A CONTRIBUTION TO THE KNOWLEDGE OF THE PROPERTIES OF MIXTURES OF DIPHTHERIA TOXIN AND ANTITOXIN WITH DIPHTHERIA FORMOL TOXOIDS AND OF THE APPLICATION OF THESE PROPERTIES FOR THE MEASUREMENT OF TOXOIDS.

By

Vida Johnston Perry, M.B., Ch.B.

(This work was carried out in the Emil von Behring Institute for Experimental Therapy, Marburg-Lahn, Germany).

C O N T E N T S.

Introduction.

On the discovery of diphtheria toxin by Roux and Yersin in 1887 and the demonstration of diphtheria antitoxin by Behring and Kitasato in 1890 is based the foundation of modern serum therapy.

Since the discovery that toxin could be neutralised by antitoxin the nature of this toxin-antitoxin combination has remained a problem up to the present day. It was first thought, that toxin was actually destroyed by antitoxin: it was found, however, that this was not the case but that, on the contrary, a definite combination took place between the two substances.

In order to explain the nature of the toxin-antitoxin union several theories have been advanced. The first of these was propounded by Ehrlich, 1898-1900, and was applied by him to serum reactions in general, and embodies the ideas of his famous "side-chain theory." According to his theory, the union of toxin and antitoxin is a purely chemical reaction. When brought together they combine with each other instantly, each having a very strong affinity for the other. This compound of toxin and antitoxin always remains the same no matter what proportions of antigen or antibody are used.

In order to explain the various phenomena which occur on the neutralisation of toxin by antitoxin, such as the Danysz phenomenon and the relation of the L^+ to the Lo dose, Ehrlich postulated the presence of various substances in the toxin such as (1) Toxone, directly secreted by the bacillus and whose affinity for antitoxin is relatively low, (2) the toxin proper which is also a primary product of the bacillus, but which in contrast to "Toxone" is very strongly toxic and has a very strong affinity for antitoxin. Under the influence

of age the "Toxones" are transformed into "Toxonoides" and the toxins into "Toxoides." These two substances although in themselves non-toxic are capable of binding antitoxin. A toxic filtrate therefore on Ehrlich's hypothesis is a very complex mixture and, as further data accumulated, still more components had to be introduced in order to make his conception of toxin agree with the observed facts. The toxin fraction of the filtrate was, for example, assumed to be divisible into three components - protoxin, deuterotoxin and tritoxin according to its affinity for antitoxin.

Antitoxin on the other hand is a much more constant and less complex substance than the toxin and always presents the same specific properties towards the latter, irrespective of the serum in which it is contained.

It will be seen then, that in his theory of the manner in which toxin and antitoxin combine, Ehrlich assumed toxin to be a substance with a highly complex constitution and postulated the presence of substances the existence of which one is unable to prove.

The second theory on the neutralisation of toxin by antitoxin was propounded by Arrhenius and Madeson and avoids many of the difficulties of Ehrlich's hypothesis. They assume that the combination between toxin and antitoxin is a reversible reaction and is governed by the law of mass action, so that some free antitoxin will always be present in toxin-antitoxin mixtures together with the toxin-antitoxin compound. The formula giving the concentration of free toxin, free antitoxin and toxin-antitoxin compound in any particular mixture would be:-

(toxin) x (antitoxin) = K. (toxin-antitoxin)

where brackets denote concentration and K is the equilibrian constant. These authors found that many of the observed results agreed closely with the formula on the assumption that

one molecule of toxin combined with one molecule of antitoxin to form two molecules of toxin-antitoxin.

Although the figures recorded by Arrhenius and Madeson gave close approximations to the theoretical results over a considerable part of the total range of observations, definite discrepancies occurred, particularly where the ratio of toxin to antitoxin became high. The Danysz phenomenon is not readily explicable on the hypothesis of the reversible action and although the authors have offered an explanation based on assumed analogies between chemical compounds of known composition such as an acid and a weak base, this argument has not been generally accepted.

The decisive test of this theory is the actual degree to which the toxin-antitoxin reaction is reversible. In the period immediately following the admixture of the reagents there is a definite degree of reversibility but it is not of the kind demanded by the theory, for it requies special procedures to bring it about. The degree of dissociation of the toxin-antitoxin following simple dilution, which should occur if the reaction were of the ordinary reversible type, has not so far been demonstrated.

The third theory is that of Bordet, who regards the combination of toxin and antitoxin as the adsorption of one colloid by another. According to this view the reagent that is adsorbed will not form a compound with merely a portion of the adsorbing substance, but will be distributed evenly among its particles. If the adsorbing substance is present in sufficient excess the amount of the other reagent left unadsorbed will be minutely small. As the ratio of the two reagents varies adsorption compounds of varying composition will be formed. Bordet considers that it is very likely that the various compounds will differ in their biological activity according to the ratio of toxin to antitoxin.

Von Krogh in 1911 carried out experiments on the adsorption of toxin by colloidal iron and found that such adsorption does actually take place; the adsorption compound, however, is only slightly less toxic than the toxin itself. Thus the physical process of adsorption would appear to be common to the action exerted upon toxin by the non-specific colloidal iron and by the specific antitoxin.

In this theory of Bordet's one would appear to be on much safer ground in as much as one does not have to regard the toxin-antitoxin compound as a chemical composite with a definite formula and a formula moreover which is a highly complicated one, for after all no one up to the present has been in the position of being able to analyse either toxin or antitoxin, and the enumeration of the various constituents of toxin as made by Ehrlich rest merely on supposition. Bordet's theory certainly explains much better the Danysz phenomenon, for by means of the adsorption theory one can compare the happenings which occur in the above phenomenon with what occurs in the process of dyeing and staining. The only point that does not accord with the idea of adsorption is, that if one reverses the process, that is, instead of adding toxin in fractional doses to antitoxin one adds the latter to the former, the phenomenon does not occur.

The theory of adsorption is also supported by the fact that time and temperature play a large part in the combination of toxin and antitoxin. According to Ehrlich the union of toxin and antitoxin is instantaneous, but it has been shown that in a toxin-antitoxin mixture which has stood for some hours at room temperature it is much more difficult to bring about dissociation than it is in a mixture which has stood for twenty minutes at the same temperature. In other words, the longer a toxin-antitoxin mixture stands the firmer becomes the union between the components.

Temperature also plays a very important part in the combination of toxin-antitoxin mixtures. For instance a mixture which is placed in the ice chest for some considerable time is much more easily dissociated than one which has stood at room temperature or at 37°C., and a neutralised one shows a much stronger combination after one hour at 40°C. than does a neutralised one which has stood for twenty two hours at room temperature.

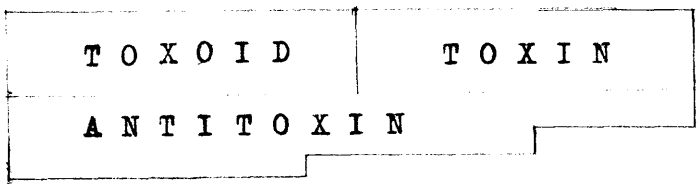
The internal construction of a toxin-antitoxin mixture in its three phases - under-neutralised, neutralised and over-neutralised - presents a very difficult problem. The question to be considered is, what is the relation of each component to the other? Is there ever either free toxin or free antitoxin present? In the older conception of a toxin-antitoxin complex it was believed that in an under-neutralised mixture there was not enough antitoxin present to combine with the toxin so that there was a definite proportion of the latter free in the solution. In a neutralised mixture the constituents were considered to be so nicely balanced that they fitted exactly, like two pieces of wood dovetailed to each other. Where the mixture was over-neutralised it was thought that there was a surplus of antitoxin, and having no toxin with which to combine it remained free in the mixture.

It is now generally held that this conception is an erroneous one. There is probably at no time either absolutely free toxin or absolutely free antitoxin present. The state of affairs obtaining in a toxin-antitoxin mixture is probably to be explained by Bordet's conception of the union of toxin and antitoxin as being a process of adsorption. In this conception, however, the existence of a modified form of toxin such as "toxoid" must be assumed in order to explain the decrease of toxicity in a toxin without an accompanying decrease in its combining power with antitoxin. Following the behaviour of

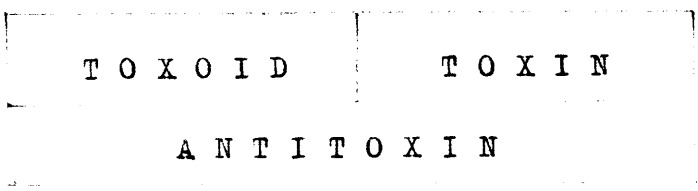
colloids in general we can anticipate that toxin and antitoxin will unite in variable proportions according to their concentration and will form different adsorption compounds; the construction of these compounds will depend directly on whether the mixture is under-neutralised, neutralised, or over-neutralised.

The possible construction of a toxin-antitoxin mixture in its three phases might perhaps be described diagrammatically thus.

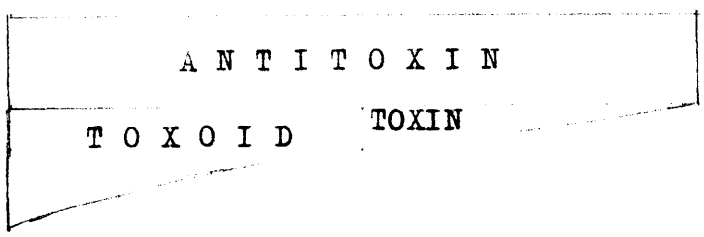
I. Under-neutralised.



II. Neutralised.



III. Over-neutralised.



In diagram I the toxin is in excess and although bound to the antitoxin is in part only lightly bound and is capable of quite rapid dissociation. In diagram II the toxin and antitoxin are present in equal amounts and the combination is a much more stable one, so that dissociation does not take place with such ease or rapidity. In diagram III the antitoxin is loosely bound to the toxin and can be easily split off. These figures are of course purely diagrammatic and merely serve the purpose of indicating the possible relationship of toxin to antitoxin.

Toxin and antitoxin would not appear to "combine" in a chemical sense such as one sees in the case of a chemical salt, for the toxin-antitoxin compound is, under certain circumstances, e.g., by the addition of toxoid, capable of dissociation.

The question of the dissociability of a toxin-antitoxin compound is a very important one. In 1904 Morgenrot found that a toxin-antitoxin mixture, which when injected subcutaneously into a guinea pig caused no death, would cause death when injected directly into the cavity of the heart. In this experiment insufficient time had been allowed for the combination between the toxin and the antitoxin to become firm and the mixture thus contained imperfectly neutralised toxin. In the subcutaneous tissue where absorption was slow time was given for the toxin and antitoxin to become more strongly bound, but when the mixture was injected into the blood stream no time was given and dissociation of toxin and antitoxin took place at once. The result of this experiment, it is true, can be explained in another way. It is possible that in both cases an equal amount of toxin became free, but in the case of the intracardial injection the toxin reached those cells (the intoxication of which was the cause of death) sooner than did the toxin from the subcutaneous injection; therefore less toxin was required by the former method of injection, for in the latter method, i.e., the intra-muscular, the action of the leucocytes lessens the amount of toxin which can act on the vital cells.

It is this factor of dissociation which plays the important rôle in Ehrlich's measurement of toxin and antitoxin, for here a toxin-antitoxin mixture undergoes dissociation in vivo and a mixture that is slightly under-neutralised will in the process of dissociation become sufficiently toxic to cause death. The possibility therefore of dissociation taking place

in vivo is very important. In the early stages of the reaction between toxin and antitoxin a firm irreversible action does not take place but in the later stages the combination becomes very firm and can only be dissociated by chemical and physical agencies such as are not known to exist in the animal body.

It will therefore be seen that time plays a very important part in dissociation. This influence of time is most marked when a fresh serum is used; the fresher the serum the stronger is its binding power. In discussing the time factor the question of avidity must also be taken into consideration. One serum may have a greater avidity for a given toxin than another serum, so that in experiments with different sera and the same toxin carried out under similar conditions one may have quite different results. The longer a toxin-antitoxin mixture stands the firmer becomes the combination, so that a mixture which originally caused death in a guinea pig in, say, four days, after it has stood for some time might not cause death at all.

Temperature also has a definite influence on dissociation. A mixture, for instance, which has stood in the ice chest for some time will undergo dissociation much more quickly than one which has stood for the same period of time at room temperature or at 37°C.

The degree of neutralisation of a mixture will also have an influence on dissociation, for according to the modern conception of the internal construction of a toxin-antitoxin mixture there is probably never at any time absolutely free toxin or free antitoxin but the two ingredients are bound to each other more or less firmly according to the degree of neutralisation. For example in an under-neutralised mixture dissociation can take place much more quickly for, in such a mixture, the toxin is at some point less firmly bound to the

antitoxin and can be liberated much more quickly than would be the case in a mixture where it was more firmly bound as, for example, in a neutralised mixture.

The study of toxin-antitoxin mixtures as a whole and of their power of dissociation has been much facilitated since the introduction of Anatoxin or Toxoid, that is a toxin whose toxicity has been destroyed by the combined action of formalin and heat, but whose binding power (i.e., whose antigenic power) remains unaltered. This modification of toxin was first made by Löwenstein with tetanus toxin and later by Ramon with diphtheria toxin and called by him Anatoxin. In England and America anatoxin is known as toxoid but it must be understood that in using this word toxoid that this substance is quite different from the toxoid of Ehrlich which is formed in old broth cultures of toxin. The latter has a reversible action as shown by Walburn as well as by Schmidt and Scholz, whereas Anatoxin or Toxoid has up to the present been found to have no reversible action; it exhibits strong antigenic powers and when added to toxin or antitoxin flocculation takes place; the degree of flocculation is a criterion of the antigenic power possessed by the anatoxin or toxoid.

