THE IMMUNOCHEMISTRY OF TOXINS AND TOXOIDS

VI. The Crystallization and Characterization of Tetanal Toxin*

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It is desirable to obtain all functional proteins in a pure state to study their nature, properties, and mode of action. This is especially true for bacterial toxins. At present, little or nothing is known concerning the primary chemical or physiological effects of bacterial toxins; even the tissue cells affected are for the most part unknown. Thus, information on either the nature or the mode of action of bacterial toxins would permit a better understanding of the specific physiologic functions which are disturbed during the course of infections and would perhaps suggest improved immunotherapeutic or specific chemotherapeutic measures.

Several exotoxins have been isolated in highly purified form. Both Eaton (1) and Pappenheimer (2) have prepared purified diphtherial toxin. Two groups (3, 4) working at Camp Detrick have reported the crystallization of *botulinum* toxin (type A). Scarlatinal toxin (5) and streptolysin (6) have been highly purified. Although these toxins have been characterized as heat-labile proteins, physical and chemical analyses have as yet offered no explanation for their extreme toxicity.

Pickett, Hoeprich, and Germain (7) prepared tetanal toxin (Mueller) in a highly purified state by neutral salt precipitation and adsorption on cadmium compounds. Their best preparation contained 0.023×10^{-6} mg. N per M.L.D. which represented a product of about 100 times greater purity than had been previously reported (8). These authors made no claim that they had reached the limits of purity for this toxin.

In a preliminary communication, Pillemer, Wittler, and Grossberg (9) reported the crystallization of tetanal toxin. The purpose of the present paper is to describe in detail the methods for the purification and crystallization of tetanal toxin. Certain chemical and physical properties of the crystalline toxin are also presented.

EXPERIMENTAL

Materials.—The parent tetanal toxins[1] employed in this study were prepared according to the method of Mueller and Miller (10) and contained between 20 and 40 Lf units and between

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[1] Supplied by Lederle Laboratories and Wyeth, Inc.

200,000 and 500,000 M.L.D. per ml. It is doubtful whether the present study could have been carried to completion if this toxin had not been available, because attempts to crystallize tetanal toxins prepared on veal infusion-proteose-peptone medium had not been successful.

All chemical reagents were either C.P. or the best grade obtainable. The acetate buffer used for pH adjustments was composed of 2 M acetic acid and 0.4 M sodium acetate. Diluted 40-fold with distilled water, this buffer gave a pH of 4.

General Methods.—Methods for the determination of minimal lethal dose (M.L.D.) of tetanal toxin have been reported elsewhere (11). It has been found that strict adherence to these methods gives results which are reproducible to within 10 per cent. The combining capacity (Lf) and the time required for optimal flocculation (Kf) were determined by methods previously described (11). In most of the current experiments, increasing amounts of toxin were added to a constant amount of antitoxin. All other conditions were maintained as previously described. Since the antitoxin was maintained constant, the Kf of different samples was comparable. The criteria of Moloney and Hennessy (12) were rigidly followed in order to establish the true zone of flocculation. Special flocculating serums (13) were also employed to minimize the occurrence of false zones.

The techniques employed here for electrophoretic analysis have been reported (14). Ultracentrifugal studies were carried out by Dr. Dan H. Moore, of Columbia University, employing techniques in use in his laboratory. Solubility determinations were carried out according to Northrop (15). Isoelectric points were determined by a modification of the method of Michaelis and Rona (16). Hydrogen ion determinations were made on the glass electrode after warming the sample to 25° and before the addition of methanol. After adjustment of the pH and the addition of methanol, the ionic strength was calculated from the valence of the ions present and from their concentration. Centrifugation was carried out in a refrigerated centrifuge (International, PR-1). Constant temperature baths were employed to maintain the desired temperature of the solutions during fractionation.

Immunological Considerations.—The classical studies of Ramon have established the validity of the flocculation test for the determination of the combining units of tetanal toxin and toxoid. However, the flocculation test does not distinguish between toxin and toxoid, both of which combine with antitoxin. Therefore, in studies on the purification of toxins, caution must be observed in interpreting data based solely on flocculation tests. The intermediate or final products may represent mixtures of varying proportions of toxin and toxoid.

The toxic properties of toxins are due to groups or configurations on the molecule distinct from those groups responsible for flocculation. The determination of the M.L.D. of the toxin offers the best proof that toxin has not been converted to toxoid during fractionation or processing. The ratio of M.L.D. to Lf per mg. of nitrogen or per ml. of sample also offers evidence of the integrity of the toxin molecule and distinguishes between spontaneous "toxoiding" and denaturation during handling. A decrease in this ratio indicates that toxin is being converted to toxoid. A loss of Lf units concomitant with a decrease in this ratio is due to denaturation with a resulting loss of toxic properties and combining capacity. Any increase in this ratio indicates that toxoid is being separated from the toxin. The exact nature of the conversion of toxin to toxoid is unknown. However, certain evidence presented below and reported elsewhere (17) suggests that tetanal toxoid is a dimer of tetanal toxin molecules

which have apparently interacted or condensed through their toxic groups. Toxin which has spontaneously converted to toxoid exhibits immunological and physicochemical properties which differ from those of toxin. Thus, the presentation of data on a "pure toxin" may be misleading unless it is definitely established that the toxin is free of toxoid or that the toxoid has properties identical in all respects with those of toxin.

