FURTHER NOTES ON MODIFICATION OF DIPHTHERIA TOXIN BY FORMALDEHYDE.

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IN a former paper (Glenny and Hopkins, 1923) it was shown that diphtheria toxin could be so modified by the action of formaldehyde that the specific toxicity was greatly reduced while the binding power, *i.e.*, affinity for antitoxin, remained. Such modified toxin proved of considerable utility as an immunising agent. That toxin could be so modified by formalin has been known for many years; modified toxin was used by one of us in 1904 on a large scale for the immunisation of horses for the routine production of antitoxin. Modern methods of testing have yielded more accurate observation, and the adoption of measures for the active immunisation of children against diphtheria has given considerable impulse to the study of modified toxin as an immunising agent. In our former paper we stated that further work was being carried out along the following lines: (1) purification of toxin by methods of concentration; (2) using toxin so further modified that it is not toxic even in large doses. We have recently prepared modifications of diphtheria toxin containing no specific toxin, and considerable work has been done to determine the methods most suitable for reducing specific toxicity without affecting the binding unit content.

The amount of formaldehyde necessary to produce a given effect upon a batch of toxin depends greatly upon its amino-nitrogen content. To a toxin of which the Van Slyke nitrogen was 2·8 mgrs. (and total nitrogen, 17·5 mgrs.) per 10 c.c., 0·1 per cent. of formaldehyde was added; after keeping for twenty-four hours at a temperature of 37° C. an intradermic reaction was no longer obtained when the toxin was diluted more than 250 times. Another toxin, with a Van Slyke figure of 19·6 mgrs. (and total nitrogen, 51·4 mgrs.), after similar treatment gave a reaction when diluted 10,000 times; this toxin needed at least 0·3 per cent. formaldehyde to reduce the toxicity to the same level as that reached by adding 0·1 per cent. to the other toxin. The amounts of formaldehyde added in all the experiments recorded were based on the assumption that commercial formalin is a 40 per cent. solution of formaldehyde; the actual strength of most samples is slightly lower than this.

In a given toxin the amount of modification produced by a given

concentration of formaldehyde depends upon the temperature and time of exposure. To a certain toxin 0·3 per cent. formaldehyde was added and different samples after twenty-four hours' exposure to temperatures of 30°, 33°, 36°, and 39° C. were found to contain 1250, 500, 250, and 25 M.R.D. per c.c. respectively. Another batch of toxin was kept at 30° C. after the addition of 0·4 per cent. formaldehyde. After two days, 1/500 c.c. still caused an intradermic reaction; after six days, 1/25 c.c. contained 1 M.R.D., and only after nine days did the toxin fail to cause a reaction when injected undiluted.

After modification of toxin, many of the tests for combining power can no longer be made. Partially modified toxin may no longer kill a guinea-pig and so the L+ value cannot be determined, but sufficient œdema may be produced that an Lo test may be made; on further modification the toxoid will no longer produce a reaction when injected intradermally, nor œdema when injected subcutaneously. In the absence of these indicating symptoms, Lo and Lr determinations cannot be made. It is still possible, however, to obtain indications of combining power by animal test.

If antitoxin be added to modified toxin that is no longer capable of producing any symptoms in a guinea-pig, titrations made some hours later fail to detect all the antitoxin added. Obviously some antitoxin must have combined with the modified toxin. This phenomenon is only an extension of the Danysz effect, and must be left to a later paper for fuller discussion. A toxin, J. 3485, with an Lo dose of 0·18 c.c., was tested in this manner before and after modification. From the Lo dose, it follows that all toxic effects are neutralised upon the addition of 5·5 units of antitoxin to 1·0 c.c. of toxin. Various amounts of antitoxin in excess of this quantity were added, and after four hours at room temperature, residual antitoxin was titrated. In accordance with the Danysz phenomenon it was found that more than 5·5 units of antitoxin had combined with the toxin. The actual figures obtained were as follows:—

Number of units of antitoxin added to 1 c.c. of toxin	15	20	30	40	50
Number of units detected after four hours	5	8	18	28	38
Number of units combined	10	12	12	12	12

It appears, therefore, that when 1 c.c. of the toxin has combined with 12 units of antitoxin a saturation point is reached, and no further combination occurs. This value we at present refer to as the *total combining power.* After treatment with formaldehyde the toxin gave the following figures:—

Number of units of antitoxin added to 1 c.c. of toxin	15	20	30	40	50
Number of units detected after four hours	10	14	22	31	42
Number of units combined	5	6	8	9	8

