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The Measurement of the Combining Power of Diphtheria Toxin and Toxoid with Antitoxin in Relation to their Antigenic Efficacy

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Introduction.

Terminology.

The word "*toxin*" as used by immunologists possesses more than one interpretation. Used in a general sense the term indicates the active filtrate of a broth culture; used in a specific sense the term signifies a definite substance which is one of the active principles of the filtrate and which has not yet been isolated. Whenever any ambiguity may arise we make use in this paper of such expressions as "batch of toxin" or "toxin brew" to indicate the active filtrate, and "specific toxin" for the active principle responsible for pathological symptoms viz., death, œdema and inflammatory skin reactions in animals. There exists at least one modification of this active principle; there may possibly be many. For the present we are using the expression "toxoid" for that modification of specific toxin which causes no pathological symptoms in animals, but is capable of combining with antitoxin and of stimulating the production of immunity. The view we wish to bring forward is that the active principle of a brew of toxin ordinarily consists of two distinct specific substances, toxin and its modification toxoid, though it may be so modified that all the active principle becomes toxoid. As in the case of toxin, the term "*toxoid*" may be used in a general sense to indicate a modified batch of toxin or in a specific sense to signify the modified active principle of a toxin brew. Where any ambiguity may arise, we use the expression "modified toxin" when referring to the altered brew and the term "toxoid" or "specific toxoid" for the modified active principle. Recently, Ramon (1924) has suggested the term "anatoxine" without defining whether this term should be applied generally or specifically and without making clear whether the word replaces "modified toxin" or "specific toxoid" or both. The introduction of a new word where an old one is in use appears undesirable unless a careful review is made of all existing terms and a new series of words coined to remove all ambiguity.

The term "toxin" is sometimes used in a third sense to denot the total active principle consisting of both specific toxin and toxoi The last section of the following quotation from *Diphtheria* (Medica Research Council, 1923, p. 130) suggests the use of the word in thi sense. "Suffice it to repeat that 'toxin' is the name given to th sterile filtered culture-fluid of the diphtheria culture of suitable ag In addition to toxin (exotoxin) it always contains some disintegration products of dead bacilli, the residue of the original fluid culture medium, and doubtless some excretory products of the bacilli othe than toxin. It is, nevertheless, convenient, and sufficiently true fo practical purposes, to regard it as pure solution of 'toxin.'" I passing, we point out that this statement cannot be taken literally present methods of concentration show that all the active principl is contained in less than 2 per cent. of the nitrogenous materia present in a batch of toxin, and in the large scale production o antitoxin we frequently find differences in the non-specific conten of different batches of toxin, which vary greatly in their harmfu effect on horses. It is this third interpretation which is given t the word "toxin" as it occurs in the terms "antitoxin" and "toxin antitoxin" mixture. In both cases toxin is used as synonymous wit antigen and refers to specific toxin *plus* toxoid.

It is our belief, in close accord with Ehrlich, that the antigeni values of specific toxin and toxoid are equal, and that the total specifi content of a batch of toxin may therefore be measured by its combinin power with antitoxin. We therefore refer to the total specific conte of a batch of toxin as the "binding unit content."

Discussion.

Different brews of diphtheria toxin vary both in their degree toxicity and in their power to stimulate the production of antitoxi It is of importance therefore to be able to measure both the toxicit of a brew and its immunising value. Until recently, the only test available were those measuring the specific toxicity in guinea-pig and the power of combining with antitoxin, and in all serum-makin laboratories the value of toxin for the immunisation of horses wa judged either on the M.L.D. or the L+ (or Lo) dose. *It has been ou experience that it is possible to prepare toxin which, when judged by th above tests, has given an extremely low value but has proved to be of hig immunising efficiency.* This failure of animal tests to measure on of the most important functions of toxin needs detailed investigatio

The relations of toxicity to antigenic efficiency and to combinin power with antitoxin have provided material for the three classic hypotheses; that of Ehrlich which stimulated so many researche the "weak acid—weak base" suggestion of Arrhenius and Madse and the "adsorption" hypothesis of Bordet. Since these hypothes

were enunciated certain advances have been made in methods of testing bringing to light new relationships. The intracutaneous method of testing has given a new measure of combining power differing slightly from those measures determined by subcutaneous methods. The recent establishment of the flocculation test (Ramon 1922, 1923 and 1924) gives us another new and important method of measuring the strength of a toxin. This will at once be evident when we state that some toxins have given an M.L.D. and L+ value so low that without some other indication of utility no serologist would have used them for the immunisation of horses in the past and yet they proved to have a high value by the Ramon flocculation test and produced, when injected into horses, high grade antitoxic serum. It is probable on the evidence we have now that we, and probably the staffs of almost every other serum-making laboratory in the world, have discarded and destroyed in the past large quantities of toxin that appeared at the time of preparation of low potency according to M.L.D. and L+ determinations, which however would have shown high flocculating value and would have produced good antitoxin in horses. We have known for many years past that toxin showing a high value by animal tests made soon after preparation may after the passage of a considerable time or after treatment with formaldehyde appear to have become much weaker or even useless according to animal tests and yet, as we show later in this paper, be of great antigenic value.

The importance of a clear understanding of the relationships between toxin and toxoid content and antigenic value has been increased by the widespread use for active immunisation against diphtheria of mixtures of toxin and antitoxin (V. Behring 1913, Park 1913), toxoid antitoxin (Glenny and Hopkins 1923) and toxoid only (Glenny and Sudmersen 1921, Glenny and Hopkins 1923, Park 1923, Ramon 1924, and Glenny, Hopkins and Pope 1924). A complete knowledge of diphtheria toxin will naturally aid future study of other toxins. Already in these laboratories the application of knowledge gained from our continual study of diphtheria toxin has enabled us to provide a mixture of tetanus toxin and antitoxin (Buxton and Glenny 1921) which gives complete protection in horses and other animals against the subsequent injection of actively growing tetanus cultures, and to prepare similar mixtures which give complete protection against the ordinary "gas gangrene" organisms.

None of the three great hypotheses appear to supply a complete explanation of the new knowledge established by the flocculation test. It appears to us that some additional theory is needed to enable us to compare the various measures of combining power, to establish the relationship between these measurements and to account for the high antigenic efficiency of some mixtures which, judged by ordinary animal tests, would be called "over neutralised" and the comparative

failure of others. All experimental data accumulated during our own experience can be explained on Ehrlich's assumption that the total amount of toxin and toxoid neutralised in any quantity of the toxin solution is directly proportional to the antitoxin added. Ehrlich postulated many forms of toxoid and also suggested such modifications as toxones and epitoxonoids. The view we wish to bring forward is that all observed phenomena can be explained by the presence of toxin and toxoid and by the fact that toxin has a much greater affinity for antitoxin than has toxoid. There may be qualitative differences between different toxoids produced in different ways, but it appears unnecessary to assume that there is a definite series of sharply defined substances. We make no endeavour to criticise in detail Ehrlich's conception of the plurality of constituents of "toxin" because we consider that certain observed phenomena, *e.g.* paralysis, may be explained without reference to a series of modifications of toxin and toxoid.

The object of the present paper is to compare the various animal tests for diphtheria toxin with one another, to contrast them with the *in vitro* test and to establish the best test of antigenic efficiency.

Methods of measurements.

Titration in vivo.

The determination of the specific toxicity and also of the combining power of diphtheria toxin with antitoxin depends upon the production of certain toxic symptoms in guinea-pigs. Diphtheria toxin injected subcutaneously will kill guinea-pigs more or less rapidly; sublethal doses cause local œdema. Toxin injected intracutaneously even in high dilution gives rise to an inflammatory reaction. From these reactions certain units have been fixed. The chief unit of specific toxicity is called the minimal lethal dose or "M.L.D."; in practice this is the smallest amount of toxin which will kill a guinea-pig of convenient size (250 grm.) in a reasonable time (5 days); it is not strictly a minimal dose. The corresponding intracutaneous unit is the minimal reacting dose, "M.R.D.," which Glenny and Allen (1921) have defined as the smallest quantity of toxin which will produce a reaction when injected into the skin of a guinea-pig. For convenience in the following argument we introduce another unit, the "minimal œdema producing dose" or "M.Œ.D." which is the smallest amount of toxin which, injected subcutaneously, will produce detectable œdema in the guinea-pig. The following ratios appear to hold approximately true for an average toxin: 1 M.L.D. = 20 M.Œ.D. = 1000 M.R.D.

