THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

1. Production, Purification and Properties of the Toxin.

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The literature of the neurotoxin of *Shigella shigae* has recently been reviewed by Engley (1952), and it remains only to summarise those observations that are immediately relevant to this paper. Rough strains of *Sh. shigae* contain a neurotoxic protein which is often referred to as a "true" toxin or an "exotoxin," because it may be liberated in the medium on autolysis of the organism and because it is neutralised by antitoxin in stoicheiometric proportions. It is particularly toxic to rabbits and produces paralysis. Smooth strains of the organism contain both this neurotoxin and a considerably less potent polymolecular enterotoxic complex of phospholipid, polysaccharide and protein which is identical with the dominant O somatic antigen. The existence of two toxins has been questioned from time to time but there remains little doubt that they both exist.

Most of the literature dealing with the neurotoxin refers to crude preparations and few attempts have been made to purify it. Anderson, Brown and Macsween (1945) obtained preparations containing 44–87 Lf/mg. and about 2 LD_{50}/mg. per kg. mouse. Dubos and Geiger (1946), who showed that the presence of iron in the culture medium inhibited the production of toxin, obtained a preparation containing 20 LD_{50}/mg. per kg. mouse and 1500–3000 LD_{50}/mg. per kg. rabbit. These preparations, however, could not have contained more than 1–4 per cent of the toxin, since this paper describes a preparation containing 750 LD_{50}/mg. per kg. mouse, 1,150,000 LD_{50}/mg. per kg. rabbit, and 5,500 Lf/mg. Engley (1952) mentions, but does not describe, preparations containing about 200 LD_{50}/mg. per kg. mouse and 30,000 LD_{50}/mg. per kg. rabbit. Shiga neurotoxin is therefore one of the most toxic substances known, being as toxic as tetanus and botulinus neurotoxins. On the other hand, it occurs in extremely low concentration in cultures of the organism, and consequently its purification presented some difficult problems.

METHODS.

The Organism.

The work was carried out with the rough strain of *Sh. shigae* used by Dubos and Geiger (1946) and kindly supplied by Dr. R. J. Dubos. It gave large flat rough colonies on the surface of nutrient agar and formed almost completely stable suspensions in 0·5 per cent saline. It was partially agglutinated by normal rabbit serum at a dilution of 1/50, but with specific O antiserum (titre 1/1600) at dilutions of 1/100–1/1600 the agglutination observed was no more than that in saline controls. Absorption of this serum at 1/100 with 10^{10} organisms/ml. failed to reduce the titre for the homologous smooth organism, showing that O antigen was absent.

At every harvesting a check was kept on the purity of the culture by microscopic examination and plating. On some occasions it was tested by fermentation reactions which were typical for *Sh. shigae*.

In order to obtain as uniform an inoculum as possible throughout the work, the growth from an overnight culture on the surface of nutrient agar was lyophilised by Stamp's (1947) method in a series of tubes. For use, one tube was opened and subcultivated on casein hydrolysate yeast agar (Gladstone and Fildes, 1940), allowed to grow overnight and then kept in the refrigerator. From this master culture, which was renewed every 7–10 days, a fresh 18-hour subculture was made for each experiment.

The Medium.

It was found that the CCY broth of Gladstone and Fildes (1940) supported vigorous growth of the organism, and produced high yields of toxin when iron was removed by adsorption on calcium phosphate in the manner described in another paper (van Heyningen and Gladstone, 1953). This resulted in a medium containing about 0·05 μg. Fe/ml. and an insufficient level of magnesium to support optimal growth: 20 μg. Mg/ml. and 0·1 μg. Fe/ml. were then added to the medium. The level of 0·15 μg. Fe/ml. is that at which the greatest yield of toxin per unit volume of culture is obtained (about 14 Lf/ml.); below this level a greater yield of toxin per organism is obtained but growth is less.

Cultures.

For cultures on a laboratory scale (1–3 l.) a vertical tubular vessel was used made up mainly of sections of standard "Pyrex" industrial glass pipe line of 2″ (5 cm.) internal diameter (J. A. Jobling & Co., Sunderland). These sections are united by means of standard watertight flanged joints and can be built up into any length, limited only by the size of autoclave necessary to sterilise the apparatus and by ease of handling. The maximum length used was 6 ft. To the lower end of the pipeline a funnel made of a short length of 2″ pipe is joined with the standard flanged joint. Fused across the funnel is a No. 3 sintered glass filter disc, and below this disc the pipe tapers rapidly down to a 0·5 cm. tube attached to an air line. About 0·5 cm. above the disc a short side tube of 0·5 cm. diameter is attached for draining the culture. With the pipeline held vertically and filled to three-fourths of its length with medium, the culture can be very effectively aerated by forcing air through the filter disc. The upper end of the vessel consists of a short section of modified pipeline attached by a flanged joint, and terminating in a dome-shaped glass seal into which are fused 3 glass tubes 0·5 cm. diameter. One of these is attached by rubber tubing to an aspirator containing sterile medium, another to a small funnel for inoculating and for additional constituents of the medium, and the third is an air exit which leads into a trap containing 1 per cent $HgCl_2$ + 0·5 per cent tributyl citrate. The air effluent is finally filtered through a tube 20 cm. long × 2·5 cm. diameter, packed with cotton-wool. The air supply is from a cylinder, and the rate of flow is maintained at 100 ml./min. at 20 cm. Hg pressure. It is sterilised by passage through cotton-wool packed in a tube similar in size to that used for the effluent air, and enters the apparatus at the lower end of the sintered glass funnel through a vertical glass tube at least as tall as the apparatus. Any leakage of culture back through the sintered disc is thus prevented from wetting the cotton-wool filter.

