CXII. NOTE ON THE POSSIBLE MECHANISM OF DIPHTHERIA TOXOID FORMATION.

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When diphtheria toxin is incubated with formaldehyde the toxic properties disappear [Glenny and Sudmersen, 1921], leaving the antigenic function practically unimpaired [Ramon, 1925, 1928]. In these laboratories toxins have been prepared with an M.L.D. as low as 0·0005 cc. and Lf. of 0·024 cc. and after incubation with 0·4 % formalin the M.L.D. is considerably greater than 5 cc. whilst 90 % of the antigenic activity (as measured by the flocculating power) is retained.

The mechanism of this process is of considerable interest both from the theoretical and practical points of view since toxoid is largely used in the preparation of diphtheria antitoxin and is being employed increasingly in prophylaxis.

Formaldehyde reacts with amino-groups forming methylimino-compounds. It appears possible therefore that the toxoiding process is due to the removal of free amino-groups in the toxin which are responsible for toxicity but play no part in eliciting antibodies or combining with antitoxin.

Kissin and Bronstein [1928] claim that all free NH_2-groups in the toxin molecule are combined during the toxoiding process, but later [1930] modify this view and claim that one-half of the free NH_2-groups disappears when the toxin is treated with 0·4–0·5 % of formalin. They conclude therefore that there are two sets of amino-groups in the toxin molecule. One set is responsible for toxicity and, conveniently, disappears during the toxoiding process; and the other set, responsible for combining with antitoxin and eliciting antibodies, remains unattacked by formaldehyde.

Experiments are described in this communication to test this and other hypotheses which might explain toxoid formation.

Experimental.

Method of toxoid preparation.

Directions for preparing diphtheria toxoid given by various authors are as follows: the toxin is incubated at 36 to 42° with 0·3 to 0·5 % of formalin for periods ranging from 2 weeks to 2 months.

In these laboratories the following standard conditions have been found most suitable.

0·4 % of 40 % formaldehyde is added to the toxin and the mixture is incubated at 38° in a hot room for 4–6 weeks. The loss of antigenic power (as measured by flocculation methods) is of the order of 10 % only, and the toxoid produces no local or general reaction when inoculated subcutaneously in 5 cc. doses into 300 g. guinea-pigs.

To give a typical example: a 5 litre batch of toxin (M.L.D. 0·0005 cc., Lf. 0·024 cc.) was incubated at 38° with 20 cc. of 40 % formaldehyde (A.R.) for 6 weeks. 5 cc. were then inoculated subcutaneously into a 300 g. guinea-pig. No local swelling or other symptom was observed and the animal steadily gained weight. The Lf. was now 0·028 cc. Thus the toxicity had diminished at least 20,000 times, whilst 90 % of the flocculating power was retained.

The following experiment shows the effect of varying the conditions of the toxoiding process.

Original toxin, Lf. 0·09 cc., 5-litre batches used; after 4½ weeks' incubation under the conditions described, subcutaneous inoculations of 5 cc. were made into 300 g. guinea-pigs with the results shown.

Table I. *Effect of amount of formalin added on toxoid formed.*

(4½ weeks' incubation at 38°)

Formalin added %	Effect on guinea-pig: Change in weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
0·3	− 5	Large firm
0·4	+ 15	Trace
0·5	+ 25	Practically nil

Table II. *Effect of temperature of incubation on toxoid.*

(0·4 % formalin)

Temp. ° C.	Effect on guinea-pig: Weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
36	− 35	Extensive firm
38	+ 15	Trace

Table III. *Effect of* p_H *after addition of formalin.*

(0·4 % formalin at 38°)

p_H when incubation commenced	Effect on guinea-pig: Weight after 1 week (g.)	Effect on guinea-pig: Amount of local swelling
8·0	± 0	Practically nil
7·3	+ 15	Trace

In another experiment one batch of toxin was incubated with 0·4 % of formalin for 6 weeks at 40–41° and another batch at 38°. In this case practically no difference was observed between the effects on guinea-pigs of toxoids prepared at 38° and 40°, but whilst the flocculating titre of that prepared at 38° had dropped some 8 %, that incubated at 40° had fallen nearly 25 %. It is

inadvisable and unnecessary therefore to incubate at temperatures higher than 38°.

