The effect of serum-sensitiveness and precipitin formation upon the efficacy of diphtheria toxoid and toxin-antitoxin mixtures in promoting antitoxin production / A.T. Glenny, Barbara E. Hopkins and Hilda Waddington.

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THE EFFECT OF SERUM-SENSITIVENESS AND PRECIPITIN FORMATION UPON THE EFFICACY OF DIPHTHERIA TOXOID AND TOXIN-ANTITOXIN MIXTURES IN PROMOTING ANTITOXIN PRODUCTION.

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EARLIER work on the subject of passive immunity has shown that antitoxin given to a sensitive animal is rapidly lost from the circulating blood. The rate of disappearance has been traced in rabbits previously sensitised by small doses of serum such as are present in 1·0 c.c. of toxin-antitoxin mixtures used for human immunisation (Glenny and Hopkins, 1922). It became evident that this rate of loss might have an important bearing upon the effect of an injection, in a sensitised animal, of a toxin-antitoxin mixture, causing possibly the rapid elimination of either the antitoxin alone or the mixture as a whole. It was also considered that, apart from the question of direct loss of material injected, the cellular activity and the precipitin formation which follow the injection of serum into a sensitive animal might inhibit or interfere with the immunising power both of toxoid and toxin-antitoxin mixtures as primary and secondary stimuli. In this paper, we shall deal with experimental work directed to investigate these two aspects of the subject.

The possibility of a rapid elimination of the antitoxin from a toxin-antitoxin mixture when injected into a serum sensitive animal was investigated on guinea-pigs. These animals were given toxin-antitoxin mixtures in doses sufficiently large that if the antitoxin alone were lost, there would remain in the circulation several lethal doses of toxin; guinea-pigs not previously sensitised were used as controls. The serum-sensitive animals did not succumb to the injection, and produced no larger swellings than did the control pigs. It was therefore concluded that if any elimination had taken place, both the toxin and antitoxin had been eliminated from the circulation.

Table I. and charts 1 and 2 show the results of an experiment upon the influence of precipitin formation upon the antigenic effect of a toxin-antitoxin mixture (T.A.M.) given as a secondary stimulus.

Previous experiments had shown that in a normal rabbit precipitin production begins about the seventh day and is thus less rapid than in a sensitive animal in which rapid production begins four days after the injection of serum, so that the injection of a toxin-antitoxin mixture, preceded four days by serum, would probably be unable to produce any immunity response if rapid elimination

occurred. A rabbit, G. 39, was made sensitive to horse serum and actively immune to diphtheria toxin by the injection of a diphtheria prophylactic mixture. Seven months later, a secondary stimulus produced a rise in antitoxic titre. After a further rest of another two weeks, the rabbit received 0·5 c.c. of normal horse serum ; after a further four days, when precipitin formation would be expected, a secondary stimulus was given consisting of 5 c.c. toxin-antitoxin. It can be seen that the effect of the stimulus was very small indeed, the antitoxic content of the blood only rising from its original 0·22 unit per c.c. to 0·3 unit per c.c. The second injection was given after a pause of thirty days when the antitoxic content had dropped to 0·09 unit per c.c. and no normal serum was given beforehand. At the end of eight days, the antitoxic value of the blood had risen to 0·70 unit per c.c. Twenty-three days later another dose of toxin-antitoxin was given preceded by horse serum and twenty-four days after that toxin-antitoxin alone. The results obtained from the third and fourth injections were very similar to those obtained from the first and second respectively, the third stimulus having very little effect, the fourth producing a large rise in antitoxic value. The same mixture and the same dose were employed for all four injections.

TABLE I.

Showing the effect upon the secondary stimulus response of two rabbits of the intravenous injection of serum four days before the subcutaneous injection of a toxin-antitoxin mixture.