The side arm in the funnel just above the sintered disc is attached to a stop cock through which samples can be withdrawn and the culture harvested.

After smearing the inner wall over the upper quarter with "Silicone" grease to prevent frothing, the empty apparatus is detached from the medium reservoir and effluent trap and sterilised by autoclaving with all flanged joints loosened. The medium, usually 10 l., is autoclaved in the aspirator at 120° for 30 min. The joints in the apparatus are tightened and it is set up in a hot room at 37°. The aspirator is then united with the apparatus with aseptic precautions and a known volume of medium, usually 2 l., as determined by a mark on the outside of the culture vessel, is run in. The culture is inoculated and any further addition to the medium made through the inoculating funnel. After growth the culture is reaped, leaving 50–100 ml., which allows for 2–5 per cent reinoculation when fresh medium is run in. In this way two reapings of 2 l. can be made in 24 hr., and 5 runs made without dismantling. More runs may be made if a fresh reservoir of medium is attached.

Production on a much larger scale was undertaken by Mr. Macsween at the Microbiological Research Department of the Ministry of Supply Station at Porton. The method,

details of which have not yet been published, consisted essentially of the use of a continuous flow of agitated and aerated broth culture which allowed organisms only 1 hr. old to be reaped. Over a course of 7 weeks nearly 2 kg. (dry wt.) of organisms were produced.

Bacterial growth was measured in the way described by van Heyningen and Gladstone (1953).

Assay of Toxin.

The purification of a bacterial toxin is almost impossible unless a rapid and reasonably accurate method of assay is available for testing the numerous fractions prepared. For this reason the Lf was generally determined in routine assays of toxin, since this value could be obtained *in vitro* within 30 min. or less and with greater accuracy than is possible in assays *in vivo*. From time to time the L+ and the LD_{50} were also determined. The Lf was generally equivalent to about 0·7 L+, and about 7 mouse LD_{50} and about 70 rabbit LD_{50}. All dilutions of toxin were made in 1 per cent NaCl containing 0·1 per cent gelatin.

Antitoxin.

The antitoxin used throughout was Anti-Dysentery Serum kindly supplied by Dr. C. L. Oakley of the Wellcome Research Laboratories, Beckenham. It was labelled as containing 50,000 units per 4·5 ml. ampoule and was assumed to contain 11,111 units/ml.

Lf determinations.

For routine Lf determinations seven to ten 0·5 ml. volumes of an appropriate dilution of toxin are pipetted into test-tubes (0·7 cm. internal diameter × 7·5 cm. long), and to these are added falling volumes of a fairly concentrated antitoxin solution with an "Agla" microsyringe. The largest volume of antitoxin is 0·1 ml., the spacing of antitoxin concentration between successive tubes is 10 per cent, and the amount of antitoxin in the middle tube is about 30 units. After mixing the tubes are placed in a water bath at 45° and observed. The water bath is fitted with a glass window in front, and the tubes are illuminated from below by means of a Perspex prism placed in the bath in apposition to a horizontal strip light in front of the window. A black background is placed behind the tubes. The order of flocculation in the tubes can readily be distinguished.

Crude extracts of *Sh. shigae* contain many antigens, and since the antiserum was prepared against crude toxic extracts numerous zones of flocculation may be observed in toxin-antitoxin mixtures. When several 0·5 ml. volumes of a crude preparation of toxin extracted

TABLE I.—*Flocculation in Mixtures of Antitoxin and Crude and Purified Toxin.*

Units antitoxin per ml. mixture.	Flocculation time (min.). Crude pH 11 extract. 17 L+/ml. 25 Lf/ml.		Purified toxin solution. 150 L+/ml. 200Lf/ml.	
1600	—		∞	
800	∞	Antigen I (4000)	∞	
400	10		∞	
200	80		82	Toxin (1200)
100	50	Antigen II (5000)	12	
50	120		45	
25	+		∞	
12·5	70	Toxin (875)	∞	
6·25	120		—	
3·13	+		—	

— = Not observed.
\+ = Flocculation overnight.
∞ = No flocculation overnight.

These results show the presence of three flocculating antigens in crude toxin preparations and a single antigen in a purified preparation. Each tube contains 0·5 ml. toxin solution and 0·5 ml. antitoxin solution in falling concentrations. The figures in brackets are the products: units antitoxin per ml. × flocculation time.

from the organisms at pH 11 were mixed with equal volumes of falling 2-fold dilutions of antitoxin and incubated at 45° the tubes containing 400, 100 and 12·5 units of antitoxin per ml. flocculated before any of the others (Table I, col. 2). Since each of these three mixtures was adjacent to mixtures that took longer to flocculate, it was clear that the toxin solution contained at least three flocculating antigens. Assuming that one of these was the toxin, it appeared that the solution contained either *c.* 800, 200 or 25 Lf/ml. Since the solution was found to contain 17 L+/ml. it followed that the true Lf value was more likely to be 25 Lf/ml. than 200 or 800 Lf/ml. When the non-toxic antigens were removed by purification only one flocculation zone appeared (Table I, col. 3). When the toxin was extracted at a higher pH two further non-toxic antigens appeared, as is evident from Table II.