Amino-nitrogen of toxins.

Veal or horse-flesh infusion broth containing 2 % of Difco proteose peptone is used for toxin production. The amino-nitrogen of the medium (determined by the Van Slyke and Sørensen methods) varies between 0·065 and 0·1 %. The toxin filtrates prepared by 8–10 days' growth of Park-Williams 8 strain of *C. diphtheriae* have generally a slightly higher content of amino-nitrogen, but lower values than those of the original medium have been observed. The limits observed have been 0·06 % and 0·11 % of amino-nitrogen.

In view of Hartley and Hartley's [1922] observation of the deleterious effect of overheating the medium required for toxin production, comparisons were made of toxin production in broths sterilised by autoclaving and by candle-filtration respectively. Batches of broth were divided into two parts, both being treated in precisely the same way, save that one part was autoclaved and the other filtered through a candle to effect sterility. In each case better toxin production and higher amino-nitrogen content were observed in the batches which had not been autoclaved. No direct relationship, however, could be traced between toxin production and the amino-nitrogen content of the medium.

Table IV. *Effect of method of sterilisation of broth on properties of toxic filtrate.*

	Filtered		Autoclaved	
Batch no.	Flocculation units per 1 cc.	Amino-nitrogen %	Flocculation units per 1 cc.	Amino-nitrogen %
821	42	0·10	18	0·05
824	28	0·09	8	0·06

Action of formaldehyde on amino-compounds.

Amino-groups are determined in the Sørensen method by addition of excess of formaldehyde and titration of the acidity developed. The acid liberated is quantitatively equivalent to the amino-nitrogen combined.

Differential Sørensen titrations were performed in the following way. A small quantity of formaldehyde (approximately the theoretical quantity required to combine with all the amino-groups present) was added to the amino-compound and the acidity developed was titrated; increasing quantities of formaldehyde were then added and the acid liberated was determined after each addition.

In the following tables are given typical results obtained with a simple amino-acid (glycine), peptone, peptone infusion broth, a diphtheria toxin filtrate and a detoxified (formalinised) toxoid: 10 cc. of the neutralised solution were used in each case.

Table V. *Differential Sørensen titrations.*

40 % HCHO added (cc.)	0·1 *N* NaOH required to neutralise (cc.)		
	0·5 % glycine	2 % peptone	2 % peptone infusion broth
0·05	1·0	0·70	1·1
0·10	2·0	1·13	2·1
0·20	3·4	1·45	3·2
0·40	4·3	1·75	3·8
0·80	5·1	2·00	4·2
1·60	5·8	2·25	4·6
3·20	6·4	2·55	5·1

The equilibrium constant $K = \frac{[\text{glycine}]\,[\text{HCHO}]}{[\text{methylimino-compound}]}$ may be calculated from the above data. The mean value is 0·28 and divergences from this value are relatively small considering the nature of the experiments (limits 0·20 to 0·32).

Table VI.

Diphtheria toxin T. 800; Lf. 0·053 cc.		Toxoid F.T. 800; Lf. 0·056 cc.	
40 % HCHO added (cc.)	0·1 *N* NaOH to neutralise (cc.)	40 % HCHO added (cc.)	0·1 *N* NaOH to neutralise (cc.)
0·05	1·0	(0·04)*	(0)*
0·10	2·5	0·10	0·8
0·20	3·9	0·20	2·2
0·40	4·7	0·40	3·3
0·80	5·3	0·80	4·2
1·60	5·9	1·60	4·8
3·20	6·6	3·20	5·3

* Already present.