In order to prevent constant repetition, this substance anatoxin or toxoid will throughout this work be referred to as toxoid in accordance with English nomenclature.

Toxoid shows almost the same affinity for antitoxin as toxin does and gives a flocculation similar to that given by toxin. On account of its affinity for antitoxin, toxoid can bring about dissociation of a toxin-antitoxin compound so that by the addition of toxoid a neutralised toxin-antitoxin mixture can be rendered toxic. This fact has been demonstrated independently by H. Schmidt and Sholz and by S. Schmidt and later by Ramon. On this property of causing dissociation in a toxin-antitoxin mixture is based the Kraus experiment which is used

for the measurement of the strength of toxoid. This measurement of toxoid will be discussed later on in the work.

The relationship of the constituents of a toxin-antitoxin-toxoid mixture is a very complicated one. A study of this relationship is made in a later part of this work where it is hoped this matter may be made somewhat clearer as a result of experimental work.

Much work has recently been devoted to the phenomenon of flocculation which takes place both in toxin-antitoxin and toxoid-antitoxin mixtures and which is constantly being utilised for the estimation of the strength of antitoxin serum and the antigenic action of toxin and toxoid. The floccules themselves have been recommended for use in prophylactic immunisation by H. Schmidt, Hartley, Aldershoff and others.

The phenomenon of flocculation underlies the observation made by Kraus that a bacterial immune serum forms a precipitate with a homologous bacterial bouillon culture. Flocculation with snake venom and its antitoxin was first observed by Calmette and Massol, later Nicolle and his co-workers, also Georgi working independently found a method of causing flocculation between diphtheria toxin and antitoxin, but the present technique of flocculation for the carrying out of various estimations is based mainly on the work of Ramon and W. Sholz. The technique is as follows. Varying amounts of either serum, toxin or toxoid are brought together with a constant amount of toxin, toxoid or serum depending on which constituent is known and which constituent is to be measured. After some time, the period of which depends on affinity, as is claimed by S. Schmidt and Ramon (but the correctness of which claim is still under discussion) and on temperature which up to 55°C. accelerates the flocculating action, flocculation takes place. It first appears in one tube - the so-called indicating tube - and in this tube the mixture is said to be completely neutralised and

on the basis of this assumption all calculations of antigenic units of toxin or toxoid or of antitoxin units of serum are made, and the result obtained is known as the Lf value of the toxin, toxoid or serum as the case may be. H. Schmidt and W. Sholz recommend as the Lf that amount of toxin which gives with one antitoxin unit the optimal flocculation whereas Ramon takes the reciprocal value, that is, the number of antitoxin units which are bound by 1 cc. of toxin at the optimal flocculation point.

Following flocculation in the indicating tube, a zone of flocculation is formed and spreads through the tubes until a point is reached where the mixtures are either too over- or too under-neutralised for flocculation to take place, so it will be seen that even in very toxic, that is, in under-neutralised mixtures, flocculation can occur. That the floccules contain toxin has been proved by their power of immunisation: it has also been proved by the observations of H. Schmidt and W. Sholz who showed that washed floccules which were non-toxic could be made toxic by the addition of toxoid. That the floccules also contain antitoxin has been demonstrated by Ramon who succeeded in producing a pure preparation of antitoxin after he had destroyed the toxin by means of careful heating and the addition of acid.

This phenomenon of flocculation is exceedingly complicated and its real significance is still to some extent unknown. With some toxins, toxoids and sera a double flocculation is found; the nature of one of these flocculations has been revealed as a precipitation reaction by H. Schmidt and W. Sholz, who succeeded in experimentally producing the double flocculation in vitro as well as in vivo by immunising horses with toxin containing specially prepared diphtheria protein. The nature of the other flocculation on which the calculation of the Lf value is based still remains obscure; this much is

certain that sera which possess no globulin do not give flocculation although still rich in antitoxin. The purer a toxin and antitoxin are, the weaker is their power of flocculation. Therefore it is not yet certain whether a pure toxin-antitoxin flocculation exists or whether an albumen-anti-albumen reaction is involved in which the toxin and antitoxin participate only secondarily.

This question of flocculation can now be more easily studied since we have in toxoid a means of analysing a toxin-antitoxin flocculation. By the addition of toxoid we can study the whole process of flocculation - the pre-flocculation period, the point of flocculation, and the post-flocculation period.

The reports of the experiments and their results are found in the following parts.

Part I.

General Considerations with regard to the Reactions of Toxin-Antitoxin Mixtures to which Toxoid has been added.

The use of toxoid in the analysis of toxin-antitoxin mixtures is based on the fact that toxoid possesses a definite affinity to antitoxin and the addition of the former to a toxin-antitoxin mixture causes a cleavage between the toxin and the antitoxin; a portion of the latter combines with the toxoid and in consequence a certain portion of the toxin is rendered free.

The interaction of toxoid with toxin and antitoxin as far as the liberation of toxin is concerned depends on several factors:-

(1) The general relation of the amount of a toxin-antitoxin mixture and the degree of its neutrality to the amount of toxoid which is necessary to render the mixture toxic.

(2) The influence which time exerts on the binding of toxin and antitoxin and its relation to the amount of toxoid which it is necessary to add in order to render the mixture toxic.

(3) The influence of temperature on a toxin-antitoxin mixture with regard to the amount of toxoid which is necessary to render the mixture toxic.

1. The general relation of the amount of a toxin-antitoxin mixture and the degree of its neutrality to the amount of toxoid which is necessary to render the mixture toxic.

A series of toxin-antitoxin mixtures was made, beginning with a strongly under-neutralised one; in each succeeding mixture the antitoxin constituent was increased until the point of neutrality was reached, then the amount of antitoxin was

further increased so that over-neutralised mixtures were produced. All the experiments were carried out on guinea pigs and as far as possible the weight of the animals was kept constant. Except where otherwise mentioned, the experiments were carried out at room temperature, the antitoxin was added to the toxin, the mixture was allowed to stand for half an hour, the toxoid was then added. The mixture stood for a period of twenty minutes and was then injected subcutaneously in a volume of 4 cc.

In the following first experiment two mixtures were used, Mixture I consisting of 50 cc. of toxin 506 (Lf = 0.15) and 6 cc. of 1/10 dilution of serum 5830 (strength 450);

Mixture III consisting of 50 cc. of the same toxin and 10 cc. of 1/10 dilution of the same serum.

Mixture I was strongly under-neutralised and Mixture III was just neutralised.

Each mixture was first injected alone in order to test its toxicity. It was again injected after the addition of toxoid. The following tables show the result.

Experiment 1.

Mixture I.	Toxoid.	Result.
0.1 cc.	-	Death on 2nd day
0.1 cc.	0.1 cc.	Death on 2nd day
Mixture III		
2 cc.	-	-
0.25 cc.	0.25 cc.	Death on 3rd day

Experiment 2.

Mixture I.	Toxoid.	Result.
0.05 cc.	0.05 cc.	Death on 2nd day
0.1 cc.	0.1 cc.	Death on 2nd day
0.25 cc.	0.25 cc.	Death on 2nd day
Mixture III		
0.05 cc.	0.05 cc.	-
0.125 cc.	0.125 cc.	Death on 5th day
0.25 cc.	0.25 cc.	Death on 3rd day

It will be seen from the above tables that the effect produced by adding toxoid to a toxin-antitoxin mixture is to render the mixture definitely toxic. The effect of the toxoid is more clearly demonstrated the nearer the mixture is to the neutral point. For instance in table 1 in the case of mixture I which is strongly under-neutralised the same dose, namely 0.1 cc. causes death in two days whether injected alone or with an equal quantity of toxoid, whereas in mixture III, which is just neutralised, 2 cc. of the mixture alone does not cause death, while one eighth of the same amount, i.e., 0.25 cc., causes death in three days when toxoid is added.

In table 2 the results with mixture I (that is with the under-neutralised mixture) are all the same even in the first dose where the amount of toxin is only one fifth as large as in the third dose and the toxoid correspondingly reduced; whereas in mixture III, which is neutralised, there is an entirely different result. The first dose does not cause death, but the third dose, which is (as was the case in mixture I) five times as strong, causes death in three days when toxoid is added. It would therefore appear that the action of toxoid on a toxin-antitoxin mixture is directly dependent on the degree of neutralisation of the mixture.

A further experiment was made with another mixture - Mixture II - which was also underneutralised but not to such a degree as mixture I. Mixture II consisted of 50 cc. of toxin 506 and 8 cc. of a 1/10 dilution of serum 5830, that is, the same toxin and serum as in the two previous mixtures. The following table gives the result.

Experiment 3.

Mixture II	Toxoid	Result
2 cc.	-	Death on 4th day.
0.2 cc.	0.2 cc.	Death on 4th day.

In an under-neutralised mixture, as seen in table 3, death will take place without the addition of toxoid, but by adding toxoid one can cause death with a much smaller dose of the mixture. As is seen in the above table (experiment 3) 2 cc. of the mixture alone caused death in four days; on the other hand, when toxoid was added it only needed one tenth of the original dose to bring about the same result. In the case of a strongly under-neutralised mixture such as that of mixture I (see experiment 1) it does not matter whether toxoid is added or not, death takes place in the same time.

Within certain limits the dose of toxoid plays a very definite part in the result obtained. The time of death stands in direct relation to the dose of toxoid; a progressive increase in the dose produces a progressive decrease in the time of death until a point is reached where even with an increase of toxoid the time of death remains the same.

A definite period of time must elapse before a toxin can produce its lethal effect: once this period is reached it will make no difference if still more toxoid be added - the time of death will remain the same, so that the relation-

ship of the dose A. of toxoid to the time of death T. may be expressed by the following equation:-

$$T \times A = \text{a constant.}$$

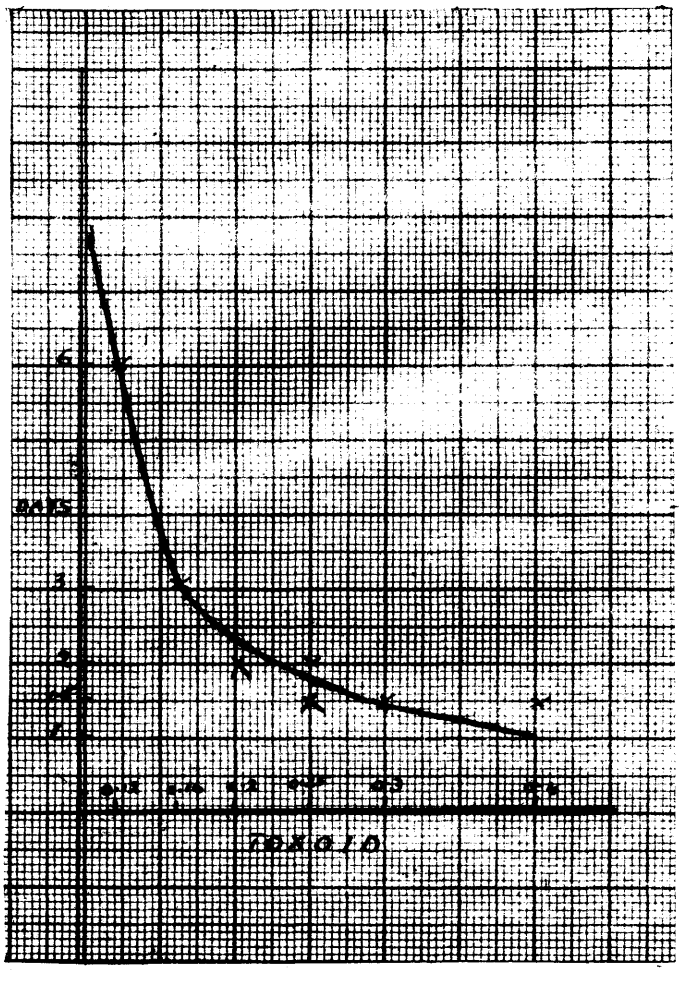
This equation can only be applied to a given toxin-antitoxin mixture and only within certain limits. For instance in a mixture which is very under-neutralised death would take place without the addition of toxoid, and in one which was too over-neutralised, as will be shown in the fourth part of this work, death would not take place even with large doses of toxoid.

The following experiment was carried out. Two antitoxin units were taken and toxoid in increasing amounts was added. These mixtures stood at room temperature for one hour, then one Lf dose of toxin was added to each; after standing half an hour at the same temperature as before the mixtures were injected and the following results were obtained.

Experiment 4.

Toxoid.	Result.
0.1 cc.	-
0.12 cc.	Death on 6th day
0.16 cc.	Death on 3rd day
0.2 cc.	Death on 2nd day
0.25 cc.	Death in 1.5 days
0.3 cc.	Death in 1.5 days
0.4 cc.	Death in 1.5 days

The relation between the amount of toxoid and the toxid effect produced in this experiment may be graphically expressed thus:-



Here the curve descends somewhat steeply, then gradually flattens out until the point is reached where an increase in the amount of toxoid does not appear to alter the direction of the curve which continues in the form of a straight line. In this particular experiment the limit of the toxic action of the toxin appears to have been reached with the dose of 0.25 cc. of toxoid: more toxin is undoubtedly liberated with the doses of 0.3 cc. and 0.4 cc. of toxoid but the amount thus liberated does not seem to be sufficient to cause an appreciable acceleration in the time of death. Probably if the experiment were carried out in great detail, and hourly instead of daily observations were made, a difference in the time of death with the higher doses of toxoid would be observed until the point was finally reached where the increase of toxoid would make no difference in the result obtained. In this experiment the measurement of time was in days not hours, so that for all intents and purposes with the dose of 0.25 cc. of toxoid the limit of the action of the toxoid is reached and the curve continues in the form of a straight line.