When quantities of antitoxin are successively added to a toxin a point is soon reached at which the time of death is delayed, and if more antitoxin is added we reach a point where the mixture is non-lethal to guinea-pigs. Obviously this affords us a suitable end point

for the titration of toxin against antitoxin; the survival or death of the test animal may be taken as indicating a definite relation between the toxin and antitoxin injected. If we accept the unit of antitoxin as an arbitrary standard then we can enunciate our first definition of one of the units of combining power. The "L+ dose" of toxin (Ehrlich) is that amount of toxin which injected subcutaneously together with 1 unit of antitoxin will kill a guinea-pig in 5 days. Experience has shown that survival or death on the fifth day forms a more convenient as well as a more certain end point than ultimate survival or death. If slightly less toxin is added to 1 unit of antitoxin (or slightly more than 1 unit of antitoxin is added to an L+ dose of toxin) another end point is reached where no œdema is produced. This has given rise to another unit of measurement of combining power of toxin, the "Lo dose" (Ehrlich) which is that amount which when injected together with 1 unit of antitoxin just fails to produce œdema. If such a mixture be injected intracutaneously it is found that a definite inflammatory reaction is produced due to free toxin not detected by subcutaneous injection. If more antitoxin be added to such a mixture the end point will be reached when no intracutaneous reactions will be produced. This gives a third unit of measurement of combining power. The "Lr dose" (Glenny and Allen 1921, Hartley and Hartley 1922) of toxin is that amount which when injected intracutaneously together with 1 unit of antitoxin will just produce an inflammatory reaction. The volume injected intracutaneously is always 0·2 c.c.: since one cannot inject an unlimited volume intracutaneously it is obvious that with a weak toxin a full Lr dose cannot be injected. In practice, the Lr dose is that quantity of toxin which when mixed with 1 unit of antitoxin produces a mixture of which 0·2 c.c. will just cause a reaction.

It follows that the various units of measurement that have been adopted vary with the toxic symptoms chosen as indicators. If varying amounts of toxin are mixed with 1 unit of antitoxin different amounts of specific toxin remain uncombined. If the amount uncombined is:—

exactly one minimal lethal dose (M.L.D.) the mixture contains the L+ dose of toxin,

just less than one minimal œdema producing dose (M.Œ.D.) the mixture contains the Lo dose of toxin,

exactly one minimal reacting dose (M.R.D.) the mixture contains the Lr dose of toxin.

Intracutaneous titrations are often made for convenience of experiment at lower levels and only 1/500th of a unit of antitoxin is used. The amount of toxin which just produces a reaction when injected with this amount of antitoxin is termed the "Lr/500 dose"

(Glenny and Allen 1921, Hartley and Hartley 1922). Such a mixture contains 1 M.R.D. of toxin uncombined. If the mixture were not diluted to the Lr/500 level but contained 1 unit of antitoxin in place of 1/500th there would be 500 M.R.D.s free.

We have found with the few toxins we have tested that the minimal œdema dose is about 1/20th of an M.L.D. while approximately 1/50th of an L+ mixture will just produce œdema: it is difficult to determine these values with accuracy but these ratios appear approximately correct. If as we believe a single body, toxin, causes both œdema and death, then œdema is produced by a definite fraction of an M.L.D., and because there is just 1 M.L.D. of toxin free in an L+ mixture, this same fraction of an L+ mixture should just cause œdema. But these fractions are not identical, for the different ratios mentioned above are well outside experimental error. We suggest that when a fraction of an L+ mixture between 1/20 and 1/50 is injected, sufficient toxin may become dissociated to produce œdema. Thus the various units of measurement depend for their estimation not upon fixed amounts of toxin being left free but upon the amount of toxin which is free or *becomes free* from a toxin antitoxin combination and is then absorbed. In the following argument we have assumed the amount of toxin absorbed and the amount left uncombined to be sufficiently near that the difference can be ignored.

The Lo dose is not always defined as above. According to Ehrlich (1903) "the Lo dose of a toxin is that amount of poison which is completely neutralised by one immune unit" and further, in the Lo mixture "all the constituents of the poison are completely neutralised so that not only the single amount but also high multiples of this can be injected into guinea-pigs without causing a trace of a local or general reaction." The Lo dose as usually determined is that amount of toxin which when mixed with 1 unit of antitoxin and injected fails to produce a reaction. In practice we find that "high multiples" of such a mixture may produce œdema and even death.

It follows that the neutralising value assigned to a toxin depends to a certain extent upon the sensitiveness of the test applied; the most sensitive test for detecting traces of unneutralised toxin consists in injecting 5 c.c. or more of the mixture subcutaneously into guinea-pigs. With a toxin of average strength the intracutaneous injection of 0·2 c.c. of the mixture is almost as sensitive a test and more sensitive than that of a single Lo dose mixed with 1 unit of antitoxin. It follows that the Lr value of a toxin determined intracutaneously records a higher strength (*i.e.* a smaller volume for the unit) for the toxin than the Lo value determined subcutaneously, unless the latter value has been obtained by injecting "high multiples" of the mixture. When Lr/500 titrations are made, the mixtures are diluted so that only 1/500th of the normal quantities are injected;

this method is again less sensitive than the Lo method and again a lower neutralisation level is given to the toxin.

Titration in vitro.

It has been demonstrated by Ramon (1922) that flocculation takes place when diphtheria toxin and antitoxin are mixed in certain proportions. In a series of experiments each consisting of a number of tubes containing a given toxin and a given antitoxin in different proportions, the ratio between the toxin and antitoxin in the mixtures in which flocculation first takes place will be the same in each experiment. Ramon (1923) states that in his experience the ratio is the same as that in a mixture exactly neutral to animals. Glenny and Okell (1924) have found that with the majority of toxins and antitoxins tested at these laboratories, flocculation first occurs in mixtures slightly over-neutralised according to animal test. They have introduced a new term, the "Lf dose," which is that amount of toxin which is equivalent to 1 unit of antitoxin as determined by the flocculation test.

Summary of units.

The various units now employed in measuring the strength of a toxin may be summarised as follows:—

	Measures of specific toxicity.	Measures of combining power.	
		Tested against 1 unit of antitoxin.	Tested against a fraction of a unit of antitoxin.
In vivo—			
Subcutaneous method .	M.L.D.	Lo L+	... ...
Intracutaneous method .	M.R.D.	Lr	Lr/500 or other convenient fractions.
In vitro—			
Flocculation method .	...	Lf	...

We thus find that there are six units of measurement used in recording the strength of a toxin. We shall show later that none of these measures of combining power necessarily bear the same relationship in two toxins; the M.L.D. and the M.R.D. alone can be deduced approximately one from the other. The relationship of the various measures of combining power is seen in table I. which gives the experimental results for a typical toxin.

The difference between L+, Lo, Lr and Lf can be seen in the series of mixtures containing different volumes of toxin varying from 0·22 to 0·14 c.c. each with 1 unit of antitoxin, tested in three different ways. By allowing the mixtures to stand we obtained the flocculation phenomenon of Ramon; flocculation may be hurried by exposure to higher temperature. In the table we show

TABLE I.

Experimental results showing relationship between various measurements of combining power of a typical toxin.

