STUDIES ON TETANUS TOXIN AND TOXOID

IV.* INTERACTION OF FORMALDEHYDE WITH TETANUS TOXIN

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Summary

The binding of formaldehyde with tetanus toxin under mild detoxification conditions (0·1M phosphate buffer, pH 7·0, 0·005–0·06M HCHO, 20–37°C) has been studied quantitatively by using [^{14}C]formaldehyde. When detoxification was followed with time (0·005M HCHO, 37°C) the toxin showed a characteristic immediate drop in lethality (~ 80%), followed by a slower detoxification, whereas the combination of the HCHO was a more gradual process. When a fixed time period was used (5 days, 20 and 37°C) at least 0·02M HCHO was required to destroy the lethality of the toxin towards mice, and at this concentration approximately 60 moles of HCHO were bound per mole of toxin. Fractionation of a partially detoxified toxin indicated the presence of a range of molecular species with varying formaldehyde binding and lethality.

The effect of introducing L-lysine and L-leucine into the formaldehyde–toxin interaction was also examined and the use of ^{3}H-labelled lysine or leucine allowed an estimate of the amount of amino acid bound to the toxin to be made. The chemical nature of the formaldehyde–toxin interaction was also studied indirectly by substituting poly-L-lysine and poly-L-glutamic acid for tetanus toxin in detoxification conditions. The results have been discussed in terms of the concepts of protein–formaldehyde interaction.

I. INTRODUCTION

For many years formaldehyde has been used successfully in empirical procedures to convert bacterial toxins, cultures, and viral agents into safe vaccines. However, the complexity of the resulting mixtures has made it difficult to determine the reasons for the potency variations that occur. In the case of protein toxins the difficulties can now be largely removed, since it is now possible to isolate purer and thus more specific agents and this permits study of the toxin–formaldehyde reaction under more defined conditions.

In the present study [^{14}C]formaldehyde has been used to determine quantitatively the amount of HCHO that is stably bound to pure tetanus toxin (moles per mole of tetanus toxin) when the conditions simulate those used in detoxification. The changes in the amounts of bound HCHO brought about by the introduction of amino acids in reaction mixtures were also determined, and the use of ^{3}H-labelled amino acids gave a measure of the amount of the amino acid condensation that occurred.

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Some relationships between the amount of bound HCHO and detoxification were also examined. Thus, in one set of conditions the decrease in lethality of a toxin sample was followed with time and was compared with the amount of stably bound HCHO that also occurred. In another experiment, the lethality of toxin molecules occurring in a partially detoxified toxin sample was compared with their amount of bound HCHO by examining samples obtained by fractionating the toxin on a DEAE-cellulose column. In order to examine some aspects of the chemical nature of the HCHO interaction with toxin occurring in detoxification conditions, experiments were carried out using poly-L-lysine and poly-L-glutamic acid as model compounds.

II. Materials and Methods

(a) *Materials*

All radioactive isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham. [^{14}C]HCHO was obtained as paraformaldehyde and HCHO (specific activity 4–15 mCi/m-mole) and was diluted appropriately (10–100-fold) with unlabelled HCHO (AnalaR) prepared in 0·1M phosphate buffer, pH 7·0. Before each experiment both labelled and unlabelled HCHO solutions were heated at 110°C for 1·5 hr in the absence of air, in order to ensure maximum conversion to monomer. When the buffered HCHO solution was dried in air at 100°C, 10% of the total radioactivity remained as residue; this was assumed to be [^{14}C]formate and appropriate corrections were made in all calculations. Experiments in which the [^{14}C]HCHO made up 0·6–10·0% of the total HCHO (0·005M) showed that the amount of radioactivity was proportional to the activity added. Similar results were assumed for all selected dilutions. [^{3}H]-L-lysine (specific activity 141 mCi/m-mole) and [^{3}H]-L-leucine (specific activity 1000 mCi/m-mole) were diluted 100- and 1000-fold respectively with unlabelled L-lysine and L-leucine (BDH).