TABLE II.—*Flocculation in Mixtures of Antitoxin and Toxin Extracted at pH* 11 *and* 13.

Units antitoxin per ml. mixture.	Flocculation time (min.) pH 11 extract. 240 L+/ml. 320 Lf/ml.		Flocculation time (min.) pH 13 extract. 200 L+/ml. 256 Lf/ml.	
200	12	Toxin (1020)	∞	
160	7		12	Toxin (896)
128	8		7	
102	12		6	
82	∞		6	Antigen III ? (396)
66	∞		6	
52	∞		5	
42	∞		5	Antigen IV ? (190)
38	∞		5	
26	∞		5	

∞ = No flocculation after 60 min.

Shows appearance of two further non-toxic antigens in pH 13 extract. Each tube contains 0·5 ml. toxin solution and 0·5 ml. antitoxin solution both in falling concentrations.

In this experiment the antitoxin dilutions were closer, *viz.*, 1·25-fold instead of 2-fold, and consequently the different zones are less clearly defined.

In order to distinguish the toxin flocculation zone from the non-toxin zones Anderson *et al.* (1945) did a corresponding L+determination for each Lf determination. This laborious and time-consuming procedure nullifies the advantage of the comparatively accurate and quick *in vitro* Lf determination. It soon became clear to us, however, that the " false " zones were so far removed from the " true " zone, in pH 11 extracts by factors of 8- and 32-fold, that in practice there was little danger of confusion. With a little experience and with a knowledge of the likely range of toxin concentration to be encountered the true toxin zone could be readily distinguished. In practice the false zones were never encountered after the preliminary work reported in Tables I and II because the toxin concentration in a given solution was never over-estimated by a factor as great as 8-fold, and consequently the antiserum concentration used in flocculation tests was never high enough to embrace the false zones. Zones III and IV (Table II) were also never again encountered because there was no virtue in extracting the toxin at pH 13.

A further guide to the true zone lies in the product, units antitoxin per ml. toxin-antitoxin mixture × flocculation time (min.). These values are given in brackets in Tables I and II. For the toxin zone it was always near 1000, whereas it was considerably higher or lower for the false zones.

The validity of the Lf was from time to time checked by a determination of the Lf:L+ ratio and this was never found to be below 0·6; generally it was between 0·65 and 0·75. In any event, the problem of the false zones became academic as soon as the non-toxic antigens were removed in the purification of the toxin.

LD_{50} determinations.

The LD_{50} is always predicted from the Lf, and serial 2-fold dilutions of toxin solution, equally spaced about the predicted value, are injected into the animals. With mice (20 g.)

0·5 ml. volumes of 7 dilutions are used and each dilution is injected intraperitoneally into 8 animals. With rabbits (*c.* 2·5 kg.) 0·2 ml. volumes of 5 dilutions are used and each dilution is injected into the ear veins of 4 animals.

The animals are observed for seven days, and those that survive this period are assumed to have survived the injection. The value of the LD_{50} is calculated by the method of Reed and Muench (1938).

L+ determinations.

The L+ is also predicted from the Lf and is determined at the "5 unit level." Seven mixtures, each containing 10 units antitoxin/ml. and amounts of toxin falling in a ratio of 1·25, are made up and 0·5 ml. volumes of each mixture are injected intraperitoneally into 8 mice. These animals are also observed for 7 days. The value of the L+ is also calculated by the method of Reed and Muench (1938).

Electrophoresis.

Zone electrophoresis on filter-paper (barbital buffer, pH 8·6, $\mu = 0{\cdot}1$) was carried out by different methods according to the objects in mind. For the determination of electrophoretic homogeneity the filter-paper was horizontally suspended in a chamber in a method generally similar to that of Plückthun and Götting (1951). After staining with bromphenol-blue the paper strips were scanned at 2·5 mm. intervals through a 3 mm. slit in a photo-electric device (van Heyningen, 1952, unpublished).

This method of electrophoresis gives good separation of components, and on scanning of the filter-paper strips neat diagrams can be obtained. These are useful, provided the limitations of the technique are understood. The most important of these is the fact that rate of migration of bands is not uniform owing to evaporation of water from the strip and consequent alterations in the field strength. For the determination of electrophoretic mobilities the method of Kunkel and Tiselius (1951) was used. In this technique the strip of filter-paper is clamped between sheets of glass. Evaporation and overheating are thus avoided and the rate of migration of protein spots on the paper is uniform. For incompletely explained reasons large spots or bands of protein become distorted as they migrate, and therefore lead to a broadening of the areas they occupy. For the determination of electrophoretic mobility, however, only small spots of protein solution need be placed on the paper, and these do not present difficulties.

Other Estimations.