Rabbit . . .	39.				7.		
Interval after last injection .	18 days	30 days	23 days	24 days	6 mths.	5 weeks	6 we
Volume of normal horse serum injected 4 days before toxin-antitoxin mixture	0·5 c.c.	--	0·5 c.c.	–	–	0·5 c.c.	–
Volume of toxin-antitoxin mixture injected (B. 543)	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c
Units of antitoxin per c.c.							
Before injection of toxin-antitoxin mixture	0·22	0·09	0·18	0·14	0·003	0·035	0·0
1 day after							
2 days after . . .	0·18	0·09	0·18	0·12	0·003	0·035	0·0
3 ,,							
4 ,, . . .	0·18	0·25	0·22	0·30	0·22	0·05	0·1
5 ,,							
6 ,, . . .	0·22	0·60	0·35	0·70	0·55	0·16	0·5
7 ,,							
8 ,, . . .	0·30	0·70	...	0·90	...	...	0·5
9 ,, . . .	...	...	0·35	...	0·50	0·20	...
10 ,, . . .	0·30	...	...	1·1	...	...	0·5
11 ,, . . .	...	0·50	0·35	...	0·40	...	...
12 ,, . . .	...	...	...	...	...	0·20	...
13 ,, . . .	0·30	...	0·30	0·9	0·35	...	0·5
14 ,, . . .	...	0·40	...	...	...	...	...
15 ,,							
16 ,, . . .	0·22	...	0·25	...	0·20	...	0·3
17 ,,							
18 ,, . . .	...	...	0·18	...	...	0·11	...
19 ,, . . .	...	...	...	...	0·18	...	...
20 ,,							
21 ,,							
22 ,,							
23 ,, . . .	...	...	...	...	0·14	...	...

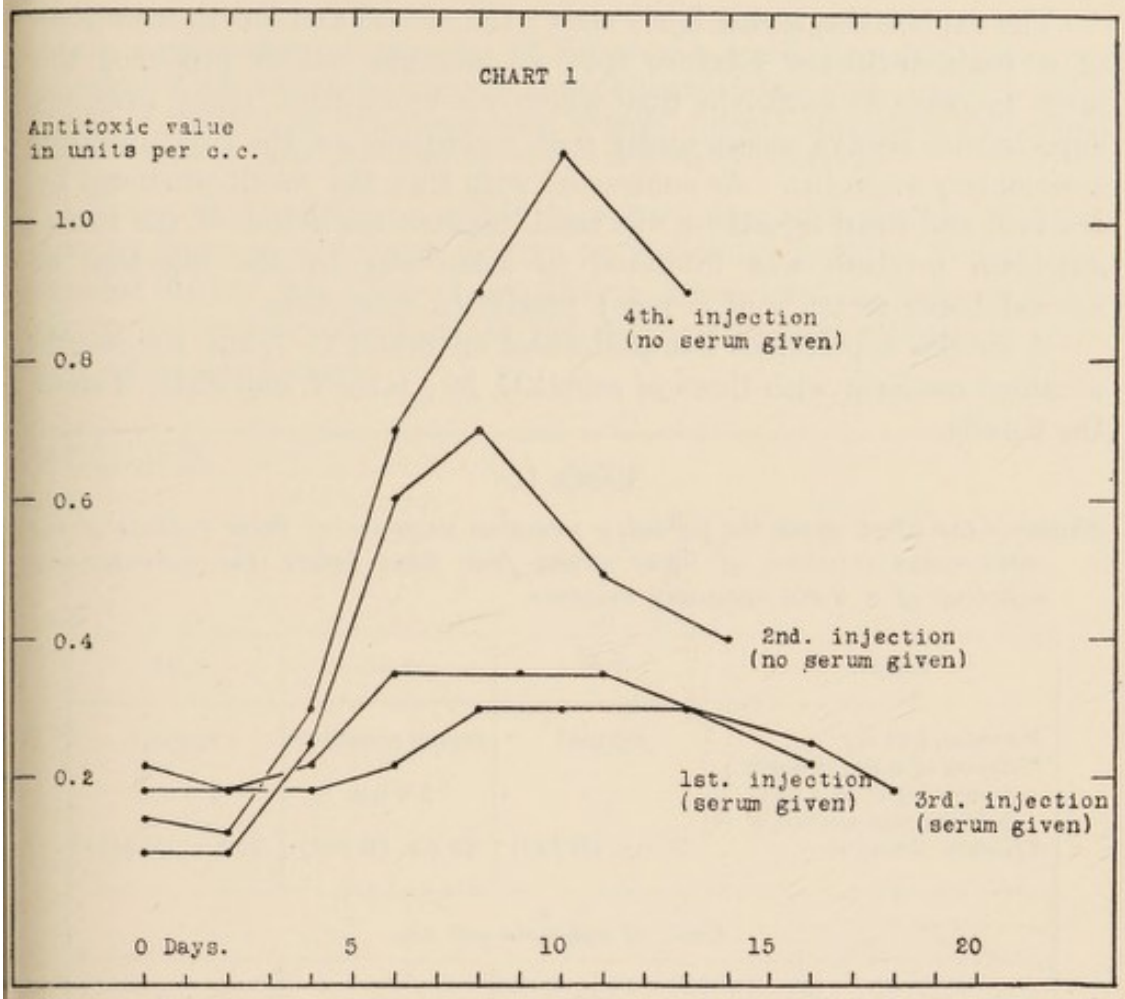
CHART 1
Antitoxic value
in units per c.c.
1.0
0.8
0.6
0.4
0.2
4th. injection
(no serum given)
2nd. injection
(no serum given)
1st. injection
(serum given)
3rd. injection
(serum given)
0 Days.
5
10
15
20