In this experiment (carried out after the manner

adopted by Kraus) it is not a case strictly speaking of the dissociation of a toxin-antitoxin combination by means of toxoid but rather of the arrest of the affinity of toxin to antitoxin. On the other hand the first mentioned experiments are concerned with the dissociation of toxin and antitoxin by means of toxoid. The third part of this work will go more deeply into the principal differences shown in these experiments.

By means of an experimental arrangement with the following formula

$$\left(\left[\text{Antitoxin} + L_{+}\ \text{Toxin}\right]^{\frac{1}{2}\ \text{hr.}} + x\ \text{Toxoid}\right)^{1\ \text{hr.}} \longrightarrow \text{Death } y$$

one might perhaps attempt to demonstrate what the relationship would be between the amount of toxoid and the time of death when one took a toxin-antitoxin mixture in which the amount of toxin remained constant but in which in each succeeding experiment more antitoxin was added. Even if such an experiment is not carried out in a consecutive series, nevertheless the results of single experiments, and especially the results of those which will be mentioned in the fourth part of this work, would allow one to sketch the result to some extent as follows.

Starting with a completely under-neutralised toxin-antitoxin mixture such as L_{+} toxin and one antitoxin unit, we find that the injected animal dies in four days even without the addition of toxoid to the mixture, but the addition of increasing doses of toxoid can so accelerate the time of death that the animal finally dies within the shortest possible time, that is, in one to one and a half days. Between a fully under-neutralised and a neutralised toxin-antitoxin mixture other mixtures are found which cause death in six, eight, nine, etc. days and which finally only cause illness but no death. Such a mixture which caused death in nine days may be taken as an example. In this mixture the addition

of small doses of toxoid caused a decrease in the time of death until a point was reached where an increase in the dose of toxoid was without further effect.

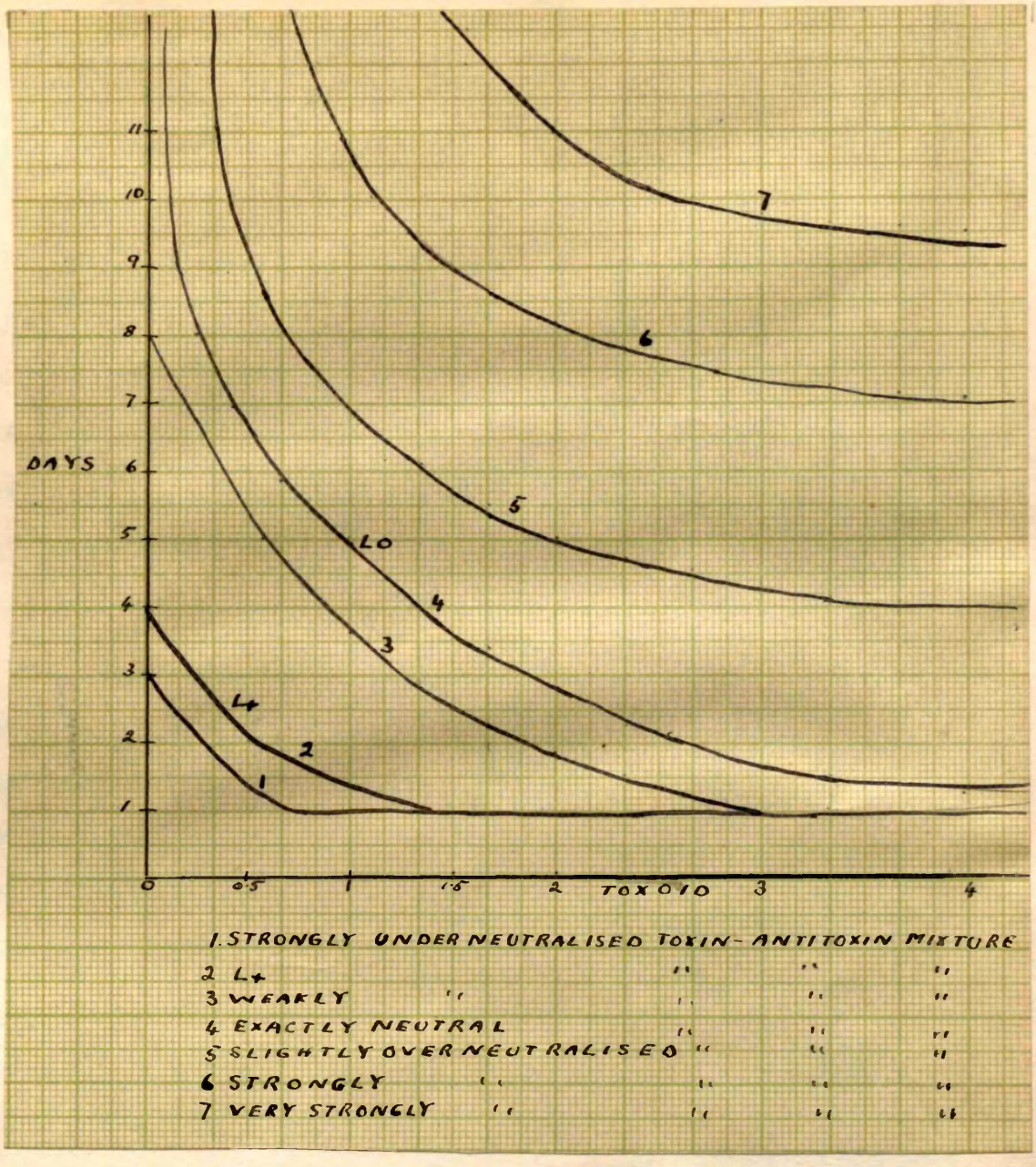
As the next example a fully neutralised mixture may be taken. By the addition of toxoid this mixture could also be rendered toxic and by increasing the dose of toxoid the mixture could be made quite as toxic as a lethal dose of pure toxin.

In a mixture in which the toxin is over-neutralised the amount of toxoid necessary to split up the toxin-antitoxin combination depends on the degree of over-neutralisation; it also depends on the temperature at which, and the time during which, the toxin-antitoxin mixture stands. It is not possible to render very strongly over-neutralised mixtures toxic by the addition of toxoid for in the case of a guinea pig the amount of fluid that one can conveniently inject is restricted to not more than 5 cc.

Between this extreme degree of over-neutralisation and the actual point of neutralisation are to be found over-neutralised toxin-antitoxin mixtures which may be made toxic by the addition of toxoid, but with each increase in over-neutralisation a point is soon reached where, owing to the restricted volume of fluid that it is possible to inject into a guinea pig, the amount of toxoid is insufficient to render the mixture sufficiently toxic to cause death.

The following diagram may serve to show, in a purely diagrammatical fashion, what has just been discussed and the limitations of the previously mentioned relationship -

The time of death x amount of toxoid = constant.



In an over-neutralised mixture the amount of toxoid which can be conveniently injected is not sufficient to cause death, it causes nevertheless a definite reaction in the animals as the following experiment shows.

A toxin-antitoxin mixture was taken consisting of 10 cc of toxin ($L_+ = 0.15$) and 1 cc. of serum (strength 450) i.e. a strongly over-neutralised mixture. The mixture was diluted 1 in 50 with saline solution, a constant amount of the mixture was taken and increasing doses of toxoid were added, then the resulting mixture was injected in a volume of 4 cc. The following table shows the result, the + sign indicates the reaction.

Experiment 5.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	2 cc.	+
"	3 cc.	+
"	4 cc.	+ +

It is seen that with the two lowest doses of toxoid there is a slight reaction; in the highest dose the reaction is more pronounced but the animal survives. Possibly if a concentrated toxoid were used, even in a strongly over-neutralised mixture it might be possible to cause death, but even here the amount would eventually be restricted.

In discussing the question of the influence of the degree of neutrality of a mixture on the result obtained when one adds toxoid to it, the question of the avidity of the antitoxin in question must be taken into consideration, at any rate in the case of neutralised or over-neutralised mixtures. When one is working with uniform amounts of antitoxin and with a serum which shows a low avidity, one would tend to get a stronger reaction from the addition of toxoid for the union between the toxin and antitoxin would not be such a strong one. But on the other hand if the serum showed a weak affinity to the toxin it might also show a weak affinity to the toxoid so that the state of affairs in the toxin-antitoxin-toxoid compound in spite of the weak affinity of the serum might remain unaltered.

Summary.

The possibility of making a non-toxic mixture of diphtheria toxin and antitoxin toxic by means of the addition of toxoid has been demonstrated.

The greater the degree of over-neutralisation of the toxin-antitoxin mixture, that is, the greater the amount of antitoxin it contains, the greater is the amount of toxoid which must be added to render the mixture sufficiently toxic to cause death. In more strongly over-neutralised mixtures the amount of toxoid injected does not actually cause death.

In very strongly over-neutralised mixtures it is not possible to bring about toxic effects owing to the restricted volume of fluid which can be conveniently injected into a guinea pig.

2. The influence which time exerts on the binding of toxin and antitoxin and its relation to the amount of toxoid which it is necessary to add in order to render the mixture toxic.

Time plays a very important part in the combining of toxin and antitoxin. After the mixture has stood for some time it is found that the same amount of toxoid renders the mixture less toxic than it did when the mixture was fresh. This is illustrated by the following experiment.

A toxin-antitoxin mixture (mixture 4) was taken, consisting of 50 cc. of toxin 503 and 11 cc. of 1/10 dilution of serum 5830, that is, it was a neutralised mixture. After being freshly made up it was injected, then the mixture was kept for a month at room temperature and was then tested by the same method as before, i.e., by the addition of the same amounts of toxoid. The following table shows the result.

Experiment 6.

Mixture 4.		Toxoid.	Result.
Fresh	0.2 cc.	0.2 cc.	Ill but survived.
	0.4 cc.	0.4 cc.	Death on 3rd day.
1 month old	0.2 cc.	0.2 cc.	No reaction.
"	0.4 cc.	0.4 cc.	Ill but survived.

Another experiment was carried out with mixture 3, consisting of 50 cc. of the same toxin and 10 cc. of 1/10 dilution of the same serum, i.e., less strongly neutralised. Here the mixture was tested as follows.

(a) Freshly made up.

(b) Ten days old.

(c) One month old.

The following table gives the result.

Experiment 7.

Mixture 3.		Toxoid.	Result.
Fresh	0.25 cc.	0.25 cc.	Death on 3rd day
"	0.375 cc.	0.375 cc.	Death in 1.5 days
10 days old	0.2 cc.	0.2 cc.	Death on 7th day
1 month old	0.2 cc.	0.2 cc.	No reaction.
"	0.4 cc.	0.4 cc.	Death on 3rd day

It has long been known that in a toxin-antitoxin mixture the length of time of the interaction of the two constituents with each other must be taken into account when using such mixtures for the titration of toxin or serum. This is specially true when fresh serum is used instead of an old standard serum. For instance if with a freshly prepared serum of 400 units per cc. a test is made regarding its strength with a standard toxin and 4 cc. of the mixture are taken containing the test dose in 2 cc. and one antitoxin unit, i.e., 2 cc. of 1/800 dilution of serum, this mixture after standing for twenty minutes will, when injected into a guinea pig, kill the animal in four days; if, however, the mixture has stood for twenty four hours, it will no longer do so.

With an old test serum the affinity to toxin is not so marked as with a fresh one and the difference seen on standing, i.e., the part played by time, is not so marked but is still observable.

It is evident that a toxin-antitoxin compound becomes much firmer with the increase of the time of interaction between the two constituents and it is clear that this will make itself observed when such over-neutralised mixtures are exposed to the splitting up action of toxoid as will be seen in the following experiment.

A constant amount of toxin and varying amounts of test serum were brought together. The first mixture, containing 0.155 cc. of toxin and 0.125 cc. of serum, was an exactly neutral one. In the first experiment the mixture of toxin and antitoxin stood for twenty minutes at room temperature before the toxoid was added and in the second experiment the mixture stood for twenty two hours at the same temperature. The following table gives the result of the experiment.

Experiment 8.

After twenty minutes:

Toxin.	Test Serum.	Toxoid.	Result.
0.155 cc.	0.125 cc.	0.05 cc.	Death on 3rd day
0.155 cc.	0.3 cc.	0.9 cc.	Death on 5th day
0.155 cc.	0.4 cc.	1.8 cc.	Death on 5th day
After twenty two hours:			
0.155 cc.	0.125 cc.	0.05 cc.	-
0.155 cc.	0.3 cc.	0.9 cc.	-
0.155 cc.	0.4 cc.	1.8 cc.	-

More exact qualitative experiments which were carried out with regard to the time factor in the combining powers of toxin and antitoxin will be found in the fourth part of the work.

When this time factor is discussed the construction of a toxin-antitoxin mixture must be taken into consideration. Even when under-neutralised or when over-neutralised there is probably never at any time either free toxin or free antitoxin present. The toxin and antitoxin, although in excess, are probably very lightly bound either to the antitoxin or the toxin, depending on whether the mixture is under- or over-neutralised, and the toxin or antitoxin is therefore capable of quick dissociation, but with the passage of time the

the union between toxin and antitoxin becomes firmer.

On the other hand, however, it has also been established that, with the passage of time, a toxin-antitoxin mixture can show an increase in toxicity. This phenomenon of increased toxicity is explained by Schmidt and Sholz on the assumption that the antitoxin content forms a gradually closer union with the toxoid (of Ehrlich) constituent of the toxin, so that the toxin itself is less strongly bound to the antitoxin. The phenomenon, however, is not always demonstrable and depends very much on the affinity of the serum to the toxin; moreover it can only be shown in mixtures which are slightly under-neutralised. With over-neutralised mixtures the difference in the relation toxoid-serum and toxin-serum does not make itself observed, for a lesser degree of over-neutralisation in a mixture is not to be distinguished from a greater degree of over-neutralisation. Nevertheless a mixture of toxin with a serum of weak affinity (such as a standard serum) which is just at the limit of neutralisation may become toxic on standing but as a rule the time of standing must be longer than the time used in this experiment.

