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Immunological Notes

XVII.-XXIV.

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IMMUNOLOGICAL NOTES

XVII.-XXIV.

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(*Continued from* vol. xxviii. p. 482.)

XVII.—The antigenic value of the toxin-antitoxin precipitate of Ramon.

An emulsion in saline of toxin-antitoxin precipitate was equally efficient as an antigen in guinea-pigs whether injected in doses of 0·001, 0·01, 0·1, *or* 1·0 *c.c.*

Sordelli and Serpa (*C. R. Soc. Biol.*, 1925, xcii. 825) have reported the antigenic value of the precipitate that occurs when diphtheria toxin and antitoxin are mixed. Park, Banzhaf, Zingher and Schroder (*Amer. Journ. Public Health*, 1924, xiv. 1047) quote without comment the toxin-antitoxin precipitate in a table of relative antigenic values. It was originally pointed out to us at the end of 1923 in a personal communication by Dr Hartley (who has just published his results, *Brit. Journ. of Exp. Path.*, 1925, vi. 112) that the precipitate was antigenic and had a higher immunising value than the supernatant liquid.

A few tests were made by us to confirm the observation by Hartley and to endeavour to attach a quantitative value to the antigenic efficiency of precipitates from various toxins and antitoxins in different proportions. The first preliminary experiments recorded below show that an emulsion of the precipitate from a toxin-antitoxin mixture was antigenic even in very small doses.

The precipitate from routine Ramon tests was collected for several months, washed twelve times with 0·5 per cent. phenol saline and then emulsified in 50 c.c. of distilled water. The total solid content of 1·0 c.c. of this emulsion was 0·0164 grm. which is roughly equivalent to the weight of precipitate obtained from 100 c.c. of toxin. Various volumes of the emulsion were injected into guinea-pigs and the immunity index determined.

The results are shown in table XXXVII. It will be seen that injection of volumes ranging from 0·01 c.c. to 1·5 c.c. all produced the same degree of immunity; the injection of 0·001 c.c. was scarcely less efficient.

These results suggest that the antigenic value of the precipitate depends on the rate of dissociation of the toxin-antitoxin complex following its injection into an animal.

Table XXXVII.

Showing the immunity index of a number of guinea-pigs injected with various volumes of an emulsion of a toxin-antitoxin precipitate.

	Immunity index for various doses.					
	1·5 c.c.	1·0 c.c.	0·1 c.c.	0·01 c.c.	0·001 c.c.	0·0001 c.c.
First test .	5	4, 6, 7	4, 5, 8	3, 4, 4	...	...
Second test	...	5, 6, x	3, 4, x	3, 6, 7	5, 5, x	...
Third test .	...	...	...	4, 4, 6	7, 9, x	8, x

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

XVIII.—Destruction of toxin by heating in the presence of phenol.

The destruction caused by heating toxin containing 0·5 *per cent. phenol to* 45° C. *is greater than that caused by heating the same toxin uncarbolised to* 60° C. *for the same length of time.*