Composition of mixture: volumes of toxin added to 1 unit of antitoxin.	0·140	0·145	0·150	0·155	0·160	0·165	0·170	0·175	0·180	0·185	0·190	0·200	0·210	0·220
Results of subcutaneous injection into guinea-pigs.								no œdema	no œdema Lo	very small swelling	medium swelling	large swelling died 10th day	died 5th day L+	died 2½ days
Results of intradermic injection into guinea-pigs.					–	–	–	± Lr	++	+++				
Appearance of mixture after thirty minutes at 50° C.														
Conclusion.				Lf				Lr	Lo				L+	

that at the end of 30 minutes' exposure to a temperature of 50° C. marked flocculation occurred in the mixture containing 0·155 c.c. of toxin to each unit of antitoxin and was beginning in the tube on either side containing 0·160 and 0·150 c.c. of the toxin; traces of precipitate could be seen forming in the tube containing 0·145 of the toxin. No other tube showed any signs of precipitate formation; the Lf value therefore of this toxin is taken as 0·155 c.c. Mixtures containing toxin increasing from 0·175 c.c. to 0·22 c.c. with 1 unit of antitoxin were injected subcutaneously into guinea-pigs and it was found that the mixtures containing 0·21 c.c. of toxin killed on the fifth day, while mixtures with more toxin killed earlier and those with less caused a late death or failed to kill. The L+ value of the toxin is therefore 0·21 c.c. In the same series of animals injected subcutaneously those receiving more than 0·18 c.c. of toxin with 1 unit of antitoxin showed local œdema, those injected with 0·18 c.c. or less gave no signs of swelling. The Lo value of the toxin is therefore 0·18 c.c. The mixtures containing from 0·16 to 0·185 c.c. of toxin were injected intracutaneously into guinea-pigs in doses of 0·2 c.c.; the Lo mixture containing 0·18 c.c. of toxin produced a very marked inflammatory reaction and the mixture containing 0·175 is taken as the Lr dose, because a small reaction was produced by this mixture and no reaction by mixtures containing less than this quantity of toxin. The mixture containing 0·20 c.c. of toxin when diluted 1 in 500 gave an intracutaneous reaction which was not caused by the injection of the same dilution of the mixtures containing less toxin; the Lr/500 dose of this toxin is therefore 1/500th of 0·20 c.c., or 0·0004 c.c. We may point out here that the Lr dose is not exactly 500 times the Lr/500 because, as we have already mentioned, the Lr/500 test is less sensitive than the Lr and therefore gives a lower neutralisation level to the toxin. We have not included this titration in table I. because we do not regard the Lr/500 dose as a definite unit; other fractions are often used by us for convenience of experiment and these fractional tests must be regarded as rough estimations or as preliminary determinations to avoid the danger of killing guinea-pigs by excess of free toxin which might result if titrations for the full Lr dose were made upon toxins of unknown strength.

Before considering the theoretical relation of these various measures one to another, and their connection with antigenic value we record a series of experiments establishing certain relationships between the toxin and toxoid content of a toxin brew, its combining power and its antigenic value.

Experimental results.

The ratio between Lf and Lr doses is not constant.

If flocculation usually occurs first in mixtures over-neutralised according to animal test, it follows that toxins appear slightly stronger in combining power when tested *in vitro* than when tested *in vivo*, and the Lf dose is less than the Lo or Lr dose. The ratio Lf/Lr in the example quoted in table I. is 0·155/0·175 *i.e.* 0·88, and in the majority of fresh batches of toxin tested against our standard antitoxin has been from 0·80 to 0·95. If flocculation indicates an exact balance between antigen and antibody this ratio indicates that no inflammatory reaction is produced when a guinea-pig is injected intracutaneously with toxin of which from 80 per cent. to 95 per cent. has been neutralised by antitoxin. If, on the other hand, the Lr

dose of a toxin be considered to indicate the true equivalent of 1 unit of antitoxin, flocculation first occurs in the mixture in which toxin is over-neutralised by antitoxin to the extent of 5 per cent. to 25 per cent. Table II. records the Lf and Lr doses and Lf/Lr ratios of twelve different brews of diphtheria toxin.

TABLE II.

Showing the relation between Lr and Lf values for twelve different brews of diphtheria toxin.

Brew of toxin.	Volume of antitoxic serum (280 units per c.c.) added to 2 c.c. of each batch of toxin to form Lf and Lr mixtures.		Lf and Lr values calculated from columns 1 and 2.		Ratio Lf/Lr.
	Lf mixtures.	Lr mixtures.	Lf.	Lr.	
1.	0·046 c.c.	0·043 c.c.	0·155 c.c.	0·166 c.c.	0·93
2.	0·064 ,,	0·058 ,,	0·112 ,,	0·123 ,,	0·91
3.	0·023 ,,	0·020 ,,	0·310 ,,	0·357 ,,	0·87
4.	0·040 ,,	0·036 ,,	0·179 ,,	0·198 ,,	0·90
5.	0·048 ,,	0·046 ,,	0·149 ,,	0·155 ,,	0·96
6.	0·036 ,,	0·032 ,,	0·198 ,,	0·223 ,,	0·89
7.	0·070 ,,	0·065 ,,	0·102 ,,	0·110 ,,	0·93
8.	0·080 ,,	0·075 ,,	0·089 ,,	0·095 ,,	0·93
9.	0·060 ,,	0·054 ,,	0·119 ,,	0·132 ,,	0·90
10.	0·035 ,,	0·028 ,,	0·204 ,,	0·225 ,,	0·80
11.	0·040 ,,	0·032 ,,	0·179 ,,	0·223 ,,	0·80
12.	0·050 ,,	0·042 ,,	0·143 ,,	0·170 ,,	0·84

Each batch of toxin was tested under identical conditions of measurement against the same serum. Mixtures were made containing 2·0 c.c. of toxin and varying amounts of antitoxin measured by means of the Trevan (1922) micro-syringe. To determine the Lf value, these mixtures were put into a water bath at 50° C., and to determine the Lr value 0·2 c.c. was injected intracutaneously into guinea-pigs. In this way the ratio Lf/Lr could be accurately determined, any small errors of measurement being common to both tests.

The first two columns in table II. give the actual volumes of antitoxin added to 2 c.c. of each of the twelve toxins to form Lf and Lr mixtures respectively; the third and fourth columns give by calculation the Lf and Lr doses, and the final column the ratio between these two doses. Other toxins prepared under different conditions have shown widely different ratios when tested against the same antitoxin.

The Lf/Lr ratio of a given toxin may vary according to the antitoxin used for titration.

The Lf/Lr ratios given in table II. were determined against a given standard serum and some were confirmed against several other samples of antitoxin. Certain other toxins however revealed quite different ratios: one toxin for example titrated at fairly wide limits against a number of special sera gave the following figures for the Lf/Lr ratios: 0·58, 0·67, 0·70, 0·86, 0·94, 1·0 and 1·18. The ratios from 0·86 and 1·00 were obtained with what we would regard as

average specimens of antitoxin, and the differences in the figures quoted may all lie within the error of this particular experiment. The Lf/Lr ratios of 18 different toxins against our standard antitoxin were between 2·1 and 2·5 times those obtained with one serum. We have thus to record that with a given standard antitoxin the Lf/Lr ratio for different toxins may vary, while for a given toxin the flocculation titres of various samples of antitoxin may vary considerably from the Ehrlich unit determined by any *in vivo* method. This serum ratio $\frac{\textit{in vitro}\text{ value}}{\textit{in vivo}\text{ value}}$ has varied from 0·4 to 2·0 in different samples we have tested. As a general rule if the Ehrlich value is considerably higher than the flocculation value, the flocculating time of the serum is very short: sera with the reverse ratio are extremely slow to flocculate and also appear to be examples of "modified antitoxin" and furthermore show considerable dissociation from combination with toxin, as will be mentioned later in this paper.