Another factor which must be considered when studying the influence of time on toxin-antitoxin mixtures is that of avidity.

It has for long been a debated point whether along with its antitoxic property the avidity which antitoxin exhibits towards toxin is a decisive factor from the therapeutic point of view. Madsen and S. Schmidt believe that there are sera which vary in their avidity and that the greater the avidity the greater is the therapeutic value of these sera and that freshly prepared sera with the same antitoxic content can in respect to avidity show very different reactions. Ramon has also associated himself with this view and bases it principally on the rapidity with which flocculation takes

place, and since the whole phenomenon of flocculation is considered by many to be based entirely on physico-chemical factors this view has not received universal recognition.

In Germany the possibility of estimating the strength of toxin and sera by means of flocculation alone is regarded with scepticism and a year or two ago Weichsel was able to show that, clinically, the "avide" serum of Madsen did not give any better results than did other serum with the same antitoxin content. Without doubt, however, there are differences between old and fresh sera and these differences may be observed not only in the rapidity with which flocculation takes place (the older the serum the slower the flocculation), but also in the closeness of the union between the toxin and antitoxin.

It is possible, then, that this question of variation in avidity must be taken into consideration if one wishes to avoid errors when experimenting with various toxin-antitoxin mixtures.

Summary.

Under otherwise similar conditions the difficulty of rendering toxic a non-toxic toxin-antitoxin mixture by means of the addition of toxoid becomes greater the longer the toxin-antitoxin mixture has stood. This influence exerted by time is particularly noticeable when the serum is fresh. It may be assumed that fresh sera show a greater avidity towards toxin than do old sera which have been in stock for some time.

3. The influence of temperature on toxin-antitoxin mixtures with reference to the amount of toxoid which must be added in order to render the mixture toxic.

Temperature appears to have a definite effect on toxin-antitoxin mixtures inasmuch as cold prevents a firm union between toxin and antitoxin from taking place, so that when toxoid is added dissociation occurs much more quickly than it would in a mixture which had stood at room or higher temperature.

Conversely heat appears to increase the degree of association between toxin and antitoxin. For instance in a series of experiments on a flocculating toxin-antitoxin mixture it was found that after standing one hour at 40°C. double the amount of toxoid was needed to bring about the same result as was obtained when the mixture was only warmed to 40°C. and then allowed to stand for the remainder of the time at room temperature. It would appear, therefore, that the association between toxin and antitoxin increases with the rise in temperature; this refers of course only to that range of temperature within which no destruction of toxin and antitoxin takes place.

The action of temperature on a toxin-antitoxin mixture is illustrated by the following more detailed experiment.

Fresh diphtheria toxin No. 541 and fresh serum No. 2223 were titrated against each other so as to obtain the optimum proportion for flocculation; this proportion was found to be 20 cc. of toxin and 0.3 cc. of serum. Then 80 cc. of a mixture of toxin and serum in accordance with the necessary proportions were prepared and divided into equal parts; each part was allowed to stand until flocculation took place, but the mixtures were placed at different temperatures as follows:-

The first portion was placed in the ice chest, the second at room temperature, the third in an incubator at 37°C. and the fourth in a water-bath at 45°C.

It was found that the lower the temperature at which the mixture stood the longer was the time during which it was necessary for the mixture to stand before flocculation took place.

After flocculation each mixture was kept at room temperature until the mixture in the ice chest had given a distinct flocculation, then all the mixtures were centrifuged, the floccules were washed in saline solution and those of each mixture were re-suspended in 5 cc. of saline solution. After that each floccule suspension and supernatant were examined with regard to the amount of toxoid it was necessary to add in order to cause death in guinea pigs by splitting up the toxin-antitoxin compound. On the fifth day after preparation of the toxin-antitoxin mixtures the experiments were carried out under exactly uniform conditions and the result is given in the following table.

Experiment 9.

Floccule Suspension.	Toxoid.	Result.
2 cc. cold.	2 cc.	Death on 2nd day
2 cc. room temp.	2 cc.	Death in 3.5 days
2 cc. 37°C.	2 cc.	Death on 4th day
2 cc. 45°C.	2 cc.	Death on 4th day
Supernatant:		
2 cc. cold.	2 cc.	-
2 cc. room temp.	2 cc.	-
2 cc. 37°C.	2 cc.	Ill but survuved.
2 cc. 45°C.	2 cc.	Death on 4th day

From this experiment it will be seen that with a rise in temperature the union between toxin and antitoxin is less easily split up. The toxoid added to the mixture which had

stood in the ice chest was therefore able to draw more antitoxin to it so that there was more toxin available to produce a toxic reaction than there was in the mixtures which had stood at higher temperatures.

In the supernatant the result is just the reverse; here the higher the temperature the more toxin is there available after the addition of toxoid to the mixture. This may be due to two causes -- either the supernatant becomes poorer in toxin and antitoxin in proportion to the length of time required for the process of flocculation at a low temperature, and one could attribute the toxicity shown by the floccules at low temperature flocculation after the addition of toxoid to the fact that they then from the start will contain more toxin and antitoxin than those floccules which have been formed by flocculation at a high temperature; or else the union between fluid toxin and antitoxin becomes stronger at a low temperature than it does at higher temperatures, and that the converse is the case with the toxin and antitoxin contained in the floccules. The first mentioned cause would seem to be the more likely and has an analogy in the Wassermann Reaction where at a low temperature the fixation of complement is a more sensitive process; a decision however can only be reached by further specialised work on the question.

Summary.

If under similar conditions with regard to time, toxin-antitoxin mixtures are placed at varying temperatures it is found that the binding power of the toxin and antitoxin increases with the rise in temperature so that the higher the temperature at which the mixture stands the greater must be the amount of toxoid that has to be added in order to render the mixture toxic, assuming of course that the temperature is not such as would injure or destroy the toxin-antitoxin content.

Part 2.

Examination of the Phenomenon of Flocculation in Toxin-Antitoxin Mixtures by the addition of Toxoid having special reference to the Distribution of Toxin and Antitoxin in the Floccules and the Fluid.

In this series of experiments an attempt was made to analyse flocculating toxin-antitoxin mixtures by means of the addition of toxoid. In order to study a flocculating mixture in all its phases the analysis was divided into three periods, viz.:-

(1) pre-flocculation period.

(2) point of flocculation.

(3) post-flocculation period.

The first and third periods were further divided so that samples of the mixture could be examined at various intervals of time.

A mixture was first chosen in which the serum gave a very slow flocculation (experiment 12). This mixture was abandoned in favour of one with a rapidly flocculating serum (experiments 10 and 11), but the results obtained at the point of flocculation with regard to the supernatant and floccules coincide with the results obtained in the second mixture and are therefore given.

The sera and toxins were first tested by the Ramon flocculation method in order to find the Lf. value; the mixtures were then made up in large quantities and placed in the water bath at 40°C. Samples were withdrawn at varying intervals of time and analysed by means of the addition of toxoid.

A detailed account in tabular form follows.

Experiment 10.

Toxin 483 (Lf. = 0.18), Serum 2026 (strength 380)

Toxoid Pasteur (tf. = 0.10)

The mixture consisted of 100 cc. of toxin and 1.45 cc. of serum.

I.

Heated to 40°C. and then examined.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	Death 1.5 to 2 days.
"	0.8 cc.	" " "
"	1.6	" " "

II.

Mixture kept at 40°C. for one hour.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 4th day.
"	0.8 cc.	Death on 2nd day.
"	1.6 cc.	Death on 2nd day.

III.

The mixture was kept at 40°C. for five and a quarter hours. At this point flocculation took place. After flocculation 50 cc. of the suspension were carefully centrifuged; the floccules thus obtained were washed in saline solution, again centrifuged and re-suspended in 50 cc. of saline solution. Both this and the clear supernatant fluid were then examined by the addition of toxoid.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 8th day.
"	0.8 cc.	Death on 3rd day.
"	1.6 cc.	Death on 3rd day.

Supernatant Fluid.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 6th day.
"	0.8 cc.	-
"	1.6 cc.	Death on 4th day.
Suspension of Floccules.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 5th day.
"	0.8 cc.	Death on 5th day.
"	1.6 cc.	Death on 3rd day.

Experiment 11.

With the same toxin 483 Lf. = 0.18 and the same serum 2026 (strength 380) a new mixture was made consisting of 500 cc. of the toxin, 7.25 cc. of the serum.

I.

Mixture warmed to 40°C. and then examined by means of toxoid.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	0.1 cc.	-
"	0.2 cc.	Death on 7th day.
"	0.4 cc.	Death on 3rd day.

II.

Mixture kept at 40°C. for one hour.

Toxin-Antitoxin Mixture.	Toxoid.	Result.
1 cc.	0.2 cc.	Death on 8th day.
"	0.4 cc.	Death on 4th day.
"	0.8 cc.	Death on 3rd day.

III.

The mixture was kept at 40°C. for five and a quarter hours, which was the point of flocculation. The mixture was then similarly treated as in experiment 10.

The Whole Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 4th day.
"	0.8 cc.	Death on 3rd day.
"	1.6 cc.	Death 1.5 to 2 days.
Supernatant Fluid	**Toxoid.**	**Result.**
1 cc.	0.4 cc.	Death on 4th day.
"	0.8 cc.	Death on 3rd day.
"	1.6 cc.	Death 1.5 to 2 days.
Suspension of Floccules.	**Toxoid.**	**Result.**
1 cc.	0.4 cc.	-
"	0.8 cc.	-
"	1.6 cc.	Death on 8th day.

IV.

The mixture was kept at 40°C. for twenty four hours and then treated as mentioned above.

The Whole Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 4th day.
"	0.8 cc.	Death on 3rd day.
"	1.6 cc.	Death on 3rd day.
Supernatant Fluid.	**Toxoid.**	**Result.**
1 cc.	0.4 cc.	-
"	0.8 cc.	-
"	1.6 cc.	Death on 4th day.

Suspension of Floccules.	Toxoid.	Result.
1 cc.	0.4 cc.	Death on 5th day.
"	0.8 cc.	Death on 4th day.
"	1.6 cc.	Death on 3rd day.

V.

The mixture was kept at 40°C. for forty two hours and then treated as mentioned above.

The Whole Mixture.	Toxoid.	Result.
1 cc.	0.4 cc.	-
"	0.8 cc.	Death 3 to 4 days.
"	1.6 cc.	Death 3 to 4 days.
Supernatant Fluid.	Toxoid.	Result.
1 cc.	0.4 cc.	-
"	0.8 cc.	Death 3 to 4 days.
"	1.6 cc.	Death 3 to 4 days.
Suspension of Floccules.	Toxoid.	Result.
1 cc.	0.4 cc.	-
"	0.8 cc.	"
"	1.6 cc.	Death 3 to 4 days.

Experiment 12.

A slow flocculating toxin-antitoxin mixture was prepared consisting of 100 cc. of toxin No. 506 (Lf = 0.15) and 2.2 cc. of Serum 5830, and the same toxoid "Pasteur" Lf. = 0.1 was used. After forty eight hours at 40°C. flocculation took place and the mixture was removed from the water bath and treated as in experiment 10, III, and then examined by the addition of toxoid.

The Whole Mixture	Toxoid.	Result.
1 cc.	1.1 cc.	Death 3 to 4 days.
"	3.3 cc.	Death 3 to 4 days.
Supernatant Fluid	**Toxoid.**	**Result.**
1 cc.	1.1 cc.	Death on 5th day.
"	3.3 cc.	Death on 4th day.
Suspension of Floccules.	**Toxoid.**	**Result.**
1 cc.	1.1 cc.	Death on 5th day.
"	3.3 cc.	Death 3 to 4 days.

When the results of these experiments are analysed it is seen that in experiment 10 where the mixture is only warmed to 40°C. one needs approximately 0.2 cc. of toxoid in order to produce death on the fourth day, but after the mixture has stood for one hour at the above temperature 0.4 cc. of toxoid is required to produce the same result, and after five and a half hours approximately 0.6 cc. is required. In experiment 11 where smaller doses of toxoid are used a similar result is seen although not so pronounced.

After twenty-four hours it will be seen that the action of the toxoid appears to be becoming weaker for with the highest dose, viz., 1.6 cc., the time of death is delayed. After seventy two hours the action of the toxoid is definitely diminished, 0.4 cc. no longer causes death, and with the two higher doses the time of death is delayed.

These observations would appear to indicate that the association between the toxin and antitoxin has become stronger so that the antitoxin constituent of the mixture is not so easily taken up by the toxoid and consequently more toxoid must be added.

That temperature plays an important part in the increase of association between toxin and antitoxin has already been shown in part one.

In a series of experiments which will be discussed in part four it was found that a neutralised toxin-antitoxin mixture after standing for twenty two hours at room temperature required double the amount of toxoid than that which was needed with a fresh serum in order to bring about the same result. But in this case the mixture only stood for one hour and yet double the amount of toxoid was required.

The increase in the combining power between the toxin and antitoxin appears to take place within the period of five and a quarter hours, that is, up to the point where flocculation takes place. At twenty four hours the degree of combining power appears to remain very much the same, and then later to increase somewhat.

The results of the analysis of the supernatant by means of toxoid are distinctly interesting. It was always assumed that with the occurrence of flocculation all the toxin and antitoxin was to be found in the floccules and that the fluid portion contained none; at all events from all outward appearance it seemed so, which also corresponded with the assumption that flocculation first took place in that mixture where the proportions of toxin and antitoxin produced complete and reciprocal neutrality and with the exception of immunisation experiments on animals (which took a long time to carry out) there was no possibility of demonstrating the presence of toxin in the fluid. This is now possible by means of the addition of toxoid and one sees now that by this means the supernatant can be shown to be strongly toxic. This is demonstrated in the above experiments.

