STUDIES ON TETANUS TOXIN AND TOXOID

II.* ISOLATION AND CHARACTERIZATION OF THE TETANUS TOXIN AND TOXOID

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Summary

Crude tetanus toxin and toxoid were prepared by methanol precipitation. The toxin was purified by a combination of TEAE-cellulose and Sephadex G-200 chromatography at pH values less than 6·0. The toxoid was purified by DEAE-cellulose at approximately neutral pH values. The nature and amount of amino acids of the culture medium which had condensed with the tetanus toxoid proteins during detoxification with formaldehyde was determined.

I. INTRODUCTION

The isolation of pure tetanus toxin by methanol precipitation and chromatography on DEAE-cellulose at neutral pH has already been reported (Dawson and Mauritzen 1967). Its lethality (minimum lethal dose/mg nitrogen) was equivalent to that found by other workers using other methods of preparation (Pillemer *et al.* 1948; Largier 1956; Turpin, Raynaud, and Pery 1959). Its sedimentation value at neutral pH was approximately 7 S in agreement with Raynaud, Turpin, and Bizzini (1960). This differs from the value reported by Pillemer *et al.* (1948) who found crystalline toxin prepared by a series of methanol precipitations below pH 6·0 to have a sedimentation value of 4·5 S. Moreover, the toxin prepared by Pillemer *et al.* (1948) changed slowly on standing at pH values above 6·0 to a non-toxic flocculating but antigenic product with a sedimentation value of 7·0 S. Since it was possible that the differences in sedimentation velocity characteristics were dependent on the pH of preparation, experiments were made to find chromatographic procedures throughout which the toxin could be kept at pH 6·0 or lower. This objective was achieved by using TEAE-cellulose and Sephadex G-200. The lethality and sedimentation values of the toxin prepared at pH values below 6 were similar to those given by toxin obtained by DEAE-cellulose chromatography. The methanol precipitation followed by TEAE-cellulose separation offers an attractive method for commercial preparation of a highly purified tetanus toxin prior to conversion to tetanus toxoid vaccine.

Crude tetanus toxoid is normally prepared by detoxifying culture filtrates of *Clostridium tetani* with formaldehyde. The product is then concentrated and substances of smaller molecular weight removed, either by ultrafiltration or by ammonium sulphate precipitation and dialysis. These products contain a large number of bacterial proteins but it is probable that only one is required for the protective response. Manufacturers conscious of this often endeavour to purify the tetanus

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toxoid fraction further by ammonium sulphate fractionation (Levine and Stone 1951). However, this is far from ideal, both with respect to the large losses involved and to the purity of the product obtained. Latham *et al.* (1965) have purified tetanus toxoid to some degree by fractionating on Sephadex G-200, subsequent to a precipitation of tetanus proteins by ammonium sulphate. However, molecular-exclusion chromatography by a single-stage process is frequently unsuitable for complete separation of multicomponent mixtures.

Since it has been shown (Dawson and Mauritzen 1967) that a very pure tetanus toxin can be obtained from culture filtrates by a combination of methanol precipitation and chromatography on DEAE-cellulose, it was felt desirable to apply similar techniques to the crude tetanus toxoid. However, the condensation of formaldehyde with the proteins caused the conditions of chromatography to be modified. The results indicate the limitations of ion exchange when used for aldehyde-treated culture filtrates. During the detoxification period the amino acids of the media become condensed to the tetanal proteins via formaldehyde links. The extent of this condensation was examined in toxoids prepared by ultrafiltration and by methanol precipitation.

II. Materials and Methods

(*a*) *Crude Tetanus Toxin and Toxoid*

These samples were taken from commercial batches prepared by the Commonwealth Serum Laboratories, Melbourne. Toxic culture filtrates of *Clostridium tetani* grown in Mueller's medium (Mueller and Miller 1947) and in a modification of this medium (Latham, Bent, and Levine 1962) were used. The crude toxoid was prepared by adding formaldehyde solution (AnalaR) to sterile culture filtrates of tetanus to give a final concentration of 0·05M, and storing at 37°C until detoxification was complete. The ultrafiltrates were combined samples of commercial preparations and represented filtrates from both media.