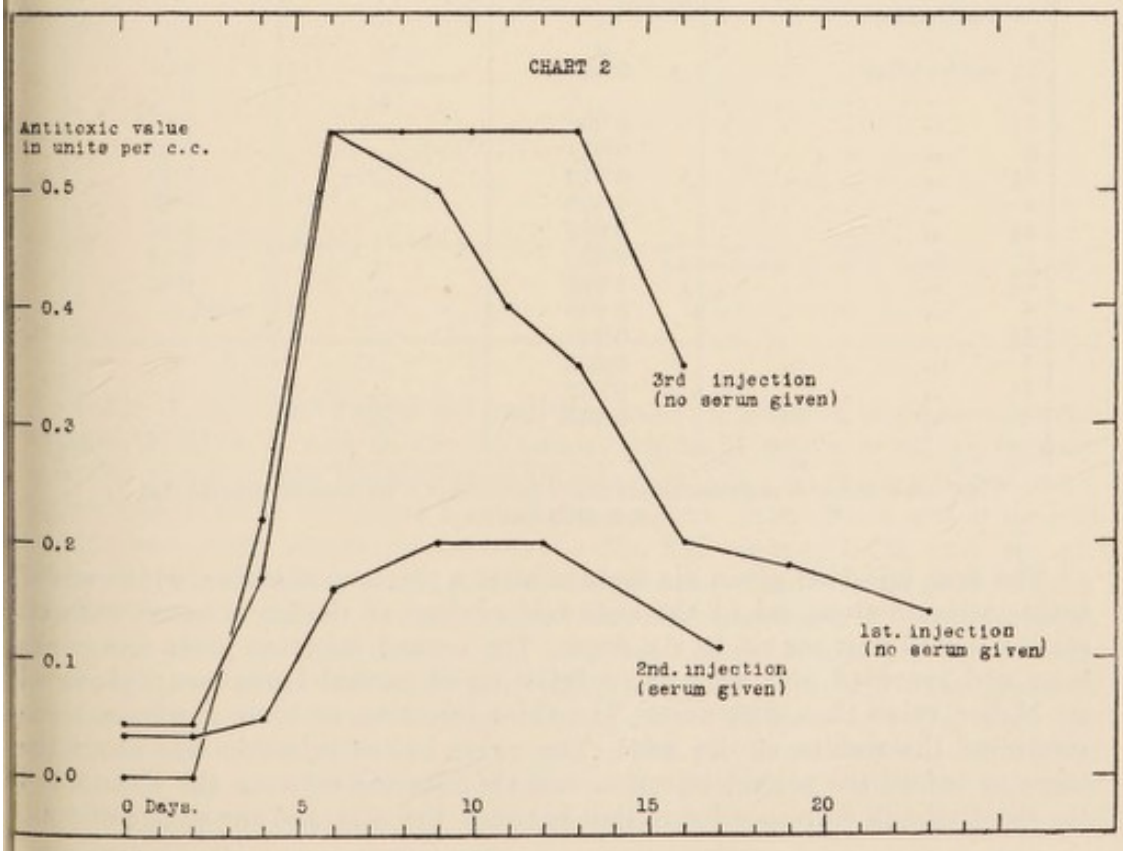
CHART 2
Antitoxic value
in units per c.c.
0.5
0.4
0.3
0.2
0.1
0.0
3rd injection
(no serum given)
1st. injection
(no serum given)
2nd. injection
(serum given)
0 Days.
5
10
15
20

The explanation seems fairly clear; the second and fourth injections of a toxin-antitoxin mixture into an immune rabbit produced the large increase in antitoxin titre which we know from many previous experiments always occurs under such conditions *i.e.* the rise following a secondary stimulus. As contrasted with this, the result produced by the first and third injections was small because the action of the toxin-antitoxin mixture was inhibited in some way by the injection of normal horse serum and sequent precipitin formation.

