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TIME OF COMBINATION OF DIPHTHERIA TOXIN WITH LIVING TISSUES

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THE intradermal injection of a small quantity of diphtheria toxin into a guinea-pig is followed by a definite inflammatory area round the point of injection. This red area is always visible within 36 hours after the injection and often may be seen within 18 hours and occasionally in less than 8 hours after injection. The time of appearance of the reaction is no indication of the time elapsing between the injection and the fixation of the toxin by the living tissues. The time of fixation is of both practical and theoretical interest.

Most of the work in the past on the time of fixation of diphtheria toxin has been done by subcutaneous injection, but Schick (1923) when working out the method of diagnosis of susceptibility to diphtheria to which his name is attached injected intracutaneously a number of children with a diagnostic dose of diphtheria toxin followed later by a subcutaneous injection of antitoxin. He found that the size of the reaction was unaffected on every occasion when antitoxin was given 9 hours after the injection of toxin; usually, the injection of toxin caused a full-sized reaction if 6 hours elapsed before antitoxin was given, and a reduction in size of reaction nearly always resulted if antitoxin was given 3 hours later. In the experiments recorded in this paper guinea-pigs were injected intracutaneously with a series of Schick doses of toxin at varying intervals of time and antitoxin was administered during or after the series of injections of toxin. In the majority of cases, the antitoxin was injected intravenously to ensure immediate distribution.

It is possible, from a consideration of the reactions produced, to decide in what time an amount of toxin injected is so completely fixed by the tissues that no amount of antitoxin given at the end of that time can affect the action of the toxin, *i.e.* the size of the reaction produced. At the other end of the scale one can find how long one can wait after the injection of an amount of toxin and yet be certain that notwithstanding the delay, one can inject antitoxin and so "catch up" the action of the toxin and ensure the suppression of any reaction. If the interval of time elapsing between

the injection of toxin and antitoxin falls between these extremes, we find a reduction in the size of the reaction produced.

Experiments were also performed in which a dose of toxin, known to be sufficient to act as an antigen and produce active immunity, was given and antitoxin was injected after varying periods of time. The object was to find the minimum period which one must allow to pass after the injection of the toxin in order to be sure that the giving of a large dose of antitoxin would not interfere with the immunising effect of the toxin.

(1) *Fixation of intradermic toxin as judged by the cutaneous reaction.*

In the first experiment three guinea-pigs were injected intracutaneously at intervals of 15 minutes with 1/500th of an Lo dose of toxin. This amount of toxin is equal to twice the Schick dose and equal for this particular batch of toxin to about 30 times the M.R.D., *i.e.* the minimum amount that will cause a reaction when injected intradermally. Between 3 and 4 hours after the first injection, the guinea-pigs received intravenously 10, 100 and 1000 units respectively of antitoxin. Table I. shows that a small reaction

TABLE I.

Showing reactions caused by the intracutaneous injection of 1/500*th of an* Lo *dose of toxin into three guinea-pigs at various intervals of time before the intravenous injection of antitoxin.*

Interval elapsing after injection of toxin before injection of antitoxin.	Reaction.		
3 hours 40 minutes . . .	+	...	...
3 ,, 25 ,, . . .	+	+	+
3 ,, 10 ,, . . .	+	+	+
2 ,, 55 ,, . . .	+	+	+
2 ,, 40 ,, . . .	+	+	+
2 ,, 25 ,, . . .	+	+	+
2 ,, 10 ,, . . .	+	+	+
1 hour 55 ,, . . .	+	+	+
1 ,, 40 ,, . . .	+	±	s
1 ,, 25 ,, . . .	±	s	s
1 ,, 10 ,, . . .	±	s	–
55 ,, . . .	±	–	–
40 ,, . . .	±	–	..
25 ,, . . .	±	–	–
10 ,, . . .	–	–	–
Amount of antitoxin injected .	10 units	100 units	1000 units

s = very small reaction. ± = reduced reaction.
+ = unreduced reaction.

was caused by toxin injected 25 minutes before the intravenous injection of enough antitoxin completely to neutralise 5000 times

the toxin injected: some toxin was so fixed in 70 minutes that 50,000 times its equivalent in antitoxin could not dislodge it, while 500,000 times its equivalent in antitoxin was not sufficient to remove from combination with the tissues the whole of the toxin injected 85 minutes earlier.