At twenty four hours, that is approximately nineteen and a half hours after flocculation had taken place, the supernatant after the addition of toxoid appears to be much less

toxic, for 0.4 cc. and 0.8 cc. of toxoid caused illness but the animals survived and it required 1.6 cc. of toxoid to cause death on the fourth day. At seventy two hours, that is, approximately sixty six and a half hours after flocculation, the supernatant again appears (by means of toxoid) to be more toxic, 0.4 cc. of toxoid does not cause death, but both the higher doses 0.8 cc. and 1.6 cc. cause death on the fourth day.

From these results it would appear that the supernatant of a neutralised flocculating toxin-antitoxin mixture is at the point of flocculation by no means devoid of toxin and antitoxin and in fact when compared with the whole mixture it appears to need very little more toxoid to render it toxic. At twenty four hours the supernatant appears to need more toxoid than it did at the flocculation point, for only with the highest dose of toxoid does it cause death. At seventy two hours the supernatant after the addition of toxoid appears to be again more toxic though less toxic than at the point of flocculation. This would seem to indicate that some time after flocculation has set in, much more of the toxin and antitoxin is contained in the floccules than was the case at the point of flocculation. Then after a period of time has elapsed some of the toxin and antitoxin becomes dissociated from the floccules and again passes into the supernatant; but in order to prove this definitely further experimental work is required. Nevertheless, one may conclude from the results of these experiments that the process of flocculation does not lead to a complete precipitation of toxin and antitoxin but rather that they are found in only a relatively small amount in the floccules and that this toxin-antitoxin compound held by the floccules can in small amounts again pass into solution.

As a result of this series of experiments several points of interest arise. Does, for instance, the heating of the

mixture have any influence on the splitting up action of toxoid? The action of high temperatures on toxin and antitoxin is a very important one and it makes a very considerable difference whether one heats the toxin and antitoxin together or separately for toxin when heated to 45°C. is considerably damaged or even destroyed and consequently loses its power of flocculating. Antitoxin, on the other hand, is not so sensitive to heat; at 50°C. it loses its power of flocculating but not its binding power; the latter is destroyed only at a temperature of 65°C. But toxin and antitoxin when already bound to each other can withstand a relatively high temperature without either of them suffering apparent deterioration, for both can be recovered from the floccules. Ramon succeeded in demonstrating the presence of antitoxin by dissolving the floccules in a weak acid and heating the solution to 45°C. and the above described experiments prove indisputably by means of toxoid that the floccules contain toxin in an active form. S. Schmidt has already proved the relative thermostabile property of a toxin-antitoxin union in both floccules and solution.

Heat (as has already been shown in part one) would appear to favour a more rapid binding of toxin and antitoxin as may also be seen in experiments eleven and twelve where, of course, the time factor also plays a part, for after the mixture had stood for one hour at 40°C. double the quantity of toxoid (i.e., 0.4 cc.) was required to obtain the same result as that which was obtained with toxoid when the mixture was only heated to 40°C. After standing for other four hours only half as much toxoid (i.e., 0.6 cc.) was required to obtain a similar result, so that the greatest increase in the binding power between the toxin and antitoxin would appear to take place within the first hour or two. The heating of a toxin-antitoxin mixture would appear then to be unfavourable

to the splitting up action of toxoid inasmuch as the action of the latter is diminished. This has already been demonstrated in the experiments given at the end of section three of the first part.

From this analysis of flocculating toxin-antitoxin mixtures it would appear to be erroneous to assume that at the point of flocculation the entire toxin-antitoxin content of the mixture is contained in the floccules. Both portions of the mixture appear to contain toxin and antitoxin but which of the two contains the more it is very difficult to say. In experiment eleven it would seem as if the floccules contain more; then again in experiment twelve it would appear as if the contrary were the case. It is possible that at the flocculation point a state of equilibrium is set up and that the supernatant and floccules contain more or less the same amount of toxin and antitoxin. Later, however, it would seem as if the supernatant contains definitely less toxin and antitoxin and it might be possible that, were one to examine the supernatant at very short intervals of time after flocculation one might strike a point at which the floccules did contain most of the toxin and antitoxin content. One may be allowed perhaps to assume that here a process of adsorption plays a part, for later on a part of the toxin-antitoxin compound can again pass into solution for, by washing the floccules in a saline solution and re-suspending them in saline and after some time again removing the saline by centrifuging, one was able by means of toxoid to demonstrate the presence of toxin in the saline solution. This point would appear to be an important one in the process of immunisation with T.A.F., for if this be the case then one is not only immunising with a T.A.F. mixture, that is with a "Depot" which acts by its slow resorption and cleavage as an immunising agent, but also at the same time by means of the saline solution (which serves

as a suspensory agent) a quickly resorbing neutral toxin-antitoxin solution is being given.

By the demonstration of the presence of toxin (by means of toxoid) in the supernatant of a flocculating toxin-antitoxin mixture one can understand how it was that in the first immunisation experiments which were carried out with T.A.F. Eberhard succeeded in some cases in immunising with the supernatant fluid alone. To-day we know that this was nothing other than immunisation with a neutral toxin-antitoxin solution.

On the other hand not all floccules can so easily be made toxic by the addition of toxoid. One had the opportunity of examining floccules where the toxin-antitoxin compound was so firmly bound that it was found impossible to split it up by means of toxoid. This may explain the fact that some clinicians have with some T.A.F's a good result and with others not so good a result. Most likely the temperature at which flocculation takes place plays a rôle as is demonstrated by the experiment described at the end of section three in part one. But this experiment is very difficult to repeat with exactly the same results. There are many other factors which play an important part in the flocculation of neutral toxin-antitoxin mixtures which only further experimental work can reveal.

Summary.

If a neutral toxin-antitoxin mixture which stands at 40°C. is exposed to the dissociating action of toxoid, then in course of time the amount of toxoid which, (ceteris paribus) is capable of rendering the toxin-antitoxin mixture toxic increases up to the point where flocculation begins. If the floccules are separated from the supernatant and after washing are suspended in saline solution then it is possible by the addition of toxoid to render both them and the supernatant

toxic. The toxin-antitoxin compound which is bound to the flocculation substance (probably through adsorption) can again undergo dissociation and pass into the surrounding fluid (saline solution or supernatant) where, by the addition of toxoid the presence of toxin can be demonstrated. To what extent this is possible depends on factors which are to be found in the individuality of the particular toxins and sera which are used for flocculation; they also depend on the time during which and the temperature at which the toxin acts on the antitoxin, longer periods of time and higher temperatures appear to strengthen the toxin-antitoxin union.

Part 3.

The Measurement of Toxoid.

1. Survey of the procedure which has been adopted up to the present.

The measurement of toxoid is, from every point of view a difficult problem and even today it has not been solved in a satisfactory manner. Since this preparation does not give the easily measured toxic reaction, all measurements of toxoid rest either on the determination of its binding power with anti-toxin or on its power of immunising.

If the toxoid is to be used for active immunisation then naturally the estimation of its power of immunising is of value. One proceeds to this estimation by using rabbits which, according to Kolle are better suited to the purpose than guinea pigs, these animals are immunised with toxoid and after a definite time for example four weeks, they are subjected either to infection with virulent diphtheria bacilli or with a toxin which through animal experiment is known to be definitely lethal, then the percentage of those animals which survive is noted. The higher this percentage is, the better (as regards the immunising action) is the toxoid. Such a method of determining the strength of toxoid has been recommended by The State Institute for Experimental Therapy in Frankfurt am Main in connection with the State testing of toxoid with regard to its efficiency.

Previous experience however has shown that the immunising power of toxoid does not need to correspond to the number of antitoxin units which are bound. There are other properties which have to be considered in connection with a good immunising toxoid, such as its reaction on the tissues, the speed with which it can be absorbed etc., points however which cannot be gone into more fully here because of the lack of sufficient experimental knowledge which we must have in

order to assess these other factors.

At all events it is certain that the estimation of the immunising power of a toxoid only determines one of its properties and is not sufficient to characterise the toxoid as a whole. The power of a toxoid of binding antitoxin must as a matter of necessity be taken into consideration. It would be very desirable to procure a method of measurement which would show the binding power of a toxoid and which would at the same time give an idea whether the toxoid were a good one in respect to its powers of active immunisation.

The following procedures are at our disposal for the measurement of the binding power of toxoid.

a. Ramon's flocculation method carried out in vitro.

This method corresponds to the measurement of toxin and antitoxin by means of mutual flocculation. But instead of toxin, toxoid is taken and depending on the proportions of toxoid and antitoxin which are found to give the optimum flocculation, the amount of toxoid in cc. is determined and is known as the L_f value which binds one antixoxin unit. If one calculates the figure which gives the number of such binding units which is contained in one cubic centimetre of the toxoid, then one gets the L_f reciprocal value upon which Ramon is accustomed to base his estimation. If, for example, the L_f of a toxoid equals 0.10 then each cubic centimetre contains ten binding units and according to Ramon that particular toxoid has a strength of 10.

When we consider that according to our present knowledge of the phenomenon of flocculation there is some doubt as to the wisdom of considering it as an absolutely reliable form of measurement for binding power (Glenny, Pope and Waddington; Bronfenbrenner and Reichert; Maloney and Beecher Weld; Zingher; H. Schmidt and W. Scholz; Asakawa), we are even less justified in looking upon the L_f value as a measurement of

antigenic power.

Nevertheless within certain limits this is possible. If toxoids are produced which have been measured by means of the unit method then one can say that the greater the number of binding units in one cubic centimetre (measured by flocculation) the better is the antigenic action of the toxoid, and up to a certain point and within the same limitations one is justified in saying that the shorter the time is in which flocculation takes place (under otherwise similar experimental conditions such as temperature etc.) the better is the immunising action of the toxoid. But by this means one cannot compare one toxoid with another which has been produced in a different way.

As is well known it is possible to produce a concentrated toxoid with respect to its powers of binding antitoxin and as far as the accompanying material from the diphtherial culture filtrate is concerned it is possible also to produce a pure toxoid (S. Schmidt and others), but if with that, the figure for the binding power rises and the time of flocculation is reduced, then as far as the experience with animals is concerned, the immunising power has not increased to a corresponding extent, in fact it can actually have decreased. (Kolle.)

b. The second procedure for determining the binding power of toxoid is the so-called Kraus' experiment. Following the method of Bächer, Kraus and Löwenstein, to every two antitoxin units a variable amount of toxoid is added and after the mixture has stood at room temperature for half an hour one L_+ dose of toxin is added to it. After a further interval of twenty minutes the mixture is injected. In that mixture which causes death in a guinea pig on the fourth day the toxoid has bound one antitoxin unit. If for example the amount of toxoid was 0.2 cc. then according to this method the toxoid has a strength of 5. Naturally the time (which is a matter of

choice) given for the binding process to take place must be strictly adhered to when one is comparing one toxoid with another. In the further experiments I have kept to the time for the binding processes which was suggested to me by Professor H. Schmidt.

The method may be expressed in the following formulae.

$$\left(\left[2AU + X\ \text{Toxoid}\right]^{1\ \text{hr.}} + L_+\right)^{\frac{1}{2}\ \text{hr.}}\ 4\ \text{cc.} \rightarrow \text{death on 4th day.}$$

The figure 0.2 is according to the proposal of H. Schmidt known as the Lba value which means Limes Bindung anterior because here the toxoid was added first to the antitoxin and the toxin only at a later stage.

The arrangement of the experiment can also be reversed, when the procedure would then have the formula

$$\left(\left[2AU + L_+\right]^{\frac{1}{2}\ \text{hr.}} + X\ \text{Toxoid}\right)^{1\ \text{hr.}} 4\ \text{cc.} \rightarrow \text{death on 4th day.}$$

It has been proposed by H. Schmidt that the value so obtained should be known as the Lbp, if, for example, it was necessary to use 2 cc. of toxoid in the 4 cc. of the mixture in order to cause death on the fourth day then the Lbp value would be 2.0 and that toxoid would have a strength of 0.5.

These two procedures for determining the Lba and the Lbp values are based on the possibility (which has been described in the first two parts of this work) of being able to split up the union between toxin and antitoxin by the addition of toxoid (Lbp), this can naturally be reversed by splitting up an antitoxin - toxoid union by means of the addition of toxin. In both cases there exists, so to speak, a rivalry or competition between the toxin and toxoid for union with the antitoxin, in the Lba this leads to partial neutralisation of toxin, in the Lbp it leads to a partial freeing of toxin.

With exception of the original Kraus procedure which

Baecher, Kraus and Lowenstein have proposed as a method for measuring toxoid, the Lbp value which has as its basis the ability of toxoid to cause dissociation of toxin, has not so far served in the actual measurement of toxoid, although Ramon found in his toxoids that the power of a toxoid to cause dissociation ran parallel with its flocculation value.

2. <u>The procedure for measuring the Lba and the Lbp</u>.

In this section it will be discussed if and how far the determination of the Lba and Lbp values can serve not only for measuring the binding power of toxoid for antitoxin but also if it be possible by means of these values to characterise or label a toxoid.

The scheme of the methods used may be set out thus -

Lf 1AU + Toxoid $\longrightarrow$ Flocculation.

Lba ([2AU + Toxoid] $^{1\ hr.}$ + L_+)$^{\frac{1}{2}\ hr.}$ 4 cc. $\longrightarrow$ death on the 4th day.

Lbp ([2AU + L_+] $^{\frac{1}{2}\ hr.}$ + Toxoid)$^{1\ hr.}$ 4 cc. $\longrightarrow$ death on the 4th day.