Poly-L-glutamic acid (lot GL50), apparent molecular weight 90,000, and poly-L-lysine HBr (lot LY36), apparent molecular weight 41,000, were obtained from YEDA (Rehovoth, Israel).

A solution of pure tetanus toxin was prepared as previously described (Dawson and Mauritzen 1967). Briefly, the method involved a precipitation of the crude toxin with methanol at low temperature from the culture filtrate prepared at pH 5·0. After preparation, the crude toxin was absorbed on a column of DEAE-cellulose and eluted by a pH–salt gradient (0·01M phosphate buffer, pH 7·2–0·05M NaH_2PO_4 and 0·05M NaCl). Tetanus toxin, chromatographically purified as above, exhibited a single precipitin band on immunoelectrophoresis, and had a toxicity (towards mice) of 7×10^7 MLD per milligram protein nitrogen and a flocculation value of $2 \cdot 7 \times 10^3$ L_f units per milligram protein nitrogen.

(b) *General Reaction Conditions*

All reactions were carried out in 0·1M phosphate buffer, pH 7·0, containing either 0·055% of tetanus toxin (200 L_f/ml) or 0·05% of polyamino acid. The molarity of the HCHO and amino acids were varied as described in the text. The reactions were performed for various times and temperatures in sealed 1-oz McCartney bottles, the total reaction volume varying from 4·5 to 10·0 ml. The reaction mixtures were transferred to dialysis sacs and dialysed against 25 volumes of buffer for 4 days at 0°C, with twice-daily changes of buffer. After this period the amount of radioactivity detectable in the dialysate had fallen to a negligible level, showing that all free HCHO or amino acid had been removed during dialysis. In general two dialysis buffers were compared, sodium phosphate buffer (0·1M, pH 7·0, containing 0·5% glycine) for neutral dialysis, and sodium citrate buffer (0·1M, pH 2·2, containing HCl) for acid dialysis. After acid dialysis the samples were subsequently prepared for radioactivity measurements by further dialysis against the neutral glycine–phosphate buffer. Any variation in the buffers is mentioned in the text.

(c) *Protein and Polyamino Acid Concentrations*

The extinction coefficient ($E^{1\%}_{1cm} = 9 \cdot 4$) of tetanus toxin at 280 nm was determined experimentally and was then used to determine protein concentrations. Only small changes in

the original concentration occurred during the dialysis procedure. Since the increase in the bound HCHO or amino acid brought about no consistent changes in absorbance, it was assumed that the bound ligand did not alter significantly the extinction coefficient. The solutions of poly-L-glutamic acid and poly-L-lysine were prepared by weighing the dry material.

(d) Toxicity and Flocculation Values

The relative changes in toxicity of the toxin solution after the addition of HCHO were measured by determining the minimum dose lethal to mice within 4 days (MLD/ml). However, less than 1 MLD per injection does not necessarily indicate complete detoxification. Complete detoxification in toxoids was assumed when an injection of 20 L_f units (approximately equivalent to 5×10^4 MLD of pure toxin) in mice had no apparent effect on their weight gain or health within 21 days. Where a partially detoxified toxin was fractionated on DEAE-cellulose, the differences in toxicity between the fractions were examined by challenging groups of 5 mice with a dose range of 120, 60, 30, and 15 μg of protein (approximately equivalent to 40, 20, 10, and 5 L_f units) and keeping them under observation for 30 days. The flocculation values were determined by the technique of Dean and Webb (1926) as previously described (Dawson and Mauritzen 1967), and these were maintained during the HCHO treatments within the expected error of the test ($\pm 10\%$).