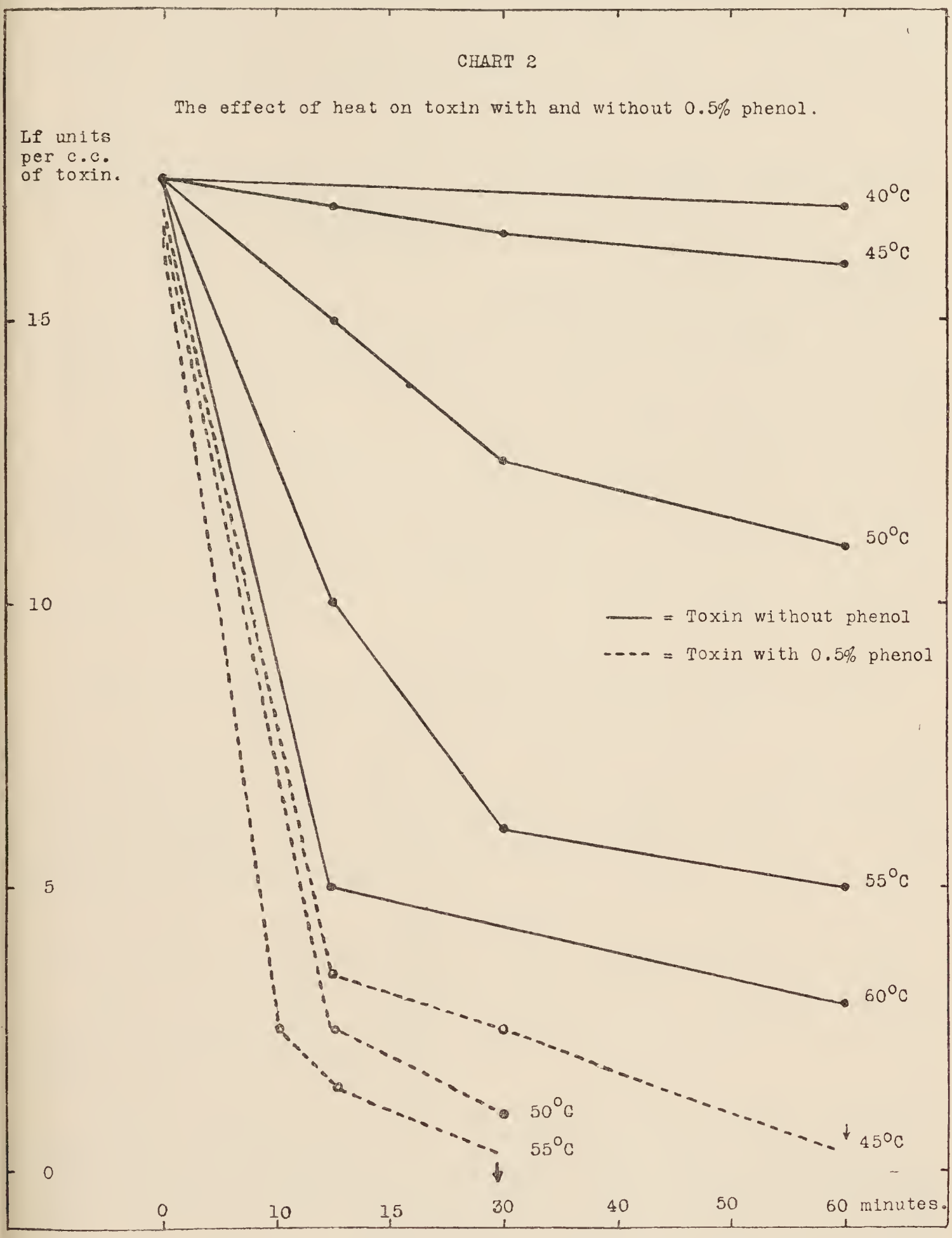
Preliminary experiments have shown that the effect of heat upon toxin varies with different batches. Most work has been done on carbolised toxin; it appeared advisable therefore to determine the effect of phenol on the heat stability of toxin.

Four bottles of the same batch of broth prepared by Watson and Langstaff from a 3-day room-temperature digest of horse muscle (method as yet unpublished) were inoculated with *C. diphtheriæ* and grown for 10 days. The contents of two bottles were mixed and filtered through Berkefeld candles; 0·5 per cent. phenol was added to the contents of the other two bottles which were filtered 24 hours later. The toxin was heated in 50 c.c. quantities in boiling tubes in a water-bath at various temperatures for 15, 30 and 60 minutes. The temperatures did not vary more than 1° C. on either side of the given temperatures. The times of heating do not include the time taken to reach the given temperatures, which was less than 5 minutes.

The strength of toxin remaining after heating was tested by the flocculation method. The Lf was determined to within 10 per cent. for all values above 10 units per c.c., to 20 per cent. for values between 3 and 10 units and to 50 per cent. for those values below 3 units. The time of flocculation greatly increased with the temperature and time of heating; it was necessary therefore to determine the Lf values by the method of blending.

Chart 2 shows that in the absence of phenol this particular toxin lost 10 per cent. of its strength after 1 hour at 40° C., 35 per cent. at 50° C., 70 per cent. at 55° C. and about 80 per cent. at 60° C. The carbolised toxin had lost over 95 per cent. after 1 hour at 45° C.

After 15 minutes at 45° C. the carbolised toxin was approximately the same strength as the uncarbolised toxin after 1 hour at 60° C.