When smaller amounts of toxin were injected, a considerably longer interval elapsed before an amount of toxin sufficient to cause a slight reaction was so fixed that antitoxin could not dislodge it, but even when enough antitoxin was injected that would completely neutralise 5,000,000 times the dose of toxin a definite reaction was produced when the toxin was injected $4\frac{1}{4}$ hours before the antitoxin. These results are shown in table II. In this series the amount of

TABLE II.

Showing reactions caused by the intracutaneous injection of guinea-pigs with different small amounts of toxin at various intervals of time before the intravenous injection of antitoxin.

Quantity of toxin injected.	Size of reactions in millimetres.								
	1/5000th of test dose.			1/2000th of test dose.			1/1000th of test dose.		
Interval elapsing after injection of toxin before injection of antitoxin.									
4 hours . . .	12×12	10×15	18×18	18×18	15×15	18×18	18×18	18×18	18×18
3 ,, 15 minutes .	8×8	10×10	10×10	12×15	10×10	12×12	12×12	12×12	15×15
2 ,, 15 ,, .	8×8	6×10	5×5	12×12	8×10	8×10	15×18	10×10	12×12
1 ,, 15 ,, .	8×8	8×8	6×10	8×8	8×8	10×10	10×10	10×10	10×10
¾ ,, 15 ,, .	trace	5×5	trace	10×10	5×5	10×10	12×12	5×5	10×10
½ ,, 15 ,, .	,,	8×8	5×5	trace	5×5	10×10	12×12	trace	8×8
¼ hour 15 ,, .	,,	trace	...	8×8	5×5	...	8×8	5×5	...
Units of antitoxin injected	10	100	1000	10	100	1000	10	100	1000

toxin injected was 1/5th, 1/2 and a full Schick dose, equivalent for that particular batch of toxin used to 3, 7 and 15 minimal skin reacting doses respectively. The reactions produced by the toxin injected 6 hours before antitoxin were less than those produced by the toxin injected 24 hours before.

In these preliminary experiments our object was to determine the interval of time before sufficient toxin was fixed to cause a definite and lasting reaction. Upon these results was based the "following dose" used by Eagleton and Baxter (1921) in their method of testing the virulence of diphtheria organisms. The batch of toxin used in these preliminary experiments was too weak in specific toxicity to be suitable for use in the Schick test. In subsequent experiments the toxin used contained twice as many minimal reacting doses in that

amount of toxin that was just neutralised by 1/1000th of a unit of antitoxin; this amount of toxin is our standard Schick test dose. Table III. shows the results obtained with two guinea-pigs injected intracutaneously with Schick doses of toxin followed by 10 and 1000 units of antitoxin intravenously. The figures recorded show that full-sized reactions occurred only when from $2\frac{3}{4}$ to $3\frac{1}{4}$ hours elapsed before 10 units of antitoxin were injected or $3\frac{1}{4}$ to $3\frac{3}{4}$ hours before 1000 units were injected. It was realised however that full-sized reactions could be produced if less than the full Schick dose of toxin was fixed by the tissues. In the next experiment therefore 1/5th only of the full Schick dose (only 6 times the minimal reacting dose and 1/5000th of the Lo of this toxin) was injected. Table IV. shows that a reduction of reaction occurs if the injection is followed within two hours by 10 units of antitoxin and between $2\frac{3}{4}$ and 3 hours when followed by 1000 units. It is also seen from this table that such an amount of toxin has been absorbed in $\frac{3}{4}$ hour that 50,000 times the equivalent in antitoxin cannot dislodge it, or in $1\frac{1}{2}$ hours that 5,000,000 times its equivalent in antitoxin cannot remove the whole of the dose of toxin from combination with the tissues. The proportion of toxin fixed at any given time was determined by comparing the size of reaction produced with standard sizes produced by known fractions of a Schick dose of the same batch of toxin in normal guinea-pigs. It was found that 1 per cent. and 2 per cent. of the full Schick dose produced no visible reactions, 3 per cent. produced a very small indefinite reaction, 4 per cent. and 5 per cent. very pale reactions of about $\frac{1}{4}$ the area of a full-sized reaction, from 6 per cent. to 9 per cent. gradually increasing but pale reaction while 10 per cent. caused reactions of half the area of those caused by the full Schick dose. The reaction produced by 25 per cent. of the full dose was indistinguishable on the second day from that caused by the full dose. Interpreting our results by means of the standard reactions we are able to say that only 20 per cent. of the full Schick dose is permanently fixed during the first three hours but that a far greater proportion of the smaller dose is fixed. A guinea-pig was injected with 1/10 of a Schick dose at frequent intervals followed by 1000 units antitoxin given intravenously. This amount of antitoxin was 10,000,000 times as much as was needed completely to neutralise the quantity of toxin given at each injection. Under 30 per cent. (the least detectable amount) was fixed in 2 hours, 50 per cent. in 3 hours and from 80 to 100 per cent. in 4 hours.