It must be made clear that the ratio Lf/Lr is a ratio of values determined against a fixed serum that we have chosen as a standard or against some other serum found to correspond in type to this same standard serum. The serum ratio $\frac{\textit{in vitro}}{\textit{in vivo}}$ is not determined against any one toxin but is the *in vitro* value determined against the Lf dose of any standard toxin, compared with the animal titration against the Lr or L+ dose of any other toxin. If the flocculating equivalent of the serum is compared with the *in vivo* equivalent of the same serum against the same toxin, the resulting ratio is the combination of the Lf/Lr ratio of the toxin and the $\frac{\textit{in vitro}}{\textit{in vivo}}$ value of the serum.

The relation between Lf and Lr values is an index of the proportion of toxin to toxoid.

Ramon (1923) has pointed out that a flocculation reaction will still be given by a toxin so modified by formalin that it can no longer produce œdema in guinea-pigs. When a toxin is not completely modified and some remains unchanged into toxoid an Lr and Lo dose can still be estimated, but it will be found that the volume of either dose is much greater for the modification than for the original toxin, and if the Lf value has remained unchanged—as it usually does—the Lf/Lo or Lf/Lr ratio approaches 0 as the modification into toxoid takes place. It would appear therefore that this ratio is some measure of the proportion of toxin to toxoid present in a batch of toxin. This proportion may vary in fresh toxins; the Lf/Lr or Lf/Lo ratio therefore of fresh toxin is not constant unless the method of preparation of the medium and the conditions of growth remain so uniform that the proportion of specific toxin to toxoid produced remains constant.

The general trend of opinion amongst immunologists in recent years has been towards the view that antigenic power can be judged from the combining value of a toxin. The main problem therefore is to compare the antigenic strength of a toxin with its combining power as determined by the different methods of testing and to find which unit of measurement gives a true indication of the close connection between the two values.

The M.L.D. is not an indication of antigenic value, and antitoxin of high potency can be obtained by immunising horses with toxin extremely weak in specific toxicity.

Experience with large scale production of diphtheria antitoxin by the immunisation of horses has shown that the M.L.D. is not an index of antigenic value and that antitoxin of high potency can be obtained by immunising horses with toxin extremely weak in specific toxicity. This was certainly our experience with toxin acted upon by formalin. During 1904 toxin was blended in batches of 100 litres, one part per 1000 formaldehyde was added and the specific toxicity thereby greatly reduced. This method of blending and modifying with formalin was continued until a store of several thousand litres was produced sufficient to last many years. Only two batches in the first thousand litres contained more than ten M.L.D.s per c.c. when first used for immunisation. The first of such blends contained in June 1904 eight M.L.D.s per c.c. when it was first used by one of us for the immunisation of horses. During 1904 horses immunised with this toxin yielded:—

5	bleedings	over 1000 units per c.c.
8	„	between 800 and 1000
13	„	„ 600 „ 800
15	„	„ 400 „ 600
6	„	less than 400.

Another toxin when first blended in September 1904 had an M.L.D. of 0·01 c.c., 12 months later the M.L.D. was 1·3 c.c., 4½ years later a horse yielded serum containing 1000 units per c.c. when immunised with this toxin alone. Several toxins were used with success after the specific toxicity has been so reduced that 5·0 c.c. would no longer kill a guinea-pig. Specific toxicity therefore is no index of antigenic power.

Toxin with a small combining power as measured by the Lo dose may be of good antigenic value.

The toxins mentioned above when first blended had an Lo dose of about 0·3 c.c.; after the addition of formalin the Lo dose increased and reached several c.c. without affecting the antigenic value. We might also mention our experience with tetanus toxin and to a less

extent with gas gangrene toxins during the war. All these toxins were titrated for combining capacity and not for minimal lethal dose and their immunising value was judged by their combining power at the time of preparation. Tetanus toxin frequently appeared when judged by L+, to possess only a fraction of its original strength a week after its preparation and yet such toxin was used to produce several million doses of tetanus antitoxin. It was our custom for many years to judge the strength of toxin intended for the immunisation of horses by means of the Lo or L+ values of fresh toxin, but to-day our judgment is based on the Lf value.

After treatment with formaldehyde the ratio Lf/Lr of a toxin decreases according to the concentration of formaldehyde used.

In order to compare the antigenic values of toxins of varying toxicity, a batch of diphtheria toxin was divided into a number of equal parts and different amounts of formaldehyde were added to each: the batches were then incubated for 24 hours at 37° C.: the various measurements of this series are recorded in table III.

TABLE III.

Showing the M.L.D. and combining values of a toxin and of various modifications of that toxin.

	Percentage of formaldehyde added to toxin.	M.L.D. in c.c.	L+ in c.c.	Lo in c.c.	Lr in c.c.	Lf in c.c.	$\frac{\text{Lo}}{\text{M.L.D.}}$	$\frac{\text{Lf}}{\text{L+}}$	$\frac{\text{Lf}}{\text{Lo}}$	$\frac{\text{Lf}}{\text{Lr}}$
J 3485	–	0·002	0·21	0·18	0·165	0·11	90	0·52	0·61	0·67*
PX 146	0·01	0·003	0·22	0·19	0·175	0·11	63	0·50	0·58	0·63
PX 147	0·03	0·01	0·22	0·20	0·175	0·11	20	0·50	0·55	0·63
PX 148	0·1	0·02	0·28	0·23	0·195	0·11	11	0·39	0·48	0·56
PX 149	0·2	0·05	0·38	0·32	0·23	0·12	6	0·32	0·37	0·52
PX 150	0·3	0·1	1·25	0·4	0·30	0·12	4	0·10	0·30	0·40
PX 151	0·4	2·0	over 5·0	1·75	0·25†	0·12	less than 1	0·02	0·07	...
PX 152	0·5	over 5·0	...	over 3·0	0·33†	0·12	...	...	0·05	...
PX 153	1·0	...	...	...	0·46†	0·12†	...	...	...	...

* This ratio was unusually low for an unmodified toxin (see table II.).

† These volumes were determined by blending with the original toxin J 3485.

With increasing quantities of formaldehyde toxin is changed into toxoid and consequently the M.L.D. increases and, as the indicating toxin decreases, the apparent combining power decreases and the Lo, L+ and Lr doses increase. The Lf value however has suffered little change.

It was impossible to obtain Lr values of PX. 151, 152 and 153 as a result of direct measurement, but on blending with the original toxin the Lr value could be deduced. This was done by mixing equal quantities of the modified toxin and the original toxin and finding how much this amount of the former reduced the Lr of the toxin; from this reduction the Lr of the unknown modified toxin was calculated.

The first measurement to be affected by formaldehyde is the M.L.D.: PX 147 has undergone a marked loss in specific toxicity indicated by the five-fold increase in M.L.D. while the L+, Lo and Lr and Lf values remain practically unchanged. Upon exposure to greater concentration of formaldehyde the L+ dose (see PX 148) shows a definite change while the other values show less change: the modification PX 149 shows a big increase in Lo dose while the Lr dose still remains fairly low. With modification PX 150 the first large increase in Lr dose occurs. For this modification the M.L.D. has increased fifty-fold, the L+ six-fold, the Lo has slightly more than doubled, the Lr has not quite doubled and the Lf has increased by only 10 per cent. This order in which the various measurements of toxin are affected when toxin becomes modified must correspond to the experience of most immunologists. In the very early days it was shown that as a toxin aged the M.L.D. suffered the greatest change and the L+ dose showed a larger increase than the Lo. Recently we have found that the Lr value is slightly more stable than the Lo but that both these titrations may fail while the Lf dose remains unaltered.

The antigenic value of toxin after modification depends upon the Lf value and not the Lr or Lo values. The antigenic values of the modified toxins in the series PX 146-153 quoted in table III. were determined by means of the immunity index (Glenny, Allen and Hopkins 1923, Glenny and Hopkins 1923), and are given in table IV.

TABLE IV.

Showing the immunity index of various doses of modified toxin.