We see first that the fundamental difference between the Lf process on the one hand and the Lba and Lbp processes on the other is, that in the first case free antitoxin takes part in a reaction with toxoid, whereas in the two later processes the reaction takes place with an already bound antitoxin. There is therefore another factor which must be taken into consideration and which for want of better understanding we call avidity or affinity.

The affinity of a toxoid for, and its power of binding antitoxin are not quite the same. Affinity comes into play with the competition which takes place between toxin and toxoid for the binding of antitoxin, whereas the actual binding power is a characteristic which is in a greater or lesser degree possessed by both toxin and toxoid and although they appear to be closely connected with each other this power of binding is exercised quite independently. It will make a great dif-

ference whether an antitoxin is presented to a toxoid when bound to toxin or as free antitoxin. A toxoid may possess quite a marked binding power for antitoxin but if the latter is already bound to toxin the affinity of the toxoid may under certain circumstances not be strong enough to split up the toxin-antitoxin union and in this way to render it toxic; and also vice versa - the affinity of a toxoid may not be a particularly marked one but if the antitoxin is presented to it as free antitoxin the latter will up to a certain degree be taken up by the toxoid, but if the binding power of the toxoid is great it will not be under certain circumstances so easy for the toxin to split up the antitoxin-toxoid compound. As regards the difference between toxin and toxoid it is very probable that on the basis of the following experiments, one may say that in general the affinity of toxin to antitoxin is greater than that of toxoid.

For the following experiments six different toxoids were used, of these, two were purified and concentrated by the adsorption process of Willstätter. With the exception of the toxoids Pasteur and Wien all the toxoids were produced in the Behringwerke.

The procedure was as follows. First the Lf value of the toxoid was ascertained by the usual Ramon flocculation method, then the Lba value was worked out, here two antitoxin units were brought together with varying amounts of toxoid the mixtures stood at room temperature for one hour, then an L_+ dose of toxin was added the mixtures then stood for half an hour at the same temperature as before and were then injected subcutaneously into guinea pigs in a volume of 4 cc. The dose of toxoid which caused death on the fourth day was taken as the Lba value. The determination of the Lbp value was carried out in a similar way but here the sequence was altered, one L_+ dose of toxin was added to two antitoxin units and after standing half an hour at room temperature, toxoid in varying amounts was added, then after standing

one hour at room temperature the whole mixture was injected in a volume of 4 cc. As before the amount of toxoid which caused death on the fourth day was taken as the Lbp value.

In all, seventy four animals were injected and the resulting measurements of the toxoids are as follows:-

Experiment 13.

Toxoid.	Lf	Lba.	Lbp.	Ratio of Lbp to Lbp.
Ramon	0.10	0.15	1.5	10.
Dänisch conc.	0.005	0.02	0.3	15.
404/490 conc.	0.02	0.045	0.3	6.7
B. W. I.	0.18	0.25	2.2	8.8
B. W. II.	0.18	0.25	2.2	8.8
Wien.	0.21	0.33	3.5	10.6

3. The Significance of $R = \frac{Lbp}{Lba}$

It will be seen from the above table that the Lbp value is much greater than the Lba, in one case the ratio "R" is actually as high as fifteen. We should like now to try and discuss what significance R has for toxoid.

Let it be assumed then that in the two formulas

Lba: 2AU + Toxoid + L_+

Lbp: 2AU + L_+ + Toxoid

there is no difference between the binding power and the affinity of toxin and toxoid, in that case then there must be no difference between the Lba and the Lbp even if the Danysz phenomenon be taken into account. Now suppose there is a difference between the binding power and affinity of toxin and toxoid but that both were added at the same time to antitoxin, then the distribution of the antitoxin between toxin and toxoid would naturally be equal for they would be

independent of any sequence so that there would then be no difference between the Lba and the Lbp values.

The value thus obtained may be called "Lb". Some preliminary experiments undertaken later with other toxoids have shown that compared with the Lba and the Lbp values the Lb value is always ~~less~~ greater than the Lba value though its difference from the Lba value is much less than that of the Lba from the Lbp. The following example will serve to show the different values of toxoid No. 540.

Lf.	0.08
Lb.	0.33
Lba.	0.166
Lbp.	1.66

The difference between the Lb value and the Lba value can be easily explained by the fact that in the Lb method free antitoxin is offered to both toxin and toxoid at the same time and here it is only a question of the binding power of diphtheria toxoids because here there is no interference by the question of affinity, but in the Lba and Lbp methods this question of affinity must be taken into consideration. The value known as the Lb value of a toxoid is naturally not the value which corresponds to the complete neutralisation of the binding power of the toxoid, but rather it represents for toxoid what the L_+ value represents for toxin. The value which corresponds to the L_0 value in toxin, would in toxoid correspond to the Lf value. Therefore the latter must be less than the Lb value.

In contrast to the Lb value, the affinity of toxin and toxoid to antitoxin must in the Lba and Lbp values be taken into consideration. Any difference therefore observed between the Lba and Lbp values must be due to differences in the binding power as well as in affinity. Both binding power and affinity are closely connected with each other.

The Lba gives the binding power of toxoid for antitoxin, for here the latter is offered to it as free antitoxin and it is a question of how much antitoxin the toxoid is capable of holding against toxin. The Lbp value on the other hand is concerned with the affinity of the toxin, for here the antitoxin is offered when already bound to toxin and the question now is, how much toxoid is necessary in order to split up the toxin-antitoxin compound and to bind antitoxin to itself?

TABLE II.

Lba			
2 AU+	0.3 Toxoid +	L+	Death on 2nd day
"	0.25 "	"	" " 4th "
"	0.2 "	"	" " 6th "
"	0.15 "	"	Survived.
Lbp			
2 AU+	L+	+ Toxoid 2.5	Death on 3rd day
"	"	" 2.2	" " 4th "
"	"	" 1.8	" " 8th "
"	"	" 1.5	Survived.
"	"	" 0.3	Survived.

The above table of the various amounts of toxoid that were added in determining the Lba and Lbp values of toxoid B.W, illustrates the very marked difference between the two values.

It must be remembered that in these methods of measurement the antitoxin and toxin are kept constant so that if there were no difference between the affinity of toxin and toxoid the result of the Lba and Lbp would be the same. But as one sees there is a difference between the two measurements

for in the above table in the Lba experiment 0.3 cc. of toxoid causes the animal's death in two days, whereas the same amount of toxoid (0.3 cc.) in the Lbp experiment has absolutely no effect whatsoever.

When we consider that it requires about ten times as much toxoid to split off one antitoxin unit from the toxin-antitoxin compound than is required to hold one antitoxin unit against the affinity of the same amount of toxin we must as a result of these experiments draw the conclusion that it is very probable that the affinity of toxin to antitoxin is greater than that of the toxoid to antitoxin, and since in all toxoids which have so far been investigated the difference between the Lba and the Lbp is relatively great, this conclusion may then be applied generally. But owing to the lack of another method quite independent of these experiments whereby the binding power of toxoid with, and its affinity to, antitoxin might be determined and compared with the above conclusions, one cannot take the above conclusions as absolutely proved but only as very likely. One may therefore be justified in looking upon the Ratio $R = \frac{Lbp}{Lba}$ as a figure which gives us a measure of the affinity of toxoid to antitoxin compared with that of a given toxin to the same antitoxin.

This affinity cannot be expressed in absolute measurement for it depends upon the test toxin that is chosen. By comparing two toxoids with the same toxin a number is obtained which in itself conveys nothing more than the fact that one toxoid has more affinity than the other when compared with the same toxin, but this number can become a measure (which can be made use of) when it refers to a standard toxoid. By the methods of comparing the Lbp and the Lba values one is in the position of being able to find the strength of a single toxoid, that is, the strength of its affinity to antitoxin.

One must not assume that a toxoid which sows a weak

or relatively weak binding power as measured by the Lf method has necessarily a weak affinity to antitoxin. This fact is illustrated in the first table where the highly concentrated "Dänisch" toxoid showed a very high binding power as shown by the Lf value (0.005) but its affinity to antitoxin was low, the ratio being 15. On the other hand toxoid "B.W" although its binding power was very much weaker than that of the "Dänisch" toxoid, its affinity was very much greater and gave a ratio figure of 8.8.

The number of experiments is still too small to allow one to draw far-reaching conclusions, but experiments carried out in the Staatsinstitut in Frankfurt am Main for testing the immunising power of toxoids have so far shown, that the concentrated and purified toxoids which showed a high binding power when measured by the Lf method are by no means the best in the immunisation of animals, while on the other hand an unpurified and non-concentrated toxoid from the Behringwerke which did not show a high binding power when tested by the Lf method gave a very good immunising result.

So far therefore as pure experience goes one may be justified in regarding a toxoid whose R value gives a low figure as a good immunising toxoid. Tests on a large scale will have to prove this statement.

Summary.

It is then clear that the Lf value of a toxoid is not the correct measurement for its action in prophylactic inoculation, for by this method one only measures the binding power of the toxoid while on the other hand the Lba and Lbp methods not only measure this but also allow the measurement of its affinity. Therefore in order to characterise a toxoid it is suggested that the Lb value should be taken as a measurement of its binding power and then the Lba and Lbp values should be

determined whose quotient $R = \frac{Lbp}{Lba}$ gives a relative measurement for the affinity and thereby also for the antigenic action. R is always greater than unity. The smaller R is, then the greater is the affinity of the toxoid to antitoxin.

Part 4.

The Quantitative Relationship in the Lbp Procedure between Toxoid and Antitoxin in presence of a constant amount of Toxin.

In the first part the question of the relationship between serum and toxoid was considered from a qualitative point of view. Now the question of relationship will be discussed in a quantitative sense.

The question to be considered here is, does a definite numerical relationship exist between the amount of serum and the amount of toxoid in a toxin-antitoxin-toxoid mixture? In other words if we knew the amount of serum contained in a neutral or over-neutralised toxin-antitoxin mixture, could we by means of a formula calculate the amount of toxoid which should be added in order to bring about a given toxic result? Or conversely, from the amount of toxoid added could we conclude what amount of antitoxin had been added to the mixture?

In the experiments which were to be undertaken in order to answer these questions there were four separate variable factors - toxin, serum, toxoid, and the time of death of the animals. Finally the time during which the several components acted upon each other could also be varied. In order to reach a functional relationship that could be used it was necessary to restrict the number of variables to two. First the amount of toxin was kept constant, for in mixtures destined for immunising purposes it is the toxin that is always kept constant and the serum that is varied when an under- or over-neutralised mixture is desired, and assuming that it were possible to obtain a formula from the results of the experiments, such a formula would be used in connection with immunising mixtures, so that toxin as the constant would appear to be a reasonable arrangement.

It was hoped also to be able to keep the time of death of the animals constant and death on the fourth day was chosen as the criterion, but in actual practice it was found difficult to maintain this as a standard owing to the variability of the reaction shown by guinea pigs even towards the same toxin-antitoxin mixtures. In order to have kept the time of death absolutely constant one would have to have used a very large number of animals, therefore the smallest amount of toxoid required to cause death was taken as the standard.

The next question was that of the neutrality of the mixtures. Here again the question of immunisation was the decisive point, for in immunisation neutral or over-neutralised mixtures are commonly used and at any rate one could not expect to obtain such accurate results when using a mixture of toxin and serum which would in itself cause death without the addition of toxoid. In the arrangement for the carrying out of the experiments the Lbp method was used, that is, the serum was added to the toxin and then after a period of standing the toxoid was added. One could equally well have chosen the Lba method where the toxoid is added to the serum and the toxin is added last. In fact it might have been more convenient had the latter method been chosen for in the experiments dealing with the Lba and Lbp values of various toxoids, it was found that the latter was always much greater than the former. In consequence in the Lbp arrangement when a non-concentrated toxoid was used one reached a stage where it was impossible to increase the amount of toxoid for the volume became too large for subcutaneous injection, therefore a concentrated toxoid was also used in order to overcome this difficulty.

As far as possible the weight of the animals was kept constant in each series of experiments. The experiments were all carried out in the same way, that is, the toxin and serum were allowed to stand for twenty minutes then the toxoid was

added and the mixture stood for another twenty minutes before being injected. A second series of experiments was also carried out with each serum and toxoid. In these experiments the toxin and serum mixture stood for twenty two hours and the toxoid was added and after standing for twenty minutes the mixture was injected. All the experiments were carried out at room temperature.

Two sera were used, the first was a test serum with a strength of 10 and the second was a serum freshly obtained from horse No. 2228 with a strength of 500. Two toxoids were used, the first came from Ramon of the Pasteur Institute and had an L_f value of 0.10, the second was a concentrated toxoid No. 233 from the Behring Werke; it was five times stronger than the first. The test serum was used with both toxoids, the fresh serum with the concentrated toxoid only.

In all a hundred and thirty four animals were inoculated. The amount of toxin used remained constant throughout. In each single series of experiments the amount of serum used was constant and varying amounts of toxoid were added in order to ascertain what was the smallest amount of toxoid which would cause death when added to the toxin-antitoxin mixture. In each succeeding series of experiments the amount of serum was increased until a point was reached where it was impossible to increase the serum as the amount of toxoid required in order to cause death was too large for the purposes of injection, for all mixtures which were injected were in a volume of 4 cc. or at most 5 cc.

In each series of experiments the least amount of toxoid which would cause death was determined and the figures for serum and toxoid thus obtained are illustrated in the form of a graph.