When the quantity of formaldehyde added is the amount theoretically sufficient to combine with all the amino-nitrogen present (usually about 0·05 cc. of 40 % HCHO added to 10 cc.) it is found that only some 15 % of the amino-nitrogen is combined, leaving about 85 % free. A very large excess of formaldehyde (over 6000 %) must be added before an approach to quantitative combination occurs. In the toxoiding process the amount of formaldehyde added (0·4 to 0·5 % of 40 % HCHO) is approximately the theoretical quantity to combine with all the amino-nitrogen. It is found, however, that even in the completely detoxified toxoid which has been incubated with formaldehyde for 6 weeks 85 % of the amino-nitrogen remains free and uncombined.

In Fig. 1 are plotted the results obtained with toxin T. 800 and the corresponding toxoid F.T. 800. It will be seen that the total titratable free amino-nitrogen of the toxoid is approximately 85 % of that of the toxin.

Furthermore, the same result has been obtained with every toxin and toxoid examined, and, not only so, but also with simple amino-compounds such as glycine, with peptone and with the medium in which the organisms are grown. It seems likely therefore that the same would be found true of the pure diphtheria toxin itself if it could be isolated. The same result was obtained when the Van Slyke method of determining amino-nitrogen was used in place

of the Sørensen titration method. It seems justifiable to assume therefore that only about one-sixth or one-seventh of the amino-groups in toxin disappear during the toxoiding process and that toxoiding may proceed by some altogether different mechanism.

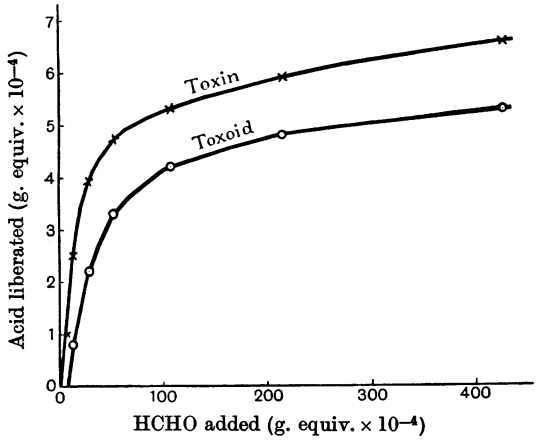

Fig. 1. Differential Sørensen titrations

This result is completely at variance with the conclusions of Kissin and Bronstein [1928, 1930] who claim that exactly 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process. Their experiments receive attention in the next section.

Acid titration curves.

Kissin and Bronstein's [1928, 1930] experiments were conducted as follows. Samples of toxin (5 cc.) were neutralised to litmus (Point I), *N* HCl was then added until the maximum precipitation was observed (Point II), then a further quantity of HCl was added until the reaction was acid to tropaeolin (Point III). If x cc. of acid were required to pass from Point I to II and y cc. to pass from II to III, the ratio x/y is known as the "acid quotient." The authors claim that the "acid quotient" of broth or of fresh diphtheria toxin is 2, whilst that of detoxified toxoid approximates to 1.

If attention is confined for the moment to the technical aspect of these experiments, a number of criticisms may be made. The use of litmus as a neutral point indicator is inadvisable owing to the wide range of colour change and the impossibility of accurate, reproducible readings. The point of maximum precipitation (II) is again not particularly well defined, there is a considerable range over which differences in precipitation are difficult to detect, and finally point III, the change of colour of tropaeolin (probably tropaeolin 00), is again susceptible to the inaccuracies involved in the observation of colour change. There is therefore considerable scope for errors in this titration method, particularly since the final "acid quotient" is the ratio of two amounts obtained by difference. A discrepancy of less than 0·1 cc. in the titrations is

sufficient to render the whole experiment useless. It must be pointed out that their experiments have not been confirmed by any other method.