Modified toxin.	Percentage of formaldehyde added to toxin.	Doses of modified toxin.							
		0·005.	0·01 c.c.	0·02 c.c.	0·05 c.c.	0·1 c.c.	0·2 c.c.	0·5 c.c.	1·0 c.c.
		Immunity Index.							
PX 148	0·1	x, x	16	4	...	...	...	...	...
PX 149	0·2	..	6, x	4	...	...	...	...	...
PX 150	0·3	...	...	6	2, 3, x	2	...	...	...
PX 151	0·4	...	...	...	2, 2, 3, 4	2, 2, 5	2, 5	1	...
PX 152	0·5	...	...	...	3, 3, 7	...	2, 3	...	1, 1, 2
PX 153	1·0	...	...	...	6, 6, 6	...	...	...	1, 1, 1

x = over 16. Each figure represents an animal test.

From these figures it would appear that the antigenic values of different batches of toxin in different stages of modification do not differ to any great extent. The modification PX 153 shows a poor index when tested in doses of 0·05 c.c. It must be pointed out however that we are not comparing modified toxins under the same conditions because the toxicity of the doses employed varied with each member of the series, depending upon the degree of modification.

We show elsewhere (Glenny, Hopkins and Pope 1924) that modifications of toxins are better antigens when incompletely toxoided and still slightly toxic. By mixing with antitoxin it was possible to compare more closely the antigenic values of the various modifications.

TABLE V.

Showing the antigenic values of mixtures of 1 *unit of antitoxin with the same dose* (0·18 c.c.) *of a series of toxins in varying stages of modification.*

Composition of mixtures.						Number of guinea-pigs showing an immunity index of—					
Antitoxin.	Modified toxin.		Mixtures in terms of Lf, Lr, Lo of each modified toxin.								
	Key No.	Volume.	Fraction of Lf.	Fraction of Lr.	Fraction of Lo.	2.	3.	4.	5.	6.	Over 6.
1 unit	PX 146	0·18 c.c.	1·64	1·03	0·95	...	1	1	...	...	...
,,	PX 147	,,	1·64	1·03	0·90	...	...	2	1	...	...
,,	PX 148	,,	1·64	0·92	0·78	...	...	1	...	...	1
,,	PX 149	,,	1·50	0·78	0·56	1	...	1	...	...	1
,,	PX 150	,,	1·50	0·60	0·45	...	2	...	...	1	...
,,	PX 151	,,	1·50	...	0·10	...	1	2	...	...	...
,,	PX 152	,,	1·50	...	0·06	...	1	...	1	1	...
,,	PX 153	,,	1·50	...	...	...	1	...	...	1	...

One unit of antitoxin was added to 0·18 c.c. of each of the modified toxins and it will be seen from table V. that the majority of guinea-pigs injected with this mixture were immune to either the third or fourth Schick test. Knowing the Lo dose of each of the modifications it was possible to calculate what fraction of this dose was contained in 0·18 c.c., and it was found that the fraction varied from 0·95 c.c. in the case of PX 146 to less than 0·06 c.c. with PX 152 and still less in the case of PX 153. If the Lo dose were a true indication of antigenic efficiency, a big variation in response to the mixtures injected would be expected. Table V. shows very little difference in the immunity index of the various mixtures, and it must be inferred that the Lo or Lr dose is not a true index of the antigenic strength of a toxin. It appears therefore that, though the action of different quantities of formalin upon toxin causes an apparent weakening in combining power judged by animal tests, the Lf and antigenic values remain unaffected. The flocculation test is thus a more exact measurement of combining power than any of the *in vivo* tests. Certain other facts must be established before the theory of the combination of toxin and antitoxin is discussed.

Toxin and toxoid may dissociate from combination with antitoxin.

The extent of dissociation of toxoid from antitoxin and its replacement by toxin is seen when attempts are made to titrate the excess of antitoxin in a mixture over-neutralised according to animal tests.

Table VI. gives the figures obtained from such an experiment on a toxin of which 1 c.c. plus 3·25 units of antitoxin constituted an Lr mixture and 1 c.c. plus 4·6 units an Lf mixture. The toxin was left in contact with varying amounts of antitoxin for four hours and at the end of this time the mixtures were titrated for excess of antitoxin by animal test. If toxin did not displace toxoid from its combination with antitoxin then no excess antitoxin would be detected in any under-neutralised mixture, and any addition, even the smallest amount of test toxin, would remain unneutralised. We see from table VI. however that in a mixture containing 1 c.c. of toxin and 3·5 units of antitoxin, *i.e.* 0·25 unit per c.c., more than that necessary to neutralise all toxin detectable by animal means, added toxin can displace as much toxoid as was in combination with 0·08 unit per c.c. With each mixture tested until well beyond the Lf value, the standard toxin added detected about one-third of the antitoxin present in excess of the Lr value.

TABLE VI.

Showing the amount of antitoxin fixed by 1 *c.c. of toxin to which varying amounts of antitoxin had been added.*

Units of antitoxin added to 1 c.c. of toxin.	Excess units of antitoxin over Lr dose.	Excess units of antitoxin over Lf dose.	Excess units of antitoxin detected.	Total units of antitoxin combined.
3·5	0·25	− 1·1	0·08	3·4
3·75	0·5	− 0·85	0·12	3·6
4·0	0·75	− 0·6	0·25	3·75
4·5	1·25	− 0·1	0·4	4·1
5·0	1·75	+ 0·4	0·6	4·6
6·0	2·75	+ 1·4	1·0	5·0

With specially modified antitoxin, as that described by one of us (Glenny 1913), dissociation can be more easily demonstrated. Such modifications of antitoxin occasionally occur naturally or can easily be prepared by exposing antitoxin to a temperature of 37° C. for a number of years. It was shown (Glenny 1913) that certain modifications of antitoxin when injected with toxin subcutaneously into guinea-pigs prevented the appearance of œdema but not death. It was suggested that the properties of this modified antitoxin seemed to warrant two conclusions:—

(1) the constituent of diphtheria toxin which is acutely lethal in its action is not identical with that which causes the local reaction at the site of injection:

(2) the power of a serum to neutralise the acutely lethal constituent of a toxin may vary independently of its power to neutralise the constituent causing local reaction.

Later work however has shown that the phenomenon of death without any precedent œdema is probably due to dissociation occurring

after the mixture has been absorbed, for such mixtures may cause no inflammatory reaction when injected undiluted into the skin of a guinea-pig and yet a marked reaction follows the injection of the diluted mixture. Thus from table VII. we see that 26 c.c. of toxin mixed with 0·5 c.c. of modified antitoxin produced no reaction when 0·2 c.c. was injected intracutaneously, nor when a 1 in 10 dilution was made, but with a dilution of 1 in 100 a positive reaction was obtained. We find that modified antitoxin will produce Lf mixtures with less antitoxin than is needed for complete animal neutralisation and therefore the serum ratio $\frac{\textit{in vitro}\text{ value}}{\textit{in vivo}\text{ value}}$ is greater than 1.

TABLE VII.

Showing abnormal reaction of guinea-pigs to subcutaneous and intracutaneous injection of mixtures of toxin with modified antitoxin.

Composition of mixture injected.		Result of injecting guinea-pigs subcutaneously with			Reactions produced as a result of injecting guinea-pigs intracutaneously with 0·2 c.c. of			
Toxin. c.c.	Modified antitoxin. c.c.	0·1 c.c.	1·0 c.c.	5·0 c.c.	Undiluted mixture.	Diluted 1 in 10.	Diluted 1 in 100.	Diluted 1 in 1000.
20·0	0·5	...	No œdema. Died 11 days.	No œdema. Died 16 days.	−	−	−	
22·0	0·5	...	No œdema. Died 20 days.	...	−	−	− +	
24·0	0·5	No œdema. Died 14 days.	No œdema. Died 4 days.	Slight œdema. Died 10 days.	− −	− −	+ + −	
24·0	0·5	..	No œdema. Died 6 days.	...				
24·0	0·5	...	No œdema. Survived.	...				
26·0	0·5	No œdema. Died 9 days.	No œdema. Died 5 days.	...	− − − −	− − − −	+ + + +	−
26·0	0·5	...	No œdema. Died 5 days.	...				
18·0	0·5	...	Slight œdema. Died 3 days.	...			+	+ +
10·0	0·5	...	Slight œdema. Died 2 days.	...	+	+	+	+

It is interesting to note that mixtures of modified toxin and modified antitoxin possess low antigenic power as shown in table VIII.; it is possible that modified antitoxin has a greater affinity for toxoid than toxin. This would account for the decreased antigenic efficiency of toxoid in the presence of modified antitoxin because since the toxoid is "neutralised" there is no "free" antigen to stimulate the production of antitoxin. The weaker affinity of modified antitoxin for toxin would also account for the reversal of the usual Lf/Lr ratio and for the intradermal reaction produced by mixtures in high dilution. On the other hand, if modified antitoxin has a weak affinity for toxin, in other words a high dissociation constant, a "unit" of modified antitoxin may appear to be far in excess of the true unit, and in table VIII.

we are comparing modified toxin partially neutralised by antitoxin with that over-neutralised by modified antitoxin.