Iron was determined in the usual way with α-α′-dipyridyl, after adsorption on calcium phosphate and nitrogen by the micro-Kjeldahl technique. Values of pH were determined electrometrically with a glass electrode. When the pH of a solution was adjusted to a value greater than 9·5, KOH was used in order to minimise deterioration of the electrode, and indeed very little deterioration was observed. High values of pH quoted in this paper, *e.g.*, pH 11, 13, are the readings obtained on the pH meter; they proved to be reproducible, but they may not represent the actual pH values. Protein content of toxin solutions was estimated by a spectrophotometric method recommended by Kalckar (1947). The optical densities of solutions at 260 mμ. and 280 mμ. were determined in a Beckmann Model DU photo-electric absorption spectrophotometer, and protein content was derived from the expression mg. protein/ml. $= 1{\cdot}45\ D_{280} - 0{\cdot}74\ D_{260}$. The dry weights of protein solutions were from time to time determined directly by evaporating 2 ml. samples on watch glasses on a water bath for 1 hr. and standing overnight in a P_2O_5 desiccator before weighing. These values always agreed within 10 per cent or less with the spectrophotometric determinations, but refractometric estimations on a solution of purified toxin showed that the dried specimens apparently retained about 25 per cent water (Baldwin, 1953). The protein concentration quoted in this paper for the purified toxin is the value obtained by the refractometric method, but other values are those determined spectrophotometrically.

EXPERIMENTAL.

Extraction of Toxin.

The neurotoxin of *Sh. shigae* is usually obtained in a soluble form by allowing the organisms to autolyse, but in our experience the yield obtained in this way

was very variable. We therefore resolved to use a method that was more readily controlled. Dubos and Geiger (1946) precipitated chilled cultures at pH 4·2, and extracted the toxin from the washed precipitate at pH 7·5 with dilute sodium carbonate or with salt solution buffered with phosphate. We found that more efficient extraction of toxin could be obtained at pH 11.

Fig. 1 shows that the optimum pH for extraction is 11. At higher pH no more toxin is extracted, but more non-toxic antigens appear, as is evident from Table II. Fig. 2 shows that the optimum time for extraction is 2-3 hr. The

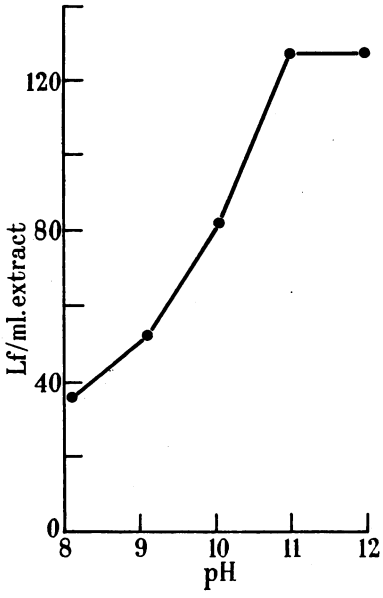

FIG. 1.—Effect of pH on extraction of the toxin.

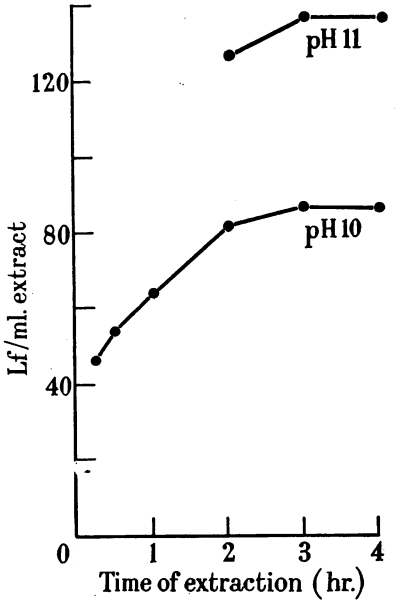

FIG. 2.—Effect of time of extraction on yield of the toxin.

yield of toxin is about 2 Lf/mg. organism, which is 50–120 per cent efficient as judged by a comparison of the LD_{50} content of the organisms and the extracts. However, when much greater volumes of extracting solutions are used 6–7 Lf may be extracted per mg. organism (*see* van Heyningen and Gladstone, 1953). The toxin does not appear to be adversely affected by the comparatively high pH of extraction, because the ratios of Lf to L+ and mouse LD_{50} (about 0·7 and 7 respectively) are no smaller than those in extracts obtained by autolysis or grinding at neutral pH (see below).

The routine method for extracting the toxin is as follows: the cultures are centrifuged down, washed with water and suspended in phosphate (potassium salts only) buffer pH 6 to a concentration of 50–60 mg. dry wt. organisms/ml. The organisms are killed by heating at 56–59° for 30 min. and the suspension is then adjusted to pH 11 with N KOH. After standing at room temperature for 3 hr. the suspensions are neutralised and spun down. The residue is re-suspended in half the original volume and re-extracted and the turbid greenish-yellow extracts are combined.

The killing of the organisms by heating at 56–59° for 30 min. is an essential stage in the extraction because no detectable toxin is extracted when this stage is omitted. We have confirmed an observation by Boivin, Delaunay and Sarciron (1940) that about 30 per cent of the toxin passes from the cells into the suspending medium on heat-killing. This process may be related to the extractability of the toxin, but neither of the phenomena is understood. Microscopic examination of smears of the heated and extracted organisms did not show any obvious evidence of destruction.

The toxin can also be extracted by grinding the cells. Packed washed cells were mixed with an equal volume of fine aluminium oxide powder (200 mesh) and ground for 3 min. by hand in a mortar. The residue of organisms and aluminium hydroxide was then extracted with phosphate buffer pH 8·6 and 60–130 per cent (LD_{50} yield) of the toxin was recovered. Heat-killing of the organisms was not necessary for this extraction. The ground organisms also did not appear to have been disintegrated. However, the alkaline extraction method is a more convenient technique for routine use.

