Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY, Vol. XXVIII., 1925

IMMUNOLOGICAL NOTES

VIII.-XVI.

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From the Wellcome Physiological Research Laboratories, Beckenham.

(*Continued from* p. 344.)

VIII.—COMPARISON BETWEEN *IN VITRO* AND *IN VIVO* METHODS OF TESTING DIPHTHERIA ANTITOXIN.

The serum ratio $\frac{\text{in vivo } value}{\text{in vitro } value}$ *is not constant for all samples of serum; successive samples of serum from the same horse often show approximately the same ratio.*

It has already been pointed out by Glenny and Okell (this *Journal*, 1924, xxvii. 187), Sordelli and Serpa (*C. R. Soc. Biol.*, 1924, xci. 1043), Glenny, Pope and Waddington (1925, *supra* p. 279) and Glenny and Wallace (1925, *supra* p. 317) that there is not always the exact agreement between the antitoxic titre of a serum determined by animal means and that established by the flocculation method as Ramon (*Ann. Inst. Past.*, 1925, xxxix. 1) maintains. Our animal determinations have been made by the intracutaneous method with occasional subcutaneous controls; those of Ramon presumably subcutaneously. The most marked discrepancy between values determined by the two methods that we have seen occurred in samples of serum taken from the same horse over a period of about twenty weeks. In all 12 samples were tested and each one appeared when tested *in vivo* at least twice as strong as when tested *in vitro;* the majority of samples were only tested to wide limits (10 or 20 per cent.), but one bleeding (15th November) was fully investigated and when tested as closely as possible (5 per cent.) gave *in vitro* and *in vivo* values of 110 and 260 units respectively, a ratio of 2·4. This same bleeding has been tested against a large number of toxins; in one series of tests the ratio determined against 18 different toxins lay between 2·1 and 2·5 in every instance. This series of tests and a number of other tests against other toxins establishes without doubt the specificity of the flocculation that occurs when mixtures are made in the proportion of 1·0 c.c. of this serum to 110 Lf doses of any toxin. The uniformity of the ratio for successive samples of sera from the same horse is seen in table XVII.

TABLE XVII.

Showing the in vitro *and* in vivo *values and serum ratios for* 12 *samples of serum from the same horse.*

Date of bleeding.	*In vitro* value.	*In vivo* value.	Ratio.
29 Oct. 1923 . . .	under 100	over 300	over 3·0
5 Nov. ,, . . .	,, 200	,, 450	,, 2·2
8 ,, ,, . . .	120	400	3·3
12 ,, ,, . . .	150	400	2·6
15 ,, ,, . . .	100	300	3·0
10 Dec. ,, . . .	175	550	3·1
14 ,, ,, . . .	200	500	2·5
7 Jan. 1924 . . .	200	450	2·2
10 ,, ,, . . .	200	450	2·2
14 ,, ,, . . .	150	300	2·0
4 Feb. ,, . . .	150	350	2·3
17 Mar. ,, . . .	250	550	2·2

The *in vivo* unit corresponds to the Ehrlich unit and agrees with the official Frankfurt and Washington standards; the *in vitro* unit is determined by titrations against toxins standardised against one Ehrlich unit of a certain antitoxin chosen by us.

Another horse showing a high serum ratio was tested at an earlier stage of immunisation; table XVIII. shows the values and ratios for 13 successive samples of serum.

TABLE XVIII.

Showing the in vitro *and* in vivo *values and serum ratios for* 13 *samples of serum from one horse.*

In vitro value.	*In vivo* value.	Ratio.	*In vitro* value.	*In vivo* value.	Ratio.
6·5	15	2·30	95	200	2·10
6·5	19	2·92	170	300	1·76
14	34	2·42	200	350	1·75
30	67	2·23	290	450	1·55
62	150	2·41	305	450	1·47
77	225	2·92	307	450	1·46
67	175	2·61			

It will be seen from this table that during the early stages of immunisation this horse maintained a serum ratio of over 2, and in the later stages the ratio dropped to about 1·5. This lowering of ratio as immunisation proceeds appears of frequent occurrence.

Table XIX. records the serum ratios of successive bleedings of 19 different horses; the *in vitro* values were all determined to within 5 per cent. and the *in vivo* values always to within 10 per cent., and often to within 5 per cent. This group of horses does not represent a number of specially chosen animals, but constitutes an entire group under observation at the same time. The horses have been grouped according to the ratio exhibited. It will be seen that there is a general

Showing the serum ratio $\frac{\text{in vivo } value}{\text{in vitro } value}$ *of successive bleedings from a number of horses.*

Horse.	Nov. 10.	Nov. 17.	Nov. 24.	Dec. 1.	Dec. 4.	Dec. 8.	Dec. 15.	Dec. 22.	Dec. 29.	Jan. 12.	Jan. 15.	Jan. 19.	Jan. 26.	Jan. 29.	Feb. 2.
1305 . .	1·00	...	0·89	...	...	0·98	...	0·95	...	...	0·80	...	0·80	...	0·75
1563 . .	0·47	...	0·82	...	...	0·90	...	0·93	...	...	...	...	...	...	...
1567 . .	1·25	...	0·80	0·82	...	0·88	...	...	...	0·79	...	0·65	...	...	...
1569 . .	1·61	...	1·07	0·81	...	0·91	...	0·86	0·76	...	0·88	...	...	...	...
1548 . .	1·85	...	1·50	...	...	1·77	...	1·35	...	...	1·38	...	1·07	...	...
1564 . .	1·40	...	1·25	...	...	1·00	...	...	...	...	...	...	...	...	...
1568 . .	...	...	1·82	1·41	...	1·25	...	...	...	...	...	...	...	1·07	...
1571 . .	...	...	1·40	1·41	...	1·35	1·08	...	...	...	...	...	...	...	...
1575 . .	...	2·03	...	1·46	...	...	0·93	...	1·00	1·00	...	...	...	...	...
1544 . .	1·20	...	1·12	...	...	1·10	...	...	...	...	...	...	...	...	...
1552 . .	1·28	...	1·35	...	...	1·33	...	1·19	...	...	1·19	...	1·25	...	...
1542 . .	1·51	...	1·20	...	1·30	...	1·46	...	...	...	...	...	...	...	...
1560 . .	1·46	...	1·12	...	...	1·28	...	1·25	...	...	...	...	...	...	...
1579 . .	...	1·50	...	1·73	1·62	...	1·30	...	...	...	...	...	...	...	...
1555 . .	2·14	(2·00)	1·92	...	...	1·67	...	1·55	...	...	...	...	...	...	...
1572 . .	...	2·20	...	1·53	...	...	1·39	...	...	...	...	...	...	...	...
1580 . .	...	1·57	...	1·59	...	...	...	...	...	...	...	1·64	...	...	...
1582 . .	...	...	...	1·80	2·03	...	1·53	...	1·18	...	...	...	...	...	...
1583 . .	...	...	...	1·87	...	...	1·50	...	1·43	...	...	...	...	...	...