XIX.—The destruction of antitoxin by heating in the presence of trikresol.

After one hour's exposure to a temperature of 60° C. *antitoxic serum containing trikresol loses from* 10 *to* 30 *per cent. of antitoxin. In the absence of trikresol the loss is from* 0 *to* 10 *per cent.*

The deterioration of diphtheria antitoxin is of considerable theoretical interest. We know that a special form of "modified

antitoxin" is produced if antitoxin is left at incubator temperature for a number of years (Glenny, *Journ. Hyg.*, 1913, xiii. 63). We also know that similar "modified antitoxin" has a low serum ratio, *i.e.* $\frac{\textit{in vivo}\text{ value}}{\textit{in vitro}\text{ value}}$ (Glenny, Pope and Waddington, 1925, this *Journal*, xxviii. 279). It may be possible therefore to study some aspects of the combination and dissociation between toxin and antitoxin by determining the effect of heat upon the serum ratio. For this ratio we have now adopted the fraction $\frac{\textit{in vivo}\text{ value}}{\textit{in vitro}\text{ value}}$ though in our earlier work we used the inverse fraction.

In our preliminary experiments recorded here, *in vivo* values only were determined as we found that the flocculating *power* (as distinct from the flocculating or *in vitro* value) of a serum was soon lost after heating (*cf. infra*, p. 40).

Table XXXVIII. shows that seven different antitoxic sera of different ages, all containing trikresol, lost from 9 per cent. to 18 per cent. of their antitoxin after 15 minutes' heating to 60° C. and from 20 to 27 per cent. after one hour at the same temperature.

TABLE XXXVIII.

Showing the effect of exposure to a temperature of 60° C. *upon seven batches of antitoxic sera containing trikresol.*

Serum.	Age in months.	Serum ratio.	*In vivo* value.			Percentage loss.	
			Before heating.	After 15 minutes at 60° C.	After 1 hour at 60° C.	After 15 minutes at 60° C.	After 1 hour at 60° C.
						Per cent.	Per cent.
A	68	1·0	280	230	220	18	21
B	34	1·2	675	610	540	9	23
C	19	3·2	310	270	225	13	27
D	8	1·3	510	430	390	15	23
F	8	4·7	340	310	260	9	23
H	6	1·5	64	55	51	14	20
K	6	2·0	28	25	21	10	25

These sera had all been obtained in a routine manner by bleeding into potassium oxalate and clotting the separated plasma by the addition of calcium chloride. Trikresol either alone or with ether was added at the same time as the calcium chloride.

The next experiment was made in order to show the effect of the method of clotting and of the antiseptic upon the rate of destruction. Five samples of serum were taken from each of two horses and two from a third horse. The different samples were as follows:—

(*a*) serum obtained from natural clot without antiseptic:
(*b*) as (*a*) with 0·3 per cent. trikresol:

(*c*) serum obtained by clotting potassium oxalate plasma; trikresol added:
(*d*) serum concentrated by the ammonium sulphate process from sodium citrate plasma without antiseptic:
(*e*) as (*d*) with 0·3 per cent. trikresol.

TABLE XXXIX.

Showing the effect of exposure to a temperature of 60° C. *upon samples of serum obtained in different ways from three horses.*

Serum.	Method of separation.	Antiseptic.	*In vivo* values.		Percentage loss after 1 hour at 60° C.
			Before heating.	After 1 hour at 60° C.	
1*a*	Natural clot . .	nil	580	580	0
1*b*	,, . .	trikresol	540	470	13
1*c*	Oxalate plasma .	,,	560	475	15
1*d*	Concentrated . .	nil	1250	1250	0
1*e*	,, . .	trikresol	950	850	10
2*a*	Natural clot . .	nil	775	760	1
2*b*	,, . .	trikresol	740	700	5
2*c*	Oxalate plasma .	,,	725	650	13
2*d*	Concentrated . .	nil	2500	2500	0
2*e*	,, . .	trikresol	2400	1750	27
3*a*	Natural clot . .	nil	450	410	9
3*b*	,, . .	trikresol	450	320	29

Table XXXIX. shows that the presence of trikresol has a marked destructive action upon antitoxin exposed to heat. Two samples of serum and two of concentrated antitoxin without preservative showed a negligible loss when heated at 60° C. for one hour.