Purification.

The crude turbid yellow-green solution obtained by extracting the organisms at pH 11, when diluted and clarified by high speed centrifugation, absorbs strongly with a maximum at 260 mμ. and presumably contains nucleic acid. The extract is rendered colourless by dialysis overnight against running tap water and then clarified by adding 0·1 vol. 0·2 M Na_2HPO_4, 0·05 vol. M $CaCl_2$, adjusting the pH to 7·6 and centrifuging the calcium phosphate precipitate after standing for 30 min. The resulting clear colourless supernatant no longer absorbs strongly at 260 mμ. but has a typical protein absorption spectrum with a maximum at 277 mμ. The yield of toxin is 80–90 per cent, with an activity of about 50 Lf/mg. protein. A typical electrophoretic analysis is shown in Curve 1, Fig. 3. At this stage the preparation is freeze-dried and material is accumulated for further purification.

Attempts at fractionating this complex mixture of proteins by precipitation with methanol at controlled temperature, pH and ionic strength, or by salting out with ammonium sulphate or sodium phosphate were not encouraging. However, it was noticed that the toxin was precipitated when a solution of the crude material was dialysed against distilled water, and this property was used in further purification. A 5 per cent solution of the freeze-dried powder in 1 per cent NaCl was dialysed against several changes of distilled water at 4°. After 10–20 days 30–90 per cent of the toxin precipitated out, with an activity of about 600 Lf/mg. protein. Zone electrophoretic analysis (Curve 2, Fig. 3) showed that the preparation was still complex. The precipitate was dissolved in 1/10 the original volume of 1 per cent NaCl, leaving an appreciable insoluble residue, and dialysed again against distilled water. A copious precipitate started forming within 30 min. and after overnight dialysis the yield of toxin in the precipitate was 60–90 per cent. After two further precipitations by dialysis, each with a 60–90 per cent yield, a preparation containing 2500–3000 Lf/mg. protein was obtained. Curve 3, Fig. 3, shows that a dominant component now appeared on electrophoretic analysis. This zone (from 5 to 9 cm. along the paper strips) was cut out and eluted with 1 per cent saline. The eluate contained 80 per cent of the toxin and no toxin could be recovered from the rest of the strip. The

barbiturate in the eluate interfered with the spectrophotometric estimation of the protein content, but the area occupied by the toxin zone corresponded to 70 per cent of the total area under the electrophoretic curve. From this it followed that electrophoretically homogeneous toxin should contain about 4000 Lf/mg.

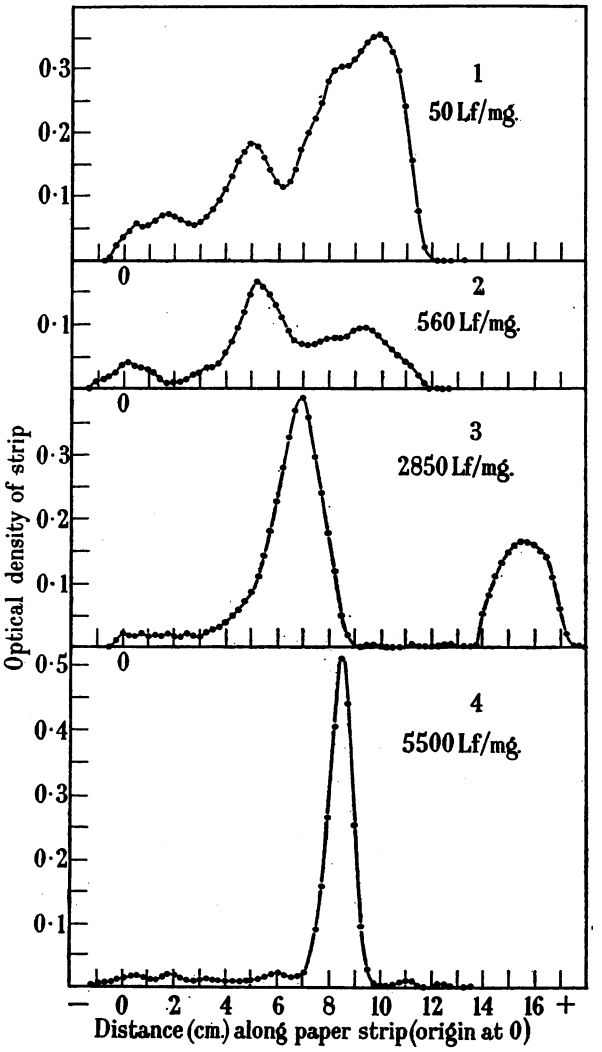

FIG. 3.—Electrophoresis (pH 8·6) of toxin preparations at different stages of purification.

protein, provided that all the proteins in the mixture adsorbed the same amount of dye.

Further precipitation by dialysis did not result in further purification, but the fast-moving component could be removed by adsorption on calcium phosphate precipitated in the manner described above. The yield at this stage was 60–90

per cent and the preparation contained 4150 Lf/mg. protein, when the protein content was determined spectrophotometrically, or 5500 Lf/mg. protein by the refractometric method. On zone electrophoresis at pH 5·6, 8·6 and 10·1 the toxin appeared to move as a single component (Fig. 4). These curves were obtained by eluting 1 cm. transverse sections of the paper strips and determining the protein content of the eluates. This technique, however, is not sensitive enough to detect very small amounts of protein.