It would appear at first that the total combining power has been considerably reduced, but it will be noticed that the saturation point appears not to be reached until considerable excess of antitoxin has been added. The effect of leaving excess of antitoxin in contact for a longer time was next tried, and the following figures were obtained:—

Number of units of antitoxin added to 1 c.c. of toxin	15	20	30	40	50
Number of units of antitoxin detected after forty-eight hours . . .	6	11	20	29	39
Number of units combined . . .	9	9	10	11	11

It would appear therefore that *the total combining power of a toxin may not be much reduced by the action of formaldehyde, but the rapidity of its combination with antitoxin may become definitely less.* This interpretation of the Danysz effect needs fuller consideration at a later date.

In the absence of the simpler animal tests for the combining power of modified toxin the flocculation test of Ramon (1922 and 1923) becomes invaluable. We (Glenny and Okell, 1924) have recently proposed the use of the symbol Lf, comparable with Lr, Lo, L+, for the amount of toxin equivalent to 1 unit of antitoxin as determined by flocculation.

By Ramon's method it is easy to detect whether the combining power of a toxin has altered during modification. Table I. shows the effect of exposing a toxin to various concentrations of formaldehyde at a temperature of 37° C. for twenty-four hours.

TABLE I.

Showing the effect of exposing a toxin to varying concentrations of formaldehyde at a temperature of 37° C. *for twenty-four hours.*

Key number.	Percentage formaldehyde added.	Specific toxicity.		Combining power.
		Number of minimal reacting doses per c.c. (Intradermic test).	Minimal lethal dose. (Subcutaneous test).	Lf. (Flocculation test).
J. 3488	nil	500,000	0·002 c.c.	0·11 c.c.
PX. 146	0·01	250,000	0·003 ,,	0·11 ,,
PX. 147	0·03	150,000	0·01 ,,	0·11 ,,
PX. 148	0·1	25,000	0·02 ,,	0·11 ,,
PX. 149	0·2	5,000	0·05 ,,	0·12 ,,
PX. 150	0·3	1,250	0·1 ,,	0·12 ,,
PX. 151	0·4	50	2·0 ,,	0·12 ,,
PX. 152	0·5	0	over 5·0 ,,	0·12 ,,
PX. 153	1·0	0	over 5·0 ,,	0·12 ,,

The figures recorded in table I. for the number of minimal reacting doses per c.c., and for the size of the minimal lethal dose are only approximate; the number of tests put up at each point was very small, but sufficient indication is given to show the course of

modification with varying concentrations of formaldehyde. When it is necessary for any purpose to make an exact comparison between the M.R.D. or M.L.D. of a toxin after exposure to varying concentrations of formaldehyde, it is necessary to test a toxin immediately after the end of a given time of exposure, because it has been found that with higher concentrations of formaldehyde modification of toxin is not complete after twenty-four hours' exposure to a temperature of 37° C., but may continue at a lower temperature. It will be seen that as the toxin becomes more modified the number of M.R.D.'s per M.L.D. becomes less. The reason for this may be (*a*) the two values were not tested at the same time, (*b*) the two tests are not measures of the same type of specific toxin, (*c*) there is present some non-specific substance unaffected by formaldehyde that greatly accelerates death in higher doses. Considerable evidence is accumulating to show that an injurious substance is present in toxin prepared from tryptic digest media. Table I. shows that even so small a concentration of formaldehyde as one part in 10,000 causes some slight modification of toxin into toxoid, while one part in 1000 reduces the amount of specific toxin present to one-tenth of the original amount. With this particular toxin, 0·5 per cent. formaldehyde was necessary before the toxin would no longer kill guinea-pigs in doses of 5 c.c. The Lf values recorded show that the total binding capacity of the toxin has not been greatly reduced, but that some slight loss has occurred. It would appear therefore, that, in addition to conversion of toxin to toxoid, either destruction has occurred or the affinity of some of the toxoid for antitoxin has been so reduced that even the Lf value is affected. This is rather suggested by the fact that the time taken for flocculation to occur increases with higher concentration of formaldehyde; with PX. 153 it was not possible to obtain flocculation even though the mixtures of modified toxin and antitoxin were left for several days at various temperatures. The Lf value of 0·12 was obtained by calculation from the Lf value of a mixture of equal parts of PX. 153 and the original toxin.