(e) Radioactivity Measurements

Two methods were used to measure radioactivity. In some experiments [^{14}C]HCHO was counted on planchets using a Nuclear Chicago gas-flow counter, model 183B, fitted with a "micromil" window. Since most of the HCHO in the reaction mixture was volatile, it was not possible to estimate the total HCHO by simple transfer of the mixture to the planchet. The HCHO of the sample (0·5 ml) was therefore converted to non-volatile sodium formate on the stainless steel planchet by treating the sample with 0·1 ml of a mixture (1:1) of 0·1M NaOH and 30% (v/v) H_2O_2 before taking to dryness.

In experiments where both ^{14}C and ^{3}H were measured, an Ansitron liquid scintillation spectrometer was used. The radioactivity of the samples containing the [^{14}C]HCHO was counted by mixing a 0·5 ml sample with 4 ml of methyl cellosolve and 5 ml of toluene containing the fluor [6·0 g of 2,5-diphenyloxazole and 0·15 g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene per litre of toluene]. For samples containing the ^{3}H-labelled amino acids, the protein was first digested by adding HCl (0·5M, 0·5 ml) to the sample (0·5 ml) in the scintillation vial and heating to dryness on a boiling water-bath. The addition and removal of acid was repeated three times. The residue was dissolved in formic acid (99%, 0·2 ml) and diluted with methyl cellosolve (2·0 ml) and toluene (5·0 ml) containing the fluor. All radioactivity measurements were corrected for background. Since within each system the samples were kept essentially identical in composition, the amount of quenching was constant. The efficiency of counting was 85 and 15% for ^{14}C and ^{3}H respectively.

(f) Calculations

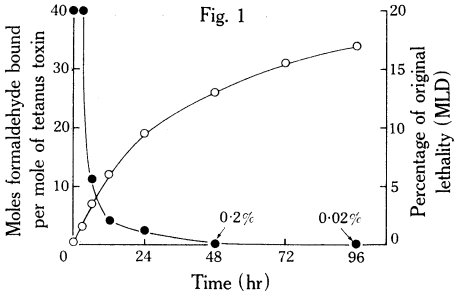
The amount of HCHO or amino acid bound to the protein in the 0·5 ml sample was calculated (as moles) by multiplying the number of moles of HCHO or amino acid in the original reaction mixture by the proportion of radioactivity retained after dialysis. By taking the molecular weight of tetanus toxin as 176,000 (Dawson and Nichol 1969), and accepting that of poly-L-glutamic acid and poly-L-lysine as 90,000 and 41,000 respectively, an estimate of the number of moles of HCHO or amino acid bound per mole of macromolecule was obtained.

III. Experimental Details and Results

(a) Rate of Binding of Formaldehyde to Tetanus Toxin and Resultant Detoxification using 0·005M Formaldehyde

Two tetanus toxin solutions in phosphate buffer which were made 0·005M with respect to HCHO, and in one case also 0·005M with respect to glycine, were incubated at 37°C. At various times from 0 to 96 hr samples were withdrawn, chilled

to slow down the reaction, and the excess HCHO removed by dialysis against phosphate buffer, pH 7. The presence of glycine did not appear to affect either the incorporation of HCHO or the rate of detoxification. Figure 1, in which the average number of moles of HCHO bound per mole of toxin are plotted against time, shows that the incorporation was not linear but fell off with time, being fairly slow after 96 hr. Figure 1 also shows the percentage of original toxicity remaining after various times and shows that there was a rapid fall in toxicity when only a few moles of HCHO had been bound. However, the residual toxicity took more than 4 days to disappear.



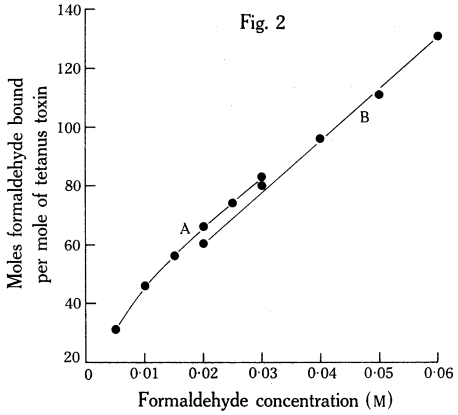


Fig. 1.—Incorporation of HCHO into tetanus toxin with time (○) and the associated decrease in lethality (●). Conditions: tetanus toxin (0·55 mg/ml) in phosphate buffer (0·1M, pH 7·0) containing 0·005M HCHO and held at 37°C.