Before going further it was decided therefore to repeat Kissin and Bronstein's experiments. It was impossible to obtain reproducible results of any value using litmus and tropaeolin 00 as indicators so electrometric titrations with the quinhydrone electrode were employed. Varying amounts of *N* HCl were added to 5 cc. samples of the toxin under examination and the p_H was determined electrometrically. A typical pair of experimental results is illustrated in the titration curves in Fig. 2. The neutral point to litmus was assumed to be p_H 6·2 (as stated by Kissin and Bronstein), that of tropaeolin 00 to be p_H 2·0, and the points of maximum precipitation of toxins and toxoids appeared to be not widely different and at about p_H 3·7. From the curves,

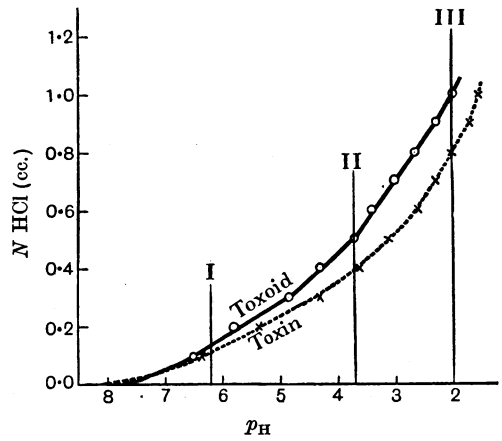

Fig. 2. Titration curves.

therefore, Kissin and Bronstein's "acid quotient" may be read off with a fair degree of accuracy. No appreciable difference between toxins and toxoids was found, in every case the "acid quotient" was between 1·3 and 1·5. Thus, when Kissin and Bronstein's experiments were performed with our toxins and toxoids under more accurate conditions their results were not confirmed.

The only other experiment reported by Kissin and Bronstein to support their view was the determination of the amino-nitrogen of toxin by the Van Slyke method before and after the toxoiding process. A 50 % diminution in free amino-nitrogen was observed. This is completely at variance with results here reported. As mentioned in the previous section a diminution of only 15 % in free amino-nitrogen was found, both by the Van Slyke and the Sørensen methods. Furthermore, this diminution occurred directly the formaldehyde was added to the toxin and was not a gradual process like that of toxoiding. It should be remarked that 3–5 minutes' shaking at 18–20° is generally considered sufficient for the completion of the reaction of nitrous acid with amino-groups. Kissin and Bronstein allow the reaction to continue for 30 minutes—

a period likely to result in hydrolysis of peptide linkings and hence to vitiate the measurement.

The theoretical interpretations placed by Kissin and Bronstein on the results of their experiments are also susceptible to critical examination. From the determination of acid quotients they conclude that the diphtheria toxin molecule contains two free amino-groups and one carboxylic acid group and that the toxoid molecule is identical but with only one free amino-group. The tacit assumption is made that in a toxic diphtheria culture filtrate there is present nothing but pure toxin, or, at any rate, that there are present no other substances likely to interfere with the titration. Of the substances in the toxic filtrate at least 99·5 % are not toxin at all, and the buffering power of peptone, phosphates, carbonates, etc. in various ranges of p_H is considerable. It is unwarrantable, therefore, to assume that the characteristics of the titration curve are attributable to the pure toxin and that conclusions regarding the constitution of the toxin molecule may be deduced therefrom. Kissin and Bronstein state that the original broth gives the same acid quotient as diphtheria toxin filtrates. This again should make for caution in drawing conclusions from the behaviour of the very complex mixture comprised in the filtrate after growth of the culture. If the toxic filtrate cannot be distinguished from the original broth by this method, then it seems unwise to attempt any deductions from the results regarding the constitution of the toxin.

An experiment with toxin purified by acid precipitation showed a diminution of amino-nitrogen from 0·04 mg. per 1 Lf. to about 0·00016 mg., showing that not more than $\frac{1}{250}$th of the amino-nitrogen in the toxic culture filtrate was attributable to the toxin itself.

Reducing effects of formaldehyde.