*Modified toxin can be so purified that the number of binding units per milligramme of nitrogen is increased about forty-fold.** Our colleagues, Watson and Wallace (1924), have described a simple method for concentrating diphtheria toxin. These workers have found that from 1 to 3 per cent. glacial acetic acid will precipitate all toxins prepared by them. Earlier workers in these laboratories used lower concentrations of acetic acid, and it was found that only a few toxins could be precipitated without the preliminary dialysis described by Glenny and Walpole (1915). The filtered precipitate, which is readily soluble in dilute alkali, retains most of the original binding capacity, but the total nitrogen content has been reduced to about one-fiftieth of that

* This has been increased in some later experiments to a concentration of over 200-fold.

of the original toxin. We have found that this concentrated toxin is easily modified by the addition of formalin, and that modified toxin can be concentrated by the process of Watson and Wallace. Since we prepared the concentrated modified toxin described below, our colleagues have successfully concentrated a number of other similar batches.

A batch of diphtheria toxin was kept for eleven days at a temperature of 30° C. after the addition of 0·4 per cent. formaldehyde. Some of the modified toxin so formed was concentrated by adding 1·0 per cent. acetic acid, filtering and redissolving the precipitate in dilute alkali. The various measurements of the original toxin (J. 3460) after modification (PX. 57) and after concentration to one-twentieth of the original volume (PX. 76) are given in table II.

TABLE II.

Showing the strength of a toxin before and after modification with formaldehyde, and after modification and concentration.

	J. 3460. Original toxin.	PX. 57. J. 3460 after treatment with formaldehyde.	PX. 76. PX. 57 after precipitation with acetic acid.
Minimal lethal dose . .	0·002 c.c.	over 5·0 c.c.	about 5·0 c.c.
Minimal reacting dose . .	0·0000015 c.c.	over 0·2 c.c.	0·1 c.c.
Lr	0·12 c.c.	...	...
Lo	0·12 c.c.	...	...
L+	0·14 c.c.	...	...
Lf	0·11 c.c.	0·14 c.c.	0·01 c.c.

Modified toxin is a more powerful antigen than toxin-antitoxin mixtures; its antigenic value can be greatly increased by concentration. The antigenic values of modified toxin before and after concentration were tested in the usual way; guinea-pigs injected with various doses of the antigen were given weekly Schick tests commencing three weeks after the initial stimulus, the number of the Schick dose that failed to produce a reaction being called the *immunity index* (Glenny, Allen, and Hopkins, 1923).

For comparison in table III. we have given the results obtained from testing a batch of toxin-antitoxin mixture containing one-tenth of an L+ dose per c.c.; this mixture was kindly supplied by Dr Park of New York. It will be seen that guinea-pigs were invariably immune three weeks after the initial injection of 5·0 c.c. of modified toxin PX. 57. The antigenic value of this modified toxin is not so good as that of the toxins only partially modified which we have tested (Glenny and Hopkins, 1923). It is possible that the original toxin of this preparation was weaker in true antigenic value than the toxin which had previously been only partially modified. On the other hand, we have considerable evidence that, unit for unit, the antigenic value of

a partially modified toxin is better than that of a toxin completely modified. We shall not discuss this point further at present because the object of this paper is mainly to show that non-toxic modifications are quite good antigens. Comparison between the results recorded in table III. shows that harmless modified toxin is a better antigen than slightly toxic toxin-antitoxin mixtures.

TABLE III.

Showing the antigenic value of modified toxin (PX. 57), *and of concentrated modified toxin* (PX. 76) *compared with a toxin-antitoxin mixture.*

Antigen.	Dose.	Number of guinea-pigs showing an immunity index of—				
		1.	2.	3.	4.	Over 4.
PX. 57 . .	0·1 c.c.	...	3	3	1	2
	0·2 ,,	...	1	2	1	...
	0·5 ,,	3	2	1	...	...
	1·0 ,,	5	4	...	...	...
	5·0 ,,	9	...	...	...	...
PX. 76 . .	0·01 ,,	...	...	1	2	...
	0·03 ,,	1	2	...	...	...
	0·1 ,,	2	1	...	...	...
T.A.M. . .	0·2 ,,	...	1	...	...	2
	0·5 ,,	...	2	1	...	1
	1·0 ,,	...	1	2	1	...
	2·0 ,,	...	3	1	...	...
	5·0 ,,	...	No	survi	vals	...