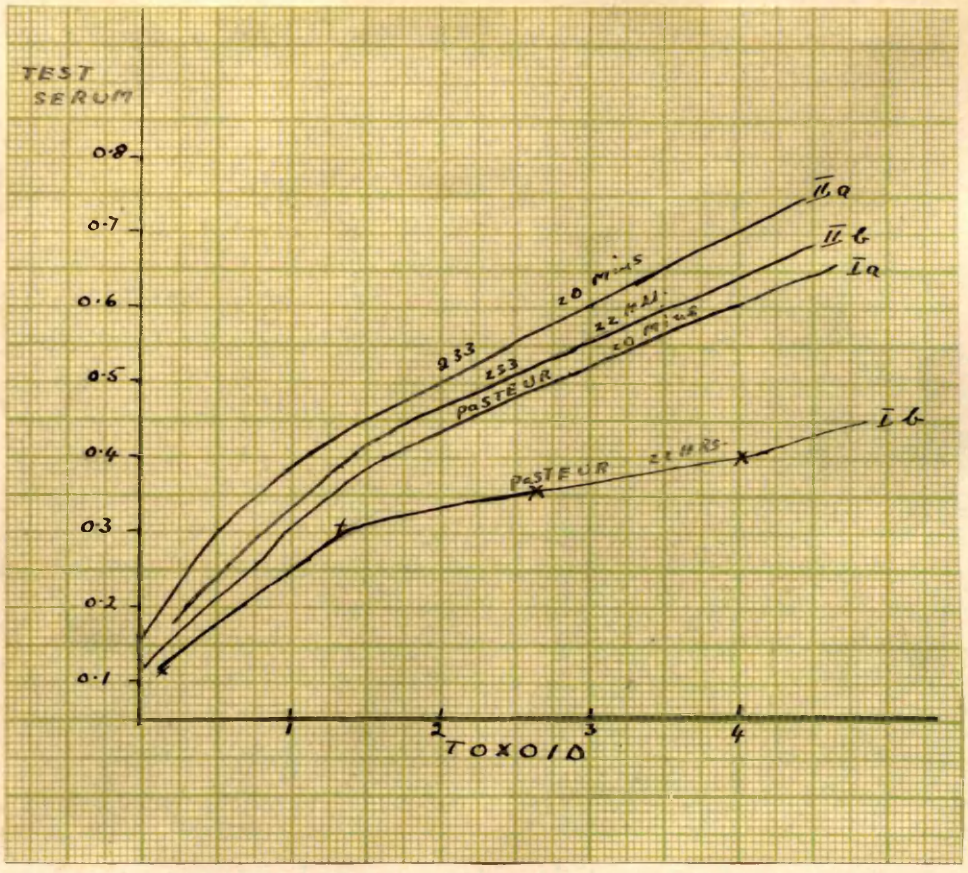
The following table shows the figures obtained.

Experiment I.

20 Minutes	Toxin 506	Test Serum	Toxoid Pasteur	Result.
at Room Temp.	0.155 cc.	0.125 cc.	0.04 cc.	Death on 4th day
	"	0.2 cc.	0.375 cc.	" " 2nd "
	"	0.25 cc.	0.70 cc.	" " 4th "
(a)	"	0.3 cc.	0.9 cc.	" " 5th "
	"	0.4 cc.	1.6 cc.	" " 5th "
	"	0.6 cc.	4.0 cc.	" " 7th "
22 Hours at Room Temp.	0.155 cc.	0.125 cc.	0.1 cc.	Death on 3rd day
	"	0.3 cc.	1.3 cc.	" " 4th "
	"	0.35 cc.	2.7 cc.	" " 6th "
(b)	"	0.4 cc.	4.0 cc.	" " 4th "

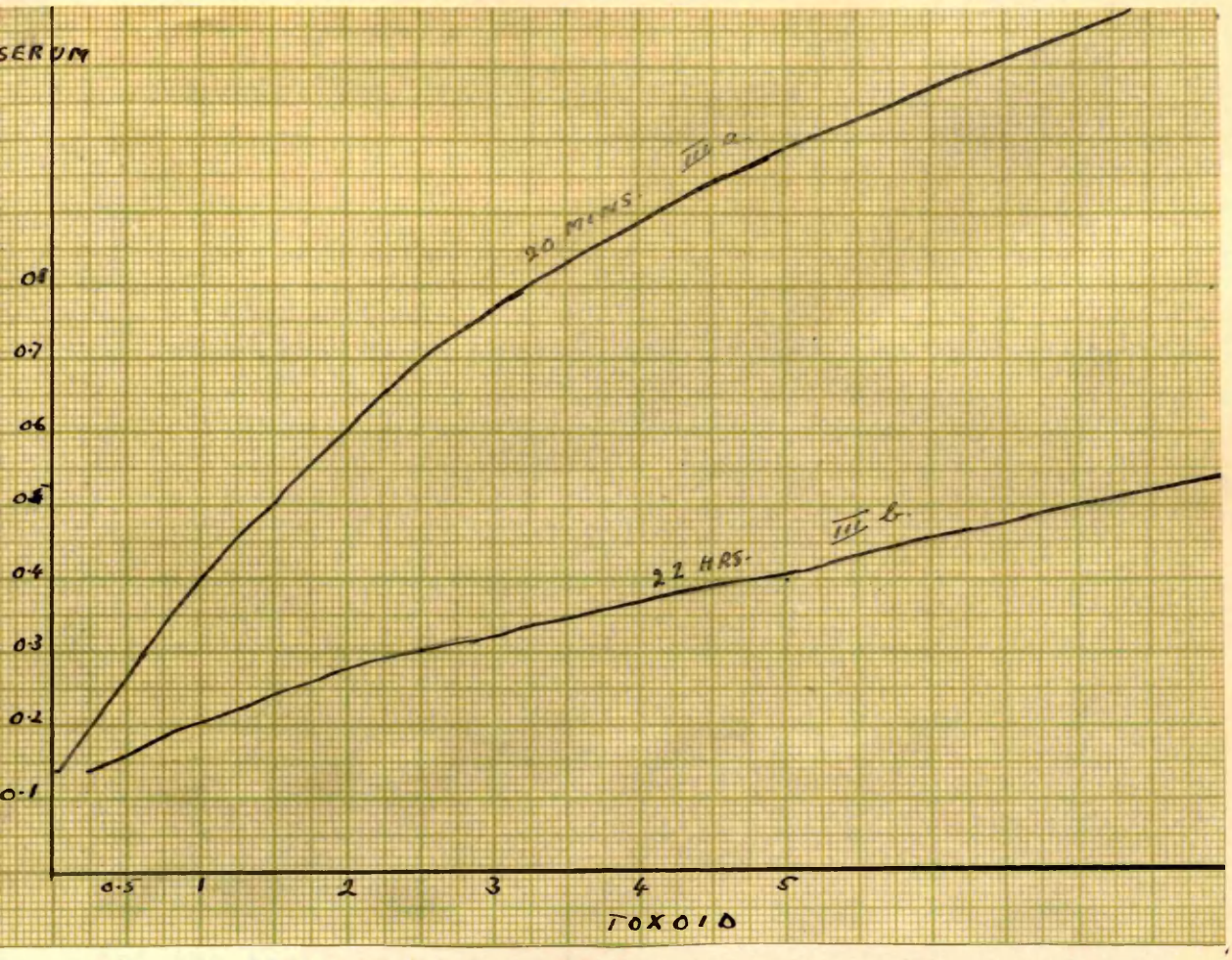
Experiment II.

20 Minutes	Toxin 506	Test Serum	Toxoid 233.	Result.
at Room Temp.	0.155 cc.	0.125 cc.	0.009 cc.	Death on 6th day
	"	0.3 cc.	0.106 cc.	" " 9th "
	"	0.4 cc.	0.23 cc.	" " 2nd "
(a)	"	0.6 cc.	0.6 cc.	" " 5th "
	"	0.7 cc.	0.9 cc.	" " 5th "
22 Hours at Room Temp.	0.155 cc.	0.2 cc.	0.05 cc.	Death on 3rd day
	"	0.6 cc.	0.68 cc.	" " 6th day
(b)	"	0.7 cc.	1.4 cc.	" " 3rd day



Experiment III.

20 Minutes at Room Temp. (a)	Toxin 506	Serum 2228	Toxoid 233.	Result.
	0.155 cc.	0.0025 cc.	0.005 cc.	Death on 8th day.
	"	0.004 cc.	0.03 cc.	" " 6th "
	"	0.006 cc.	0.125 cc.	" " 2nd "
	"	0.008 cc.	0.2 cc.	" " 3rd "
	"	0.010 cc.	0.3 cc.	" " 3rd "
	"	0.012 cc.	0.5 cc.	" " 6th "
	"	0.013 cc.	0.55 cc.	" " 4th "
	"	0.02 cc.	1.75 cc.	" " 4th "
22 Hours at Room Temp. (b)	0.155 cc.	0.0025 cc.	0.05 cc.	" " 4th "
	"	0.004 cc.	0.1 cc.	" " 4th "
	"	0.006 cc.	0.5 cc.	" " 4th "
	"	0.008 cc.	0.8 cc.	" " 4th "
	"	0.010 cc.	1.5 cc.	" " 4th "



It will be seen from the diagrams that the figures for serum and toxoid when plotted do not represent straight lines, therefore there is no direct linear relationship between the variables. It is however possible within a narrow limit of over-neutrality to express the experimental findings with a fair degree of accuracy by means of a simple linear function.

(Serum) = K. (Toxoid) + constant

where K. is a numerical (proportional) factor. If, however, over-neutralisation exceeds a certain limit, the curve decidedly bends and the function is certainly no linear one.

There appears to be a point of over-neutralisation where, within the limits of experimental procedure any amount of toxoid is no longer capable of rendering the mixture toxic. This latter point seems to be reached sooner when the toxin-antitoxin union is allowed to become firmer as is shown in the curves of the toxin-antitoxin mixture which stood twenty two hours and which therefore required more toxoid to bring about dissociation. This influence of time has already been

demonstrated in the first part of this work.

The forms of the curves make it probable that the curves represent a potential function of the type

$$S = K(T)^n$$

where K and n are constants and S and T represent serum and toxoid respectively.

In order to prove the validity of this function which, according to H. Freundlich, is the empirical formula which expresses the experimental data obtained in isothermal adsorption, the equation is expressed by means of logarithms as follows:-

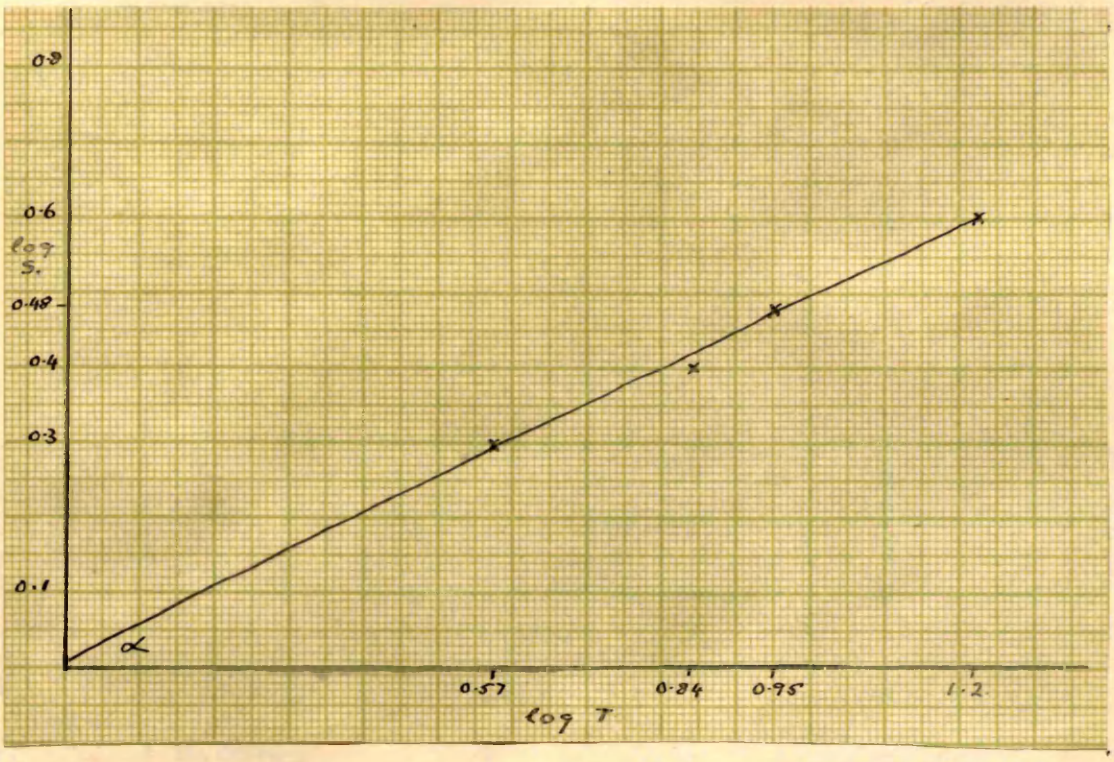
$$\log S = \log K + n \log T.$$

This equation represents a straight line cutting the log S axis at a point which gives the log of the constant K, and the inclination towards the log T axis gives an angle the tangent of which is numerically the value of the constant n. We have now to see how far the plotted points representing the logs of S and T follow a straight line.

The figures of the results obtained in experiment Ia will first be examined as the following table and diagram show.

Experiment Ia.