Fig. 2.—Increase in HCHO bound per mole of toxin occuring with increase in HCHO concentration. Conditions: tetanus toxin (0·55 mg/ml) in phosphate buffer (0·1M, pH 7·0) containing HCHO. Solutions held 2 days at 20°C and 3 days at 37°C. *A*, planchet counting; *B*, scintillation counting.

(*b*) *Effect of Formaldehyde Concentration on the Extent of Combination of Formaldehyde and the Stability of the Bound Formaldehyde*

Preliminary experiments had shown that complete detoxification of tetanus toxin in phosphate buffer could be obtained if the solutions were made 0·02M or more with respect to HCHO and held first for 2 days at 20°C and then for 3 days at 37°C. In order to study the combination of HCHO at concentrations around those required for detoxification, similar reaction mixtures were prepared in which the concentration of HCHO was varied between 0·005 and 0·03M. In earlier experiments it had been assumed that the loosely bound HCHO would be removed during dialysis and that only stably bound HCHO would be measured. To demonstrate the existence of any variation in the stability of the bound HCHO, samples were dialysed against one of three buffer solutions: (1) 0·1M phosphate, pH 7·0; (2) 0·1M phosphate, pH 7·0, containing 0·5% glycine; (3) 0·1M citrate–HCl, pH 2·2. As there were no significant differences in the radioactivity of the samples prepared by the three washing procedures, it would appear that the HCHO still present after neutral dialysis is bound quite strongly.

Figure 2, curve *A*, records the average of estimates of bound HCHO (moles per mole of toxin) obtained using the different HCHO molarities. Since the toxin treated at the lower HCHO concentrations was irreversibly precipitated when treated at pH 2·2, the solutions were clarified by centrifugation. The percentages of soluble protein recovered after 0·005, 0·01, 0·015, 0·02, 0·025, and 0·03M HCHO treatments were 39, 84, 90, 96, 100, and 100% respectively, which indicates that the amounts of HCHO bound after treatments with HCHO of concentrations of 0·02M or more prevent precipitation at pH 2·2. In another experiment using similar conditions, the HCHO concentrations were varied from 0·02 to 0·06M in order to cover the range commonly used in commercial detoxification; these samples were only dialysed against glycine–phosphate buffer. The results are given in Figure 2, curve *B*. It will be noticed that the increase in the amount of bound HCHO is approximately linear with respect to HCHO concentration over the range studied, and using 0·06M the amount bound reaches 131 moles HCHO per mole of toxin.

(*c*) *Fractionation of a Partially Detoxified Tetanus Toxin and Variation of Toxicity with the Distribution of Bound Formaldehyde*

Previous work (Dawson and Mauritzen 1968) has shown that the elution pattern of tetanus toxin from DEAE-cellulose is altered on its complete conversion to toxoid, a much higher ionic strength being required for elution of the toxoid. One would expect that the specific ionizing characteristics determining the affinity of the toxin for DEAE-cellulose will be altered according to the extent of reaction of ionizing side chains with HCHO, and it follows that it should be possible to fractionate toxin molecules which have reacted with HCHO to different extents. Tetanus toxin (20 mg) in phosphate buffer containing HCHO (0·01M) was allowed to react for 2 days at 20°C and 3 days at 37°C. The excess HCHO was removed by dialysis against 0·01M phosphate buffer (pH 7·2) and the partially detoxified toxin was absorbed on a 10-ml DEAE-cellulose column equilibrated in the dialysis buffer. Elution was carried out using a salt gradient prepared by introducing a solution of NaH_2PO_4 (0·05M) and NaCl (0·5M) into a constant-volume reservoir containing phosphate buffer (pH 7·2, 0·01M, 60 ml). The toxoid peak was collected in five fractions and the protein content, radioactivity, and toxicity determined on each. Toxicities were determined by injecting 120, 60, 30, and 15 μg of protein from each fraction into mice (using groups of five mice for each assay) and examining the death response over 30 days. The results given in Table 1 show that, in the reaction mixture used, the molecular distribution of the bound HCHO was not very wide and that toxicity decreased as the amount of bound HCHO increased.