TABLE VIII.

Showing the immunity index of mixtures of modified toxin with antitoxin and modified antitoxin.

Composition of mixtures.		Immunity index of mixtures containing	
Modified toxin.	Units of antitoxin or modified antitoxin added.	Antitoxin.	Modified antitoxin.
0·1 c.c.	0·1 unit	2, 3, 7	5, 5, 5
,,	0·2 ,,	4, 4, x	4, x, x
,,	0·3 ,,	2, 2, 3	4, 7, x
,,	0·4 ,,	2, 6, x	5, x, x
,,	0·45 ,,	4	...
,,	0·5 ,,	2, x	9, x
,,	0·55 ,,	3, 5, x	...
,,	0·6 ,,	...	x, x

x = over 10.

Other evidence exists to show that toxin and antitoxin can be dissociated from combination. We know that a neutral mixture under certain obscure conditions may become toxic when frozen (Kelley 1924, White and Robinson 1924, Kirkbride and Dow 1924, and Anderson and Leonard 1924) and further that antitoxin can be recovered from a toxin-antitoxin mixture (Ramon 1923).

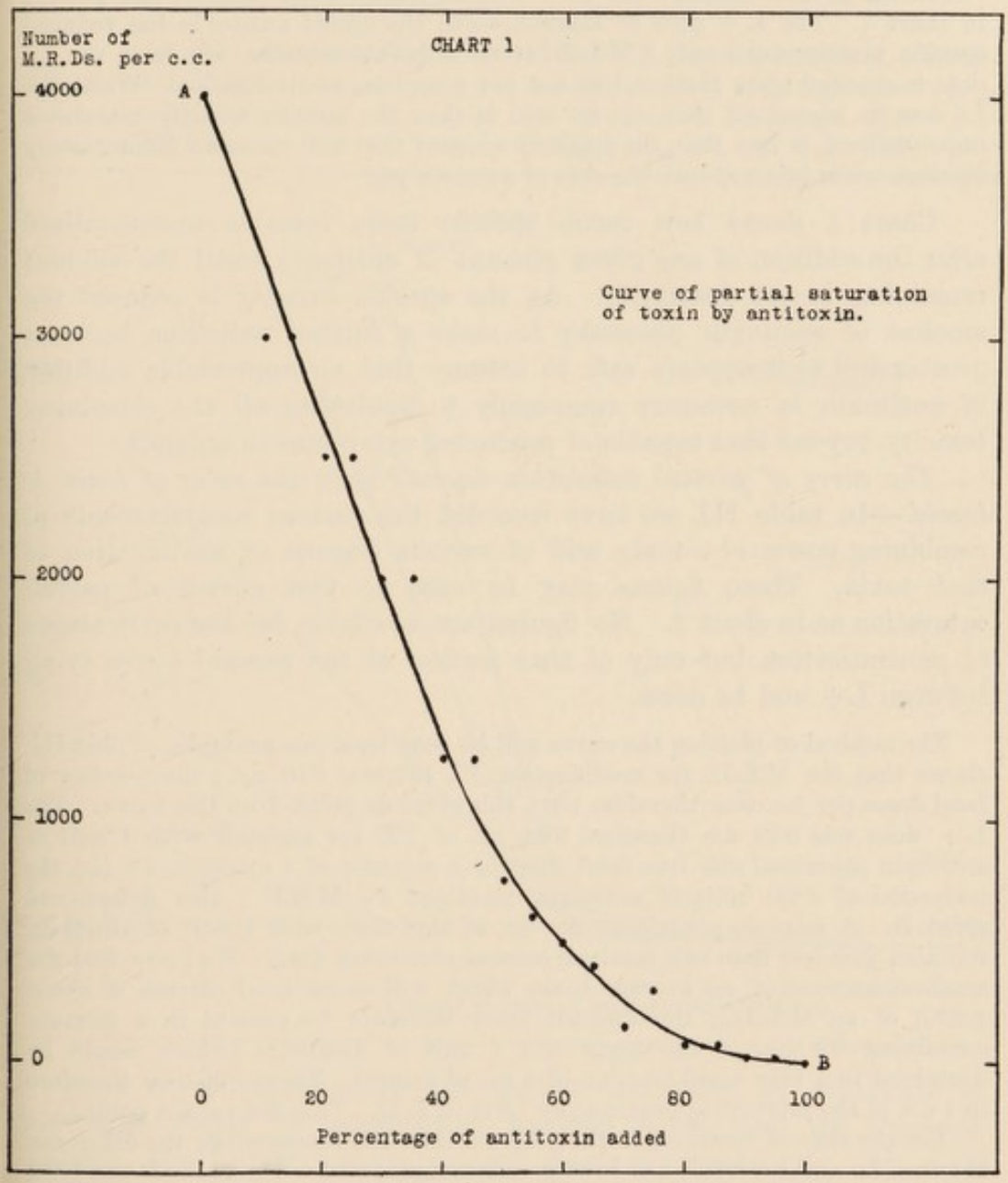
As we hope to record in other papers, we have recently succeeded in rendering an over-neutralised mixture toxic by the addition of suitable concentrations of phenol in imitation of the local concentration that occurs when a carbolised mixture is frozen. We can also by Ramon's method of heating the toxin-antitoxin precipitate in the presence of a dilute acid render a toxic mixture antitoxic. It can thus be claimed that in considering the theory of the combination of toxin or toxoid with antitoxin allowance must be made for possible dissociation.

Curves of partial neutralisation.

When considering the relationship between the various units of combining power it is necessary to consider the curve of partial neutralisation of specific toxin.

Progressive additions of antitoxin to a batch of toxin do not reduce the specific toxicity equally.—In the majority of toxins examined, the addition of say 1/10th of the total quantity of antitoxin needed completely to neutralise all effects upon animals reduces the specific toxicity by far more than 10 per cent. and successive additions apparently combine with decreasing amounts of toxin. Ehrlich believed that this neutralisation took place in stages, *i.e.* the course of

neutralisation could not be plotted as a continuous curve, but this is not supported by later work. Arrhenius and Madsen (1902) show that the curve of resulting toxicity is a continuous one. If the specific toxicity of a series of mixtures containing progressive amounts



of antitoxin be plotted against the quantity of antitoxin added the resulting curve is similar to that shown on chart 1.

The general shape of this curve agrees with those published by Madsen. Chart 1 shows the curve of partial neutralisation of an incompletely modified toxin; this curve is typical of several curves that we have prepared and has been chosen because the low toxicity of the modified toxin enabled the whole course of neutralisation to be depicted on a reasonable scale.

We first determined the least amount of standard antitoxin to be added to 10 c.c. of modified toxin so that 0·2 c.c. of the resulting mixture should cause no reaction when injected intracutaneously into a guinea-pig. We then added to a series of 10 c.c. of the toxin, 5, 10 and 15 per cent. etc. of this amount of antitoxin by means of a Trevan (1922) micro-syringe. The number of M.R.D.s remaining in each mixture was then titrated; the results obtained are plotted in chart 1. The L + dose is reached when the added antitoxin has reduced specific toxicity until only 1 M.L.D. (1000 M.R.D.s) remains; similarly the Lo dose is reached upon further, but not yet complete, neutralisation. When the Lr dose is passed all that can be said is that the specific toxicity remaining unneutralised is less than the smallest amount that will cause an inflammatory reaction when injected into the skin of a guinea-pig.