This method of testing was used to demonstrate the difference in rate of absorption of antitoxin given intravenously, intramuscularly and subcutaneously. Table V. shows that antitoxin given intravenously or intramuscularly commences to act extremely rapidly while antitoxin given subcutaneously is very slow in action.

Table III.

Showing reactions caused by the intracutaneous injection of Schick doses of toxin into guinea-pigs at various intervals of time before the intravenous injection of antitoxin.

Interval elapsing after injection of toxin before injection of antitoxin.	Size of reactions on day 2 (millimetres).	
24 hours	18 × 18	19 × 20
5 ,, 45 minutes	17 × 21	18 × 18
5 ,, 15 ,,	18 × 20	14 × 18
4 ,, 45 ,,	17 × 20	17 × 17
4 ,, 15 ,,	16 × 20	18 × 19
3 ,, 45 ,,	18 × 20	18 × 20
3 ,, 15 ,,	18 × 18	17 × 18
2 ,, 45 ,,	16 × 18	16 × 17
2 ,, 15 ,,	14 × 17	13 × 14
1 ,, 45 ,,	12 × 15	14 × 14
1 ,, 15 ,,	11 × 11	6 × 6
Units of antitoxin injected	10 units	1000 units

Table IV.

Showing the reactions caused by the intracutaneous injection of 1/5*th of the dose of toxin used in the Schick test into guinea-pigs at various intervals of time before the intravenous injection of antitoxin.*

Interval elapsing after injection of toxin before injection of antitoxin.	Size of reactions on day 2 (millimetres).	
4½ hours	14 × 14	12 × 12
4 ,,	14 × 15	10 × 14
3¾ ,,	13 × 13	9 × 10
3½ ,,	13 × 15	12 × 12
3¼ ,,	15 × 15	9 × 12
3 ,,	13 × 13	12 × 13
2¾ ,,	13 × 15	10 × 10
2½ ,,	15 × 15 (pale)	8 × 8 (indefinite)
2¼ ,,	13 × 13	9 × 9 (indefinite)
2 ,,	10 × 10	6 × 6 (indefinite)
1¾ ,,	10 × 11	5 × 5 (pale)
1½ ,,	8 × 8 (very indefinite)	4 × 5
1¼ ,,	8 × 8 (very indefinite)	nil
1 ,,	4 × 4	nil
¾ ,,	5 × 5	nil
½ ,,	nil	nil
¼ ,,	nil	nil
Amount of antitoxin injected	10 units	1000 units

TABLE V.

Showing reactions caused by the intracutaneous injection of Schick doses of toxin into guinea-pigs at different intervals of time before the injection of antitoxin given intravenously, subcutaneously, and intramuscularly.