tendency for the ratio to be higher in the earlier tests and to become constant as immunisation proceeds. A careful consideration of the figures shows that with certain of the horses (*e.g.* 1305 and 1563) the *in vivo* value is consistently lower than the *in vitro* value. With other horses (1567 and 1569) the earlier bleedings only show a higher value when tested *in vivo*. Another group (1548, 1564, 1568, 1571 and 1575) show high ratios gradually lessening to approximately 1. Others (notably 1552) maintain a fairly uniform level with the *in vivo* value from 20 to 30 per cent. higher than the *in vitro* value; others (*e.g.* 1555 and 1580) show a much higher discrepancy between their two values.

IX.—Two zones of flocculation in Ramon tests.

Certain sera have been found that exhibit two zones of flocculation when titrated against toxin by Ramon's method. Successive samples of serum from horses during immunisation may show one almost constant zone of non-specific flocculation and a specific zone altering as the immunisation proceeds.

In the early stages of immunisation of a horse, it was found that while the *in vivo* value increased from 12 to 30, 40 and then 67 units per c.c. the *in vitro* figure remained almost constant at about 10 units per c.c. The next sample taken contained 150 *in vivo* units while the Ramon test showed 8 units per c.c. with typical flocculation occurring in 30 minutes. A long range of tests, however (see table XX.), revealed a second zone of flocculation at the end o

Table XX.

Showing two in vitro *values from successive samples of serum from the same horses.*

In vitro value.		*In vivo* value.	Ratio.	Flocculation time in minutes.	
Non-specific zone.	Specific zone.			Non-specific zone.	Specific zone.
9	80	40	0·50	30	Over 150
8	100	67	0·67	35	,, 150
8	187	150	0·80	30	120
9	200	200	1·00	35	100
10	210	200	0·95	40	210
10	180	...	...	40	120
7	140	100	0·71	30	90
10	200	150	0·75	25	100
7·5	207	150	0·72	35	130
11	215	150	0·70	35	120
13	290	200	0·69	35	120
12·5	290	...	...	...	...
11	270	...	...	45	120
6·2	135	...	...	50	120
6·2	228	200	0·87	35	130
13·3	240	150	0·62	50	120
13	245	...	...	60	130

2 hours; an investigation of some of the earlier samples and many subsequent samples of serum from the same horse showed always two zones; one zone gave an apparent value of about 10 units per c.c. in from 30 minutes to one hour, while the other gave a value about 50 per cent. higher than the *in vivo* value of the corresponding sample and flocculation was much slower. Tests of different samples of serum against another toxin gave a different lower (non-specific) value but the same higher (specific) value; for example, one sample recorded as containing 7 or 140 units per c.c. with one toxin appeared to contain 32 or 135 units when tested against another toxin, and a sample recorded as 10 or 200 appeared to be 45 or 190 against the other toxin.

Table XXI. records instances of samples of serum from another horse exhibiting two zones of flocculation; again the non-specific flocculation is more rapid than the specific flocculation. Another sample of serum from the same horse was titrated against four different toxins and appeared to contain 30 or 285, 55 or 295, 65 or 295 and 30 or 295 units per c.c. Titrations were made at 50° C.; a few tests made at lower temperatures showed similar zones.

TABLE XXI.

Showing two in vitro *values from successive samples of serum from the same horse.*

In vitro value.		*In vivo* value.	Ratio.	Flocculation time in minutes.	
Non-specific zone.	Specific zone.			Non-specific zone.	Specific zone.
64	360	360	1·0	25	60
63	270	240	0·89	30	75
58	155	150	0·98	45	65
80	460	440	0·95	25	50
32	250	200	0·80	60	...
80	560	450	0·80	55	85

X.—THE RELATION BETWEEN ANTIGENIC VALUE AND DOSE INJECTED OF TOXIN-ANTITOXIN MIXTURES AND OF MODIFIED TOXIN.

Many mixtures of diphtheria toxin and antitoxin can be diluted to a considerable extent without a marked decrease in the antitoxin efficiency. This does not hold true to the same extent for unneutralised toxoid.

It has been our custom when preparing toxin-antitoxin mixtures for human immunisation to inject 6 guinea-pigs with each preparation, 3 with 1 c.c. and 3 with 5 c.c. These animals serve as a check that the mixture is not toxic and are used to determine the antigenic efficiency. An average mixture has an immunity index of 2 or 3 for 1·0 c.c. doses and 1 or 2 for 5·0 c.c. doses. It frequently happens however that no difference can be seen in the indices produced, and

with certain mixtures a wide range of doses produces a constant degree of immunity. Table XXII records the results obtained with such a toxin-antitoxin mixture.

TABLE XXII.

Showing the immunity indices (weekly Schick doses) for a series of different doses of a toxin-antitoxin mixture and of a batch of toxoid.

Dose injected.	Indices produced by a toxin-antitoxin mixture (3 guinea-pigs to each dose).			Indices produced by toxoid (2 or 3 guinea-pigs to each dose).		
0·05 c.c. . . .	10	12	over 12	6	11	11
0·1 ,, . . .	4	4	7	4	7	8
0·2 ,, . . .	3	3	5	2	4	8
0·5 ,, . . .	2	3	3	2	3	...
1·0 ,, . . .	2	3	3	2	2	...
2·0 ,, . . .	3	3	3	...	...	...
5·0 ,, . . .	2	2	3	1	1	...