Chart 1 shows how much specific toxin remains unneutralised after the addition of any given amount of antitoxin until the amount remaining is not detectable. As the specific toxicity is reduced the amount of antitoxin necessary to make a further reduction becomes greater and so it appears safe to assume that an appreciable addition of antitoxin is necessary completely to neutralise all the remaining toxicity beyond that capable of producing symptoms in animals.

The curve of partial saturation depends upon the ratio of toxin to toxoid.—In table III. we have recorded the various measurements of combining power of a toxin and of various degrees of modification of that toxin. These figures may be used to plot curves of partial saturation as in chart 2. No figures are available for the early stages of neutralisation but only of that section of the general curve lying between L+ and Lr doses.

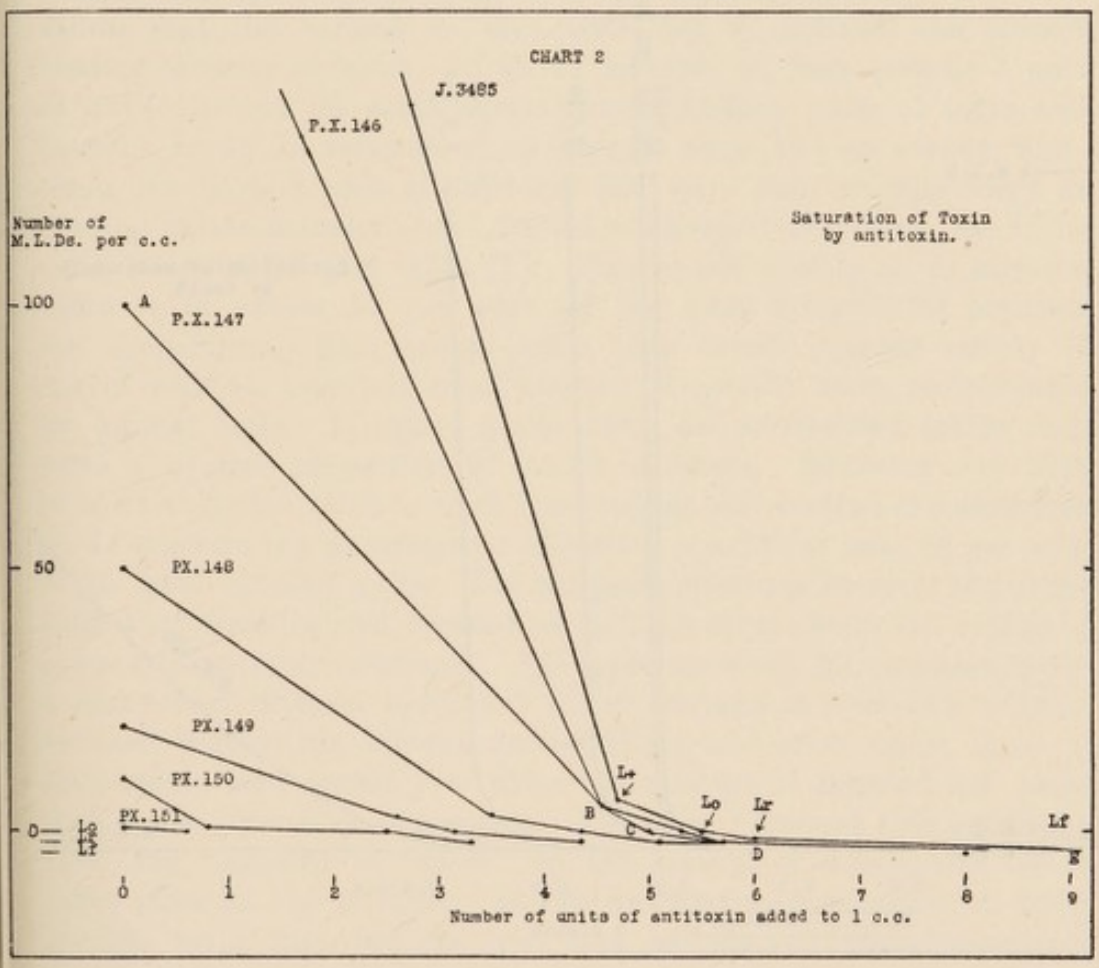
The method of plotting the curve will be seen from one example. Table III. shows that the M.L.D. for modification PX 147 was 0·01 c.c.; the number of fatal doses per c.c. was therefore 100; this gives us point A on the curve. The L + dose was 0·22 c.c. therefore 0·22 c c. of PX 147 together with 1 unit of antitoxin contained one free fatal dose, or a mixture of 1 c.c. with 4·5 (*i.e.* the reciprocal of 0·22) units of antitoxin contained 4·5 M.L.D.; this determined point B. A mixture containing 0·2 c.c. of this toxin with 1 unit of antitoxin contains just less than one minimal œdema producing dose. We know that the smallest amount of an average toxin which will cause local œdema is about 1/20th of an M.L.D.: this amount must therefore be present in a mixture containing 0·2 c.c. of the toxin and 1 unit of antitoxin (which would be contained in a very small fraction of a c.c. of serum). There must be therefore in 1 c.c. of the mixture approximately 1/4th M.L.D. This determines point C.

For the sake of clearness in the diagram the scale representing the difference between Lo and Lr levels has been greatly exaggerated. The point D has been fixed at a distance below the Lo level equivalent to two M.L.D.s in place of a very small fraction. Similarly the point E representing the Lf value has been placed at a similar level below the Lr. Our object is to consider the relationship of the Lf dose to the other units of combining power; we have therefore included this point on the curves without any definite knowledge at present whether any specific toxin remains uncombined in an Lf mixture.

Chart 2 shows how the curve for each successive modification of toxin becomes flatter as the degree of specific toxicity is reduced. An inspection of any of the curves shows that it is quite reasonable to

suggest that the points A, B, C, D and E all lie upon a continuous curve and that the Lf value represented by the point **E** probably represents complete neutralisation of specific toxicity. The various additions of antitoxin to 1 c.c. of PX 147 have neutralised the following quantities of toxin:—