A similar experiment was performed on rabbit G. 7, and the results obtained conform with those of rabbit G. 39; table I. and chart 2 show the details.

TABLE II.

Showing the effect upon the primary stimulus response of three rabbits of the intravenous injection of horse serum four days before the subcutaneous injection of a toxin-antitoxin mixture.

Rabbit . . .	G. 53.	G. 41.	G. 81.
Previous history . .	normal	serum sensitive	normal
Volume of normal horse serum injected 4 days before toxin-antitoxin	...	1·0 c.c.	1·0 c.c.
Primary stimulus . .	25 c.c. (B 543)	25 c.c. (B 543)	25 c.c. (B 543)
Units of antitoxin per c.c.			
Before injection of toxin-antitoxin	nil *	nil	nil
½ week after . . .	,,	,,	,,
1 ,, . . .	,,	,,	,,
1½ weeks after . . .	0·006	,,	0·01
2 ,, . . .	...	,,	0·015
2½ ,, . . .	0·009	,,	0·015
3 ,, . . .	0·011	,,	0·02
3½ ,, . . .	0·012	,,	0·03
4 ,, . . .	0·022	,,	0·05
4½ ,, . . .	0·022	,,	0·07
5 ,, . . .	0·025	,,	0·07
5½ ,, . . .	0·033	,,	0·07
6 ,, . . .	0·033	...	...
6½ ,, . . .	0·033	...	...
7 ,, . . .	0·033	...	...
7½ ,, . . .	0·033	...	...
8 ,, . . .	0·033	...	...

* *Nil* means either no antitoxin or less than 0·0005 unit, *i.e.* the smallest amount that we ordinarily test for.

The first injection given six months after a previous stimulus, which was of toxin-antitoxin alone, raised the antitoxic content of the blood from 0·003 unit per c.c. to 0·55 unit per c.c. in six days. The second injection given five weeks later and preceded four days by an injection of normal horse serum produced no higher value than 0·20 unit. The third injection of toxin-antitoxin alone confirmed the results of the first. The value before injection was about the same as before the second injection and the interval between the second and the third stimuli corresponded to that between the first and the second stimuli.

Again we find that the injection of homologous serum, four days before the injection of a secondary stimulus (toxin-antitoxin), into a serum sensitive rabbit which is immune to diphtheria toxin greatly reduced the effect that would otherwise have been produced by such a secondary stimulus.

These experiments all relate to a secondary stimulus in animals already immune, and seem to give very definite results. It was now decided to try the effect of sensitising a non-immune animal before giving the primary stimulus, *i.e.* the first injection of diphtheria toxin and antitoxin mixture.