Toxin-antitoxin mixtures containing 1/10 L+ dose per c.c. introduced by Park and Zingher are as efficient as those containing 30 times as much toxin. Table XXIII. shows the antigenic effect of mixtures of a

TABLE XXIII.

Showing the immunity index produced by mixtures of a toxin with antitoxin in different proportions and in different dilutions.

Composition of mixture per 1·0 c.c.		Immunity index of 1·0 c.c. of various dilutions of mixtures.			
Toxin.	Antitoxin.	Undiluted.	Diluted 1 in 40.	1 in 50.	1 in 60.
0·14 c.c.	1 unit	2	...	...	...
0·15 ,,	,,	2, 3	...	...	...
0·16 ,,	,,	1, 2	...	...	...
0·17 ,,	,,	2	...	...	...
0·18 ,,	,,	...	3	2	...
0·19 ,,	,,	...	3, 3	2, 4, x	3, 3, x
0·20 ,,	,,	...	...	...	3

x indicates that the guinea-pigs were still Schick positive at the tenth weekly test.

toxin and antitoxin in different proportions; the most toxic mixture tha was tolerated undilute by guinea-pigs contained 0·17 c.c. of toxin t one unit of antitoxin; 4 out of 6 guinea-pigs injected with mixture containing between 0·14 and 0·17 c.c. of toxin gave an immunity inde of 2; 8 out of 12 guinea-pigs injected with mixtures containing 0·18 t 0·20 c.c. of toxin diluted 40, 50 and 60 fold gave an index of 3 or less With this range of dosage there is no close relationship between th total amount of toxin injected and the degree of immunity produced.

Table XXIV. shows the antigenic effect of still higher dilutions c more toxic mixtures. Several guinea-pigs were injected with 0·0 0·005, 0·002 and 0·001 c.c. of mixtures containing toxin to which ha

been added 60, 70 and 80 per cent. of the amount of antitoxin required to produce an Lo mixture. If 7·7 units of antitoxin were added to 1·0 c.c. of toxin, the mixture became non-toxic; the mixtures tested contained 4·6, 5·4 and 6·2 units of antitoxin per c.c. of toxin.

TABLE XXIV.

Showing the immunity index of high dilutions of toxic mixtures.

Composition of mixture.		Extent of neutralisation.	Immunity index of small volumes of mixture.			
Toxin.	Antitoxin.		0·001 c.c.	0·002 c.c.	0·005 c.c.	0·01 c.c.
1·0 c.c.	4·6 units	60 per cent.	6	3, 3, 4	2, 3, x	3
1·0 ,,	5·4 ,,	70 ,,	3, x, x	4, 6	2, 3, 3	2, 2, 2
1·0 ,,	6·2 ,,	80 ,,	x, x	3	3, 4	2, 3

x indicates that the guinea-pigs were still Schick positive at the tenth weekly test.

The M.L.D. of the toxin used may be taken as about 0·002 c.c. Mixtures containing a single fatal dose of toxin partially neutralised produced a considerable degree of immunity; some immunity was produced by only half as much toxin partially neutralised. A number of undiluted mixtures containing different proportions of the same toxin and antitoxin were tested and no index better than 3 was obtained; from table XXV. it is seen that better results were obtained with non-toxic mixtures diluted 100 fold, and as good results with mixtures diluted 200 and 500 fold. There is strong evidence of qualitative differences between toxins, possibly a function of rate of dissociation; certain toxins produce good immunity over a wide range of dosage even when slightly over-neutralised, while others of apparently the same toxicity or binding power cannot be successfully employed when sufficiently neutralised to be non-lethal to guinea-pigs.

XI.—THE STABILITY OF DIPHTHERIA TOXIN USED FOR THE SCHICK TEST.

Some undiluted diphtheria toxin intended for subsequent dilution for the Schick test decanted into nine different bottles showed unequal rates of deterioration. Four bottles when tested within a year were less than half the strength of the remainder.

The routine method used by us for testing all batches of diluted toxin to be used for the Schick test consists of (*a*) a determination of the minimal reacting dose, and (*b*) a titration against antitoxin to determine its combining power. The need for two such tests has already been pointed out (Glenny, *Lancet*, 1922, vol. i. p. 228). This method of testing shows at once any change in the undiluted toxin from which the dilution is prepared; should the test for the minimal reacting dose show a low value while that for the combining

power with antitoxin remains normal it indicates that some of the toxin has been changed into toxoid, but if the values for both tests fall it shows that destruction of the toxin has taken place. When a batch of toxin has been selected for Schick work it is divided into 100 c.c. quantities under strictly aseptic conditions and tested for sterility. Subsequently a 100 c.c. bottle is subdivided into 10 c.c. bottles as required for use. These bottles are all stoppered with rubber stoppers and kept in a cool dark room. The dilute Schick toxin is prepared from a 10 c.c. bottle, the dilution being of the order of 1/600.

Recently a 100 c.c. bottle of neat toxin was subdivided into 10 c.c. quantities and one bottle was taken into routine use for the preparation of batches of dilute Schick toxin. The first dilutions successfully passed the tests and appeared to be of full strength, but dilutions prepared later were found to have undergone destruction. The remainder of the undilute toxin in the 10 c.c. bottle was therefore discarded, and the second, and later the third bottles of the series taken into routine use. These were found to be of full strength and all dilutions prepared from them passed both tests. When the fourth bottle of the series was tested it was found that marked destruction had taken place and it was decided to test the remaining bottles of the series. Of nine bottles four were found to be of full strength while the other five had undergone marked destruction.

Table XXV. shows the results. The bottles had been kept under the same conditions, and had remained unopened until tested: the P_H of the toxin in each of the nine bottles was identical and all were sterile. It is evident that there is need for constant control tests on toxin used for Schick work.

TABLE XXV.