(*b*) *Methanol Precipitation of Toxin and Toxoid*

The toxin or toxoid was precipitated with commercial methanol using a method similar to that described for toxin by Dawson and Mauritzen (1967). In brief, the toxic culture filtrate or its subsequently prepared toxoid was chilled to 0°C and adjusted to pH 5·0 by addition of acetic acid. Cooling (to less than −5·0°C) was continued while cold methanol was added to a final concentration of 40% (v/v). After storage for 24 hr, the fine buff-coloured precipitate was sedimented in an International PR2 centrifuge (1000 *g* for 40 min at −10°C). The precipitate was transferred in buffer of appropriate pH and held frozen until prepared for chromatography.

(*c*) *Column Chromatography*

(i) *DEAE-cellulose.*—Purification of the methanol-precipitated toxoid was attempted using chromatographic conditions identical with those found successful with tetanus toxin (Dawson and Mauritzen 1967). These, however, proved unsatisfactory as the bulk of the toxoid protein was still bound to the DEAE-cellulose at the end of the pH–salt gradient. The conditions finally used were as follows: The methanol precipitate was dissolved in 0·1M phosphate buffer to give a pH value of approximately 7·0. The solution was clarified by sedimentation and dialysed at 0°C against 0·005M phosphate buffer, pH 7·5. The prepared toxoid sample was loaded on the column and the non-absorbed material eluted by passage of the equilibration buffer (0·005M phosphate, pH 7·2). This step was followed by an elution with a pH–salt gradient, prepared by using a constant-volume mixing vessel originally containing 0·01M phosphate buffer, pH 7·2, and with 0·05M NaH_2PO_4 and 0·5M NaCl in the reservoir. The salt concentration and pH changes were recorded throughout the elution.

(ii) *TEAE-cellulose.*—The methanol-precipitated toxin which had been suspended in 0·1M sodium acetate, pH 5·5, was prepared for the TEAE-cellulose column by dialysis against 0·025M phosphate buffer, pH 5·9, and subsequent clarification. Columns of TEAE-cellulose were equilibrated with 0·025M phosphate buffer at pH 5·9 and operated at 2°C. A column size of 20 ml was amply sufficient to absorb the non-toxin protein of the crude sample under the conditions used. In the two experiments reported here a sample of approximately 90 ml (equivalent to 3 litres of toxic culture filtrate) was applied to the column and the unabsorbed material, which included the toxin, was collected in the effluent. The separation was completed by following the sample with an equal volume of equilibrating buffer.

(iii) *Molecular-exclusion Chromatography.*—The toxin solution obtained from the TEAE-chromatography was concentrated approximately tenfold by dialysis at 0°C against Ficoll (Pharmacia). Before application to the Sephadex column, the sample was prepared by dialysis against the elution buffer and sedimentation. The Sephadex was prepared by suspension in buffer to remove fines, and autoclaved for 15 min at 110°C. The Sephadex beds were prepared, as described in Sephadex literature, in a column 2·5 by 100 cm, volume *c.* 450 ml. The column and collecting tubes were cooled at 4°C by using a refrigerated fraction collector. In the first experiment Sephadex G-100 was used and the buffer consisted of 0·15M NaCl in 0·05M phosphate, pH 5·9. In the second experiment where Sephadex G-200 was used, the buffer was changed to 0·1M sodium acetate, pH 5·5. Samples of approximately 15 ml were applied to both columns.

