., **11**, 231–242.
45. FLEXNER, S., AND SWEET, J. E. 1906 The pathogenesis of experimental colitis and the relation of colitis in animals and in man. J. Exptl. Med., **8**, 514–535.
46. GATÉ, J., AND PAPACOSTAS, G. 1921 Antagonisme biologique entre le bacille de Loeffler et le pneumobacille de Friedländer. Compt. rend. soc. biol., **85**, 859–861.
47. GLASSMAN, H. N. 1948 Surface active agents and their application in bacteriology. Bact. Revs., **12**, 105–148.
48. GRIGORJEFF, A. W. 1892 Zur Frage der Mikroorganism bei Dysenterie. Centr. Bakt. Parasitenk., Ref. **12**, 876–879.
49. HAAS, R. 1937 Über Endo- und Exotoxine von Shiga-bazillen. Z. Immunitätsforsch., **91**, 254–272.
50. HAAS, R. 1938 Über Exo- und Endotoxine der Dysenteriebazillen. II. Weitere Studien über Endotoxine von Shiga- und Flexner-Ruhrbazillen. Z. Immunitätsforsch., **92**, 355–381.
51. HAAS, R. 1938 Über Endo- und Exotoxine von Ruhrbazillen. III. Vorkommen und Eigenschaften eines Endotoxins der E-Ruhrbazillen (Kruse-Sonne). Z. Immunitätsforsch., **94**, 239–246.
52. HAAS, R. 1938 Über Exo- und Endotoxine von Dysenteriebazillen. IV. Beitrag zur Kenntnis der Endotoxine der Ruhrbazillen. Z. Immunitätsforsch., **94**, 480–488.
53. HAAS, R. 1940 Vergleichende Untersuchungen über die toxischen und antigenen Eigenschaften von bei tiefer Temperatur gewonnenen löslichen Bakterienbestandteilen der Typhus-Paratyphus-Enteritis und Ruhr-Gruppe. Zugleich 5. Mitteilung der Untersuchungsreihe über Endo- und Exotoxine von Dysenteriebazillen. Z. Immunitätsforsch., **97**, 317–329.
54. HAAS, R. 1940 Untersuchungen zur Kenntnis des Exotoxins und Formoltoxoids von *Bact. dysenteriae* Kruse-Shiga. 6. Mitteilung über Endo- und Exotoxins von Dysenteriebakterien. Z. Immunitätsforsch., **99**, 121–141.
55. HORIMI, K. 1913 Ueber die pathogenen Wirkungen der Dysenterietoxine. Centr. Bakt. Parasitenk, Abt. I., Orig., **68**, 342–358.
56. ISTRATI, G. 1938 Action neurotrope et entérotrope de la toxine protéique thermolabile du bacille de Shiga. Action enterotrope de l'antigène glucido-lipidique du bacille de Shiga "S". Compt. rend. soc. biol., **129**, 1010–1013.
57. KOCH, P. K., AND OLITZKI, L. 1946 The action of dysentery toxins on different laboratory animals. Exptl. Med. and Surg., **4**, 54–68.
58. KRAUS, R., AND DÖRR, R. 1905 Ueber experimentelle Therapie der Dysenterie. Wien. Klin. Wochschr., **18**, 1077–1079.
59. KRUSE, W. 1900 Ueber die Ruhr als Volkskrankheit und ihren Erreger. Deut. Med. Wochschr., **26**, 637–639.
60. KURAUCHI (See ANDO, K.)
61. LARSON, W. P., AND NELSON, E. 1924 The effect of the surface tension of the medium upon bacterial toxins. Proc. Soc. Exptl. Biol. Med., **21**, 278.
62. LARSON, W. P., AND HALVORSON, H. O. 1925 The effect of concentration upon the neutralization of toxin by sodium ricinoleate. Proc. Soc. Exptl. Biol. Med., **22**, 550–552.
63. LEVADITI, C., AND VAISMAN, A. 1939 Chimiotherapie antiendotoxique. Compt. rend. soc. biol., **131**, 33–35.
64. LEVADITI, C. 1941 Action antitoxique de certains derivés aromatiques sulfures sur les endotoxines du *B. coli* et du bacille typhique. Compt. rend. soc. biol., **135**, 619–622.

65. MACFARLANE, M. G., AND KNIGHT, B. C. J. G. 1941 The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J., **35**, 884–902.
66. MCCARTNEY, J. E., AND OLITSKY, P. K. 1923 Separation of the toxins of *Bacillus dysenteriae* Shiga. J. Exptl. Med., **37**, 767–779.
67. MEYER, K., AND MORGAN, W. T. J. 1935 The relationship between the heterophile hapten and the specific polysaccharide hapten of the "smooth" form of *Bact. shigae*. Brit. J. Exptl. Path., **16**, 476–488.