(*d*) *Binding of Formaldehyde and Amino Acids during the Detoxification of Tetanus Toxin*

Tetanus toxin solutions, both with and without added amino acids, were made 0·02M with respect to HCHO and were held at 20°C for 2 days and 37°C for 3 days. The amino acids were L-lysine and L-leucine at concentrations of 0·01 and 0·04M. Where estimates of both HCHO and amino acid were required from the reaction mixture, the experiment was duplicated, one containing [^{14}C]HCHO and unlabelled amino acid, and the other containing [^{3}H]amino acid and unlabelled HCHO. All

reaction mixtures were divided and dialysed respectively against phosphate–glycine and citrate buffers. The estimates for the bound HCHO and amino acids per mole of toxin are given in Table 2.

TABLE 1

BOUND FORMALDEHYDE AND LETHALITY IN SAMPLES OF PARTIALLY DETOXIFIED TETANUS TOXIN ISOLATED BY CHROMATOGRAPHY ON DEAE-CELLULOSE

Tetanus toxin (0·55 mg/ml) in phosphate buffer (0·1M, pH 7·0) reacted with HCHO in conditions described (see text)

	Fraction				
	1	2	3	4	5
Volume (ml)	5	5	4·5	4	7·5
Protein content (mg/ml)	0·32	1·45	1·37	0·57	0·21
Moles HCHO bound per mole of toxin	47	52	54	58	55
Day of death of mice (av.) at dose of:					
120 μg	6	7	10	10	10
60 μg	7	10	10·5	11·5	10·5
30 μg	7·5	9	11	13·5	12
15 μg	7·0	9·5	10	19	19·5

TABLE 2

BINDING OF FORMALDEHYDE, L-LEUCINE, AND L-LYSINE TO TETANUS TOXIN AT DETOXIFICATION

Tetanus toxin (0·55 mg/ml) in phosphate buffer (0·1M, pH 7·0) reacted with HCHO in presence or absence of leucine or lysine in conditions described (see text). Excess reagent was removed by dialysis against buffer at pH 2·2 or 7·0

HCHO Concn. (M)	Amino Acid Addition	Moles HCHO Bound per Mole of Toxin		Moles Amino Acid Bound per Mole of Toxin	
		Acid Dialysis	Neutral Dialysis	Acid Dialysis	Neutral Dialysis
0·02	None	59	57	—	—
0·02	Lysine, 0·01M	46	46	7	8
0·02	Lysine, 0·04M	44	45	15	16
0·02	Leucine, 0·01M	60	60	12	12
0·02	Leucine, 0·04M	66	63	41	40

The binding of the HCHO was not altered significantly from the control by the presence of leucine at either 0·01 or 0·04M, but in the presence of lysine the binding was slightly reduced, although similar at both concentrations of the amino acid. The increase of amino acid concentration from 0·01 to 0·04M increased the amount of bound amino acid twofold with lysine and threefold with leucine. The binding of

amino acids appeared equally stable in neutral and acid buffers and the radioactivity of the final diffusates gave no evidence that the amino acids were being slowly released. In an earlier paper (Dawson and Mauritzen 1968), coupled amino acids had been released from crude toxoids by dialysing against citrate–HCl buffer containing 0·1% phenol at 37°C. The stability of the bound amino acids was tested therefore in some samples by similar treatment. The toxoid was irreversibly precipitated by the treatment but, whereas the bound leucine was essentially unchanged (7% loss), in the case of bound lysine 43% of the radioactivity was lost after acid treatment and dialysis, and this perhaps indicates a greater instability of binding.