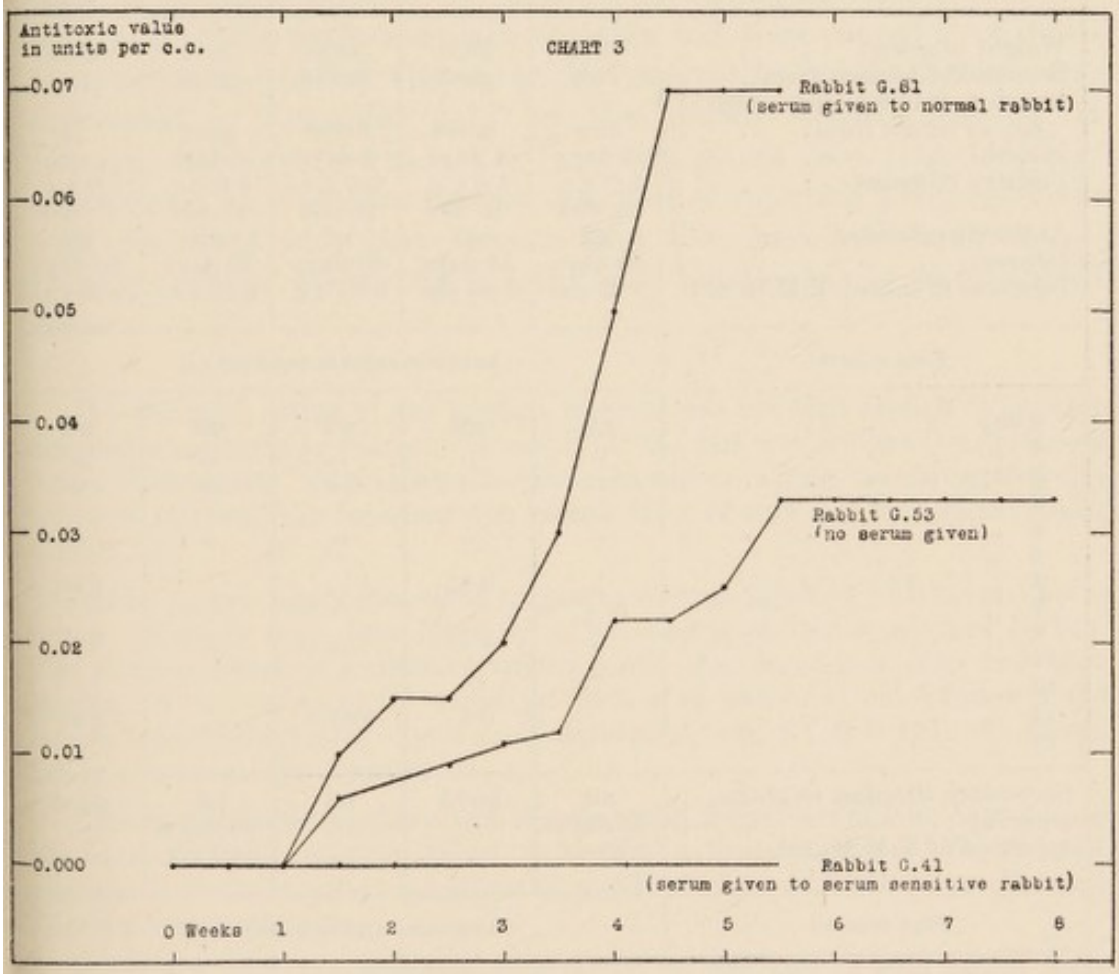

Table II. and chart 3 show the results of an experiment on three non-immune rabbits, G. 53, G. 41 and G. 81. A normal rabbit (G. 53) received as a primary stimulus 25 c.c. of the toxin-antitoxin mixture B. 543. At the end of one and a half weeks its blood contained a titratable amount of antitoxin, and at the end of five and a half weeks the antitoxic value had risen to 0·033 unit per c.c. This was a reasonable response and showed that the mixture was a good antigen. A serum-sensitive rabbit (G. 41) received the same amount of the same mixture four days after an injection of 1 c.c. of normal horse serum and the mixture produced no detectable antitoxin. This dose of toxin-antitoxin repeated six months later did not act as a secondary stimulus, showing that the rabbit had not been actively immunised by the previous injection. A normal rabbit (G. 81) also had normal horse serum four days before an injection of toxin-antitoxin but in this case the immunity production was in no way impaired.

Summarising this experiment, we have three rabbits, two normal and one serum sensitive. The two normal animals received an injection of a toxin-antitoxin mixture, but in one the mixture was preceded four

TABLE III.

Showing the effect upon the primary stimulus response of three rabbits of the intravenous injection of homologous or heterologous serum four days before the subcutaneous injection of a toxin-antitoxin mixture.