The oxidation-reduction condition of a reversible oxidation-reduction system may be observed by immersing an unattackable electrode in the solution. The potential difference set up at the electrode is dependent on the proportion of oxidised and reduced forms present of the substance studied. The more highly oxidising the system, the more highly positive is the potential and the more reducing the system, the more negative is the potential.

Formaldehyde itself does not produce an oxidation-reduction potential at an electrode. By itself it is inert electrochemically but when added, for example, to fresh milk an enzyme present has an activating effect, and the mixture develops reducing properties when incubated. These reducing properties may be observed by the reduction of methylene blue (Schardinger reaction) or by measurement of the reducing potential developed [Clark, Cohen and Gibbs, 1925].

When formaldehyde was added to diphtheria toxin and the mixture was incubated a slight reducing potential was developed. For example, in one case, 0·4 % of 40 % formaldehyde was added to a sample of toxin and the mixture was incubated. The original toxin had an oxidation-reduction potential of $E_h = 0{\cdot}34$ v. but the formalinised mixture developed slowly a

potential of $E_h = 0{\cdot}22$ v., at which level the potential was maintained for 10 days, the duration of the experiment.

It seemed possible therefore that the toxoiding process might be due to the maintenance of slight reducing conditions when the toxin was incubated with formaldehyde.

An alternative method of reduction was therefore attempted. It has been shown [Hewitt, 1930] that intense reducing conditions are established in diphtheria cultures during growth and that these are maintained after the cessation of active proliferation. After 9 days' incubation, therefore, a culture of *C. diphtheriae* (Park-Williams 8 strain) was sealed with melted vaselin, in order to prevent free access to air and to assist in the maintenance of reducing conditions. The incubation of the sealed culture was continued for 27 days. The filtrate of this culture was still highly toxic to guinea-pigs and the toxin-toxoid content, as measured by the Ramon flocculation method, had increased slightly during the incubation from 18 to 22 antigenic units per 1 cc. The flocculation time had also decreased slightly and the organisms proliferated actively when sub-cultured.

This method of reduction had therefore failed to produce the toxoiding process. That the toxoiding process is not due to the reducing action of formaldehyde is supported by the fact that although formaldehyde disappears during the toxoiding process the acidity corresponding to formic acid formation is not developed, as would be expected if the following formula expressed the process accurately:

$$\underset{\text{toxin}}{\mathrm{H.CHO} + \mathrm{T} + \mathrm{H_2O}} \longrightarrow \mathrm{H.COOH} + \underset{\text{toxoid}}{\mathrm{TH_2}}$$

If the formaldehyde, in its reducing effects, were oxidised still further to carbon dioxide,

$$\mathrm{HCHO} + \mathrm{O_2} \longrightarrow \mathrm{CO_2} + \mathrm{H_2O},$$

there should be an increase of some 0·2 % in the CO_2 content of the toxin. Actually a decrease in CO_2 content from 0·13 % to 0·12 % was observed during the incubation with formaldehyde. During the toxoiding process a considerable proportion of the formaldehyde disappears but its transformation product has not been found.

Effect of other reagents.

Sodium hydrosulphite. To 20 cc. of toxin (T. 824, Lf. 0·04 cc.) were added 0·05 g. sodium hydrosulphite and *N* NaOH to neutrality. The mixture was incubated at 40° for 2 hours and allowed to stand in the incubator for one night. The mixture was rendered sterile by filtration through a Seitz filter and 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal died in 24 hours and *post mortem* examination showed typical diphtheritic toxaemic changes. This exposure to reducing conditions had not therefore led to disappearance of toxicity.

Hydrogen peroxide. 20 cc. of toxin (T. 824) were incubated with 0·04 cc. of hydrogen peroxide (20 vol. A.R.) at 40° for 2 hours. After standing overnight on ice 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig. The animal was dead in 16 hours and *post mortem* examination revealed typical acute diphtheritic toxaemia. The oxidation, therefore, had not caused disappearance of toxic effects.