The Kf of toxin has been followed here to ascertain the extent of early denaturation of either toxin or toxoid. An increase in the Kf of a fraction compared with the preceding fraction or with the parent toxin is considered a definite indication of damage to the molecule. Any decrease in the Kf points to the removal of degraded toxin or toxoid molecules. Of course, such separations are desirable during purification and should occur if the fractionation procedure itself does not lead to irreversible changes in the labile proteins. In the present studies, the importance of Kf has been fully recognized. Any fraction showing evidence of a prolonged Kf is not employed for further fractionation or characterization.

Criteria of Purity.—The identification of a protein as a single molecular species is difficult. The commonly accepted criteria are constancy of solubility, constancy of chemical composition, and homogeneity of all measurable physical properties. When the protein has measurable functional activity, a constant correlation must exist between the above properties and all the biological functions of the protein. Such proteins as toxins, toxoids, and antitoxins must act as single substances in the flocculation test. In so far as possible, the conditions employed for physical characterizations should be within the physiologic range. This should permit a greater degree of correlation between physicochemical data and biological function of the protein. On the basis of data presented in this report, it would appear necessary to apply the above mentioned criteria to freshly prepared proteins. Identical samples should be employed for the comparison of biological activity with chemical and physicochemical data, and these measurements should be made simultaneously.

Methods of Purification.—The purification of toxins and toxoids offers many difficulties. One of the major problems is the separation of minute amounts of the active principle from large volumes of culture media and water. The instability of toxins, as well as their great affinity for surfaces and adsorbing agents, offers additional difficulties. Furthermore, contamination of the toxin by microorganisms capable of elaborating other lethal toxins and enzymes may alter the true M.L.D. value of the toxin. Finally, several closely related proteins are present as contaminants. Thus, the separation of toxins and toxoids requires fractionation systems other than salt precipitation, adsorption, or acid precipitation. The method must be reproducible and transferable from small scale laboratory experiments to the large scale production routinely employed by industry.

Theoretical considerations of the precipitation by ethanol-water mixtures

have been reviewed by Edsall (18). Cohn (19) and associates have put these theories into practice in the fractionation of plasma proteins. As pointed out by these workers, the denaturing effect of alcohol is minimized at the low temperatures employed. Dialysis, which is a major objection to neutral salt precipitations, is unnecessary in the process. The influence of pH, ionic strength, and protein concentration in this system of fractionation has been described (18).

In studies on the purification of labile toxins and toxoids (9, 11, 20–24) methanol has been found preferable to ethanol as the organic precipitating agent. Under certain conditions ethanol alters toxins even at low temperatures. It has also been noted in this laboratory that methanol may be preferable to ethanol for the fractionation of other proteins, including certain of the plasma proteins. The inherent danger of denaturation by increased temperature is greatly minimized by the use of methanol. Certain proteins which are denatured by ethanol at $-5°$ are quite stable in methanol even at 0°. Since the volatile methanol is removed from the proteins by freezing the mixture and then removing both the water and alcohol at reduced pressure, clinical objections to the use of methanol are minimized.

For the most part, the influence of pH, alcohol concentration, temperature, and protein concentration on the solubility of toxins and toxoids is similar to that described for plasma proteins. The precise balance of the charged condition of proteins (which is determined by the pH of the mixture) and the methanol concentration plays the major rôle in the separation of toxin from bacterial proteins and culture medium constituents. Protein concentration is adjusted to allow protective stabilization of the toxin molecules, by virtue of protein-protein interaction, and of their dipolar activities. The adjustment of five independent variables permits the attainment of a large number of experimental conditions for the separation of the desired proteins.

The solubility of the components of a protein system will vary as a function of pH at constant ionic strength and constant methanol concentration, or as a function of methanol concentration at constant pH and constant ionic strength. Other sets of experimental values can be obtained as functions of temperature, ionic strength, or protein concentration at constant methanol concentration or pH. Each variable is adjusted at any one step in order to obtain the highest yield and greatest purity accompanied by no deleterious changes in the toxin molecules.

The conditions for removal of impurities at any one step in fractionation are specific for that step. In the same manner, the conditions required for the separation of a particular toxin will not necessarily apply to another toxin. Such investigations involve the laborious task of physicochemical, analytical, and immunological control and do not offer an easy or simple application to the purification of all protein systems. However, the precision of the method which

conforms to the disciplines of physical chemistry, the ease in obtaining highly pure products once the exact method has been established, and the reproducibility of results make it the method of choice in the fractionation of functional proteins.