Table III. also shows how so low a dose as 0·01 c.c. of a concentrated modified toxin which is no more toxic than many toxin-antitoxin mixtures that have been used for human immunisation can produce quite good immunity in guinea-pigs. The amount of nitrogenous material present in this concentrated modified toxin is a fraction of that present in the original toxin. By the process of concentration described by Watson and Wallace (*loc. cit.*) it is possible to obtain the original binding unit content of a toxin, whether modified or not, in a solution containing less than one-fortieth of the original nitrogen content. It is obvious therefore that it is possible to produce non-toxic antigens of great efficiency by this method. Modified toxin has recently been used on a small scale for human immunisation without causing any greater reaction than the ordinary mixtures and has produced rapid immunisation.

In an immunising mixture prepared with modified toxin the amount of antitoxin present does not, within wide limits, affect the antigenic power. We have already shown in the previous paper that with modified toxin the differential region between Lo and L+ is so great that there is a very large margin of safety in the preparation of mixtures. That this is so is shown in table IV. which gives the antigenic value of five successive batches of toxin-antitoxin mixtures prepared from partially modified toxin. The amount of neutralisation varied with each batch;

the amount of antitoxin added to the same volume of the same modified toxin varied in the five batches in a proportion of 1·0, 1·25, 1·5, 1·75, and 2·0. All these mixtures, which were diluted 1 in 10, proved as good antigens as the mixture recorded in table III. The advantage of using mixtures made with modified toxin lies in the fact that such modified toxin does not need the careful titration and neutralisation within narrow limits necessary when mixtures are made with unmodified toxin.

TABLE IV.

Showing the antigenic value of 1·0 c.c. *doses of five successive batches of toxin-antitoxin mixture prepared from partially modified toxin, with varying amounts of antitoxin.*

Relative amount of antitoxin.	Number of guinea-pigs showing an immunity index of—				
	1.	2.	3.	4.	Over 4.
1·0	...	1	1	...	1
1·25	...	2	...	1	...
1·5	...	3	...	...	...
1·75	...	3	...	...	...
2·0	...	2	1	...	...

Relatively high degrees of immunity are rapidly obtained by the use of modified or of concentrated modified toxin.—The high immunity index of modified and of concentrated modified toxin suggested that immunity might be attained in guinea-pigs earlier than in three weeks. A number of guinea-pigs therefore were injected with 1 c.c. and 5 c.c. of PX. 57 and of PX. 76, and fourteen days later they were injected intradermally with a series of doses of toxin representing one, two, four, eight, sixteen, and thirty-two times the Schick dose of toxin. From the reactions resulting it was possible to detect the degree of immunity produced in each animal. Table V. shows that 1 c.c. of PX. 57 was not

TABLE V.

Showing the reactions produced by multiple Schick doses in guinea-pigs fourteen days after a single injection of modified or of concentrated modified diphtheria toxin.

Primary stimulus.		1.	2.	4.	8.	16.	32.
PX. 57	1·0 c.c.	++	++	++	+++	+++	+++
	5·0 ,,	...	S	±	±	+	+
PX. 76	1·0 ,,	–	+	++	++	++	++
	1·0 ,,	–	–	S	S	+	+
	1·0 ,,	–	–	–	–	–	S
	5·0 ,,	–	–	–	–	–	S

+++, ++, + represent reactions of decreasing magnitude.

± = a definite but very reduced reaction. S = faint indication of a reaction.

sufficient to render a guinea-pig immune to a single Schick dose given fourteen days later, 5 c.c. however produced protection against twice the Schick dose of toxin, and reduced reactions only were produced by four times and eight times the dose. The three guinea-pigs injected with 1 c.c. of the concentrated modified toxin PX. 76 gave variable results. One showed immunity to the extent of a single Schick dose, another to an eight-fold dose, while the third tolerated thirty-two times the ordinary Schick injection and showed only a faint indication of a reaction. This marked variation between individual animals is only to be expected when the animals are tested quantitatively at a time when the degree of their immunity is increasing most rapidly. It is quite probable that, if a test had been made a day later on the guinea-pig that showed immunity to the extent of a single Schick dose only, this animal would have tolerated as large a dose as any of the guinea-pigs tested on the previous day. The method usually employed for determining the immunity index as already described is free from this type of variation.

This earlier production of immunity suggested that when dealing with potent antigens, our routine method for testing antigenic values should be modified to allow the titration of more potent antigens than those showing an immunity index of one; in other words, it should be possible to detect differences between antigens capable of producing immunity in less than two weeks and those that do not produce such immunity in less than three weeks. A number of guinea-pigs were injected with 1 c.c. of modified toxin, and, starting 7, 9, 11, etc. days later, they received Schick tests every two or three days. In this way an experimental modification of the routine method was made by altering the incubation period of three weeks to seven or more days, and the interval between successive injections was also shortened. Table VI. gives the results of this experiment. It will be seen that only

TABLE VI.