(*e*) *Nature of the Formaldehyde–Toxin Bond: Experiments with Poly-*L*-lysine and Poly-*L*-glutamic Acid*

It is generally accepted (French and Edsall 1945; Fraenkel-Conrat and Olcott 1948*a*, 1948*b*; Blass 1964) that the HCHO reaction with protein involves, at least in part, the rapid conversion of the available ϵ-amino groups of lysine to unstable methylolamine compounds which in turn slowly cross-link to form stable methylene linkages.

In an attempt to demonstrate the presence of methylolamine compounds in the reaction mixture, tetanus toxin in phosphate buffer was treated at 37°C with 0·01M HCHO containing [^{14}C]HCHO. Samples were taken at various times and applied to G50 Sephadex columns equilibrated in phosphate buffer and 0·01M unlabelled HCHO. The bulk of the radioactivity, that is the unreacted HCHO, separated distinctly from the protein. The radioactivity of the protein fraction gradually increased with time and was only of an order expected for dialysed samples at the reaction time concerned. The experiment therefore gave no evidence of an immediate large formation of methylolamine compounds.

To demonstrate the formation or otherwise of certain bonds under detoxification conditions, viz. methylene bonds between two amino groups and peptide bond methylolimide, poly-L-lysine and poly-L-glutamic acid were substituted for tetanus toxin and were treated under similar conditions with HCHO. In the case of poly-L-lysine the reaction was apparently rapid since it almost immediately precipitated from solution and thus very likely restricted further reaction. The reaction mixtures were divided and dialysed against neutral and acid buffers.

[^{14}C]HCHO bound to poly-L-glutamic acid was measured by standard procedures. With poly-L-lysine, samples of the suspensions were prepared for counting by digestion in alkaline peroxide, taking to dryness, dissolving in 0·2 ml of formic acid, and finally adding 2 ml of methyl cellosolve and 5 ml of toluene containing the fluor.

The results are given in Table 3. The results of the experiments with poly-L-lysine indicate that some of the HCHO was bound in a form which is less stable to acid than to neutral dialysis. A further indication of a weaker binding was that, in contrast with all other experiments, an increase of radioactivity was found in the final diffusates, indicating slow release of HCHO and therefore reversal of the reaction. The amount of HCHO bound to poly-L-glutamic acid was small and therefore the formation of methylolimides of the peptide bond under the above conditions of test is doubtful. However, it was in excess of that expected of end-group reaction and increased slightly on increasing the HCHO concentration. To test the possibility of

condensation of methylolimides with other amino groups, lysine (0·01M) was added to a poly-L-glutamic acid mixture (containing 0·02M HCHO). In the presence of lysine the binding of HCHO was unaffected and 2 moles of lysine were bound per mole of poly-L-glutamic acid. This is of an order expected of amino-terminal reaction.

TABLE 3

BINDING OF FORMALDEHYDE TO POLY-L-LYSINE AND POLY-L-GLUTAMIC ACID IN DETOXIFICATION CONDITIONS

Polymer (0·5 mg/ml) in phosphate buffer (0·1M, pH 7·0) reacted with HCHO in conditions described

HCHO Concn. (M)	Dialysis Treatment	Moles HCHO Bound per Mole of Macromolecule	
		Poly-L-lysine	Poly-L-glutamic Acid
0·02	Acid	42	5; 5*
0·02	Neutral	49	6; 7*
0·05	Acid	61	7
0·05	Neutral	69	9

* Reaction carried out in presence of 0·01M L-lysine.