Showing the results of tests made on diluted toxins of Schick strength prepared from nine bottles of the same toxin. The neat toxin was diluted 1 *in* 600 *to form Schick strength dilution.*

	Dilution.	Bottle numbers and results of tests.								
		A. 1.	A. 2.	A. 3.	A. 4.	A. 5.	A. 6.	A. 7.	A. 8.	A.
Test for M.R.D. by intracutaneous injection of 0·2 c.c. of the following dilutions of the Schick toxin.	1 in 30	...	−	−	−	...	−	...	...	
	1 ,, 25	−	S	S	−	−	±	...	...	
	1 ,, 20	−	+	±	−	−	+	−	−	
	1 ,, 15	S	+	+	−	−	+	−	−	
	1 ,, 10	±	+	+	+	−	+	−	−	
	1 ,, 5	+	...	...	+	...	...	...	...	
Test for combining by intracutaneous injection of 0·2 of a mixture of 1·0 c.c. of Schick toxin with following amounts of antitoxin.	1/200th unit	−	−	−	−	−	−	−	−	
	1/250th ,,	−	S	−	−	−	±	−	−	
	1/300th ,,	−	+	±	−	−	+	−	−	
	1/400th ,,	±	+	+	S	−	+	−	−	
	1/500th ,,	...	...	...	+	−	...	−	−	

+ = full reaction S = small reaction
± = slightly reduced reaction − = negative.

XII.—The destruction of dilutions of diphtheria toxin of Schick strength by shaking.

Dilutions of Schick toxin in saline containing 0·5 *per cent. phenol are rapidly destroyed when shaken in the presence of air.*

We have previously reported, at a meeting of the Pathological Section of the Royal Society of Medicine (see *Lancet*, 1922, i. p. 442), that toxin diluted to Schick strength in 0·5 per cent. phenol saline is very susceptible to shaking; under certain circumstances the toxin is almost completely destroyed. As the details of our experiments were not published at the time we take this opportunity of putting them on record.

During the summer of 1921, several hundred children were Schick tested by Dr O'Brien at a school one hour's journey from the laboratories. The children were tested in small groups and at each visit the toxin dilution was tested before use. To safeguard against the failure of the test due to loss of potency of the toxin on the journey, possibly owing to some such cause as temperature, Dr O'Brien decided always to carry an additional bottle of toxin there and back; when tested afterwards the toxin on every occasion was found to be of full strength. This precaution was necessary as during a warm summer dilute Schick toxin may lose 50 per cent. of its potency in one day.

On one occasion a larger number of children were injected than had been allowed for, and the reserve bottle of toxin was partially used. This sample of Schick toxin when tested was found to have lost over 50 per cent. of its potency, and negative results in the children tested were regarded with suspicion. On the next occasion in addition to the reserve unopened bottle, a partially used bottle was also tested. The former was of full strength while the latter was under half strength. It was suggested by Dr O'Brien that the destruction might possibly have been due to shaking affecting the half bottle to a much greater extent than the full one. This point was therefore investigated experimentally.

Two bottles of toxin dilution, one full and the other half full, were shaken mechanically for 15 minutes. No loss could be detected in the full bottle, while the potency of the half full bottle had dropped to 1/10th of its original strength. We next determined whether the phenol in the diluent had any effect on the destruction caused by shaking. Table XXVI. shows the results of shaking dilutions made with various diluents. The tests were carried out in duplicate and it will be seen that where the diluent contained phenol the destruction was marked.

Twenty samples of toxin diluted with phenol saline in full and in half filled bottles were sent to various parts of the country and returned to us. We were unable to detect any loss in potency in the toxin

contained in full bottles. When however the bottle was only half filled marked destruction occurred. Similar dilutions made without phenol showed no detectable loss even in half filled bottles.

TABLE XXVI.

Showing the strength, after 15 *minutes' shaking, of Schick toxin prepared in different diluents.*

Diluent.	Percentage of original strength.	
	Test 1.	Test 2.
0·7 per cent. saline in tap water without phenol.	100 per cent.	100 per cent.
0·7 per cent. saline in distilled water without phenol.	75 to 100 per cent.	100 per cent.
0·7 per cent. saline in tap water with 0·5 per cent. phenol.	less than 25 per cent.	less than 10 per cent.
0·7 per cent. saline in distilled water with 0·5 per cent. phenol.	less than 25 per cent.	less than 10 per cent.

In a further series of experiments the connection between the volume of diluted toxin in the bottle and of the extent of destruction was studied. Diluted toxin distributed in bottles filled to varying extents was carried in a pocket for varying lengths of time. The original diluted toxin contained 150 minimal reacting doses per c.c. and the number of M.R.D's remaining after treatment are shown in table XXVII. Destruction was most marked with the bottles containing the smallest quantity of liquid, *i.e.* those bottles with the largest relative amount of air and in which the greatest amount of agitation could take place. A control set of bottles was carried in a case and every effort was made to avoid shaking. Among these bottles destruction could only be detected in the bottles one quarter filled with toxin.

TABLE XXVII.

Showing the number of minimal reacting doses remaining after Schick toxin had been carried for varying lengths of time in bottles filled to different extents.

Time of journey.	Number of M.R.D's remaining in—			
	Full bottle.	5/6 full.	½ full.	¼ full.
3 minutes	150	100	50	25
9 ,, ...	...	50 to 100	25	25
14 ,, ...	...	50	5 or less	5 or less
27 ,, ...	150	5 or less	,,	,,
27 ,, (control) . .	150	150	150	25

In a further series of experiments it was shown that toxin diluted in phenol saline was destroyed after two minutes' shaking by hand in partially filled bottles but not in full bottles which contained glass beads

to ensure agitation in the liquid. It was also demonstrated that the amount of shaking caused by carrying half filled bottles from one room to another was sufficient to produce a detectable degree of destruction in the toxin. Undiluted toxin is far less susceptible to destruction by shaking.

XIII.—The action of phenol on mixtures of toxin and antitoxin.