(*d*) *Protein Nitrogen, Flocculation Values* (L_f), *Lethality, and Immunoelectrophoresis*

These were carried out as described earlier (Dawson and Mauritzen 1967). Protein nitrogen was converted to ammonia by hot digestion in H_2SO_4 and H_2O_2, and determined by the ninhydrin method as used by Johnson, Driedger, and Marko (1964). Flocculation values (L_f) were determined in tests using constant amounts of standard antitoxin (30 L_f/ml), 2-ml volumes, 50°C, and a diluent of phosphate–saline pH 7·2. Immunoelectrophoresis was carried out essentially by the method described by Grabar (1964). The toxin preparations were tested after electrophoresis by diffusion against a horse antiserum which was capable of reacting with a large number of antigens present in the crude toxin. To differentiate between the 7 S toxin and the non-toxin components, some experiments were performed using a sheep antiserum which had been obtained from a sheep hyperimmunized with pure 7 S tetanus toxin. The lethality of the toxin was measured as the minimum lethal dose (M.L.D.) needed to kill mice weighing 18–22 g in 4 days.

(*e*) *Determination of Amino Acids Bound to Toxoids and Released at pH 2.2*

Five samples of toxoid were examined (see Table 2). Samples 1 and 2 were from culture toxoids (modified Mueller's medium) and were concentrated by methanol precipitation. Samples 3 and 4 were ultrafiltered toxoid prepared from modified Mueller's medium and from Mueller's medium culture filtrates respectively. Sample 5 comprised that part of the chromatographic elution peak (freeze-dried and redissolved) which contained the bulk of the tetanus toxoid. With the exception of the chromatographically purified toxoid, the toxoid samples were dialysed at 0°C against 0·005M phosphate, pH 7·0, to remove any free amino acids including those released at pH 7·0. In order to obtain an estimate of amino acids bound to the toxoid proteins, those released by hydrolysis at pH 2·2 were determined. This was performed by dialysis of the sample against an equal volume of citrate–HCl buffer, pH 2·2 (0·40M with respect to Na^+ and containing 0·2% phenol as preservative) for 4 days at 37°C.

The sample was first protected from microbial decomposition by standing at 4°C overnight to allow diffusion of phenol into the sample. A sample of the external diffusate was quantitatively analysed for amino acids on a Beckman model 120 B amino acid analyser.

(*f*) *Sedimentation Values*

These were determined in a Spinco model E ultracentrifuge fitted with rotor temperature indicator control. The samples were sedimented at 59,780 r.p.m. at 20°C and schlieren optics were used. All samples contained approximately 10 mg protein per millilitre.

III. Results

(*a*) *Tetanus Toxin*

M.L.D., flocculation (L_f) and sedimentation values, L_f/M.L.D. ratios, and the number of precipitin lines on immunoelectrophoresis for the various samples and elution components are recorded in Table 1. In both experiments, preparation of the toxin from TEAE-cellulose yielded a product containing approximately 70% of the L_f units applied to the column. Both these products showed marked lethality increase per milligram nitrogen over the material prepared by the methanol

Table 1

Flocculation and sedimentation values, lethality, and immunoelectrophoresis characteristics at various stages in the isolation of tetanus toxin

L_f and M.L.D. values are per milligram nitrogen. Values for minor components are given in parenthesis

Toxin Source	L_f	$10^{-7}\times$ M.L.D.	$10^{-4}\times$ (M.L.D./L_f)	10^{13}S	No. of Precipitin Lines
Experiment 1					
Methanol precipitate*	810	3·5	4·3		9
TEAE-cellulose eluate*	2380	10	4·2	6·2 (1·6)	1 (4)
Sephadex G-100 eluate					
Major peak	3000	11	3·7		
Minor peak		3		1·3 (4, 6·5)	4
Experiment 2					
Methanol precipitate*	1130	2	1·8		8
TEAE-cellulose eluate*	3180	10	3·2	6·5†	1 (3)
Sephadex G-200 eluate					
Major peak	2500	9	3·6	6·7	1
Minor peak		0·3		3·7‡	4

* As prepared for subsequent chromatography.

† Trace of slower component.