XX.—THE EFFECT OF EVAPORATION OF TOXIN-ANTITOXIN MIXTURES.

Neutral mixtures of toxin and antitoxin may become toxic upon evaporation because the increased concentration of phenol destroys antitoxin at a greater rate than toxin.

We have shown in a previous note (vol. xxviii. p. 473) that phenol in certain concentrations can cause a relatively greater destruction of antitoxin than of toxin in a toxin-antitoxin mixture, and we suggested this as an explanation of the increased toxicity which was found in some toxin-antitoxin mixtures which had been accidentally frozen. These results were obtained by adding pure phenol to a toxin-antitoxin mixture until the desired concentration was reached.

When toxin-antitoxin mixtures are frozen water is temporarily removed in the form of ice and higher concentrations of phenol are produced. A similar result may be obtained by evaporation.

The experiment was carried out by measuring into an evaporating dish 10 c.c. quantities of a toxin-antitoxin mixture containing 0·5 per

cent. phenol. The dish and contents were weighed and then placed in a vacuum desiccator. Evaporation was allowed to proceed, checked by frequent weighings of the dish and contents, until the desired reduction in volume had been obtained. The volume was then made up to 10 c.c. with saline and the mixture tested for toxicity.

TABLE XL.

Showing the increase in toxicity that occurs when toxin-antitoxin mixtures are evaporated.

Original phenol content.	Original volume.	Final volume.	Final phenol content.	Toxicity of evaporated mixtures made up to original volume.
0·5 per cent.	10 c.c.	10·0 c.c.	0·5 per cent.	Less than 50 M.R.Ds. per c.c.
0·5 ,,	10 ,,	0·99 ,,	5·0 ,,	More than 500 ,, ,,
0·5 ,,	10 ,,	0·70 ,,	7·0 ,,	500 ,, ,,
0·5 ,,	10 ,,	0·26 ,,	20·0 ,,	Less than 50 ,, ,,

It will be seen that when the phenol content was increased to 5 per cent. by evaporation there was a marked increase in the toxicity of the mixture; the increased toxicity at 7 per cent. was less, while at about 20 per cent. there was no increase.

These results corresponded with those previously obtained where pure phenol was added to give the desired concentration.

XXI.—THE EFFECT OF FORMALDEHYDE ON DIPHTHERIA ANTITOXIC SERUM.

The addition of 0·2 *per cent. formaldehyde destroys half the antitoxic value of a serum.*

R. Henley (*Journ. Biol. Chem.*, 1923, lvii. 139; *J. Agricultural Research*, 1924, xxix. 471) has reported that formaldehyde acts on sera and alters the precipitation of the proteins by ammonium sulphate. From his results it seemed possible that a concentrated antitoxin having a higher antitoxin-protein ratio might be obtained, provided formaldehyde had no action on the antitoxin. The action of formaldehyde has been investigated and it has been found that it causes great destruction of the antitoxin, the amount of destruction increasing with increased concentration of the formaldehyde.

The experiment was carried out with diphtheria antitoxic serum containing 0·4 per cent. of trikresol, to which 0·1, 0·2, 0·3, 0·4 and 0·5 per cent. of formaldehyde was added. The mixtures were examined for protein precipitation by ammonium sulphate and for antitoxin content 72 hours after preparation. The precipitating level of ammonium sulphate used was the same for each mixture, being

30 parts by volume of saturated ammonium sulphate (D. 1240 at 17° C.) to 70 parts by volume of the serum. The protein concentrations were determined refractometrically.

It will be seen from table XLI. that the formaldehyde, in increasing concentration, caused a progressive destruction of the antitoxin and marked changes in the protein precipitation by ammonium sulphate.

TABLE XLI.

Showing the effect of addition of formaldehyde to serum upon the antitoxic value and upon the amount of protein precipitated by ammonium sulphate.