The tail which can be seen in Curve 4, Fig. 3 was invisible to the naked eye on the paper strip and could be detected only with the photo-electric scanning

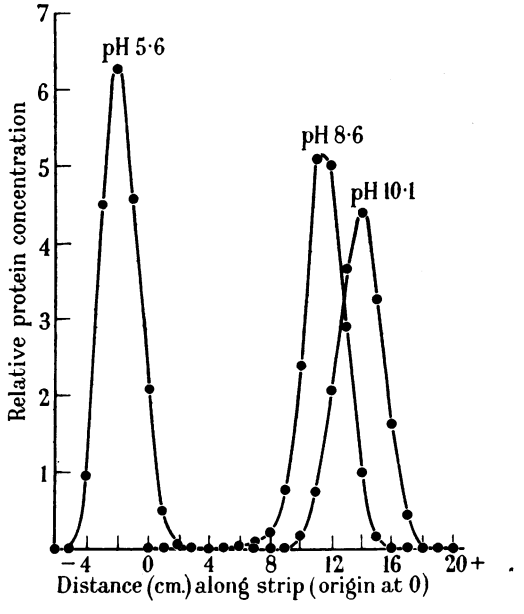

FIG. 4.—Electrophoresis of purified toxin at different pH values.

device. It may be due to retarding of the main component by adsorption on the paper, a common phenomenon in paper electrophoresis, or it may represent other components. The homogeneity of the preparation in sedimentation in the ultracentrifuge is discussed by Baldwin (1953). We were unable to crystallize the toxin.

The purification of the toxin requires at least 6 stages, *viz.*, extraction, clarification, 3 precipitations by dialysis and adsorption of impurities on calcium phosphate. At each of these stages a 90 per cent recovery of toxin was obtained at one time or another, but in any single purification from organisms to final product we never succeeded in getting six successive yields of this magnitude. On the average the stage yield was 70 per cent, and consequently the overall yield was about 12 per cent. The concentration of toxin in the organisms is about 1/1000; from 1000 l. of a culture grown on a de-ferrated medium and containing 2 mg. of organism/ml., it should be possible to recover about 100 mg. of purified toxin.

PROPERTIES.

Examination in the Ultracentrifuge.

The purified preparation was studied in the ultracentrifuge by Mr. R. L. Baldwin (1953). The sedimentation constant, diffusion coefficient, frictional coefficient and molecular weight as determined by him are given in Table V.

Nitrogen Content.

A Kjeldahl nitrogen estimation was made on a solution of the purified preparation, the protein content of which had been determined refractometrically (Baldwin, 1953). The preparation contained 15·7 per cent N.

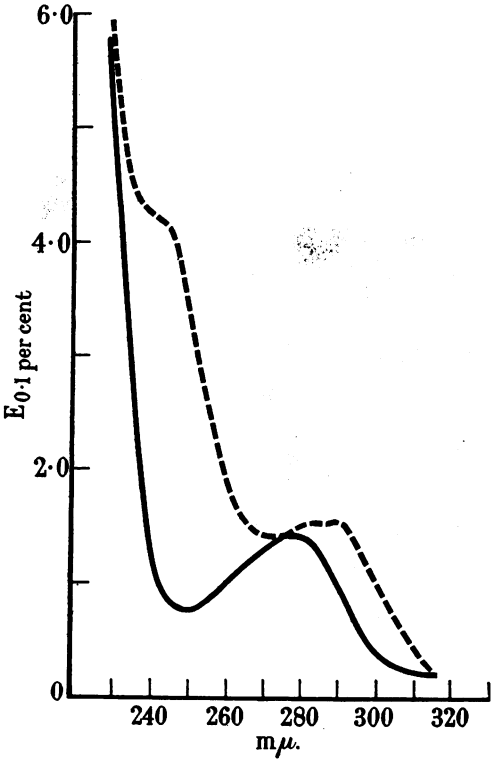

FIG. 5.—Absorption of ultra-violet light by purified toxin preparation. Continuous line, in saline pH 7; broken line, in 0·1 N NaOH.

Ultra-violet Absorption Spectra

The ultra-violet absorption spectra of the purified material dissolved in 1 per cent NaCl and in 0·1 N NaOH are shown in Fig 5. They are typical of proteins. From Holiday and Ogston's (1938) formulae, it was calculated that the tyrosine and tryptophan contents of the purified preparation are approximately 9·8 per cent and 2·5 per cent respectively.

Amino Acids.

Dr. G. G. F. Newton of this Department kindly carried out a qualitative chromatographic analysis on an acid hydrolysate of the preparation. A Pyrex glass tube containing 2 mg. with 1 ml. 6N HCl was sealed and heated at 110° for 18 hr. The HCl was then evaporated in a stream of air at 100°, and the residue taken up in water to give a solution of 300 μg. in 5 μl., of which 10 μl. were applied to Whatman No. 1 paper (18·5″ × 22·25″) and the chromatogram was first developed with butanol acetic acid (Woiwod, 1949) overnight. The