Methyl alcohol. 20 cc. of toxin (T. 824) were incubated at 40° for 24 hours with 0·04 cc. of methyl alcohol (A.R.). 1 cc. was inoculated subcutaneously into a 300 g. guinea-pig which was dead in 16 hours with typical acute diphtheritic toxaemia (*post mortem* indications). Methyl alcohol, which may be produced from formaldehyde by reduction, had therefore failed to remove the toxic properties of the toxin.

Conclusions.

In the conversion of toxin into detoxified toxoid the amount of formaldehyde added is approximately the quantity theoretically sufficient to combine with all the amino-nitrogen present. Under these conditions, however, only some 15 % of the total amino-nitrogen is combined, and even after 6 weeks' incubation some 85 % of it remains uncombined. A very large excess of formaldehyde is necessary before disappearance of free amino-nitrogen approaches completion. This is found to be the case not only with toxins and with broth but with peptone and the amino-acid, glycine. It seems probable therefore that the same would be found true of pure diphtheria toxin itself if it could be isolated. Since some six-sevenths of the amino-groups in the toxin molecule probably remain uncombined when this is converted into toxoid an alternative explanation of the effect of formaldehyde seems possible.

Kissin and Bronstein [1928, 1930] maintain that 50 % of the free amino-groups in the toxin molecule disappear during the toxoiding process and conclude that the action of formaldehyde is to remove those amino-groups (50 %) which are responsible for toxic effects but not for antigenic function or combining power. It was not found possible to confirm their results, and their experimental methods and theoretical conclusions are criticised.

As an alternative to the removal of free amino-groups, another function of formaldehyde was investigated. Formaldehyde is capable of developing reducing tendencies in the presence of a suitable activator. Oxidation-reduction potential measurements indicated some activation of formaldehyde when incubated with toxin, but another method of maintaining reducing conditions, namely, that of sealing a living diphtheria culture, failed to detoxify the toxin. Formaldehyde disappears during the toxoiding process but is not converted to formic acid, or CO_2, which is again evidence that it does not function as a reducing agent in the process.

Under the conditions employed reducing and oxidising agents and methyl alcohol failed to produce a detoxified toxoid.

Possible explanations of the toxoiding effect of formaldehyde, therefore, remain as follows.

1. The disappearance of a small fraction of the free amino-groups in the toxin molecule may result in detoxification without destruction of combining power with antitoxin, or antigenic activity.

2. The formaldehyde may cause union of two or more toxin molecules with formation of a compound of high molecular weight:

$$\underset{\text{toxin}}{TNH_2} + HCHO + \underset{\text{toxin}}{TNH_2} \longrightarrow \underset{\text{toxoid}}{THN - CH_2 - NHT} + H_2O$$

The compound, having the same general configuration as the original toxin, might possibly retain antitoxin-combining power without being able to exert toxic effects.

3. The formaldehyde may react with groups other than NH_2 in the toxin molecule.

4. The formaldehyde may itself undergo transformation, such as reduction, oxidation, polymerisation or combination with other broth constituents before reacting with the toxin. In this connection two facts are of interest, firstly, the slow and gradual nature of toxoid formation and secondly that hexamethylenetetramine, acetaldehyde and trioxymethylene sulphite have been found to produce toxoid formation, whilst paraldehyde does not [Berthelot and Ramon, 1925; Hollande and Penn, 1928].

Although these hypotheses are entirely speculative they may provide a working basis for further investigation.

Summary.

1. Of the amino-nitrogen present in fresh diphtheria toxins some 85 % remains free in completely detoxified toxoids prepared by incubation with formaldehyde.

2. Maintenance of toxins under reducing or oxidising conditions failed to produce toxoid.

3. No evidence that toxoid formation is due to disappearance of free amino-groups, to reduction or to oxidation has been obtained. Possible mechanisms are discussed.

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