Percentage amount of formaldehyde added.	Serum containing trikresol.			Serum containing no preservative.	
	Antitoxic value.	Percentage destruction of antitoxin.	Percentage of original protein precipitated at 30 ammonium sulphate.	Antitoxin value.	Percentage destruction of antitoxin.
Nil	220 units	Nil	41·6	440 units	Nil
0·1	160 ,,	27·3	50·9	300 ,,	32
0·2	100 ,,	54·6	72·1	200-250 ,,	44-55
0·3	70 ,,	68·2	88·1	200-250 ,,	44-55
0·4	60 ,,	72·8	99·1	100 ,,	78
0·5	50 ,,	77·4	...	100 ,,	78

As a serum containing trikresol was used in this experiment it was considered desirable to repeat the work, in so far as it relates to the destruction of antitoxin, using fresh serum containing no preservative. Owing to gel formation taking place before the tests were complete the results are only approximately correct, but they show the same order of antitoxin destruction with increasing concentration of formaldehyde in the serum containing no preservative as in that containing trikresol.

XXII.—THE ANTIGENIC VALUE OF PRECIPITATED TOXOID.

The antigenic value of toxoid is slightly increased when precipitated by the addition of 1 *per cent. glacial acetic acid.*

Considerable evidence has been accumulating to show that the method in which a toxin or toxoid is presented has considerable bearing upon the antigenic response of the animal. It was thought that the antigenic response would be increased by the slight delay in absorption that would occur if toxoid was injected as a suspension of a precipitate. Table XLII. shows the results of some preliminary experiments. Four different toxoids were injected in 1 c.c. doses into a number of guinea-pigs on different occasions, and at the same time a similar number of guinea-pigs were injected with the same volume

of toxoid to which 1 per cent. glacial acetic acid was added. In all cases the toxoid was contained in the precipitate so formed. All the guinea-pigs were Schick tested three times a week starting on the 10th day after the initial injection. Seven experiments in all were made, and on six occasions the antigenic efficiency of the acid toxoid as measured by the rapid immunity index was greater than that of the toxoid without the addition of acid.

Table XLII.

Showing the antigenic value of four batches of toxoid with and without the addition of 1 *per cent. glacial acetic acid.*

Batch of toxoid.	Form of presentation.	Rapid index recorded by different guinea-pigs.
A	Neutral solution .	20, 20, 20, 20, 23, x, x, x
	Acid suspension .	16, 18, 20, 23, 23, 27, x
B	Neutral solution .	27, 30, x, x
	Acid suspension .	20, 23, 25, 27
C	Neutral solution .	16, 18, 21, 23, 24, 30, 30
	Acid suspension .	16, 18, 18, 20, 23, 23, 23, 23
D	Neutral solution .	18, 20, 23, 23, 23, 27, 27, 30
	Acid suspension .	16, 16, 18, 23, 23, 25, 27
	Neutral suspension .	20, 23, 25, 26, 30, x, x

x indicates that the guinea-pig was still Schick positive on the 30th day.

Table XLII. records the summary of all the tests and shows that there is a definite tendency for immunity to result earlier after the injection of acid toxoid. One batch of toxoid was tested in three forms, the original toxoid, the acid suspension with a P_H of about four, and a neutralised suspension of which the P_H had been adjusted to 8·0 by the addition of sodium carbonate. It will be seen from the table that the neutral suspension of the acid precipitate was not a good antigen; it appeared worse than the original toxoid while the acid suspension was better.

XXIII.—The antigenic value of toxoid precipitated by potassium alum.

Toxin or toxoid may be precipitated by the addition of varying quantities of potassium alum. An emulsion of such a precipitate has high antigenic properties.

A number of preliminary experiments have shown that the addition of potassium alum to toxin causes a precipitate and the filtrate may no longer contain any toxin. With certain toxins small amounts of alum may cause a precipitate without any appreciable lowering

of the toxicity of the filtrate; further additions, however, remove all the toxin. The precipitate formed does not appear to be soluble and therefore cannot be titrated by the flocculation test. The preliminary experiments described in this note were made with two batches of toxoid. Potassium alum was added to each and the precipitate collected by filtration and then emulsified in water making up the total volume to 1/10th of the original. Toxoid "A" had an original total solid content of 2·3 grms. per 100 c.c. The weight of the precipitate after the addition of 0·4 per cent. potassium alum was 0·24 grms. per 100 c.c. of which 0·11 of a grm. was incombustible material.

Toxoid "B" had a total solid content of 4·04 grms. per 100 c.c., and 0·8 per cent. potassium alum was used in this experiment. The precipitate weighed 0·66 grms. of which 0·18 was incombustible.