RESULTS

In the present study the first step required that the toxin be removed as a precipitate with a minimum accompaniment of cytoplasmic bacterial proteins, and of proteoses, peptones, and amino acids which are constituents of the culture medium. In subsequent steps, further removal of these impurities was accomplished. Finally, the nucleoproteins, mucoids, and carbohydrates were removed, and then crystallization of the toxin was achieved. As will be seen below, the toxin may be precipitated or may be maintained in solution during the removal of impurities depending upon the limits of solubility of the protein and of the impurities, the nature of the impurities, and the convenience of the experimental conditions. It should be stressed that the conditions indicated in the text and tables must be rigidly followed for reproducibility. Small deviations in pH or other variables which may seem insignificant can result in a great decrease in yield and marked changes in the purification factors. Temperature should be controlled rigidly during the process. A cooling coil immersed in the toxin during the addition of methanol or the immersion of the toxin in cold baths with the slow addition of alcohol will greatly aid in maintaining the temperature near the freezing point or at $-5°$. Weston dial thermometers (stainless steel) make excellent stirring rods in this process because the temperature can be easily followed during processing.

Separation of Fraction T-PI.—In a series of solubility studies, the conditions for the separation of the toxin from culture medium products were determined. The general fractionation method follows.

One volume of toxin was chilled to 1° and adjusted to the desired pH with ice-cold acetate buffer or, in some instances, with acetic acid. To this mixture the calculated amount of methanol (measured at $-5°$ and chilled to $-20°$) was added slowly with constant stirring, care being taken to maintain the temperature at $-5°$ or under. The mixture was maintained at $-5°$ for 24 hours. At alcohol concentrations above 40 per cent, the temperature was maintained at $-10°$, while at alcohol concentrations of 40 per cent or under, the temperature was kept near the freezing point or at $-5°$. The precipitate was removed in a refrigerated centrifuge at the same temperature as that used during processing. The precipitate was then dissolved to the desired volume with 0.15 M sodium acetate of pH 6.8. The solution was clarified by centrifugation at 4000 R.P.M. for 20 minutes at 1°, and immediately assayed or stored at $-25°$ until needed.

Pertinent data on the behavior of the toxin in mixtures consisting of culture media, methanol, and water under varying conditions of pH, ionic strength, and temperature are summarized in Table I. Analysis of these resltus discloses the following information. Tetanal toxin is quantitatively precipitated be-

tween pH 4.5 and 5.25 in 40 per cent methanol at ionic strength 0.09 and at −5°. The highest concentration per milligram of precipitated nitrogen is obtained between pH 5.0 and 5.25. Hydrogen ion concentrations greater than 5.0 lead to the increased precipitation of constituents other than toxin. Hydrogen ion concentrations less than pH 5.25 result in an increased solubility of the toxin. Increasing or decreasing the ionic strength under the above conditions does not affect appreciably the yield or purity. Elevation of the temperature to 0° in 40 per cent methanol generally results in denaturation of the

TABLE I

Precipitation of Tetanal Toxin from Culture Medium in Methanol-Water Mixtures Varying in pH at Ionic Strength of 0.09 and at a Temperature of −5°C.

Conditions		Lf per mg. N	Kf*	M.L.D. per mg. N × 10^6	Yield of parent toxin
pH	Methanol				
	per cent		*min.*		*per cent*
4.5	40	226	30		100
4.5	25	378	25	5.0	70
4.8	40	634	18	9.3	100
4.8	25	487	20	7.1	75
5.0	40	812	12	10.3	100
5.0	25	506	15	7.4	75
5.15	40	862	10	10.1	100
5.15	25	516	15	7.2	75
5.25	40	883	10	10.4	100
5.4	40	872	10	8.7	85
6.0	40	207	30	4.1	35
6.6	40	183	30		20
Parent toxin.....		6.9	30	0.068	

* Tested at 50 Lf units.

toxin.[2] Alcohol concentrations less than 40 per cent generally result in increased solubility of the toxin. Methanol concentrations greater than 40 per cent precipitate inert culture medium products and bacterial proteins. There is no evidence that hydrogen ion concentrations between 4 and 7 under the present conditions impair the toxin, since the Kf is often shorter than that of the parent toxin over the wide pH range. Pigment is precipitated with the toxin under pH 5.4.

The above information indicates that separation of the toxin from the crude toxic filtrate occurs optimally at pH 5.0 to 5.25 in 40 per cent methanol at −5°. The charged condition of the toxin has a greater influence on its solubility than the ionic strength of the mixture. The separation of bacterial toxins

[2] Such toxins show either an absence or marked retardation of flocculation.

differs in this respect from the separation of the plasma proteins in ethanol-water mixtures, which depends largely on precise salt concentrations (19). These results compare favorably with those reported for the purification of tetanal toxin produced in veal infusion medium (11). It should be noted that a single precipitation quantitatively separates toxin containing more than 800 Lf per mg. of nitrogen. Assuming that pure toxin has between 3300 and 3600 Lf per mg. of nitrogen, this material is over 20 per cent pure. This precipitate is hereafter referred to as T-PI. The ratio of M.L.D. to Lf units per mg. N is about 1.2×10^4 and is slightly greater than that of the crude toxin.