‡ Also minor slower component.

precipitation. When chromatography of the toxin (purified by TEAE-cellulose) was attempted using Sephadex G-100 it was found that most of the protein was excluded from the gel matrix and emerged around the void volume (V_0) of the column. When separated on Sephadex G-200, only a small amount of the protein was excluded from the gel and most of the protein eluted as one symmetrical peak together with smaller amounts of more diffusible protein [Fig. 1(*a*)]. In sedimentation-velocity experiments the toxin prepared from TEAE-cellulose was found to contain a major component with a sedimentation value of approximately 6·5 S along with smaller amounts of components of lower sedimentation values (Fig. 2, lower pattern).

The major component (80%) of the elution pattern gave a single precipitin line on immunoelectrophoresis, and M.L.D., flocculation, and sedimentation values corresponding to those obtained from tetanus toxin (Dawson and Mauritzen 1967).

The number of immunoelectrophoretic precipitin lines found for the toxin prepared on TEAE-cellulose and for the minor peaks of the Sephadex elution indicate considerable heterogeneity. However, these immunoelectrophoretic results are purely qualitative and the detection of precipitin lines was assisted by using a high concentration of the antigen. By using sheep antiserum specific only to the 7 S toxin, it was demonstrated that none of the precipitin lines obtained with the minor peak fraction (Sephadex G-200) corresponded to a 7 S toxin (see Fig. 3).

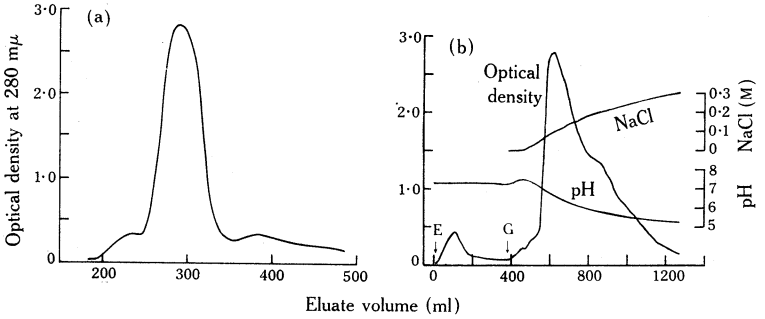


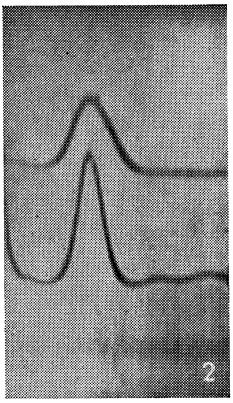
Fig. 1.—(*a*) Fractionation of tetanus proteins on Sephadex G-200. Sample: 15 ml concentrate of tetanus proteins obtained from TEAE-cellulose column. Column size *c.* 450 ml (2·5 by 90 cm); elution with 0·1M sodium acetate, pH 5·5; flow rate 2 ml/cm²/hr. (*b*) Chromatography of tetanus toxoid proteins (methanol-precipitated) from DEAE-cellulose. Equilibrating buffer (*E*) 0·005M phosphate, pH 7·2; gradient (*G*) 0·01M phosphate buffer, pH 7·2, 0·05M NaH_2PO_4–0·5M NaCl; column size 40 ml (1·3 by 30 cm); *c.* 200 mg tetanus protein; flow rate 30 ml/hr.

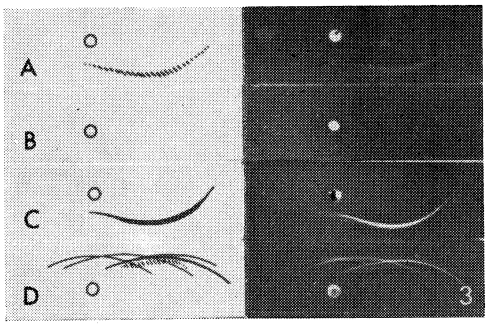
(*b*) *Tetanus Toxoid*

As in the case of tetanus toxin, the toxoid proteins were precipitated by 40% methanol and, when the fine buff-coloured precipitate was collected by sedimentation, it was found to contain more than 80% of the original flocculation units (L_f). On chromatography, as in the case of toxin, a small amount of the toxoid protein was not absorbed by the DEAE-cellulose. As mentioned above, when a pH–salt gradient similar to that used for the elution of the toxin was run, it was found that the protein began to elute in quantity only at the end of the gradient.