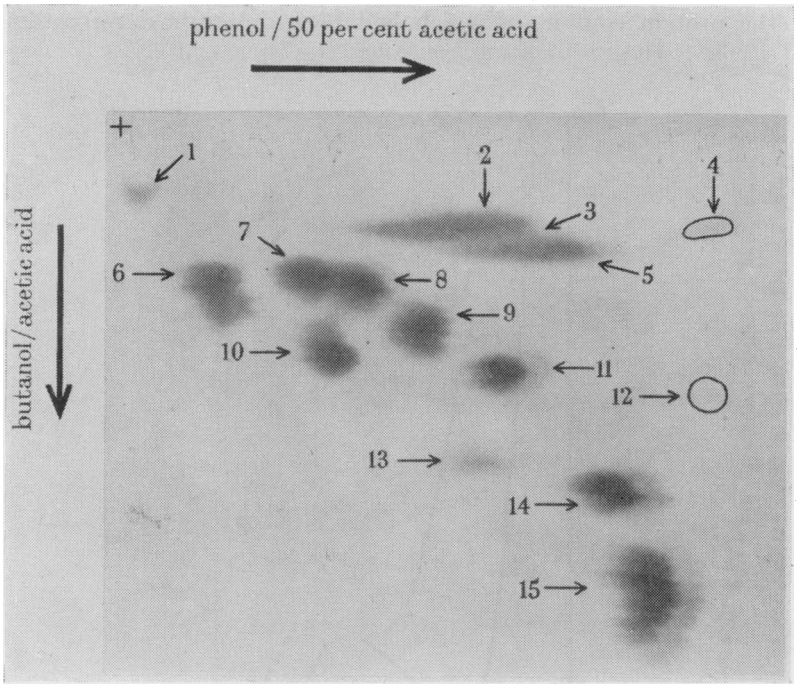

FIG. 6.—Paper chromatogram of acid hydrolysed purified toxin preparation. 1, cysteic acid (= cysteine); 2, lysine and/or ornithine; 3, histidine; 4, unidentified; 5, unidentified; 6, aspartic acid; 7, serine; 8, glycine; 9, threonine; 10, glutamic acid; 11, alanine; 12, proline and/or hydroxyproline; 13, tyrosine; 14, valine and/or methionine; 15, leucine and/or isoleucine and/or phenylalanine.

paper was then dried in a stream of air at 80° for 30 min. and developed in a second dimension with 80 per cent phenol in an atmosphere of 50 per cent acetic acid (Dent, 1948). The phenol was removed in a stream of air at 30–40° for 6–8 hr., the paper was sprayed with 0·2 per cent ninhydrin solution containing 0·2 per cent collidine (Woiwod, 1949), and the colour developed by heating at 105° for 5 min. The resulting chromatogram is shown in Fig. 6. In addition to the 15 or 16 amino acids seen in the chromatogram the ultra-violet absorption spectra suggest that the preparation contains about 2·5 per cent tryptophan which would be destroyed during the acid hydrolysis.

Solubility.

The toxin is insoluble in distilled water and soluble in aqueous salt solutions. This is evident from the method of purification described above. The solubility of the toxin in salt solution has a high temperature coefficient. If a 1 per cent solution of the crude toxin (after the first calcium phosphate clarification) is rapidly cooled to $-5°$ a precipitate is formed. If this precipitate is collected and re-suspended in 1 per cent NaCl a major portion of the toxin will dissolve, leaving an appreciable insoluble residue. The activity per unit dry weight of the resultant toxin solution may be increased by a factor of 10-fold, but the yield is poor.

Electrophoretic Mobility.

The electrophoretic mobility of the toxin was determined by the method of Kunkel and Tiselius (1951). Samples of the toxin and of human serum albumin were laid on a single strip of paper (Whatman No. 1) and allowed to move simultaneously in an electric field. The experiment was done at different pH values and corrections were made for the endosmotic effect by observing the movement of glucose spots. Since the mobility of albumin in free solution is known the corresponding values for the toxin could be calculated. These are shown in Table III. At pH 8·8 the toxin has about the same mobility as human serum β-globulin.

TABLE III.—*Electrophoretic Mobilities of Shiga Neurotoxin on Filter-paper and in Free Solution at Different pH Values.*

pH.	Buffer ($\mu = 0{\cdot}1$).	Electrophoretic mobility, $-u_3$° (cm.² sec.$^{-1}$. volt^{-1} × 10^5).	
		On Whatman No. 1 paper.	Calc. for free solution.
10	Barbital	3·30	3·76
8·8	,,	2·44	2·78
7·0	Phosphate	1·21	1·38
6·0	Phthalate	0·81	0·92
5·7	,,	0·58	0·66

Since the toxin was strongly adsorbed on the filter paper at pH values below 5·7 it was not possible to determine the iso-electric point by this method. The toxin appears to be least soluble at about pH 4.

Toxicity.

The purified preparation containing 5500 Lf/mg. was found to contain 750 LD_{50}/mg. per kg. mouse, 1,150,000 LD_{50}/mg. per kg. rabbit (see Table IV), and 4200 L+/mg. measured at the 5 unit level. Since the Lf : L+ and Lf : LD_{50} ratios of the purified material were not significantly different from those of crude extracts it was clear that no toxoiding had taken place during purification.

Like botulinus and tetanus neurotoxins Shiga neurotoxin is slow in its action. Rabbits and mice generally died on the 3rd and 4th day after injection of the toxin. Mice did not show any obvious signs before dying, but rabbits showed marked paresis. They became paralysed first in the forelimbs, then in the hind limbs, and finally became comatose. Several rabbits injected with sub-lethal doses of toxin showed almost complete paralysis on the 4th day after injection,

TABLE IV.—*Protocols for LD_{50} Determinations with Purified Shiga Neurotoxin* (5500 *Lf/mg.*, 4200 *L+/mg.*).