TABLE II

Precipitation of Tetanal Toxin from Fraction T-PI in Methanol-Water Mixtures Varying in pH, Ionic Strength, and Temperature

Conditions				Lf per mg. N	Kf*	M.L.D. per mg. N $\times 10^6$	Yield from T-PI
pH	Methanol	Ionic strength	Temperature				
	per cent		°C.		*min.*		*per cent*
3.5	25	0.057	−5	283	120		
3.5	10	0.068	−3	212	120		
4.0	25	0.057	−5	341	20	1.5	60
4.0	10	0.068	−3	364	20	1.5	15
4.5	20	0.06	−5	812	15	9.3	100
4.5	10	0.068	−3	974	15	12.6	83
5.0	20	0.06	−5	1113	12	18.3	100
5.0	10	0.068	−3	1282	12	22.0	100
5.5	25	0.037	−5	1150	12	20.6	100
5.5	15	0.024	−3	1390	12	24.2	100
6.0	25	0.057	−5	741	20	13.6	36
6.0	15	0.064	−3	1122	15	20.6	22

* Tested at 50 Lf units.

Solubility of Toxic Principle in Fraction T-PI.—Several large lots of crude tetanal toxin were processed to fraction T-PI. The initial separation of tetanal toxin from culture medium, conducted at pH 5.15 ± 0.05 in 40 per cent methanol at −5°, has been found to be reproducible for all crude toxins studied here. Fraction T-PI was dissolved to one-tenth of the parent toxin volume in 0.15 M sodium acetate. The behavior of this fraction in methanol-water mixtures was then observed over a wide range of experimental conditions. The results of these studies are summarized in Table II.

The toxin in fraction T-PI is quantitatively precipitated between pH 4.5 and 5.5 under appropriate conditions. It is insoluble in 20 per cent methanol at pH 4.5, in 10 per cent methanol at pH 5.0, and in 15 per cent methanol at pH 5.5. The removal of culture medium substances and bacterial impurities reduces the amount of methanol required to precipitate the toxin. It will be noted from the prolonged Kf that pH 3.5 apparently alters the toxin molecule

or precipitates degraded toxin or toxoid molecules. Hydrogen ion concentrations less than pH 5.5 result in increased solubility of the toxic principle under the observed conditions. The ionic strength apparently plays little or no rôle in the precipitation of toxin in this step. In view of the above results, the toxic principle is reprecipitated at either pH 5.5 in 15 per cent methanol or at pH 5.0 in 10 per cent methanol, at ionic strength between 0.025 and 0.075, and at a temperature of $-3°$ to $-5°$. These fractions have an Lf content of about 1300 per mg. of N and about 20 million M.L.D. per mg. of N. The ratio of M.L.D. to

TABLE III

Solubility of Tetanal Toxin from Fraction T-PII in Methanol-Water Mixtures and Water Varying in pH, Ionic Strength, and Temperature

Conditions				Lf per mg. N	Kf*	M.L.D. per mg. N × 10^6	Yield from T-PII
pH	Methanol	Ionic strength	Temperature				
	per cent		°C.		*min.*		*per cent*
3.5	7.5	0.075	−5		60		90
3.5	5.0	0.075	0		60		90
3.5	0.0	0.075	0		60		90
4.0	10.0	0.075	−3	1712	5		80
4.0	10.0	0.025	−3	1483	5		60
4.0	7.5	0.075	−3	2080	5	42.1	92
4.0	7.5	0.05	−3	1562	5	31.9	86
4.0	7.5	0.025	−3	1100	5	27.8	60
4.0	7.5	0.15	−3	1538	15		92
4.0	5.0	0.075	0	1743	10		86
4.0	0.0	0.15	0	1320	15		92
4.0	0.0	0.075	0	1612	15		92
4.0	0.0	0.025	0	1612	15		86

* Tested at 50 Lf units.

Lf units per mg. of N is about 1.8×10^4, which is considerably greater than the ratio in T-PI. This fraction is designated T-PII.

Solubility of Toxic Principle in Fraction T-PII.—Fraction T-PII was dissolved to one-twentieth of the parent toxin volume in 0.075 M sodium acetate. Observations over a wide range of experimental conditions revealed that the separation of the toxic principle as a precipitate from the contaminating materials did not materially improve purification. Experiments were then designed to remove the bacterial proteins as a precipitate while leaving the toxin in solution. The results of an experiment with one large batch of T-PII are given in Table III. All determinations given in this table were performed on the supernatants after the removal of the precipitated material.

It will be seen that most of the tetanal toxin remains in solution between pH 3.5 and 4.0 under appropriate conditions. Close analysis of the data reveals that at pH 3.5, with methanol concentrations of 7.5 per cent or lower, only a

slight precipitation of the toxin occurred. The Kf of the soluble toxin, however, is markedly prolonged indicating damage to the toxic principle. At pH 4.0 in either aqueous solution or in methanol up to 10 per cent, a large part of the toxic principle remains soluble with a sharp decrease in the Kf, indicating the removal of degraded toxin or toxoid molecules. It will also be observed that in the absence of methanol at this pH, the degree of purity is lower. For this batch of toxin, optimal separation of the toxin from bacterial protein occurs at pH 4.0, methanol concentration 7.5 per cent, ionic strength 0.075, at a temperature of $-3°$. Increasing the alcohol concentration at this pH results in a decreased yield of toxin in the supernatant, and decreasing the alcohol at this pH leads to incomplete removal of the impurities. The ionic strength at this step is of great importance. Whereas an ionic strength of 0.075 yields a toxic solution containing 2000 Lf per mg. of N, decreasing the ionic strength to 0.05 results in a product 25 per cent less pure. Further decrease of the ionic strength to 0.025 leads to a precipitation of toxin, and the supernatant has a purity of only 1100 Lf per mg. N. It is interesting that the removal of bacterial proteins under appropriate conditions markedly decreases the Kf of the resulting purified toxin.