Interval elapsing after injection of toxin before injection of antitoxin.	Size of reactions on day 2 (millimetres).			
4 to 4¼ hours . .	17 × 17	17 × 17	...	...
3¾ ,, 4 ,, . .	17 × 17	16 × 16	...	...
3½ ,, 3¾ ,, . .	18 × 18	16 × 16	...	...
3¼ ,, 3½ ,, . .	18 × 18	16 × 19	...	...
3 ,, 3¼ ,, . .	18 × 18	16 × 16	...	...
2¾ ,, 3 ,, . .	15 × 16	15 × 15	...	...
2½ ,, 2¾ ,, . .	15 × 17	15 × 15	...	...
2¼ ,, 2½ ,, . .	16 × 16	13 × 13	...	...
2 ,, 2¼ ,, . .	16 × 18	12 × 14	15 × 15	16 × 16
1¾ ,, 2 ,, . .	15 × 15	12 × 12	12 × 17	14 × 14
1½ ,, 1¾ ,, . .	15 × 15	10 × 10	11 × 11	13 × 15
1¼ ,, 1½ ,, . .	14 × 14	7 × 7	11 × 12	13 × 16
1 ,, 1¼ ,, . .	14 × 14	9 × 9	8 × 11	16 × 18
¾ ,, 1 ,, . .	14 × 14	7 × 7	9 × 11	15 × 15
½ ,, ¾ ,, . .	11 × 11	5 × 6	8 × 8	14 × 14
¼ ,, ½ ,, . .	9 × 9	5 × 6	nil	14 × 14
0 ,, ¼ ,, . .	5 × 5	nil	4 × 4	...
Injection . . .	10 units	1000 units	1000 units	1000 units
Route	Intravenous	Intravenous	Intramuscular	Subcutaneous
0 to ¼ hour after .	...	...	...	7 × 7
¼ ,, ½ ,, .	...	...	...	7 × 7
½ ,, ¾ ,, .	...	...	...	7 × 7
¾ ,, 1 ,, .	...	...	...	nil

(2) *Fixation of intradermic toxin as judged by the production of antitoxin.*

In the next section of the work we investigated the time taken for toxin injected into immune rabbits so to be fixed that the intravenous injection of antitoxin could not prevent the toxin from causing an antigenic response. We have shown that the Schick dose of toxin will act as a Secondary Stimulus (Glenny and Allen, 1922). By using so small a dose of toxin we could be sure that any failure to dislodge toxin from the tissues was not due to the use of insufficient antitoxin. In the first experiment recorded in table VI. three actively immune rabbits were injected intracutaneously with Schick doses of diphtheria toxin followed 1, 2 or 3 days later by the intravenous injection of 10 units of antitoxin, *i.e.* 10,000 times the quantity of antitoxin necessary completely to neutralise the toxin injected.

The rabbits had received injections of toxin antitoxin mixtures several months previously rendering them actively immune to diphtheria toxin and sensitive to horse serum. From the injection of Schick toxin we expected an active production of antitoxin. From our recent work on passive immunity (Glenny and Hopkins, 1922, 1923, 1924) we know the course of loss of antitoxin passively administered

to "horse sensitive" rabbits. By the fifth day after the injection of antitoxin we expect all antitoxin to be lost, and if no active immunity results from the injection of Schick toxin the antitoxin value of rabbits should have reached its original level. We find however that in the three rabbits the antitoxin level 10 days or more after injection is still well above the original level. There occurs also a

TABLE VI.

Showing the antitoxic content of actively immune rabbits injected with Schick toxin at different intervals of time before the intravenous injection of antitoxin.

Rabbit.	G. 22.		G. 23.		G. 31.	
Interval between toxin and antitoxin.	24 hours		48 hours		3 days	
Units of antitoxin given.	10 units intravenously		10 units intravenously		10 units intravenously	
Interval after toxin.	Interval after antitoxin.	Antitoxic content of blood.	Interval after antitoxin.	Antitoxic content of blood.	Interval after antitoxin.	Antitoxic content of blood.
0 day	...	0·003	...	0·003	...	0·0005
1 ,,	...	0·003	...	0·003	...	0·0005
	15 mins.	0·09	...	...	...	...
2 days	1 day	0·045	...	0·003	...	0·0005
			15 mins.	0·08	...	...
3 ,,	2 days	0·033	1 day	0·05	...	0·0005
					15 mins.	0·09
4 ,,	3 ,,	...	...	...	...	...
5 ,,	4 ,,	0·022	3 days	0·12	2 days	0·03
6 ,,	5 ,,	0·045	4 ,,	0·16	3 ,,	0·002
7 ,,	6 ,,	0·055	5 ,,	0·16	4 ,,	0·0025
8 ,,	7 ,,	0·04	6 ,,	0·14	5 ,,	0·0017
9 ,,	8 ,,	0·04	7 ,,	0·11	6 ,,	0·0017
10 ,,	9 ,,	0·035	8 ,,	0·09	...	...
11 ,,	10 ,,	...	...	...	...	...
12 ,,	11 ,,	0·033	10 ,,	0·09	9 ,,	0·0012