Figure 1(*b*) shows the elution pattern obtained when a gradient of higher ionic strength was used. Although many different protein species can be expected to be present in the crude toxoid, only one major peak with a pronounced shoulder was obtained in the gradient elution. The fractions comprising the main peak were combined (550–750 ml) and found to contain 60% of the flocculation units originally applied to the column. Some evidence of its purification was suggested by the decreased flocculation time: 22 min as against 75 min for the crude toxoid applied to the column. Additional evidence of its purification was obtained by immunoelectrophoresis which indicated the presence of one main and two minor components (Fig. 4). The remainder of the elution peak when bulked contained most of the colour and had only small and slow flocculating ability. In this fraction seven components were demonstrated by immunoelectrophoresis (Fig. 4).

Table 2 shows the type and amount of amino acids found combined in toxoids but released at pH 2·2 in toxoids prepared by methanol precipitation, by ultrafiltration, and by chromatography. These represent a cross-section of the amino acids that one would expect to be available in the culture medium. Considerable





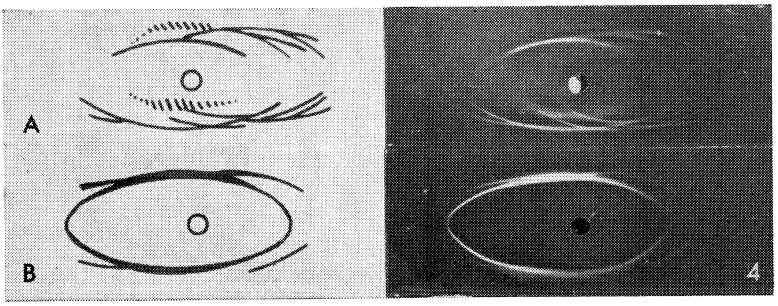


Fig. 2.—Sedimentation velocity pattern (lower pattern) found for the sample of tetanus proteins obtained by chromatography on TEAE-cellulose and used for Sephadex G-200 chromatography. Speed 59,780 r.p.m.; bar angle 60°; 20°C; protein concentration *c.* 1%; sedimentation value of main peak 6·5 S. Sedimentation from right to left.

Fig. 3.—Immunoelectrophoretic patterns given by tetanus proteins obtained from Sephadex G-200 chromatography [see Fig. 1(*a*)]. *A*, *B*, main and minor peaks, respectively, tested against sheep antitetanus globulins (specific for 7 S toxin). *C*, *D*, main and minor peaks, respectively, tested against horse antitetanus globulins (commercial product).

Fig. 4.—Immunoelectrophoretic patterns given by tetanus toxoids obtained by DEAE-cellulose chromatography [see Fig. 1(*b*)]. *A*, seven precipitin lines, given by bulked fractions from latter part of elution peak. *B*, three precipitin lines given by fractions from the central part of the elution peak (550–750 ml), the main precipitin band probably representing the tetanus toxoid species.

ammonia was also present in condensation. Although not large, the amount of amino acids and ammonia combined in the methanol-precipitated toxoid (1300 and 1900 n-moles/mg protein nitrogen) was much greater than that found in the ultrafiltered toxoid, (290 and 360 n-moles/mg protein nitrogen). In all cases the basic amino acids were predominant, though histidine was absent, tyrosine was present only in traces, and sulphur amino acids were present only as oxidized derivatives.