The step which has just been described is of utmost importance in the purification of tetanal toxin. It has been noted that various lots of T-PII behave differently at this step. It is, therefore, necessary to ascertain on an aliquot of T-PII the optimal conditions for the separation of the bacterial proteins from the toxin. While this is a laborious procedure, it is imperative in order to obtain unaltered and highly purified toxin. For the adequate removal of the inert precipitate from T-PII from twelve different batches of toxin, it has been observed that the conditions at this step vary from pH 3.8 to 4.2, from methanol concentrations of 5 per cent to 12 per cent, and from 0.05 to 0.15 ionic strength at temperatures between 0° and $-5°$. These fractions are designated T-SIII. The ratio of M.L.D. to Lf per mg. of N for these fractions is about 2×10^4.

Solubility of Toxic Principle in Fraction T-SIII.—Fraction T-SIII was maintained at its freezing point and studies were carried out to remove other contaminants which may have remained in solution during separation of fraction T-SIII. It is still desirable to maintain the toxic principle in solution at this step. It was noted that the removal of nucleoproteins and other materials with low solubility at pH 4.0 allowed the addition of greater concentrations of methanol at this pH with little or no precipitation of the toxin. The results of these studies are given in Table IV. 90 per cent of the toxin remains soluble at pH 4.0 when the alcohol concentration is increased to 30 per cent. Increasing the pH leads to the precipitation of the toxic principle. On the basis of these studies, fraction T-SIII was adjusted to pH 4.0 and the alcohol concentration increased to 30 per cent. After several hours a flocculent precipitate settled out. The material was allowed to stand for 24 hours, and the supernatant was

collected by centrifugation at −5° and designated fraction T-SIV. This solution generally contains approximately 2500 Lf per mg. N.

Precipitation of Tetanal Toxin from Fraction T-SIV.—Various experimental conditions have been employed for the separation of tetanal toxin as a precipitate from fraction T-SIV. The general procedures employed for the separation of fraction T-PII were followed. At this step, high ionic strengths should be avoided. It was observed that the precipitation of tetanal toxin at this point at ionic strengths greater than 0.025 resulted in irreversible changes in the toxin molecule. Furthermore, hydrogen ion concentrations less than pH 6.0 should also be avoided in order to retard the spontaneous conversion of tetanal toxin

TABLE IV

Solubility of Tetanal Toxin from Fraction T-SIII in Methanol-Water Mixtures Varying in pH

pH*	Methanol	Lf per mg. N‡	Yield from T-SIII
	per cent		*per cent*
4.0	40	2800	60
4.0	30	2520	90
4.0	20	2118	100
4.2	25	2314	70
4.2	15	1982	80
4.5	25		40
4.5	15		40
5.0	25		10
5.0	15		10
5.5	25		
5.5	15		

* pH of T-SIII diluted with two parts of water.

‡ Supernatants tested.

to a flocculating atoxic protein. The most suitable conditions encountered so far for the precipitation of the toxin at this step are pH 5.4, 10 per cent methanol, ionic strength 0.02 at a temperature of −5°. A large quantity of sodium hydroxide is necessary to raise the pH of fraction T-SIV to 5.4. This, of course, increases the ionic strength of the solution. In order to avoid local excess of salt and hydroxyl ions, the alkali should be added in a dilute form in a methanol-water mixture to attain the above mentioned conditions. This addition should be made slowly with constant stirring. The material insoluble under these conditions is almost pure tetanal toxin. At times, some crystallization of the toxin occurs at this step. The precipitate was collected by centrifugation at −5° and was dissolved in 0.075 M sodium acetate to an Lf concentration of about 12,000 per ml. This fraction is designated T-PV and usually contains approximately 3000 Lf and 50 million M.L.D. per mg. of N.

Precipitation of Tetanal Toxin from Fraction T-PV.—Fraction T-PV, which

is almost pure tetanal toxin, was subjected to varying experimental conditions in order to remove traces of impurities prior to the crystallization of the toxin. It was found that reprecipitation of this material at pH 4.9 and ionic strength of 0.01 at 0° in the absence of methanol yielded the purest product. Therefore, fraction T-PV was diluted with ice-cold distilled water to an ionic strength of 0.01, the pH was adjusted to 4.9, and the material was allowed to stand for 24 hours at 0°. This precipitate was generally amorphous; however, crystals were often observed at this step. This fraction was designated T-PVI and was dissolved in 0.05 M sodium acetate to a concentration of about 12,000 Lf per ml. This solution contains about 3200 Lf and 60 million M.L.D. per mg. of N.