Rabbit	87.	88.	109.	118.	128.
Weight in grams	2920	2920	2920	2010	1900
Sensitised to serum from	cow	goat	horse	goat	...
Injected intravenously with 1 c.c. of serum from	cow	horse	horse	goat	...
Interval	4 days	4 days	4 days	4 days	...
Primary stimulus	5·0 c.c. B. 654	5·0 c.c. B. 654	5·0 c.c. B. 654	5·0 c.c. B. 654	5·0 c.c. B. 654
Antitoxin produced	nil	nil	nil	nil	nil
Interval	30 days	30 days	30 days	30 days	30 days
Injection of toxoid Y.M.B. 101	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.	0·05 c.c.
Time interval.	**Antitoxic value in units per c.c.**				
0 day	nil	nil	nil	nil	nil
1 ,,	,,	,,	,,	,,	,,
2 days	,,	,,	,,	,,	,,
3 ,,					
4 ,,	,,	,,	,,	,,	,,
5 ,,					
6 ,,	,,	0·55	,,	,,	0·06
7 ,,					
8 ,,	,,	1·75	,,	,,	0·35
9 ,,					
10 ,,					
11 ,,	,,	0·8	0·01 *	,,	0·2
12 ,,					
Secondary stimulus response	nil	good	nil	nil	good
Interval	33 days	...	...	33 days	...
Injection of Y.M.B. 101	0·05 c.c.	...	...	0·05 c.c.	...
Time interval.	**Antitoxic value in units per c.c.**				
0 day	nil	...	...	nil	...
1 ,,	,,	...	...	,,	...
2 days	,,	...	...	,,	...
3 ,,					
4 ,,	0·05	...	...	0·12	...
5 ,,					
6 ,,	0·25	...	...	0·55	...
7 ,,					
8 ,,	0·4	...	...	0·45	...

* 0·05 unit thirteenth day, and 0·06 unit eighteenth day, indicating that a primary response to toxoid had occurred.

days by normal horse serum. Both animals produced good immunity. The serum-sensitive rabbit, given normal horse serum followed four days later by a toxin-antitoxin mixture, failed to respond. Thus

absence of immunity occurs when toxin-antitoxin is given at the time of precipitin formation. In a normal rabbit precipitin begins to appear at about the seventh day and had rabbit B. 81 received its dose of toxin-antitoxin seven or eight instead of four days after the normal horse serum the former would have been a less effective stimulus, but given on the fourth day the antigen was able to produce a maximum response undisturbed by precipitin accumulation.

The number of animals in this experiment was too small to do more than give slight support to our general conclusions, and it was not possible to decide whether the horse serum during its rapid elimination took with it the toxin-antitoxin complex and thus caused a reduction in response, or whether the antigenic response to diphtheria toxin was suppressed or "crowded out" by the already existing activity of response to horse serum. In the next group (see table III.) of experiments we therefore studied the effect of injecting toxin-antitoxin mixtures made with horse serum, into cow, goat, and horse-serum-sensitive rabbits, previously injected with homologous or heterologous serum.

The antigenic value of the mixture injected was not high enough to produce detectable antitoxin in the control rabbit G. 128, but was sufficient to produce a "ground immunity" or primary stimulus response for a large secondary stimulus response followed the injection one month later of 0·05 c.c. of modified toxin, Y.M.B. 101.

Rabbit 88 previously sensitised to goat serum was injected with normal horse serum, followed four days later by a toxin-horse-antitoxin mixture B. 654. The mixture acted as a primary stimulus, and that basal immunity had been established was shown by the rapid production of antitoxin which followed the injection thirty days later of 0·05 c.c. of diphtheria toxoid Y.M.B. 101—the toxoid acting as a secondary stimulus.

Rabbit 109, horse-sensitive and injected with horse serum four days before the toxin-antitoxin mixture failed to become actively immune. The delayed response to the subsequent injections of modified toxin was such as would occur after a primary stimulus.

Rabbits 87 and 118, sensitive respectively to cow and to goat serum, were injected with cow and goat serum. No antitoxin was produced as a result of an injection of toxin-horse-antitoxin mixture, and the absence of a secondary stimulus response to an injection of toxoid one month later showed that no active immunity had resulted from the injection of toxin-antitoxin. A later injection of toxoid Y.M.B. 101 acted as a secondary stimulus showing that the rabbits were not refractory and that the first injection of toxoid had acted as a primary stimulus, although the earlier injection of toxin-antitoxin had failed.

It must be pointed out that the normal horse, cow and goat serum used throughout these experiments did not contain any "detectable" antitoxin. The smallest amount of antitoxin that we can detect with any degree of certainty is 0·0005 unit per c.c.: it is not probable that the presence of less than this amount of antitoxin would have any specific effect upon the antigenic values of either the mixture or the modified toxin used.