Showing the reactions produced by Schick test doses in guinea-pigs injected every two or three days, commencing seven or more days after a single initial dose of 1 c.c. *of modified diphtheria toxin.*

Days after primary stimulus.	Reactions produced by Schick dose of toxin.											
7	++	++	...	...	...	...	...	...	...	...	...	...
9	++	++	++	++	...	...	...	...	...	...	...	...
11	−	++	++	++	++	++	...	...	...	...	...	...
13	...	+	+	+	++	++	?+	++	...	...	...	...
14	...	...	...	...	...	...	...	...	S	++	...	...
16	...	−	−	−	−	±	−	++	...	++	−	−
18	...	...	...	...	...	±	...	−	...	++	...	...
20	...	...	...	...	...	−	...	...	...	−	...	...

one guinea-pig was Schick negative in less than fourteen days, but the majority of the animals tested were negative on the sixteenth day. The

secondary stimulus effect of the earlier Schick doses is not very apparent; it would appear therefore that the guinea-pigs injected with this antigen are not actively immune or capable of responding to a secondary stimulus until about fourteen days after the primary stimulus; at this stage the antitoxin produced increases so rapidly that most animals are Schick negative by the sixteenth day even without a secondary stimulus. Sixteen days may be taken as an index of the rapidity with which guinea-pigs may be immunised by a single injection of 1 c.c. of this modified toxin PX. 57. Table VII. gives the results of a

TABLE VII.

Showing the reactions produced by Schick test doses in guinea-pigs injected every two or three days commencing seven or more days after a single initial dose of 5 c.c. *of modified diphtheria toxin.*

Days after primary stimulus.	Reactions produced by Schick dose of toxin.									
7	++	++	...	...	...	...	...	...	...	...
9	++	++	++	++	...	...	...	...	...	...
11	−	−	−	++	−	++	...	...	...	...
13	...	...	...	++	...	−	−	±	−	−
14	...	...	...	...	...	...	...	...	...	...
16	...	...	...	−	...	...	...	−	...	...

similar experiment upon guinea-pigs injected with 5 c.c. of the same antigen. With this dose easily detectable immunity is produced in about eleven days. In a further modification of this experiment guinea-pigs, all injected with 1 c.c. of PX. 57, received Schick tests at slightly wider intervals of three or four days commencing on the 3rd, 4th, 5th, 6th, 7th, and 8th days. The results are very similar to those recorded in table V.; no guinea-pig tested earlier than the 13th day was immune, five out of the thirteen tested between the 13th and 15th days were Schick negative, while eleven out of thirteen tested on the 16th day gave no reaction. It would appear again, therefore, that with this differently spaced series the same period of immunity of about sixteen days occurs.

This production of immunity in normal guinea-pigs by a single injection of a non-toxic antigen suggested the possibility of still further reducing the latent period following a primary stimulus. A number of rabbits were injected subcutaneously with various doses of modified toxin and of concentrated modified toxin, and samples of blood were withdrawn daily and tested for antitoxin. It was found that *a single injection of a non-toxic antigen may produce detectable antitoxin in a normal rabbit within nine days.* This is the shortest latent period following a primary stimulus in a normal animal that we have yet encountered. Two normal rabbits injected with 5.0 c.c. of modified toxin PX. 57, and a third rabbit injected with the same volume of another batch of modified toxin produced detectable antitoxin nine

days after this single injection. The same results were obtained with 1·0 c.c. of concentrated modified toxin PX. 76, while 0·1 c.c. produced detectable antitoxin in eleven days.

Modified toxin and concentrated modified toxin can be boiled without destroying entirely their antigenic properties. Boiling for at least five minutes is necessary before all antigenic value is destroyed.

A recent paper by Ramon (1924) shows that he is now studying the antigenic value of modified toxin. The flocculation test has proved invaluable in our work and has given a great impetus to the study of modified toxins which, although frequently used by one of us during the past twenty years, were not closely studied until three years ago. It was not possible until the publication of the flocculation method for this study to be carried out to any great extent.

SUMMARY.

(1) Diphtheria toxin so modified by formalin that it has become non-toxic possesses high immunising powers.

(2) Such modified toxin can be concentrated so that the antigenic power can be increased about forty-fold for the same nitrogen content.

(3) It is possible by the use of these antigens to produce immunity so rapidly that antitoxin may be detected in the blood of a normal rabbit nine days after a single injection and guinea-pigs become Schick negative in eleven days.

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