Crystallization of Tetanal Toxin.—The crystallization of tetanal toxin from fraction T-PVI is dependent on the purity of this fraction. Traces of nucleoprotein will interfere with crystallization. Furthermore, if even partial spontaneous detoxification of the toxin has occurred during processing, crystallization is retarded. It is obvious that if the special precautions indicated in the above procedures are not followed to avoid denaturation of the toxin, crystallization will not be accomplished.

A number of experimental conditions were employed to achieve crystallization. Tetanal toxin readily forms an amorphous precipitate in aqueous solutions between pH 4.5 and 5.4 resembling a "euglobulin" in the classical terminology. However, in very dilute solutions, under these conditions of pH and in the presence of optimal concentrations of methanol, the toxin will crystallize out of solution. This procedure was employed early in the experimental work. While this procedure yields adequate quantities of crystals, it involves the handling of large volumes of material and is time-consuming. Attempts have been made to establish more practical conditions for the crystallization of tetanal toxin. Crystallization of the toxin does not occur readily at a pH acid to its isoelectric point. However, if the hydrogen ion concentration is sufficiently low (between pH 5.8 and 6.2), the addition of alcohol up to 20 per cent concentration does not produce an amorphous precipitate. Fraction T-PVI was adjusted to a 1 per cent protein solution at pH 6.0, methanol concentration of 20 per cent, ionic strength of 0.02, and a temperature of −5°. Crystallization of the toxin occurred within a few days, and on standing for several weeks, a good yield of crystals was obtained. Attempts to obtain crystals at hydrogen ion concentrations less than pH 6.0 were not highly successful, since at these hydrogen ion concentrations tetanal toxin is readily detoxified. Hydrogen ion concentrations greater than pH 6.0 resulted in a precipitate of both crystalline and amorphous material. A summary of the degree of purity and yield of tetanal toxin at each step is given in Table V. The conditions of pH and methanol concentration employed at each step are shown graphically in Fig. 1.

The crystals obtained can be redissolved in sodium acetate and recrystallized under the above stated conditions. The final product can be dissolved either

in 0.15 M sodium chloride at pH 5.5 or, for greater stability, in 0.3 M glycine at pH 5.8. This material can be stored at −25°, dried from the frozen state, or

TABLE V

Degree of Purity and Yield of Tetanal Toxin at Various Fractionation Steps

(Average Values)

Fraction	Lf per mg. N	M.L.D. per mg. N × 10^6	Yield
			per cent
Parent	6–10	0.06–0.1	
T-PI	800–1000	10–12	95–100
T-PII	1200–1400	18–24	90–100
T-SIII	1800–2400	36–45	70–80
T-SIV	2200–2600		70–80
T-PV	2800–3200	40–50	60–80
T-PVI	3000–3400	50–60	50–70
T-CVII	3300–3600	60–70	10–40
T-CVII-4	3300–3600	60–70	5–35

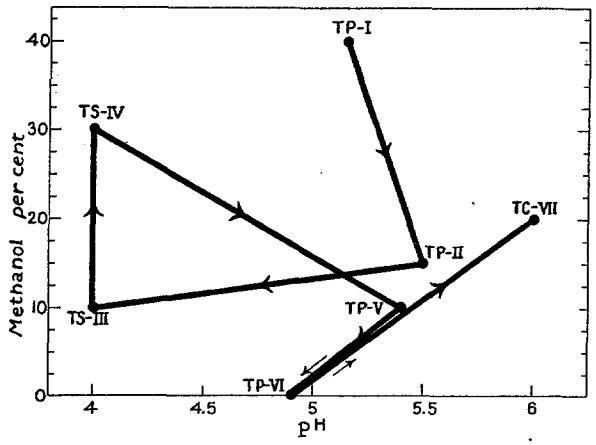

FIG. 1. A schematic diagram of the conditions of pH and methanol concentration at different steps of the purification of tetanal toxin. P, toxin precipitated under described conditions; S, toxin soluble under described conditions; C, toxin crystallized under described conditions.

maintained at 0° in the presence of 0.3 M glycine. A photograph of the crystals has been published previously (9).

Properties of Crystalline Tetanal Toxin

Crystalline tetanal toxin in a 1 per cent solution is pale yellow. It is very stable in the presence of glycine between pH 5.0 and 6.0. It is relatively stable

in the presence of neutral salt at this pH. However, hydrogen ion concentrations less than 6.0 lead to the spontaneous conversion of the toxin to a flocculating atoxic protein.

TABLE VI

Chemical Composition of Crystalline Tetanal Toxin

Nitrogen, *per cent*	15.7
Phosphorus, *per cent*	0.065
Sulfur, *per cent*	1.04
Carbohydrate	—

TABLE VII

Properties of Crystalline Tetanal Toxin

Mobility in veronal buffer, pH 8.6, 0.1 ionic strength	2.8×10^{-5}
Sedimentation constant, $s^{1\%}_{20}$, *w*	4.5
Optical rotation, $[\alpha]^{25}_{D}$, *degrees*	−63
Isoelectric point	5.1 ± 0.1
Lf per mg. N	3600
Kf_{50}, *min*	10
M.L.D. per mg. N	6.6×10^{7}

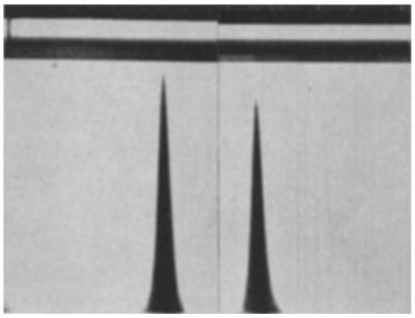

FIG. 2. Electrophoretic pattern of crystalline tetanal toxin. Time = 18,000 seconds.