68. MOORE, F. J., AND MARMORSTON, J. 1946 Relative activity of sulfonamides against dysenteric bacilli and their toxic filtrates. Am. J. Path., **22**, 655.
69. MORGAN, W. T. J. 1936 Studies in immunochemistry; preparation and properties of specific polysaccharide from *B. dysenteriae* (Shiga). Biochem. J., **30**, 909–925.
70. MORGAN, W. T. J. 1937 Studies in immunochemistry. II. The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). Biochem. J., **31**, 2003–2021.
71. MORGAN, W. T. J., AND PARTRIDGE, S. M. 1939 Bacterial antigens. Nature, **143**, 1025–1026.
72. MORGAN, W. T. J., AND PARTRIDGE, S. M. 1940 Studies in immunochemistry. 4. The fractionation and nature of antigenic material isolated from *Bact. dysenteriae* (Shiga). Biochem. J., **34**, 169–191.
73. MORGAN, W. T. J., AND PARTRIDGE, S. M. 1941 Studies in immunochemistry. 6. The use of phenol and of alkali in the degradation of antigenic material isolated from *Bact. dysenteriae* (Shiga). Biochem. J., **35**, 1140–1163.
74. MORGAN, W. T. J., AND SCHUTZE, H. 1943 Prophylactic inoculation with the O antigen of *Bact. shigae*. Lancet,**ii**, 284.
75. MUELLER, J. H., AND MILLER, P. A. 1940 Tetanus toxin production on a simplified medium. Proc. Soc. Exptl. Biol. Med., **43**, 389–390.
76. MUELLER, J. H. 1941 The influence of iron on the production of diphtheria toxin. J. Immunol., **42**, 343–351.
77. MURTHUM, J., AND GLENK, M. 1944 Die Sulfonamidwirkung auf Bakterientoxine im Tierversuch. Klin. Wochschr., **23**, 316–318.
78. NETER, E. 1942 The effects of anti-microbial substances of biological origin upon bacterial toxins. Science, **96**, 209–210.
79. NETER, E. 1945 Effects of penicillin, clavacin and streptomycin upon tetanus toxin. J. Infectious Diseases, **76**, 20–23.
80. OKELL, C. C., AND BLAKE, A. V. 1930 Dysentery toxin (Shiga): notes on its preparation with a discussion of its position as an endotoxin. J. Path. Bact., **33**, 57–63.
81. OLITSKY, P. K., AND KLIGER, I. J. 1920 Toxins and antitoxins of *Bacillus dysenteriae* Shiga. J. Exptl. Med., **31**, 19–33.
82. OLITZKI, L., AND LEIBOWITZ, J. 1935 Toxicity and antigenic properties of different fractions of *B. dysenteriae* (Shiga). Brit. J. Exptl. Path., **16**, 523–531.
83. OLITZKI, L., BENDERESKY, J., AND KOCH, P. K. 1943 Studies on the toxins of *Shigella dysenteriae* (Shiga). J. Immunol., **46**, 71–82.
84. OLITZKI, L., AND KOCH, P. K. 1943 Production of potent toxins by *Shigella dysenteriae* (Shiga) in a synthetic medium. Nature, **151**, 334.
85. OLITZKI, L., AND BICHOWSKY, L. 1946 The preparation of a potent toxin of *Shigella dysenteriae* (Shiga) on a semi-synthetic medium and its use for the preparation of an alum-precipitated toxoid. J. Immunol., **52**, 293–300.
86. PAPPENHEIMER, A. M., JR., AND JOHNSON, S. J. 1936 Studies in diphtheria toxin production. I. The effect of iron and copper. Brit. J. Exptl. Path., **17**, 335–341.
87. PAPPENHEIMER, A. M., JR., LUNDGREN, H. P., AND WILLIAMS, J. W. 1940 Studies on the molecular weight of diphtheria toxin, antitoxin and their reaction products. J. Exptl. Med., **71**, 247–262.
88. PAPPENHEIMER, A. M., JR. 1948 Proteins of pathogenic bacteria. Advances in Protein Chem., **4**, 123–153.

89. PETERMANN, M. L., AND PAPPENHEIMER, A. M., JR. 1941 The ultra-centrifugal analysis of diphtheria proteins. J. Phys. Chem., **45**, 1–9.
90. PETHERICK, M. H., AND SINGER, E. 1945 Studies on the detoxication of toxins: catalytic breakdown of crystalline ovalbumin. Australian J. Exptl. Biol. Med. Sci., **23** 171–176.
91. PFEIFFER, R., AND LUBINSKI, H. 1927 Ueber die Wirkung des Formalins auf Endotoxin. Centr. Bakt. Parasitenk., Abt. I, Orig, **102**, 459–470.