Certain strengths of phenol around 5 *per cent. cause a greater relative destruction of dilutions of antitoxin than of toxin, and the addition of this amount of phenol to a non-toxic mixture may render it toxic. The local concentration of phenol that occurs when mixtures are frozen probably causes a similar effect.*

Early in January 1924 it was reported (Kelley, *J. Amer. Med. Assoc.*, lxxxii. 567) that toxin-antitoxin mixtures that had become frozen had caused severe reactions on injection. The action of freezing upon toxin-antitoxin mixtures was then investigated by White and Robinson (*ib.*, 1924, lxxxii. 1675), Kirkbride and Dow (*ib.*, p. 1678), and Anderson and Leonard (*ib.*, p. 1679). Two of these pairs of workers reported an increase of toxicity as a result of freezing toxin-antitoxin mixtures, but Kirkbride and Dow froze 16 lots of 3 L+ mixtures, 3 lots of 1/10th L+ mixtures and one batch of 1 L+ mixture without being able to demonstrate an increase in toxicity. On the other hand White and Robinson obtained a ten-fold increase in toxicity after freezing a 1 L+ mixture for 18 hours but they obtained conflicting results when 1/10th or 3 L+ mixtures were frozen. Anderson and Leonard found no increase in toxicity after freezing mixtures containing heat treated concentrated antitoxic serum; 3 L+ mixtures made with unconcentrated diphtheria antitoxin and those made with concentrated antitoxin that had not received heat treatment during concentration all showed an increase in toxicity on freezing. They found however that 1/10th L+ mixtures became less toxic after freezing.

Toxin-antitoxin mixtures both 3 L+ and 1/10th L+ were frozen by us at two temperatures, (1) at about $-6°$ C. by means of salt and ice, and (2) at $-30°$ C. with carbon dioxide snow and ether. Two out of five of the mixtures at the higher temperature and all tested at the lower temperature precipitated after freezing and thawing. We did not find any increase in toxicity in any of these mixtures after freezing.

It would appear that different effects were produced by freezing mixtures according to differences in the constitution of the mixtures. The simplest explanation of increased toxicity occurring in any given mixture after freezing appeared to us to be that the local concentration that occurred on freezing and thawing a solution brought the toxin-antitoxin complex into contact with a high concentration of phenol or other substances which destroyed the antitoxin or rendered it insoluble.

We know however that toxin is partially destroyed by high concentrations of phenol. If our explanation is correct it should be possible to demonstrate that concentrations of phenol such as would occur on freezing a mixture originally containing 0·5 per cent. phenol must destroy antitoxin to a greater extent than toxin.

A toxin-antitoxin mixture was prepared containing 3 L+ doses of toxin so neutralised with antitoxin that the mixture was almost non-toxic to guinea-pigs: an intradermic reaction was produced by 0·2 c.c. of the undiluted mixture but not by the mixture diluted 1 in 10. To this mixture we added various amounts of phenol so that from 1 to 9 per cent. phenol was present in the mixture. Mixtures to which 1, 2, 3 and 4 per cent. phenol had been added appeared slightly less toxic than the original mixture but those mixtures to which more phenol had been added became very toxic, increasing in toxicity with the amount of phenol added from 5 to 7 per cent. The mixture to which 7 per cent. phenol had been added was found to contain 25,000 minimal reacting doses per c.c. A toxin dilution was made containing the same quantity of toxin as the mixture and also an antitoxin dilution of the same strength. Varying amounts of phenol were added to these two solutions which were then tested at wide limits for toxin and antitoxin remaining. A reference to table XXVIII. giving the details of all these experiments shows that little destruction of antitoxin occurred until 4 per cent. phenol was added. Marked destruction of toxin was produced by the addition of 2 per cent. phenol but no further destruction occurred until up to 7 per cent. phenol was added.

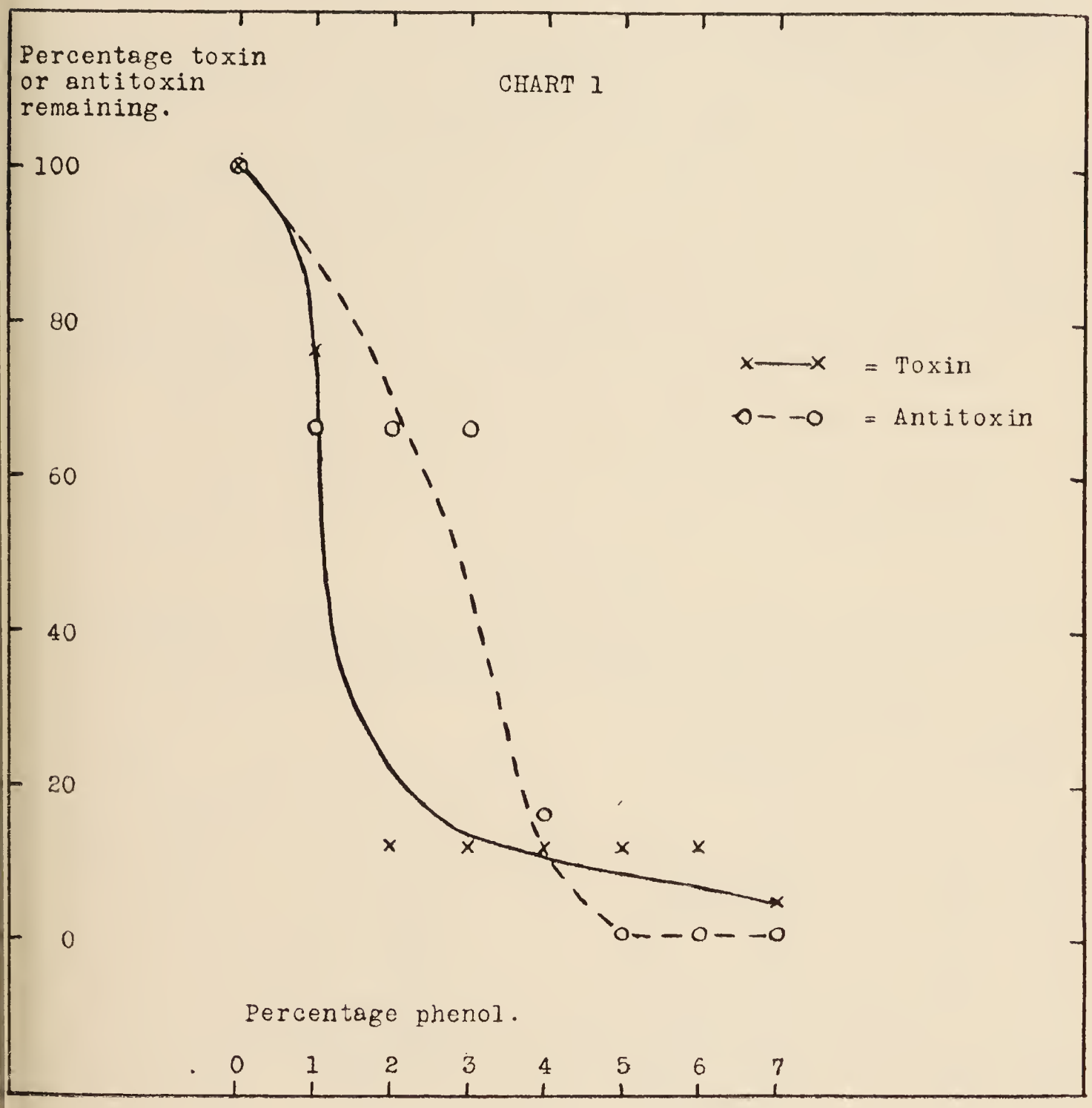
Table XXVIII.