IV. DISCUSSION

The reaction of HCHO with proteins and model compounds has been extensively studied and discussed (French and Edsall 1945; Fraenkel-Conrat and Olcott 1948*a*, 1948*b*; Blass 1964; Saidel, Satzman, and Elfring 1965). Three groups of observations are of importance in considering the reactions of HCHO with protein under the mild conditions used in detoxification of tetanus toxin. Firstly, non-protonated amino groups of amino acids rapidly combine with HCHO under mild reaction conditions to form methylolamines which are unstable to dialysis and acid pH (French and Edsall 1945). Secondly, under similar conditions methylolamines combine slowly with the amide, guanidyl, phenolic, or imidazole groups of amino acids to form methylene links which are resistant to dialysis or acid pH (Fraenkel-Conrat and Olcott 1948*a*, 1948*b*). Thirdly, although Fraenkel-Conrat and Olcott (1948*a*, 1948*b*) were unable to demonstrate direct combination of HCHO with the above functional groups under these conditions, amide and peptide groups have been shown to react with HCHO under more rigorous conditions to form acid-stable compounds (Saidel, Satzman, and Elfring 1965). Consequently, we could assume that most of the reactions occurring under our mild conditions of HCHO treatment of toxin would be restricted to the first two types, and that the HCHO bound to the toxin after dialysis would be mainly in the methylene form.

Direct application of results from model systems to proteins can be questionable, but two comments may be made from our observations. At neutral pH, most of the free amino groups could be expected to be in the protonated form and, in addition, not all are necessarily available for immediate reaction, although they may become

available with time and as the reaction proceeds. Indeed, this could be the explanation for the failure to observe evidence of methylolamine formation in the separation of a reaction mixture on Sephadex, although this result could also be explained by a rapid exchange of combined labelled HCHO with the unlabelled HCHO in the buffer. Moreover, after 5 days of treatment it was observed that the increase in the amount of bound HCHO was linear with respect to HCHO concentration and did not fall off as the number bound approached the total number of lysine residues (126) in the toxin molecule (which is essentially the maximum number of methylolamine groups possible). This suggests that products of monofunctional reactions of HCHO, in addition to methylolamines, are produced in toxin under the reaction conditions used for detoxification, and these make up a proportion of the bound HCHO stable to acid dialysis.

One reaction of this type, viz. the reaction of HCHO with the peptide bond, was examined using poly-L-glutamic acid as a model. HCHO reaction with this compound can occur only with the terminal amino or the peptide group. The number of HCHO groups bound per mole of poly-L-glutamic acid, although in excess of the amino-terminal end-groups, was small, and this estimate depends upon the weight average molecular weight given by the YEDA Co. More credence may be placed on the fact that there was a slight increase in the amount of bound HCHO with increase in the molarity of the HCHO and therefore it is possible that peptide bonds do react slowly with HCHO under detoxification conditions.

In the reaction of HCHO with poly-L-lysine, combination could be expected to be restricted mainly to methylolamine and methylene link formation with the side-chain amino groups. There was, in fact, a large uptake of HCHO, but this fell short of the number expected if all the amino side chains were available for reaction. It is presumed that all methylolamine compounds were hydrolysed during the dialysis procedure and that only those which subsequently reacted further to produce methylene cross-links survived dialysis treatment. Thus, although Fraenkel-Conrat and Olcott (1948*a*) were unable to demonstrate the formation of such cross-links using model compounds, it seems likely that such links will form within protein molecules where side chains are suitably orientated.