IV. Discussion

In the chromatography of tetanus toxin on DEAE-cellulose (Dawson and Mauritzen 1967) it was found that whereas the toxin was absorbed at pH 7·5 in 0·005M phosphate, it was fully eluted when the pH and the molarity of the salt gradient reached approximately 6·2 and 0·05M respectively, and that most of the

Table 2

AMINO ACIDS AND AMMONIA RELEASED FROM VARIOUS TETANUS TOXOIDS BY HYDROLYSIS AT pH 2·2

Amino acid concentrations are expressed as n-moles per milligram protein nitrogen

Amino Acid	Sample 1*	Sample 2	Sample 3	Sample 4	Sample 5
Lysine	147	201	28	21	46
Histidine					
Ammonia	681	939	258	204	509
Arginine	95	132	36	18	55
Cysteic acid	84	165	14	6	19
Aspartic acid	11	27	3	10	14
Threonine	5	3			1
Serine	9	5	1	6	3
Glutamic acid	10	9			4
Proline	36	45			1
Glycine	17	21	9	8	11
Alanine	21	27	1	1	6
½Cystine					
Valine	58	69	1		6
Methionine sulphoxide	21	36	3		4
Isoleucine	53	72	3	3	7
Leucine	66	93	4	4	8
Tyrosine	†	15			
Phenylalanine	†	45	4	6	9
Total	1310	1910	360	290	700
Lysine+arginine (%)	18	17	17	14	14
Ammonia (%)	52	49	71	71	72
Acidic+neutral amino acids (%)	30	34	12	15	13

* Samples 1–5 prepared as described in Section II(*e*).

† Abnormal peaks.

colour and the majority of the protein did not elute till the pH was lower and the ionic strength higher. From these observations one could expect a considerable purification of the methanol-precipitated toxin if it was prepared at pH 6·2 in 0·05M phosphate and subsequently passed through DEAE-cellulose prepared similarly. Under these conditions the eluate should contain predominantly tetanus toxin protein. It was also considered possible that by using a more basic absorbent such as TEAE-cellulose, conditions of pH less than 6·0 could be found which would allow maximum absorption of non-toxic tetanus proteins but also permit the tetanus toxin to be present in the eluate. By simple experimental trials, a pH of 5·9 and a molarity of 0·025M phosphate buffer was found to be suitable for this purpose. Since this semipurified tetanus toxin

was now suitable for molecular-exclusion chromatography, complete chromatographic separation below pH 6·0 was possible. As the isolation of 4 S toxin was sought, Sephadex G-100 was chosen. However, when it was found that most of the protein was excluded, Sephadex G-200 was used.

Although the 6·5 S pure tetanus toxin obviously made up the major part of the protein applied, the minor peaks of both columns were toxic but considerably less so than the pure toxin, the toxicity probably being due to the component with a sedimentation value of *c.* 2 S (Dawson and Mauritzen 1967). The overall result, therefore, of isolation of tetanus toxin by chromatography below pH 6·0 is similar to that found for DEAE-cellulose-prepared toxin, i.e. the major toxic component of tetanus toxin has a sedimentation value of approximately 7 S, and there is no evidence of a toxic component with a sedimentation value of 4 S.

By reason of its effectiveness, the use of tetanus vaccine is encouraged and tetanus toxoid has become an accepted component of the multiple vaccines used for the immunization of children. However, with the increased use of toxoid and subsequent booster dosing, the ability of tetanus toxoid to induce allergic reactions (particularly those local to the site of injection) has become manifest in recent years (Levine, Ipsen, and McComb 1961; Brindle and Twyman 1962; Schneider 1964). While the nature of these sensitizing antigens is unknown, it is possible that they occur in either the non-toxin bacterial proteins or in the sterilized basic media. In view of this, the need for the purification of tetanus toxoid is well recognized. One method of achieving this aim is to detoxify the pure toxin prepared by DEAE-cellulose chromatography (Dawson and Mauritzen 1967). However, it would be more practical and possibly quite adequate to prepare a less pure toxoid by using toxin prepared by TEAE-cellulose chromatography. When a purified toxin is used, a better control of the detoxification process and a better-defined product can be obtained than in the case of the crude preparation. It was necessary, however, to investigate the possibility of separating a purified toxoid from the crude product. Some degree of purification has been obtained by using Sephadex G-200 (Latham *et al.* 1965). To date no completely satisfactory method for its purification has been offered. In the experiments described here, despite careful chromatographic procedure with slow changes in pH and ionic strength, it was not possible to separate the individual bacterial proteins on DEAE-cellulose, presumably because condensation of formaldehyde with lysine residues had reduced differences in net charge between the individual proteins. The main tetanus toxoid activity, however, was present in the first part of the elution peak. When a portion of this peak was arbitrarily selected, it gave a product of considerable purification and represented approximately 50% of the flocculating units originally used. Since the method described above could be adapted readily to commercial application, it should be considered as an alternative to those methods already in use.