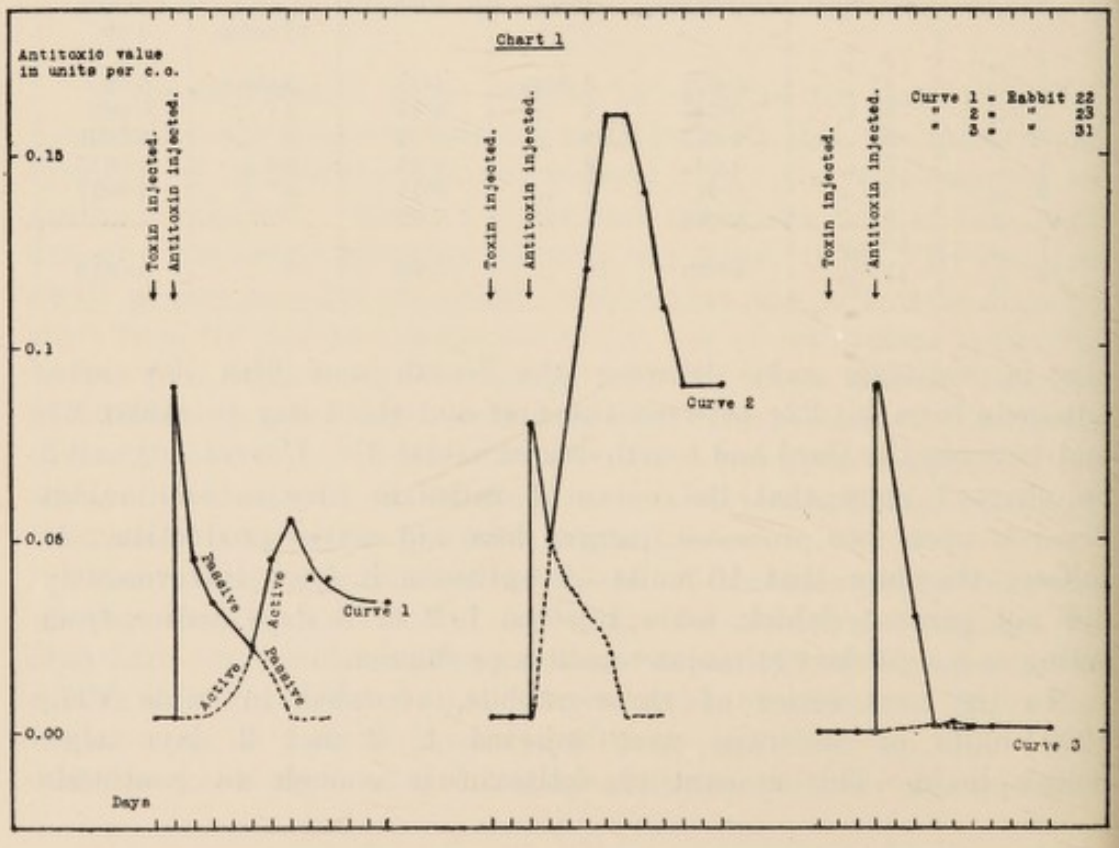
rise in antitoxic value between the fourth and fifth day after antitoxin in rabbit 22; between the first and third day in rabbit 23, and between the third and fourth day in rabbit 31. Curves 1, 2 and 3 on chart 1 show that the curve of antitoxin titre in each rabbit depends upon two processes, passive loss and active production. It follows therefore that 10 units of antitoxin injected intravenously did not prevent Schick toxin injected 1, 2 or 3 days earlier from acting as a secondary stimulus to active production.

In the next series of three rabbits, recorded in table VII., 1000 units of antitoxin were injected 1, 2 and 3 days after Schick toxin. This amount of antitoxin is enough to neutralise

Table VII.

Showing the antitoxic content of actively immune rabbits injected with Schick toxin at different intervals of time before the intravenous injection of antitoxin.