TABLE XLIII.

Showing the antigenic value of toxoid before and after precipitation with potassium alum.

Material.	Dose injected.	Lf units injected.	Number of days after injection before pigs became Schick negative.
Toxoid "A" original .	1·3 c.c.	8 units	15, 15, 15, 15
Alum filtrate . . .	4·0 ,,	8 ,,	13, 15, 16, 18
Alum precipitate emulsion	2·0 ,,	80 ,,	11, 11, 13, 14
	1·0 ,,	40 ,,	12, 12, 12, 12, 12, 12
	0·1 ,,	4 ,,	12, 14, 14, 14
Toxoid "B" original .	1·0 ,,	6 ,,	18, 27, 27, 40
Alum precipitate emulsion	1·0 ,,	60 ,,	10, 11, 12, 12, 13, 13, 18, 21
	0·1 ,,	6 ,,	14, 14, 17, 17

Table XLIII. shows the antigenic value of the original toxoid the filtrate and the emulsified precipitate. The Lf value of the precipitate from toxoid "A" was calculated from a knowledge of that of the original and of the filtrate, it being assumed that no destruction had occurred; the precipitate of toxoid "B" was assumed to contain all the toxoid since the Lf of the filtrate was less than 1 unit per c.c. Various doses of toxoid precipitate were injected into a number of guinea-pigs, and on the 9th or 10th day afterwards the guinea-pigs were injected with a Schick dose of toxin and this was repeated every two days. It will be seen from the table that the antigenic value of the emulsion of precipitate appeared greater than that of the toxoid from which it came. This may be due to the delayed rate of absorption of the precipitated material or to the absence of non-specific interfering substances.

XXIV.—The antigenic value of toxoids that will not flocculate in the presence of antitoxin.

Some batches of toxoid of high antigenic efficiency may fail to flocculate, but the absence of flocculating power does not necessarily indicate the absence of combining power. An Lf value for such toxoid may be determined by the method of blending.

An Lf dose of toxin has been defined (vol. xxviii. p. 317) as "the amount of toxin per unit of a certain chosen antitoxin in the mixture that flocculates more rapidly, under the same conditions, than all other mixtures containing other amounts of the same toxin per unit of the same antitoxin." It follows therefore that an Lf value of 0 cannot be assigned to a preparation as a result of a direct titration. An Lf value of 0 can only be given if the Lf value of a mixture of equal parts of the preparation under test and of a known toxin gives an Lf value of half that of the known toxin.

Zingher (1925, *Proc. Soc. Exper. Biol. and Med.*, xxii., 454) concludes that "the flocculation reaction does not appear to be an index of the antigenic value of diphtheria toxoid (anatoxin)." This conclusion is based mainly upon the fact that certain toxoids have been given an Lf value of 0 because they failed to flocculate. We have, however, frequently found batches of toxin that lose their power to flocculate with antitoxin after they have been modified by the action of formaldehyde. This loss of flocculating *power* does not necessarily indicate an absence of combining power, and such toxoids can be titrated by blending with the original toxins.

A certain batch of toxoid was titrated against three different antitoxins. A series of mixtures was kept at room-temperature and another in a water-bath at 50° C. for 14 days: no flocculation occurred in either series with any of the antitoxins. The toxoid was then mixed in equal proportions with a toxin of known value and the value of the mixture titrated. The Lf of the known toxin against the three sera was 11·5, 11·5, 11·25; 2 c.c. of the mixture were found to contain 14·25, 14·25, 14·75 units; so that the calculated Lf value of the toxoid lies between 2·75 and 3·5 units per c.c. This toxoid has been frequently injected into guinea-pigs and has been shown to be highly antigenic.

The absence of flocculation in any given toxin cannot be taken as indicating complete loss of flocculating power. Another toxoid which appeared to have no flocculating power was titrated by blend and its Lf value found to be 3·2 units. A series of mixtures of the toxoid alone with antitoxin was then made up with small differences round this value and at the end of 14 days at 50° C. a slight but definite flocculation appeared, showing the Lf value to lie between 3·0 to 3·3 units. This toxoid had just sufficient flocculating power left for the Lf value to be determined by direct titration after 14 days; but this same value could be determined in 8 hours by blend with the original toxin.

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