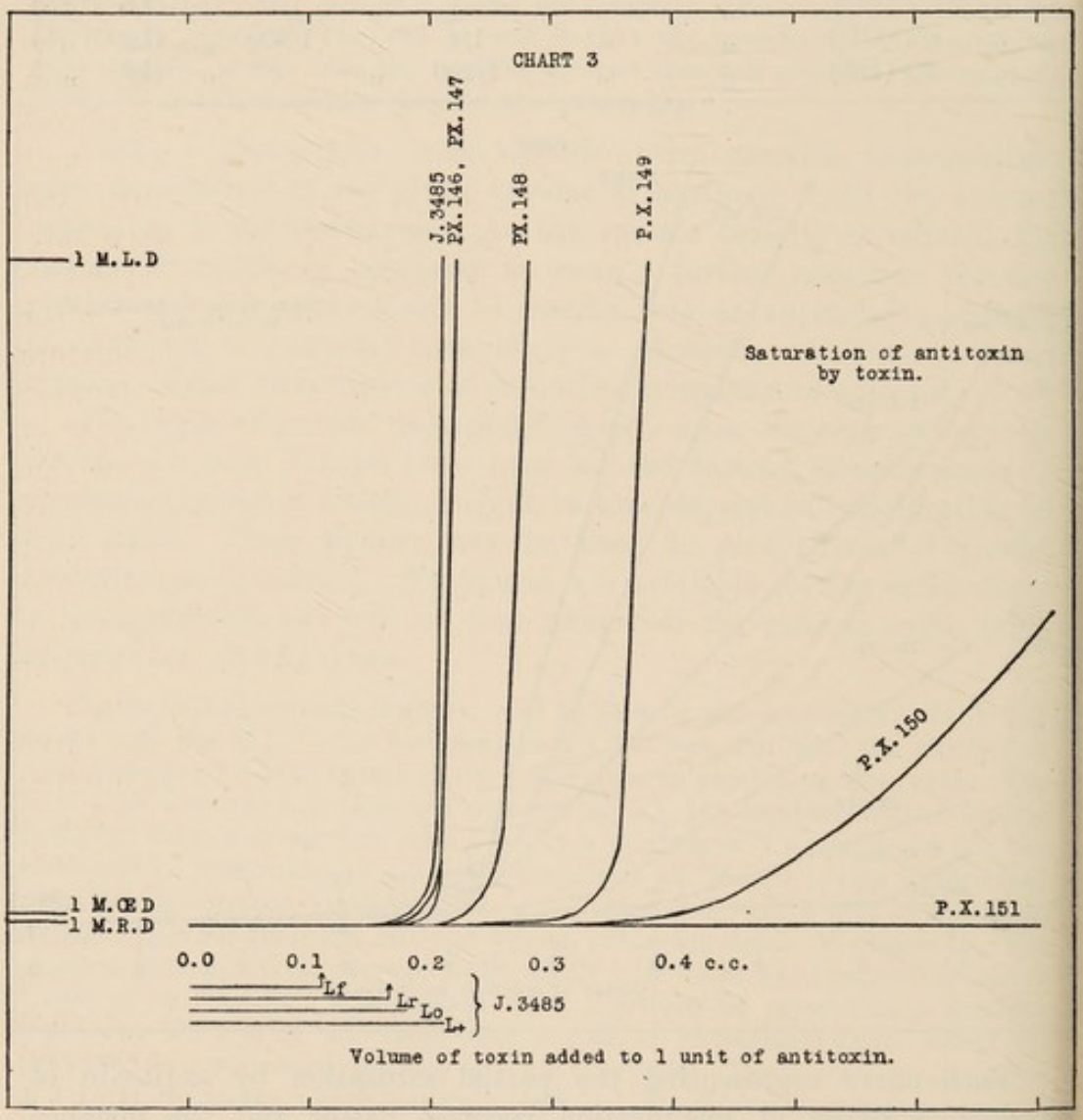
	units							
	4·5 (AB)	of antitoxin have reduced	100	M.L.D.s to	4½	M.L.D.	(L+	level)
a further	0·5 (BC)	„	„		4½	„ 1/4	„ (Lo	„)
„	0·7 (CD)	„	„		1/4	„ 1/200	„ (Lr	„)
„	3·3 (DE)	„	„		1/200	„ 0	„ (Lf	„)



Each curve representing the partial saturation by antitoxin of successive stages of modification becomes flatter and the distance between the animal measurements becomes greater, *i.e.* for each modification more antitoxin is required to pass from the L+ to the Lo and Lr levels and again more before the Lf point is reached. The original toxin appeared to contain an unusually high proportion of toxoid because the Lf/Lr ratio was 0·67 instead of 0·8 to 0·95 as with the majority of toxins.

The relationship of the different units of measurement and their dependence upon the amount of specific toxin left unneutralised may also be shown in a different type of curve. The curves in charts 1 and 2 have been plotted to show the specific toxicity free after the

addition of different amounts of antitoxin to a fixed volume of toxin. Chart 3 has been prepared from the L+, Lo, Lr and Lf values of the various modifications of toxin given in table III. to connect residual toxicity with the amount of toxin added to 1 unit of antitoxin. The curve of J 3485 can be visualised as built up from figures like those given in table I. working from left to right along the table. Each curve can



be regarded as a "mirror image" of the corresponding curve in chart 2. These curves show that no free toxin exists until more than the Lf dose of toxin has been added to 1 unit of antitoxin. If more toxin is added some small quantity of specific toxin may be free but until the Lr dose is reached the amount free is not enough to produce a reaction when injected intracutaneously into a guinea-pig. This form of curve affords a convenient method of showing the course of neutralisation within the differential region. Ehrlich used this phase to indicate the region between Lo and L+ but we extend the phase to cover the range from Lf to L+. It appears that it is reasonable to suggest that the

L+, Lo, Lr and Lf doses of toxin all lie upon a smooth curve of partial saturation of specific toxicity representing the point at which 1 M.L.D., 1 M.Œ.D., 1 M.R.D. and no toxin is free.

Theoretical considerations.

It is reasonable to assume that if the flocculation phenomenon has any significance, flocculation occurs first in the mixture in which the antigen and antibody are present in equivalent amounts. We have shown that the various *in vivo* tests fail to indicate the correct binding capacity of toxin. In an Lf mixture we have present 1 unit of antitoxin and its exact equivalent in binding units of toxin and toxoid. In an Lr mixture of an average toxin and an average antitoxin we have 1 unit of antitoxin and *more* than its equivalent in binding units—actually about 1·1 times its equivalent since the Lf/Lr ratio is about 0·9 (see table II.). This means that in an Lr mixture there is in excess 10 per cent. of the total toxin-toxoid required for equilibrium. This excess toxin plus toxoid consists mainly of toxoid with an infinitely small amount of specific toxin, undetectable by animal tests. Mixtures made from old or modified toxins may have a greater proportion of toxoid in excess. Mixtures owe part of their antigenic value to their free binding unit content and therefore an Lr mixture has an antigenic efficiency equal to at least 10 per cent. of its total binding units. The antigenic efficiency however is greater than that stated above because as we have shown toxin and antitoxin are not inseparably combined. We must therefore, for clearness, make a distinction between toxin and toxoid *free* and in *excess* in a mixture because, besides the amount unneutralised *and so in excess*, there is some toxin and toxoid *free* when the mixture is injected and more may *become free* after absorption. Thus the "amount free" is larger than the "amount in excess" by the amount of dissociation which takes place in the mixture. If this dissociation is excessive, so much specific toxin may become free that we observe the phenomenon associated with "modified antitoxin," *i.e.* acute death occurring without local œdema. If there is less dissociation, animals may die of paralysis. It is probable that the differences we have observed between sera in their *in vitro*/*in vivo* ratios may be due to differences in their dissociation constants.

Summary.

The different units of measurement of toxin are discussed; it is pointed out that only the flocculation test is a true measure of the combining capacity of a toxin. The indicating mixture or mixture containing the "precipitate indicateur" consists of exact equivalents of toxin *plus* toxoid and antitoxin.

The following facts are established experimentally:—

(1) The ratio between Lf and Lr doses is not constant.
(2) The Lf/Lr ratio of a given toxin may vary according to the antitoxin used for titration.
(3) The relation between Lf and Lr values is an index of the proportion of toxin to toxoid.
(4) The M.L.D. is not an indication of antigenic value and antitoxin of high potency can be obtained by immunising horses with toxin extremely weak in specific toxicity.
(5) Toxin with a small combining power as measured by the Lo dose may be of good antigenic value.
(6) After treatment with formaldehyde the ratio Lf/Lr of a toxin decreases according to the concentration of formaldehyde used.
(7) The antigenic value of toxin after modification depends upon the Lf value and not the Lr or Lo values.
(8) Toxin and toxoid may dissociate from combination with antitoxin.
(9) Progressive additions of antitoxin to a batch of toxin do not reduce the specific toxicity equally.
(10) The curve of partial saturation depends upon the ratio of toxin to toxoid.

Conclusions.

1. *The combining capacity of a toxin can be fully determined by the flocculation test: the* in vivo *measurements indicate partial neutralisation only.*

2. *Antitoxin has a greater affinity for toxin than for toxoid.*

3. *Toxin and antitoxin can dissociate from combination: the amount of dissociation which takes place in a mixture may vary with different samples of antitoxin.*

4. *Both specific toxin and toxoid are antigenic.*

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