Chemical composition of the toxin is summarized in Table VI. While the crystalline toxin gives the usual protein reactions and contains 1 per cent sulfur and a trace of phosphorus, it is entirely lacking in carbohydrate. Amino acid analyses of the crystalline protein are being conducted by Dr. Max S. Dunn and will be reported later.

Table VII summarizes the physicochemical character of freshly dissolved crystalline tetanal toxin. The toxin is electrophoretically homogeneous (Fig. 2) with a mobility of 2.8×10^{-5} in veronal buffer of 0.1 ionic strength at pH 8.6. The optical rotation of $-63°$ differs from the optical rotation of $-45°$ for diphtherial toxoid (21).

The isoelectric point was estimated from the minimum solubility of both total N and the combining activity of the crystalline toxin. The toxin was adjusted to different hydrogen ion concentrations with acetic acid at 0.2 pH intervals in distilled water at constant volume and at 1°. The results of this experiment

TABLE VIII

Solubility of Crystalline Tetanal Toxin in Distilled Water as Function of pH

pH*	Lf precipitated	Protein N precipitated
	per cent	*per cent*
4.3	0	0
4.5	30	30
5.0	90	90
5.2	90	90
5.5	40	40
6.0	20	20

* Determined on supernatants after equilibration with the precipitates for 18 hours at 1°

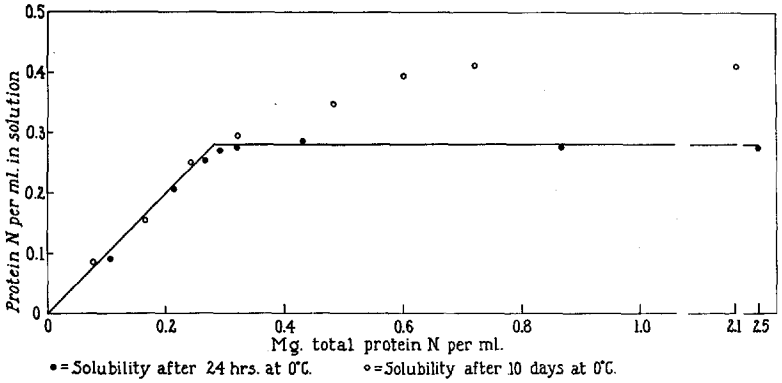

FIG. 3. Solubility curves of crystalline tetanal toxin. The protein nitrogen per milliliter in solution is plotted against the total protein nitrogen concentration. The solid line is the theoretical curve for a solid phase of one component.

are given in Table VIII. It will be noted that, judged both by N and by combining capacity, the toxin has a minimum solubility at pH 5.1 ± 0.1.

As pointed out above, crystalline tetanal toxin, on standing at 0° rapidly loses toxicity with no loss in its ability to flocculate with tetanal antitoxin. This phenomenon complicated the determination of the exact sedimentation constant and true constant solubility of the crystalline protein. Inspection of Fig. 3 reveals that the solubility of the freshly prepared crystalline protein is almost constant in 1.4 M ammonium sulfate at pH 7.0 and acts as though it were essentially a solid phase of one component. However, on standing for 10 days at

0°, at least 50 per cent of the material shows a change in solubility and the solution appears to contain two molecular species. This change is accompanied by a loss of about 75 per cent of the toxicity of the solution while the flocculating capacity remains unaltered. This would indicate that the solubility of freshly