LD_{50} in mice (20 *g.*):

Dose (μg.)	0·25	0·125	0·0625	0·0313	0·0157	0·0078
Deaths.	8/8	7/8	8/8	6/8	0/8	0/8

LD_{50} (Reed and Muench, 1938) = 0·027 μg. per mouse
= 1·35 μg. per kg. mouse.

LD_{50} in rabbits (2·65 kg.):

Dose (μg.)	Deaths.	Remarks.
0·003	3/6	1 dead in 2 days, 2 dead in 3 days, 1 paralysed and recovered.
0·0015	2/4	2 dead in 3 days, 1 paralysed and recovered.
0·00075	0/4	1 paralysed and recovered.
0·00038	0/4	—

LD_{50} (Reed and Muench, 1938) = 0·0023 μg. per rabbit.
= 0·00087 μg. per kg. rabbit.

and would have died of thirst and hunger had they not been carefully nursed. They gradually recovered and after a further 8–10 days appeared to be quite normal.

Like botulinus and tetanus neurotoxins, but unlike most bacterial toxins, Shiga neurotoxin has no general histotoxic effect. A rabbit injected intracutaneously with nearly 400 LD_{50} showed no dermonecrosis and died 4 days after injection. Nor does it appear to be toxic by mouth; when 1000 intraperitoneal lethal doses were fed to mice no harmful results were observed. This has not yet been tested on rabbits.

Summary of Properties.

The properties of the purified preparation of Shiga neurotoxin are listed in Table V.

DISCUSSION.

The purified preparation of toxin appears to move essentially as a single zone on filter-paper electrophoresis, but thus far no protein which has been reported homogeneous in electrophoresis has in fact been shown to be so by the test of

TABLE V.—*Properties of Shiga Neurotoxin.*

Toxicity, per kg. mouse	750 LD_{50}/mg.
,, per kg. rabbit	1,150,000 LD_{50}/mg.
N content	15·7 per cent
Electrophoretic mobility, $-u_{8^\circ}$ (barbital buffer, pH 8·8, $\mu = 0·1$)	$2·78 \times 10^{-5}$ cm.^{2} sec.$^{-1}$. volt^{-1}
Average sedimentation coefficient, Sm_{20}, w.*	4·80 ± 0·05 S
Diffusion coefficient, D_{20}, w.*	$5·7 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ ± 5 per cent
Frictional coefficient, f/fo.*	1·26
Average molecular weight.*	82,000

* From Baldwin (1953).

boundary spreading (Alberty, Anderson and Williams, 1948). Although the material has been carefully separated and gives a single fairly symmetrical boundary in the ultracentrifuge (like the other purified toxins in Table VI) it has not been found by Baldwin (1953) to be homogeneous with respect to its sedimentation coefficient.

The physical heterogeneity of our preparation may mean that a homogeneous active material is still contaminated with other inactive material, or that a degree of heterogeneity has been produced in the course of its separation; it may be on the contrary, that the active material was physically heterogeneous in its original state. There is no good reason for supposing that the toxin must be homogeneous.

The toxicity of the purified material was surprisingly high. Table VI shows that it is considerably more toxic than diphtheria toxin and as toxic as the two

TABLE VI.—*Comparison of Toxicity of Highly Purified Bacterial Toxins.*

Toxin	LD_{50}/mg. per kg. animal.	Animal.
Diphtheria	3·5	Mouse
	3,500	Guinea-pig
Botulinus Type A	620,000	Mouse
	1,200,000	Guinea-pig
Tetanus	200,000	Mouse
	1,200,000	Guinea-pig
Shiga neurotoxin	750	Mouse
	1,150,000	Rabbit

(For references to diphtheria, botulinus and tetanus toxins, see Pappenheimer (1948).)

most toxic substances hitherto known, botulinus and tetanus toxins. Dubos and Geiger (1946) reported that per unit weight rabbits were 75–150 times more susceptible to the toxin than mice, but in our experience this ratio was greater than 1000. The concentration of the toxin in the organism is only about 1/1000, and this accounts for the fact that cultures of *Sh. shigae* are not considered particularly toxic when compared with other toxin-producing organisms.

There can be little doubt that the highly neurotoxic protein of *Sh. shigae* is a different substance from the considerably less toxic polymolecular complex of the O antigen of the smooth strains. The view may still be held that the neurotoxin is the protein component of the O antigen, but in that event its toxicity would have to be considerably reduced by the attachment of the phospholipid and polysaccharide components. That this is not altogether impossible is perhaps suggested by the work of Boivin, Delaunay and Sarciron (1940), who found that the O antigen content of autolysates of smooth strains remained constant on standing while the neurotoxin content increased up to 20-fold. This is not inconsistent with the view that a negligible amount of O antigen may have decomposed to liberate a very small amount of extremely potent neurotoxin.

SUMMARY.

The neurotoxin has been extracted from a rough strain of *Sh. shigae* and obtained in an electrophoretically homogeneous state. The purified preparation contains 5500 Lf/mg., 4200 L+/mg., 750 LD_{50}/mg. per kg. mouse and 1,150,000 LD_{50}/mg. per kg. rabbit. Its physical and chemical properties have been investigated.

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