These results may be summarised as follows:—

Rabbit.	Sensitive to serum from	Serum injected.	Probable precipitin formation.	Antigenic response to toxin-antitoxin made with horse serum.
128	–	–	–	+
88	goat	horse	–	+
109	horse	horse	horse	–
87	cow	cow	cow	–
118	goat	goat	goat	–

The formation of precipitin to cow and goat serum prevented an antigenic response to the injection of a toxin-antitoxin mixture with horse serum. We infer from this that the failure of a toxin-horse-antitoxin mixture to produce immunity in animals producing precipitin to a heterologous serum must be due to an interference of activity by the precipitin formation and not an elimination of the antigen.

This interference of activity should also be made manifest when the secondary stimulus contains no antitoxic serum. The next experiments were therefore performed to see whether this "crowding out"

TABLE IV.

Showing the effect of the intravenous injection of normal horse serum upon the primary stimulus response of rabbits to toxoid.

Rabbit	82.	49.	55.
Sensitive to serum from . . .	–	horse	horse
Injected intravenously with serum from	horse	horse	horse
Interval	4 days	4 days	4 days
Primary stimulus of Y.M.B. 101 .	0·1 c.c.	0·05 c.c.	0·1 c.c.
Time interval.	Antitoxic value in units per c.c.		
0 day	nil	nil	nil
4 days	,,	,,	,,
7 ,, ...	,,	,,	,,
10 ,, ...	0·6	,,	,,
14 ,, ...	0·32	,,	,,
25 ,, ...	0·25	,,	,,
32 ,, ...	0·12	,,	,,
Interval	...	33 days	33 days
Secondary stimulus of Y.M.B. 101	...	0·05 c.c.	0·05 c.c.
Time interval.	Antitoxic value in units per c.c.		
0 day	...	nil	nil
2 days	...	,,	,,
4 ,, ...	...	0·04	0·6
6 ,, ...	...	2·75	3·75
8 ,, ...	...	2·75	2·25
11 ,, ...	...	1·75	1·75
13 ,, ...	...	1·12	1·25

of immunity response occurred when diphtheria toxoid was used in place of toxin-antitoxin mixtures as a primary stimulus and also as a secondary stimulus. Table IV. shows the results of injecting diphtheria toxoid as a primary stimulus during or before formation of precipitin to horse serum. The number of animals used in this experiment is so small that the results are not very convincing.

Rabbit 82 without previous treatment was injected intravenously with horse serum four days before the subcutaneous injection of modified toxin. A large response occurred giving rise to an antitoxic titre of 0·6 unit in ten days. This response is one of the greatest that we have ever recorded : eleven rabbits were at different times injected with either 0·05 c.c. or 0·1 c.c. of this same modified toxin and only one reached a higher titre than 1/100th unit. The other two rabbits 49 and 55 were already horse-sensitive and were injected with horse serum four days before an injection of toxoid. No detectable antitoxin was produced but a second injection of toxoid thirty-three days later was followed by a rise in antitoxic value showing that some immunity had resulted from the first injection. The experiments show that the injection of diphtheria toxoid into rabbits actively making precipitin to horse serum produces some immunity response, though probably the toxoid is of lessened efficiency under these conditions.

A more striking result is obtained when diphtheria toxoid is given as a secondary stimulus to serum-sensitive animals. The most satisfactory method of comparing the secondary stimulus response in a number of animals subjected to different treatment is to repeat the injections on several occasions over a long period of time and to observe the behaviour of a group as a whole rather than each animal individually.

A series of rabbits immune to diphtheria and sensitive to horse serum was injected on four occasions with a dose of toxoid and on alternate occasions this dose was preceded four days by an injection of normal horse serum (see table V.) ; on each occasion, some of the rabbits received normal horse serum and afterwards toxoid while others had toxoid only ; thus the rabbits fell into two groups, 1 and 3. An additional group 2 was formed by two rabbits that were not serum sensitive at the commencement of this experiment : their primary stimulus to diphtheria had consisted of toxoid alone and not of a toxin-antitoxin mixture containing horse serum. The initial antitoxic content of the blood immediately before the secondary stimulus was found and also the maximum value reached six, eight and ten days subsequent to the injection.