Showing the increased toxicity of a toxin-antitoxin mixture and the relative amounts of destruction of toxin and antitoxin after the addition of various amounts of phenol.

Phenol added.	Toxin-antitoxin Mixture.	Toxin Dilution.	Antitoxin Dilution.
	Toxicity : M.R.D's per c.c.	Toxicity : M.R.D's per c.c.	Units per c.c.
0	10	200,000	3
1 per cent.	less than 10	150,000	2
2 ,,	,, 10	25,000	2
3 ,,	,, 5	25,000	2
4 ,,	,, 5	25,000	less than 0·5
5 ,,	2,500	25,000	,, 0·01
6 ,,	5,000	25,000	,, 0·001
7 ,,	25,000	10,000	,, 0·001
9 ,,	5,000	...	...

A reference to chart 1 will show that the percentage destruction of antitoxin is greater than the percentage destruction of toxin when 4 per cent. or more phenol is added. This point corresponds fairly

closely with that obtained when phenol was added to the mixture of toxin and antitoxin. It is probable that the marked differences that are obtained by different observers with different mixtures are due to the amount of protein present and other factors influencing the relative degrees of destruction of antitoxin or of toxin by the local concentration of phenol that occurs when these mixtures are frozen.



XIV.—The stability of toxin-antitoxin mixtures.

No toxin-antitoxin mixture that we have prepared has shown any marked increase in toxicity upon keeping and no decrease in antigenic efficiency has been observed.

A number of different batches of toxin-antitoxin mixtures of different constitution have been tested from time to time and in only one case did we observe an apparent increase in toxicity. This increase was not very great and may have been due to difference in the time of year in which the tests were made. The batch concerned

consisted of a 3 L+ mixture prepared in May 1922 and tested again in December and a year or more later. The results are shown in table XXIX. It is suggested that the apparent increase in toxicity indicated by the result of the 5 c.c. tests in December 1922 compared with those of the previous May was within experimental error, taking into consideration the time of year at which the tests were made. Even with the December 1922 test, the batch would still pass the U.S.A. official test.

TABLE XXIX.

Showing the results of four tests for toxicity at different times upon the same toxin-antitoxin mixture.

Date of test.	Result of injecting guinea-pigs with—	
	1·0 c.c. Number paralysed (all survived).	5·0 c.c. Day of death.
May 1922 . . .	2 out of 3	11, 22, and 24 days.
December 1922 . .	1 out of 3	4, 8, and 12 days.
February 1924 . .		6 and 17 days.
March 1924 . . .	0 out of 3	19, 20, and 29 days.

TABLE XXX.

Showing the results of retests upon guinea-pigs of different batches of toxin-antitoxin mixtures when over two years old.

Date of preparation of mixture.	Original toxicity tests on guinea-pigs.	Toxicity tests 29th Nov. 1924.	Age.
Mar. 1920	5·0 c.c. small swelling survived	5·0 c.c. no reaction	4 yrs. 8 mths.
July 1920	5·0 c.c. no reaction	5·0 c.c. no reaction	4 ,, 5 ,,
Oct. 1920	0·1 c.c. killed in 3 days	0·1 c.c. large swelling survived	4 ,, 1 ,,
Oct. 1920	5·0 c.c. small swelling survived	5·0 c.c. small swelling survived	4 ,, 1 ,,
April 1921	1·0 c.c. large swelling survived	1·0 c.c. large swelling survived	3 ,, 7 ,,
Nov. 1921	5·0 c.c. small swelling survived	5·0 c.c. no reaction	3 ,,
Mar. 1922	5·0 c.c. no reaction	5·0 c.c. no reaction	2 ,, 8 ,,
May 1922	5·0 c.c. medium swelling survived	5·0 c.c. medium swelling survived	2 ,, 6 ,,
May 1922	5·0 c.c. killed in 4 and 6 days; one survived	5·0 c.c. medium swelling survived	2 ,, 6 ,,
May 1922	5·0 c.c. small swelling survived	5·0 c.c. no reaction	2 ,, 6 ,,
June 1922	5·0 c.c. medium swelling survived	5·0 c.c. small swelling survived	2 ,, 5 ,,
July 1922	5·0 c.c. killed in 4 days	5·0 c.c. large swelling survived	2 ,, 4 ,,
Aug. 1922	5·0 c.c. small swelling survived	5·0 c.c. no reaction	2 ,, 3 ,,
Aug. 1922	5·0 c.c. small swelling survived	5·0 c.c. no reaction	2 ,, 3 ,,

Table XXX. gives results of retests upon 14 different batches tested in November 1924, when all batches were at least two years old. No increase in toxicity could be detected in any batch. Mixtures were kept at temperatures ranging from just above 0° C. to room temperature. In a further experiment a mixture containing 1/10th L+ was tested after three months and eleven months at 0° C., room temperature and 37° C., and no increase in toxicity could be seen. The toxicity tests on each sample were made on three guinea-pigs on each occasion. Similar tests made on toxoid only (diluted 1 in 10) after 3, 7 and 11 months showed no toxicity whatever.