Rabbit.	G. 33.		G. 34.		G. 35.	
Interval between toxin and antitoxin.	1 day		2 days		3 days	
Units of antitoxin given.	1000 units		1000 units		1000 units	
Interval after toxin.	Interval after antitoxin.	Antitoxic content of blood.	Interval after antitoxin.	Antitoxic content of blood.	Interval after antitoxin.	Antitoxic content of blood.
0 day	...	0·001	...	0·015	...	0·0018
1 ,,	...	0·001	...	...	...	0·0018
	15 mins.	8·0	...	...	...	...
2 days	1 day	4·5	15 mins.	7·5	...	0·0018
3 ,,	2 days	3·5	1 day	3·5	...	0·0018
					15 mins.	7·0
4 ,,	3 ,,	2·25	2 days	2·5	1 day	3·75
5 ,,	4 ,,	0·03	3 ,,	1·87	2 days	2·75
6 ,,	5 ,,	0·055	4 ,,	1·37	3 ,,	2·25
7 ,,	6 ,,	0·055	5 ,,	0·055	4 ,,	0·014
8 ,,	7 ,,	0·055	6 ,,	0·10	5 ,,	0·018
9 ,,	...	...	7 ,,	0·10	6 ,,	0·018
10 ,,	9 ,,	0·05	8 ,,	0·09	7 ,,	0·014
11 ,,	...	...	9 ,,	0·09	...	...
12 ,,	...	...	...	...	...	...
13 ,,	12 ,,	0·045	...	...	...	...



1,000,000 times the dose of toxin given. Again there is definite evidence of active production of antitoxin between the fourth and fifth day after antitoxin was given to rabbit 33, between the fifth and sixth day in rabbit 34, and between the fourth and fifth day in rabbit 35. In all three rabbits the antitoxic level 10 or more days after injection was greater than the level at the commencement of the experiment. The passive immunity given is of the order of 8 units and the active immunity induced about 1/10 unit per c.c. It is necessary therefore to depict the course of antitoxin content on