92. PFEIFFER, R., AND UNGERMANN, E. 1909 Zur Antitoxinfrage bei der Dysenterie. Centr. Bakt. Parasitenk., Abt. I, Orig., **50**, 534–541.
93. PILLEMER, L. 1946 The immunochemistry of toxins and toxoids. I. The solubility and precipitation of tetanal toxin and toxoid in methanol-water mixtures under controlled conditions of pH, ionic strength and temperature. J. Immunol., **53**, 237–250.
94. PILLEMER, L., AND ROBBINS, K. C. 1949 Chemistry of toxins. Ann. Rev. Microbiol., **3**, 265–288.
95. PUTNAM, F. W., LAMANNA, C., AND SHARP, D. G. 1946 Molecular weight and homogeneity of crystalline botulinus A toxin. J. Biol. Chem., **165**, 735–736.
96. RIBEIRO, F., AND GUIMARAES, L. M. 1942 Estudo sobre a acao da clorofila. Rev. faculdade med. Vet., Univ. São Paulo, **2**, 3–11.
97. SCHMIDT, S. 1932 Studien über den Einfluss verschiedener aliphatischer und aromatischer Verbindungen auf das Diphtherietoxin. Biochem. Z., **256**, 158–189.
98. SHIGA, K. 1898 Ueber den Erreger der Dysenterie in Japan. Centr. Bakt. Parasitenk., Abt. I, **23**, 599–600.
99. SHIGA, K. 1898 Ueber die Dysenteriebacillus (*Bacillus dysenteriae*). Centr. Bakt. Parasitenk., Abt. I, **24**, 817–828.
100. SINGER, E. 1945 Natural resistance against diphtheria toxin. Australian J. Exptl. Biol. Med. Sci., **23**, 165–169.
101. SOUTO, A. B., AND LIMA, C. 1939 Acao da vitamina C (acido l-ascorbico) sobre das toxinas da gangrena gazosa. Mem. inst. Butantan, (São Paulo), **12**, 265–296.
102. STEABBEN, D. 1943 A study on bacteriological lines of the antigens derived from *Bact. dysenteriae* and of their antisera in protective tests against the living organisms. J. Hyg., **43**, 83–95.
103. TAKITA, J. 1939 Production of a strong toxin of the Shiga dysentery bacillus by bouillon cultures. Kitasato Arch. Exptl. Med., **16**, 174–181.
104. TAL, C. 1950 Differences in toxicity of the S- and R- variants of *Shigella dysenteriae*. J. Immunol., **65**, 221–227.
105. TODD, C. 1904 On a dysentery toxin and antitoxin. J. Hyg., **4**, 480–494.
106. TOPLEY, W. W. C., AND WILSON, G. S. 1946 *Principles of bacteriology and immunity*. 3rd edition. The Williams and Wilkins Co., Baltimore, Md.
107. TREFFERS, H. P. 1946 The detoxification by acetylation of soluble antigens from *Shigella dysenteriae* (Shiga) and *E. typhosa*. Science, **103**, 387–389.
108. TREFFERS, H. P. 1946 The detoxification of dysentery and typhoid-soluble antigens by chemical means. J. Bact., **51**, 642.
109. VAILLARD, L., AND DOPTER, C. 1903 La dysenterie épidémique. Ann. inst. Pasteur, **17**, 463–491.
110. VELLUZ, L. 1939 Importance des phénomènes d'oxydation dans l'inactivation des toxines, *in vitro*. Compt. rend. soc. biol., **131**, 602–603.
111. VINCENT, H. 1926 Sur les propriétés générales des cryptotoxines, en particulier de la cryptotoxine tétanique. Compt. rend., **182**, 1307–1310.
112. WAALER, E. 1936 Studies on the toxin production of the Shiga bacilli. J. Exptl. Med., **63**, 1–15.
113. WAGNER-JAUREGG, T. 1940 Die Endotoxine der Bakterien. Angew. Chem., **53**, 319–321.

114. WEIL, A. J. 1943 Progress in the study of bacillary dysentery. J. Immunol., **46**, 13–46.
115. WEIL, A. J. 1947 Medical and epidemiological aspects of enteric infection. Ann. Rev. Microbiol., **1**, 309–332.
116. WEIL, A. J. 1947 Dysentery—a progress report for the years 1942–1946. J. Immunol., **55**, 363–405.
117. WILDFÜHR, G. 1947 Über die Toxinbildung der Tetanusbazillen unter der Einwirkung von Vitamin H (p-aminobenzoesäure). Zentr. Bakt. Parasitenk., Abt. I, Orig., **152**, 96–98.
118. ZAHL, P. A., HUTNER, S. H., AND COOPER, F. S. 1944 Effect of sulfonamides on toxic and antigenic actions of endotoxins of certain gram-negative bacteria. Proc. Soc. Exptl. Biol. Med., **55**, 4–7.