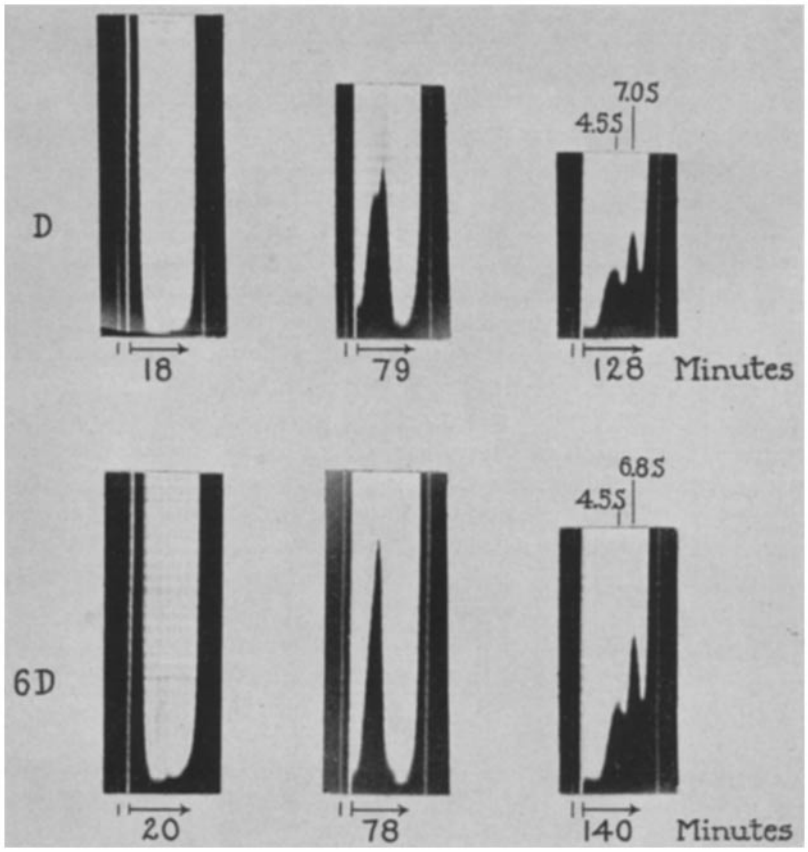

FIG. 4. Sedimentation patterns of crystalline tetanal toxin after standing for 10 days at 0°. *D*, once crystallized toxin. 6 *D*, four times recrystallized toxin.

prepared crystalline toxin is constant and that the changes observed on standing are caused by the condensation of the pure toxin. This hypothesis was substantiated by the results of ultracentrifugal studies (17). The unavailability of an ultracentrifuge in this laboratory at present made it impossible to study freshly prepared crystalline toxin. Samples were forwarded to Dr. Dan H. Moore, Columbia University, who conducted the ultracentrifugal studies. It will be noted that 55 to 60 per cent of the molecules had a sedimentation constant of 7.0 and the remaining molecules sedimented at 4.5 S (Fig. 4). Immu-

nological analysis of these fractions revealed that the toxin was associated with the molecules sedimenting at 4.5 S. Both fractions flocculated with antitoxin. These results suggest that crystalline tetanal toxin is spontaneously converted to a flocculating atoxic dimer. Work will be carried out to determine the mechanism of this reaction. In reviewing the work of other investigators on the purification of toxins, little or no reference is made to the possibility that their final toxin preparations may have contained toxoid. The spontaneous conversion of crude toxin to toxoid (natural toxoiding) is well known. It seems highly improbable that the usual methods employed for the fractionation of toxins involving dialysis and extraphysiologic conditions of pH, salt concentration, and temperature would not have caused some conversion of the toxin to toxoid. That such a conversion might have taken place is indicated by the absence of constant solubility data for purified toxins. The recognition of the possibility that toxin may easily be converted spontaneously to a toxoid should lead to a better understanding and to more exact physicochemical data on these substances. On the basis of the present studies it appears that tetanal toxin has a sedimentation constant of 4.5 Svedberg units.[3]

Crystalline toxin is not precipitated by an anti-*Clostridium tetani* rabbit serum. The toxin is almost immediately detoxified by very small amounts of formalin (0.01 to 0.001 per cent). This detoxified material is highly antigenic and can be used for immunization. However, the inherent technical hazards to personnel during the processing of the toxin makes it advisable to employ crude toxoid for the parent material when a purified toxoid is desired. The conditions described in the text for the purification of tetanal toxin are the same for the purification of tetanal toxoid with the following exception: in the removal of T-PI the pH should be adjusted to 4.85 ± 0.05.

SUMMARY

Methods for the purification and crystallization of tetanal toxin are described. The methods consist of the multiphase fractionation system involving methanol as the organic precipitating agent under controlled conditions of pH, ionic strength, protein concentration, and temperature.

Crystalline tetanal toxin has an electrophoretic mobility of 2.8×10^{-5} in veronal buffer of 0.1 ionic strength at pH 8.6. The solubility of freshly prepared toxin is essentially constant. The isoelectric point is 5.1 ± 0.1. The crystalline toxin contains 1 per cent sulfur, traces of phosphorus, and gives the usual protein reactions. It does not contain carbohydrate.

[3] Since this paper has gone to press, samples of crystalline tetanal toxin have been stored at 0° in the presence of homogeneous human albumin (4.5 S). It has been noted that no loss either in the toxicity or in the combining capacity of the toxin occurred after standing for 20 days. Ultracentrifugal analysis of this mixture revealed that the sample was homogeneous with a sedimentation constant of about 4.5. This substantiates the view that tetanal toxin has a sedimentation constant of 4.5.

The crystalline toxin does not precipitate anti-*Clostridium tetani* rabbit serum. The final product contains between 3400 and 3600 Lf and about 6.6×10^7 M.L.D. per mg. N.

Crystalline tetanal toxin is spontaneously converted to a flocculating atoxic dimer upon standing at 0°. This change is accompanied by the appearance of another molecular species as judged by constant solubility tests. Ultracentrifugal analysis of these fractions reveals that tetanal toxin has a sedimentation constant of 4.5 Svedberg units while the atoxic flocculating dimer sediments at 7 S.

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