A number of tests made on various types of mixtures, including the standard mixture that we now issue consisting of partially neutralised toxoid diluted 1 in 10, showed no toxicity or increased toxicity on any occasion. Some of the tests were made on samples that had been sent abroad, *e.g.* to Shanghai and the Federated States of Malay. The total number of tests of this series were

10 retests after less than 6 months.
10 retests after less than 12 months.
9 retests after more than 12 months.

TABLE XXXI.

Showing the results of tests for antigenic value of a batch of toxin-antitoxin mixture kept for different lengths of time at different temperatures.

	Immunity index determined on different guinea-pigs, injected with 1·0 c.c.
Original mixture A (1/10th L+) .	2, 2, 3
After 3 months at 12° C. . . .	2, 3, 3
,, ,, R° C. . . .	2, 2, 2
,, ,, 37° C. . . .	2, 3, 3
After 11 months at 0° C. . . .	2, 3
,, ,, R° C. . . .	2, 7
,, ,, 37° C. . . .	2, 2

R° C. = room temperature, usually between 15° C. and 20° C.

TABLE XXXII.

Showing the results of tests for antigenic value of a batch of diluted toxoid kept for different lengths of time at different temperatures.

	Immunity index determined on different guinea-pigs injected with 1·0 c.c.
Original preparation B (toxoid 1 in 10)	2, 4, 6
After 3 months at 37° C. . . .	9, over 11, over 11
,, 7 ,, 0° C. . . .	5, 11
,, 7 ,, R° C. . . .	8, 9, 12
,, 7 ,, 37° C. . . .	10
,, 11 ,, 0° C. . . .	2, 7, 7
,, 11 ,, R° C. . . .	7, 9, 13
,, 11 ,, 37° C. . . .	15

A number of batches were tested for antigenic value at the end of different periods. Table XXXI. shows that a 1/10th L+ mixture kept for 3 and 11 months at different temperatures retained its antigenic properties. Table XXXII. however suggests that diluted toxoid is less stable.

The results of other retests on antigenic value are given in table XXXIII. It will be seen for example that batch E, consisting of 3 L+ mixture, retained its antigenic value for 29 months at room temperature. The results of tests on batch L consisting of dilute

TABLE XXXIII.

Showing retests for antigenic value of different batches of diphtheria prophylactic.

		Immunity index determined on different guinea-pigs injected with—	
		1·0 c.c.	5·0 c.c.
C.	Original 3 L+	2, 2	2, 2
	Posted to Shanghai and back	2, 2, 4	2, 2, 2
	Kept 3 months at Shanghai	5, 7, x	3
D.	Original 3 L+	2, 4, 5	2
	Kept for 7 months at R° C.	3, 3, 4	2
E.	Original 3 L+	2, 2, 2	...
	Kept for 7 months at R° C.	2, 3, 3	...
	,, 12 ,, ,,	2, 2, 5	...
	,, 22 ,, ,,	2, 3, 6	...
	,, 29 ,, ,,	1, 2, 3, 4	...
F.	Original 3 L+	4, 6	1, 2, 3
	Kept for 7 months at R° C.	4	...
G.	Original 3 L+	7, x	3, 11, x
	Kept for 7 months at R° C.	5, 7	...
H.	Toxoid-antitoxin mixture diluted 1 in 10	2, 2, 2	2
	Kept for 3 months at 12° C.	2, 3	1, 1, 3
K.	Original 3 L+	2, 2, 3	2, 2
	Kept for 3 months at R° C.	3	1
L.	Toxoid only, diluted 1 in 10	2, 3, 3	1, 2, 2
	Kept for 7 months at R° C.	3, 3, 5	2, 2, 2
M.	Toxoid-antitoxin mixture diluted 1 in 10	2, 2, 3	3, over 4
	Kept for 4 months at R° C.	...	1, 3, 3
N.	Toxoid-antitoxin mixture diluted 1 in 10	4, 5, 5	3, 4, 6
	Kept for 4 months at R° C.	...	4, 9, 10
	,, 7 ,, ,,	5, x, x, x	...
P.	Toxoid-antitoxin mixture diluted 1 in 10	2, 2, 10	1, 2, 3
	Kept for 12 months at R° C.	2, 3	1, 2, 3
Q.	Toxoid-antitoxin mixture diluted 1 in 10	2, 4, 4, 5	2, 2, 2, 3, 4
	Kept for 6 months at R° C.	2, 5, 7	2, 2, 2
	,, 12 ,, ,,	2, 4	2, 3, 3

x indicates that the guinea-pig was Schick positive at the tenth test.

toxoid again suggest that this preparation may be unstable. Batch N appeared unstable; all the other batches remained good antigens at the end of 6 months or more.

XV.—Some discrepant *in vivo* titrations of antitoxin.

The relative strength of two batches of antitoxin may vary according to toxin used for titration.

It has always been accepted that the relative strength of two batches of antitoxin determined by any *in vivo* method was independent of the toxin used. It occurred to us that, since there were apparent differences in the relative dissociation rates between different batches of antitoxin and toxin or toxoid, it was conceivable that all toxins (differing as they do in toxoid content) may not give identical "unit" determinations with different antitoxins. Antitoxin A appeared weaker than antitoxin B on each of six occasions when they were tested side by side by the intradermic method, toxin 1 being used as a test toxin. The reverse was the case on each of the five occasions that toxin 2 was used as the standard.

From table XXXIV. it will be seen that an intradermic reaction was always obtained upon injecting a mixture of 0·15 c.c. of toxin 1

Table XXXIV.

Showing the intradermic reactions produced by mixtures of 0·15 c.c. *of standard toxin* 1, *with varying volumes of antitoxin A and B.*

No. of test.	*Antitoxin A.*						*Antitoxin B.*					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Vol. of antitoxin—												
0·0030 . .	...	+	...	...	...	...	...	±	+	+	+	...
0·0032 . .	...	...	+	...	...	...	...	...	±	+	+	+
0·0033 . .	...	+	...	...	...	...	...	S	...	...	...	...
0·0034 . .	...	...	+	+	...	...	S	...	−	+	S	?S
0·0035 . .	±	...	...	...	...	...	...	...	...	...	...	...
0·0036 . .	...	S	+	+	+	+	−	−	...	±	−	−
0·0038 . .	...	...	+	+	+	−	−	...	...	...	−	−
0·0040 . .	−	...	...	+	−	−	−	...	...	...	...	−
0·0042 . .	...	...	...	+	−	−	...	...	...	...	...	...
0·0044 . .	...	...	...	...	−	...	...	...	...	...	...	...