The interaction with proteins, amino acids, and comparable model systems is well documented (Levy and Silberman 1937; French and Edsall 1945; Fraenkel-Conrat and Olcott 1948*a*, 1948*b*; Milch 1965; Saidel, Satzman, and Elfring 1965) and therefore it can be postulated that when formaldehyde is added to the culture

filtrate, condensation of formaldehyde with the bacterial protein and with free amino acids will occur. On the other hand it would be expected that the free amino acid–formaldehyde compounds (methylolamines) would react with available sites on the protein to form amino acid–protein combinations by methylene linkages but would have to compete with methylene links formed internally. Further, when such combinations did occur it might be expected that these would be resistant to acid hydrolysis. However, at least in part, this does not appear to be the case. Linggood *et al.* (1963) found small amounts of condensed amino acids when detoxification of diphtheria toxin was carried out in the presence of amino acids. They released these acids by Soxhlet extraction with boiling water (this method of hydrolysis was found in earlier experiments to be a convenient substitute for hydrolysis with 0·1M HCl at 37 °C). They did not observe any release of the amino acids during the sample washing procedures.

In the present work, small amounts of condensed amino acids of the same order as found by Linggood *et al.* (1963) were released by hydrolysis at pH 2·2. Since these samples were commercially prepared toxoids, one might expect these amino acids to be a representative sample of the type available in the culture filtrates. The amount of amino nitrogen per milligram protein obtained from the methanol-precipitated samples was approximately four to five times that obtained in the ultrafiltered samples. The ultrafiltered samples differed from the material precipitated by methanol in that it had received more extensive dialysis at neutral pH. Although this evidence is indirect it does indicate that at least part of the amino acid combination is slowly reversible at neutral pH. Moreover, the differences in the ratios of bound basic amino acids plus ammonia to the acidic plus neutral amino acids occurring between methanol-precipitated and ultrafiltered toxoids (Table 1) indicate that the neutral and acidic amino acid combinations are more readily reversible than those of lysine and arginine and that ammonia is even more firmly bound. Whether this is due to stronger molecular bonding or due to ring closure resulting from bifunctional activity is not known. As might be expected from its preparation, the amount of bound amino acids obtained from the sample purified by chromatography was intermediate to that found from the two other sample types.

The absence of histidine, cystine, and small amounts of tyrosine in combination is at first surprising, considering the requirement of histidine peptides in media (Mueller and Miller 1956) and that tyrosine and cystine are added to the medium. The absence of these amino acids is probably due to their immobilization, by formation of internal methylene linkages with formaldehyde. The oxidation product cysteic acid is not able to do this and is, therefore, found condensed with the protein.

Although one of the objects of the present work was to attempt the direct isolation of purified tetanus toxoid from the formalin-treated culture filtrate, the results have suggested that the non-specific combination of formaldehyde with the bacterial proteins renders them less susceptible to fractionation than when in the native condition. The preparation of pure toxoid is, therefore, best achieved by prior isolation of pure toxin by a combination of methanol precipitation and ion-exchange chromatography.

V. Acknowledgments

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