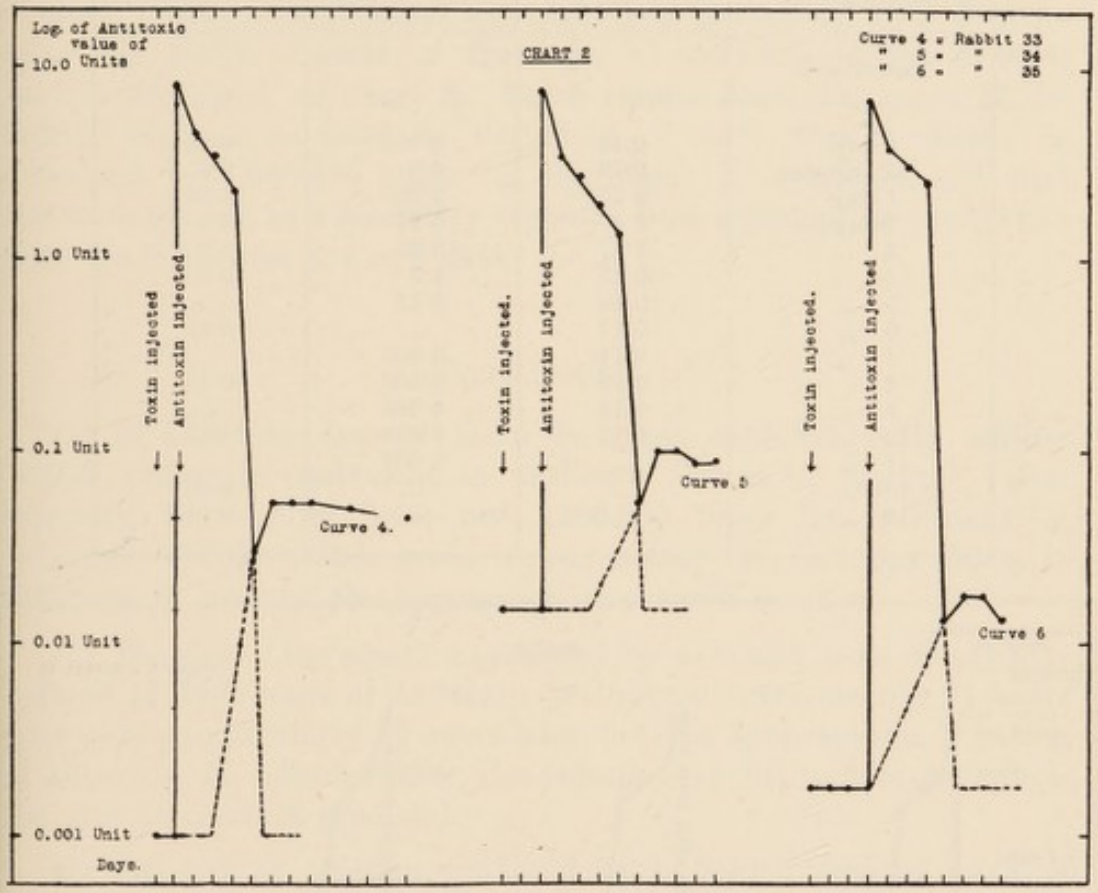


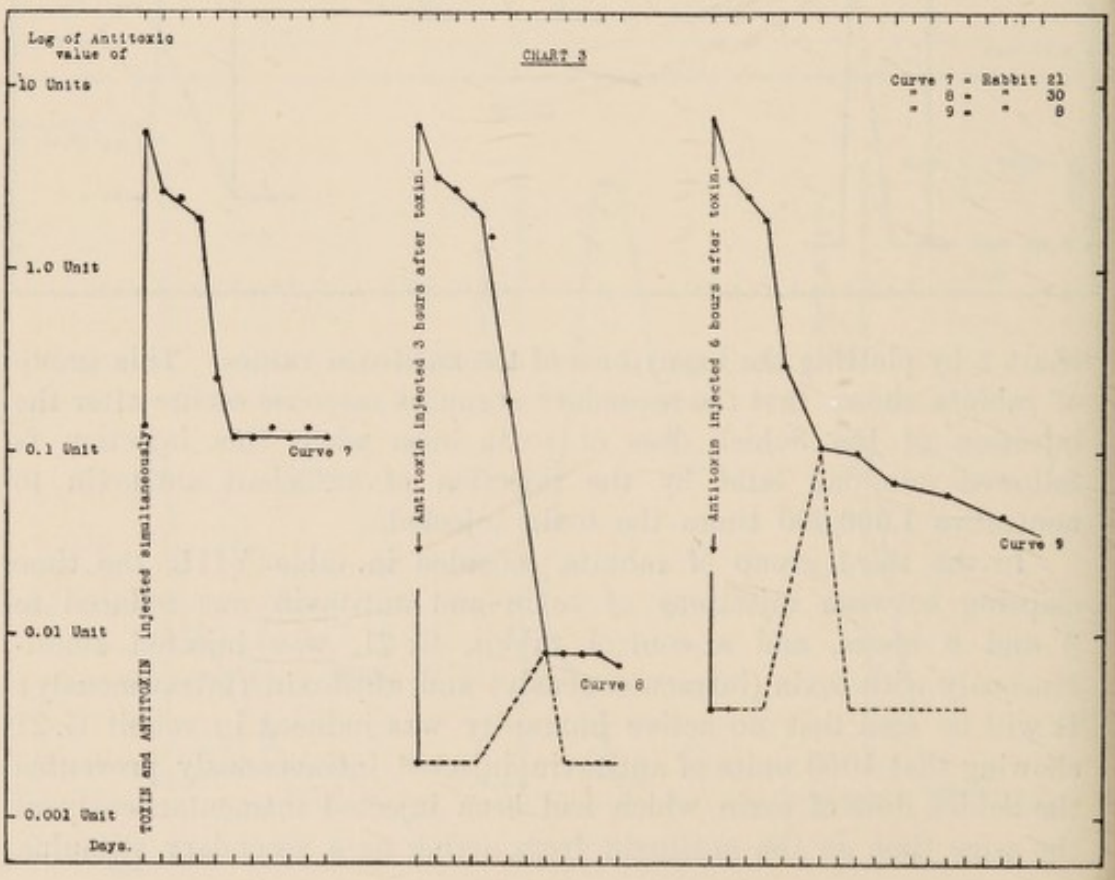
chart 2 by plotting the logarithms of the antitoxin values. This group of rabbits shows that the secondary stimulus response occurs after the injection of the Schick dose of toxin even when this injection is followed one day later by the injection of sufficient antitoxin to neutralise 1,000,000 times the toxin injected.