+ = large reaction S = slight reaction
± = reduced reaction − = no reaction.

and 0·0036 c.c. antitoxin A; a reaction was obtained three times out of four when the mixtures contained 0·0038 c.c. of antitoxin A and only once in five times was any reaction produced when 0·0040 c.c. was used. A consideration of all the tests both for this antitoxin and for antitoxin B on the one occasion (test 4) when a reaction was produced with 0·0040 c.c. shows that some error was made and all

mixtures were too toxic. The results therefore show that 0·0039 c.c. should be taken as one unit of antitoxin A. If we now consider antitoxin B, we find that reactions were always produced with 0·0032 c.c. of serum, that border line reactions occurred with 0·0034 c.c. and only one (discrepant) reaction with 0·0036 c.c. of serum mixed with 0·15 c.c. of toxin 1. One unit of antitoxin B must therefore be taken as contained in 0·0034 c.c. Comparing the tests day by day we see that on every occasion less volume of antitoxin B than of antitoxin A was able to prevent the appearance of any reaction. When toxin 2 was used for titration we find that more of antitoxin B was required to prevent a reaction than of antitoxin A.

Table XXXV.

Showing the intradermic reactions produced by mixtures of 0·15 c.c. *of standard toxin 2 with varying volumes of antitoxin A and B.*

No. of test.	*Antitoxin A.*					*Antitoxin B.*				
	1.	2.	3.	4.	5.	1.	2.	3.	4.	5.
Vol. of antitoxin—										
0·0030	+	...	...	...	...	+	...	...	...	...
0·0032	...	...	+	...	...	...	...	...	...	...
0·0033	...	...	...	...	...	...	...	...	...	...
0·0034	...	−	+	+	+	...	...	+	±	+
0·0035	+	...	...	...	...	+	...	...	...	...
0·0036	...	−	+	+	−	...	±	+	+	S
0·0038	...	−	−	S	−	...	−	+	+	−
0·0040	−	−	−	−	−	±	−	−	−	−
0·0042	...	...	−	−	−	...	−	−	−	−
0·0044	...	...	...	...	...	...	...	...	...	...

+ = large reaction S = slight reaction
± = reduced reaction − = no reaction.

Table XXXV. shows that 0·0036 of antitoxin A or 0·0038 c.c. of antitoxin B is equivalent to 1 unit determined against toxin 2. The results may be summarised as follows:—

0·15 c.c. toxin 1 is neutralised by 0·0039 c.c. antitoxin A or 0·0034 c.c. of antitoxin B.
0·15 „ toxin 2 is neutralised by 0·0036 c.c. antitoxin A or 0·0038 c.c. of antitoxin B.

The evidence is not by any means conclusive but it is strongly suggestive when we consider that six out of six tests made with one toxin show one antitoxin to be about 10 per cent. stronger than the other, while with another toxin the same antitoxin appeared to be about 5 per cent. weaker than the other on five occasions out of five. When investigating this particular point, these two sera A and B were intentionally chosen, A as a usual type of antitoxin and B as an antitoxin with a very high $\frac{in\ vivo}{in\ vitro}$ serum ratio. Of the

toxins, number 1 was of the usual type while number 2 contained a very high proportion of toxoid. To avoid possible fallacies due to the volume of material injected or other causes the two toxins were so chosen that although differing so very much in toxoid content the same intradermic test dose could be used for both, and the antitoxins were chosen so that although differing so much in serum ratio the unit value was almost the same. Further investigations will be carried out to ascertain whether these animal titrations are affected in any way by the time of combination between toxin and antitoxin.

XVI.—A METHOD OF PRODUCING TETANUS ANTITOXIC SERUM OF HIGH POTENCY.

Horses are given a few primary immunising injections and then rested from all injections of tetanus toxin for at least a month, after which they respond rapidly to immunisation and produce higher grade antitoxin than those not similarly rested.

During the war when tetanus antitoxin was produced on a very large scale, it was at first our custom to inject new horses with relatively large doses of tetanus toxin over-neutralised by antitoxin. The extent of over-neutralisation was reduced with successive injections, and after three or four injections of mixtures very small but gradually increasing amounts of toxin were injected. Most workers followed this method or injected a dose of tetanus antitoxin followed by successive doses of toxin.

Our normal horses did not contain any tetanus antitoxin naturally produced, their immunisation was therefore compared with that of such diphtheria horses as are found without natural antitoxin. If such horses received mixtures of diphtheria toxin-antitoxin no active immunity could be demonstrated for some weeks and early injections of toxin would be useless, because no increased response could be expected until active immunity as a result of a primary stimulus had been produced. The early injections were not only a source of danger to the animals but also the cause of lowered condition owing to the injection of considerable quantities of toxin, which always contained some non-specific material. We therefore applied this principle to the immunising of tetanus horses with the result seen in table XXXVI. It will be seen that 14 horses in one particular group averaged 400 units per c.c. and more than half of these horses contained over 500 units. A corresponding group of 10 horses which were given similar mixtures of toxin and antitoxin to begin with, and then gradually increasing doses of the same toxin without any rest failed to produce any serum over 300 units; half of the horses had to be discarded before they produced more than 60 units per c.c.

Table XXXVI.

Showing the highest titre of tetanus antitoxin reached in six months by each of a group of horses according to the time elapsing between their initial primary stimulus and their subsequent continuous course of injections.

Each figure represents the highest number of units per c.c. reached by a single horse.

Interval . .	None.	1 month.	2 months.	3 months.
Useless . . .	20	...	...	...
	40	...	...	...
	50	...	...	...
	60	...	...	...
	60	...	...	...
Low value . .	120	...	120	...
Medium value . .	300	200	200	300
	300	250	...	...
	300	300	...	...
	300	...	...	...
Good value . .	...	400	500	600
	...	500	600	600
	...	600	600	...
Number of horses .	10	6	5	3
Average value . .	155	450	400	500

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