Table V. shows that the injection of normal horse serum four days before the injection of toxoid caused a definite reduction in immunity response. In the first group the first injection of toxoid was preceded by one of normal horse serum. There was considerable variation in response to this injection but all four rabbits showed a greater response to the next injection of toxoid not preceded by horse serum. The second group consisted of two non-sensitised animals and there was no marked difference between the results from the first and second injection of toxoid ; one rabbit showed an increased and one a decreased response. In the third group one rabbit showed a decreased response to the second injection preceded by serum. Comparing the responses to the second and third injections we may include groups 1 and 2 together because the injection of horse serum in April rendered the rabbits in group 2 serum sensitive. Although the dose of toxoid injected in July was increased to 5 c.c. only one

rabbit, G. 218, out of six showed a big increase in response to the third injection and three responded to a smaller extent to an injection of 5 c.c. of toxoid preceded by horse serum than to the previous injection of 1 c.c. not preceded by serum. In the third group however the injection of toxoid in July, not preceded by horse serum, produced an increased response in all four rabbits.

Similar results are seen when the third and fourth injections are compared. In groups 1 and 2, horse serum was not given before the September injection and an increased response was produced in all six rabbits while the inclusion of horse serum in group 3 caused two rabbits to show a lessened response while the remaining two remained at the same value.

TABLE V.

Showing the antitoxic value of the serum of ten immune rabbits after each of four injections of diphtheria toxoid preceded on alternate occasions by an injection of normal horse serum.

Date of injection.	April.	June.	July.	September.
Toxoid injected . . (same for all 3 groups)	1·0 c.c.	1·0 c.c.	5·0 c.c.	5·0 c.c.
GROUP 1.—*Rabbits serum-sensitive at start.*				
Normal horse serum .	5·0 c.c.	nil	5·0 c.c.	nil
Rabbit 217 . .	2·5 units	5·5 units	4·5 units	7·0 units
,, 218 . .	1·0 ,,	1·75 ,,	4·0 ,,	10·0 ,,
,, 222 . .	0·2 ,,	1·75 ,,	2·0 ,,	5·0 ,,
,, 225 . .	0·2 ,,	1·25 ,,	0·8 ,,	5·0 ,,
GROUP 2.—*Control rabbits not previously serum-sensitive.*				
Normal horse serum .	5·0 c.c.	nil	5·0 c.c.	nil
Rabbit 215 . .	(2·5) (*a*) units	1·0 units	1·5 (*b*) units	5·0 units
,, 221 . .	(2·5) (*a*) ,,	5·5 ,,	3·5 (*b*) ,,	8·0 ,,
GROUP 3.—*Rabbits serum-sensitive at start.*				
Normal horse serum .	nil	5·0 c.c.	nil	5·0 c.c.
Rabbit 216 . .	1·5 units	0·9 units	2·5 units	2·5 units
,, 219 . .	0·8 ,,	1·25 ,,	5·0 ,,	3·5 ,,
,, 223 . .	1·5 ,,	2.5 ,,	7·0 ,,	7·0 ,,
,, 224 . .	1·5 ,,	3·5 ,,	12·0 ,,	3·5 ,,

(*a*) Rabbits not serum-sensitive therefore figures not comparable with group 1.
(*b*) Rabbits now serum-sensitive from injection of serum in April and figures now comparable with group 1.

Summarising the results, we find that on all fourteen occasions the response to an injection not preceded by horse serum (in sensitised rabbits) was greater than the response to the previous injection preceded by horse serum, while six out of fourteen injections preceded

by horse serum produced a smaller response than the previous injection without horse serum and on two other occasions there was no increased response. It is clear therefore from the figures given, that the injection of normal horse serum into horse sensitive immune rabbits four days before the injection of toxoid lessens the response produced. Therefore, precipitin formation interferes with the action of toxoid as a secondary stimulus.

Conclusions.

1. If a rabbit be sensitised with horse serum and is later given an injection of horse serum, rapid precipitin formation follows; if an injection of diphtheria toxin-horse-antitoxin mixture be given during this stage of active precipitin formation, the mixture fails as a primary and a secondary stimulus.

2. It is believed that this observed phenomenon is entirely due to a "crowding out" of cellular activity by precipitin formation, and it is shown in support of this view that:—

(*a*) Precipitin formation prevents the action of toxin-horse-antitoxin mixture even when heterologous cow or goat serum precipitin is being formed.

(*b*) The formation of precipitin to horse serum has a marked inhibitory action on the antigenic power of diphtheria toxoid.

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