In the third group of rabbits, recorded in table VIII., the time elapsing between injections of toxin and antitoxin was reduced to 3 and 6 hours, and a control rabbit, G. 21, was injected simultaneously with toxin (intracutaneously) and antitoxin (intravenously). It will be seen that no active immunity was induced in rabbit G. 21 showing that 1000 units of antitoxin injected intravenously prevented the Schick dose of toxin which had been injected intracutaneously at the same time as the antitoxin from acting as a secondary stimulus.

TABLE VIII.

Showing the antitoxic content of actively immune rabbits injected with Schick toxin at different intervals of time before the intravenous injection of antitoxin.

Rabbit.	G. 21.	G. 30.	G. 8.
Interval between toxin and antitoxin.	Simultaneously	3 hours	6 hours
Units of antitoxin given.	1000 units intravenously	1000 units intravenously	1000 units intravenously
Time interval.			
0 day	0·14	0·002	0·004
15 minutes	5·75	6·0	6·5
1 day	2·75	3·25	3·25
2 days	2·5	2·75	2·5
3 ,,	1·87	2·25	1·9
4 ,,	0·25	1·5	0·3
5 ,,	0·12	0·11	...
6 ,,	0·12	...	0·11
7 ,,	0·14	0·008	...
8 ,,	0·12	0·008	0·10
9 ,,	0·14	0·008	...
10 ,,	0·12	0·008	0·07
11 ,,	...	0·007	...
13 ,,	...	...	0·06
16 ,,	...	...	0·045



This rabbit lost all its passive immunity by the fifth day, and after that day the antitoxic level remained constant within the limits of experimental error. We know from our previous work on passive immunity that in serum sensitive rabbits phase C, the phase of rapid elimination, occurs about the third or fourth day and all passive immunity is lost in the next two days. Rabbits G. 30 and G. 8 did not reach their original value in this time showing that rabbit G. 30 had responded to a very slight extent to the stimulus of Schick toxin given 3 hours before antitoxin, and rabbit G. 8 responded to a much greater extent when antitoxin was not given until 6 hours after the toxin. Curves of the logs. of antitoxin values of these rabbits are given on chart 3. These results show that some of the Schick toxin is so fixed by tissues in 3 hours that it cannot be dislodged by 1,000,000 times its equivalent in antitoxin, and that the toxin can act as a secondary stimulus even although no productive response is seen for 3, 4 or 5 days.

Conclusions.

(*a*) If a Schick dose of toxin be given intradermically neither 10,000 times its equivalent in antitoxin (10 units) injected intravenously 15 minutes later nor 1,000,000 times its equivalent in antitoxin (1000 units) given intravenously 30 minutes later is sufficient to prevent the appearance of a small reaction.

(*b*) The size of the reaction produced by a Schick dose of toxin is reduced if 1000 units of antitoxin be injected intravenously $2\frac{1}{2}$ hours later or intramuscularly $1\frac{3}{4}$ hours later, but the subcutaneous injection of antitoxin 15 minutes after the injection of toxin fails to reduce the size of reaction produced.

(*c*) The size of reaction produced by a Schick dose of toxin is reduced to half its usual diameter if 10 units of antitoxin be injected intravenously $\frac{1}{2}$ hour later, or if 1000 units be injected intravenously $1\frac{1}{2}$ hours later, intramuscularly $\frac{3}{4}$ hour later or subcutaneously $\frac{1}{4}$ hour earlier.

(*d*) A reaction equivalent to that produced by half the quantity of toxin injected will be produced by 1/10th of a Schick dose given intradermically followed 3 hours later by 10,000,000 times its equivalent of antitoxin given intravenously.

(*e*) If a Schick dose of diphtheria toxin be given to an immune rabbit it will act as a secondary stimulus causing an increase in antitoxic titre of the blood even although 1000 units of antitoxin be given intravenously 3 hours after the injection of the toxin.

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