On complete detoxification of tetanus toxin (i.e. treatment with 0·02M HCHO under the prescribed conditions) there were approximately 60 moles of bound HCHO per mole of toxin and this value may be compared with those found in the presence of amino acids. When leucine is added, the amino acid, or perhaps its methylolamine derivative, could be expected to combine with the methylolamine derivatives of the lysyl side chains of the protein and perhaps also with amide, phenolic, or imidazole amino acid side chains (Fraenkel-Conrat and Olcott 1948*a*, 1948*b*). The presence of leucine might therefore be expected to bring about a reduction in cross-linking and also an increased uptake of HCHO. However, the addition of leucine to the reaction mixture did not, in fact, increase the binding of HCHO to any appreciable extent. The addition of lysine to the reaction mixture should cause similar reactions to those expected for leucine, but in addition one might expect more extensive cross-linking. However, added lysine decreased the number of HCHO molecules bound to the toxin. It is important to note that in the case of both leucine and lysine the concentration of amino acid does not appear to influence significantly the binding of HCHO. One

explanation is to postulate that the concentration of the methylolamine of the α-amino group is too small in the reaction conditions to be an effective reactant and that the methylolamine of the ϵ-amino group of the protein lysyl groups controls the reaction. The reduced binding in the case of lysine could be due to an increased lability of an amine $\rightarrow$ amine methylene bond which may be inferred indirectly in the work of Fraenkel-Conrat and Olcott (1948*a*) and which has been indicated experimentally in our observations.

It has already been demonstrated that crude toxoids prepared by adding HCHO to culture filtrates couple to the amino acids present (Linggood *et al.* 1963; Dawson and Mauritzen 1968). Linggood *et al.* (1963) have also shown that the immunological properties of a purified diphtheria toxoid can be varied by coupling different amino acids. The present work indicates some of the chemical variation that may occur in such circumstances and allows a tentative interpretation of their results. Thus the increase in potency of the diphtheria toxoids brought about by the introduction of amino acids may be due to a reduction in the conformational rigidity and a substitution of weaker linkages for the stable Mannich-type linkages presumed to be normally occurring (Fraenkel-Conrat and Olcott 1948*b*; Blass, Bizzini, and Raynaud 1967). The decrease in storage stability of the glycine–toxoid may be due to reduced cross-linking, and finally the better absorption of the lysine–toxoid on $AlPO_4$ may be due to the introduction of free ionizing groups.

When a partially detoxified reaction mixture was fractionated on DEAE-cellulose, the five fractions ranged in value from 47 to 58 moles of HCHO per mole of toxin and exhibited accordingly decreasing toxicity. It would be expected that this degree of reaction would change considerably the affinity of the toxin for DEAE-cellulose and that unreacted toxin would separate from it. Moreover, the toxicity in even the maximum mouse doses (120 μg) would represent less than 10^{-5} μg of toxin. It is therefore concluded that the residual toxicity shown by the toxoid was not due to free toxin but to molecules which had greatly reduced effectiveness in toxin action.

One interesting feature of the present work was that when the detoxification of tetanus toxin was followed with time, there was a very rapid fall in the lethality of the toxin when only a few moles of HCHO had been bound; thereafter the residual lethality was only slowly lost although the amount of bound HCHO increased greatly. This characteristic has been demonstrated by other workers using crude culture filtrates (Lowenstein 1909; Cheyroux 1954). One possible explanation is that combination of HCHO with the toxic site considerably reduces toxicity but that complete loss of lethality of the toxin molecule only occurs when there is cross-link formation and an increase in conformational rigidity. In this connection, it is perhaps noteworthy that the toxoid is more stable (i.e. no longer forms an irreversible precipitate at pH 2·2) after treatment with HCHO concentrations of 0·02M or greater.

Although the foregoing experiments have investigated the extent of the reaction of HCHO with toxin during detoxification, they have provided very little information as to the nature of detoxification. However, we feel it is reasonable to assume that detoxification will depend on one or more of the following effects: (1) the reaction of HCHO with one or more groups of the toxic site; (2) the conformational rigidity

induced in the molecule by cross-linking; and (3) the ability of the animal species to remove bound formaldehyde and "retoxify" the molecule prior to or during its catabolism.

V. Acknowledgments

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