Amount of Serum multiplied by ten.	Logarithm	Logarithm abbreviated.	Amount of Toxoid multiplied by ten.	Logarithm	Logarithm abbreviated.
1.25	0.0969	0.1	0.4	0.60206-1	0.6-1
2.	0.30103	0.3	3.75	0.57403	0.57
2.5	0.39794	0.4	7.	0.84510	0.84
3.	0.47712	0.48	9.	0.95424	0.95
4.	0.60206	0.6	16.	1.20412	1.2
6.	0.77815	0.8	40.	1.60206	1.6



It will be seen that most of the points with the exception of the first (serum 0.125 and toxoid 0.04) lie upon a straight line, which in this case (as we shall see later) passes by chance through the starting point of the co-ordinates, i.e. the origin. Therefore K = 1 and

$$n = \tan\alpha = \frac{\log S}{\log T}$$ results in the value 0.5

so that the function reads thus

$$\log S = 0.5 \log T$$

$$S = T.^{0.5} = \sqrt{T.}$$

If conversely we start from the equation $S = T^{0.5}$ by the insertion of the values of T, we find for S the following values:-

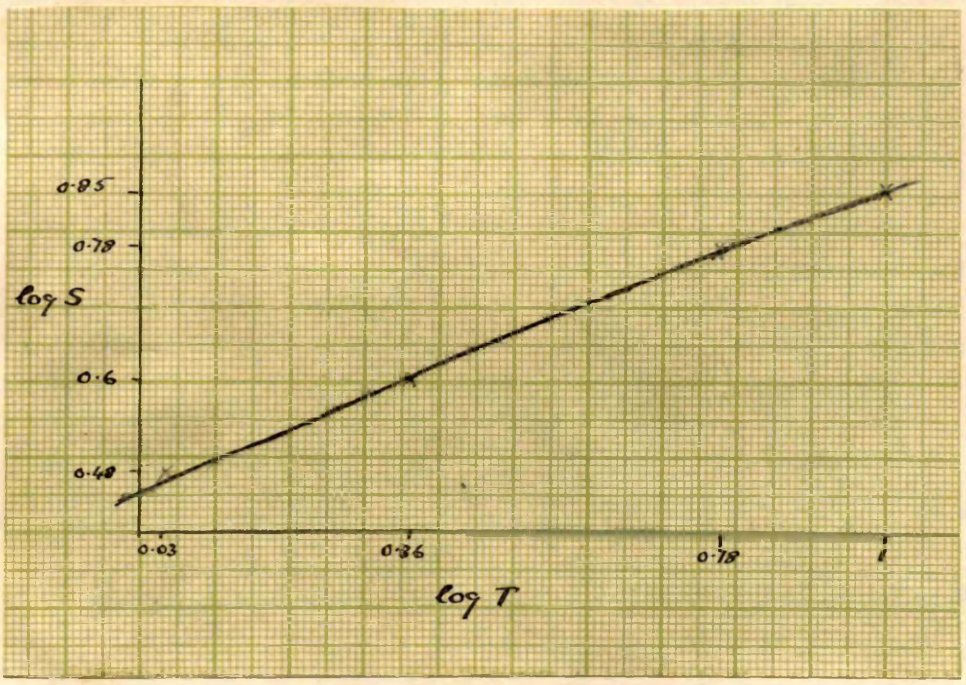
S. calculated.	S. found experimentally.
0.063	0.125
0.193	0.20
0.264	0.25
0.300	0.30
0.400	0.40
0.631	0.60

with the exception of the first value the figures agree with each other very well.

In experiment IIa where the test serum is used with the concentrated toxoid No. 233 similar calculations can be made.

Experiment IIa.

Amount of Serum multiplied by ten.	Logarithm	Logarithm abbreviated.	Amount of Toxoid multiplied by ten.	Logarithm	Logarithm abbreviated.
1.25	0.09691	0.1	0.09	0.95424-2	0.0-1
3.	0.47712	0.48	1.06	0.02531	0.03
4.	0.60206	0.6	2.3	0.36173	0.36
6.	0.77815	0.78	6.	0.77815	0.78
7.	0.84510	0.85	9.	0.95424	1.05



Here again when the logarithms are plotted the various points lie very nearly on a straight line whose tangent $\alpha = n = 0.40$ and the log of K = 0.465, consequently K = 2.917. The function here then reads $S = 2.917 \times T^{0.40}$

The calculation of S. according to this formula gives values which agree very closely with the experimental data as is seen in the following table.

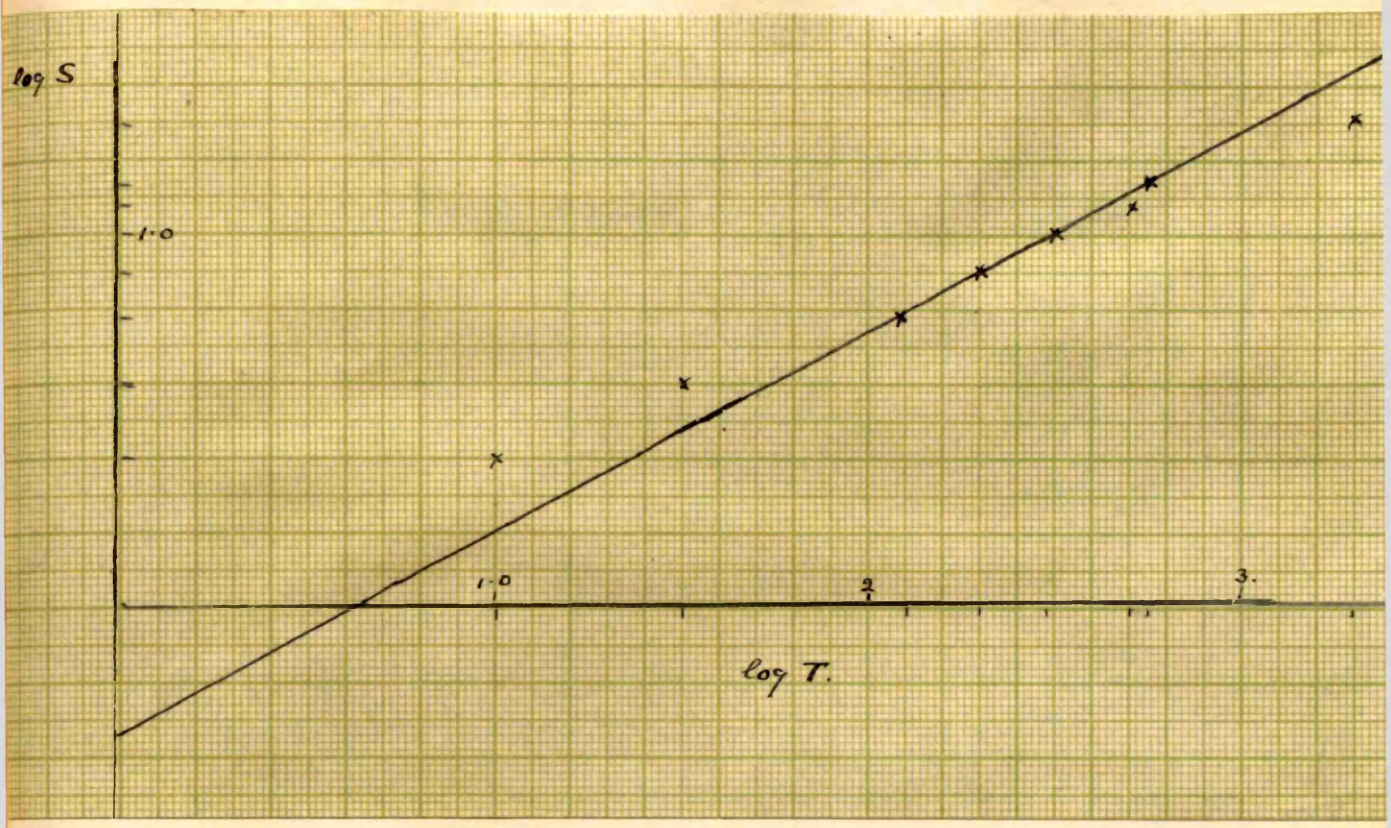
S. calculated.	S. found experimentally.
0.111	0.125
0.298	0.3
0.407	0.4
0.597	0.6
0.702	0.7

Finally the calculation for the fresh serum and concentrated toxoid is now given.

Experiment IIIa.

Amount of Serum multiplied by 1,000.	Logarithm	Logarithm abbreviated.	Amount of Toxoid multiplied by 1,000.	Logarithm	Logarithm abbreviated.
2.5	0.39795	0.398	10	1.0	1.0
4.	0.60206	0.602	30	1.47712	1.48
6.	0.77815	0.778	125	2.09691	2.10
8.	0.90309	0.903	200	2.30103	2.30
10.	1.0	1.0	300	2.47712	2.48
12.	1.07918	1.08	500	2.69897	2.70
13.	1.13394	1.13	550	2.74036	2.74
20.	1.30103	1.30	1750	3.24304	3.24

It will be seen that the first figure 10 of the toxoid does not correspond with the figure 0.005 of the toxoid in experiment III on page 58. This discrepancy is due to the fact that with the dose 0.005 of toxoid the guinea pig did not die until the 8th day and in the interval the calculation had been made on the figure 0.01 which was the next lowest dose of toxoid.



Here the calculation of the constants from the graph of experiment IIIa results in the following functional relationship between the amounts of serum and toxoid:

$$S = 0.468 \times T^{0.54}$$

and the calculation of S. with the help of this relationship from the T value found experimentally gives once again as the following table shows a satisfactory agreement.

S. calculated.	S. found experimentally.
0.0016	0.0025
0.0029	0.0040
0.0062	0.0060
0.0081	0.0080
0.010	0.010
0.0130	0.012
0.014	0.013
0.026	0.020

One must take into consideration that the greatest difference between the "calculated" and the "found experimentally" figures only amounts to 0.006 cc. of serum. Since that serum had a strength of 500, then this amount of serum (0.006 cc.) corresponds to only three antitoxin units and in the cases where the difference appears only in the fourth decimal it corresponds then to a difference of less than half an antitoxin unit.

It can then be established that the relationship between antitoxin and toxoid is not a linear one but rather (to judge by the formula which these relationships give in a satisfactory manner when calculated) that it follows a power function which is very often to be seen in adsorption processes. It will not do however to look upon this as a proof that the union is an adsorption process, but nevertheless it would appear to be very probable.

When applied to the example presented of a toxin-antitoxin-toxoid mixture with reference to the Lbp arrangement, one may say that antitoxin is bound to toxin by means of a process of adsorption during which the specific toxin removal action of the toxoid is a secondary proceeding. This union is at first very firm and only that portion of the antitoxin which is in excess of the toxin is relatively lightly bound. If a second adsorbent for antitoxin in the form of toxoid is added, then from the outset on account of the competition between the adsorbents there must be an excess in order that the primary adsorption can be made reversible. This is seen again in the Lbp method where, unlike that of the Lba, more toxoid is necessary to produce the same result, and the conclusion that the affinity of toxoid to antitoxin is weaker than that of toxin to antitoxin should in the meantime be regarded with caution.

The more toxoid that is added, the greater is the amount

of antitoxin that can be withdrawn from the toxin-antitoxin mixture, but the more antitoxin that there is in excess and which is held by toxin, then the more toxoid must there be added. Theoretically there is according to the formula no limit, that is, be the amount of antitoxin present ever so great, nevertheless there must be an amount of toxoid be it ever so large which is capable of rendering the mixture toxic. In practice however a limit is soon reached, for the volume of the mixture becomes so large that it cannot be injected. By using intracutaneous methods this limit can be considerably extended but will eventually be overstepped.

Can this formula be put to practical use? In the meantime only when one wishes to analyse toxin-antitoxin mixtures in which one knows the amount of toxin per cc. which is present. If both components are unknown then naturally the addition of toxoid will render the given mixture toxic, and by means of that, prove qualitatively the presence of toxin, but at present one is not in the position to analyse quantitatively an unknown mixture. This investigation which is presented is naturally not complete, but is a statement of experimental findings upon which further work can be based which would have for its object, for example, the calculation of the amount of toxin present in a toxin-antitoxin mixture with a given amount of antitoxin, or help in general with the carrying out of examinations of toxin-antitoxin mixtures; it could also be extended to other toxins such as tetanus and gas gangrene. Specially necessary is the explanation of the significance to be attached to the two constants in the above formula and what characteristics of the three components (which react upon each other) find their expression in these figures.

Summary.

If, in a neutral toxin-antitoxin mixture or in one of varying degrees of over-neutralisation one knows the amount of toxin used in the production of the mixture, then through dissociation of the compound by means of toxoid one can determine how much antitoxin is present in the mixture per cc. The relationship between antitoxin and toxoid is, however, not a linear one, but follows a formula which has the following form:-

$$(\text{Serum}) = \text{a constant } (\text{Toxoid})^{n},$$

which makes it probable that a process of adsorption underlies the processes of binding and dissociation.

I have to acknowledge the help given me by Professor H. Schmidt in the working out of the formula which, owing to my lack of mathematical knowledge, I could not have evolved alone.

SUMMARY.

The possibility of rendering toxic a non-toxic toxin-antitoxin mixture by the addition of toxoid is confirmed. The more antitoxin the mixture contains, the greater must be the amount of toxoid added in order to render the mixture toxic. When the excess of antitoxin is too great it is technically impossible to bring together the necessary amount of toxoid with the toxin and antitoxin in a volume that would allow of subcutaneous injection of a guinea pig.

Time has an influence on the dissociative action of toxoids. Under otherwise similar conditions the difficulty of rendering toxic a non-toxic toxin-antitoxin mixture by means of the addition of toxoid becomes greater, the longer the toxin-antitoxin mixture has stood. This influence exerted by time is particularly noticeable when the serum is fresh. It may be assumed that fresh sera show a greater avidity towards toxin than do old sera which have been in stock for some time.

In the dissociative action of toxoid on toxin-antitoxin mixtures the temperature at which the binding process takes place plays a part; if for example, under similar conditions with regard to time, toxin-antitoxin mixtures are placed at varying temperatures it is found that the binding power of the toxin and antitoxin increases with the rise in temperature, so that the higher the temperature at which the mixture stands the greater must be the amount of toxoid that has to be added in order to render the mixture toxic, assuming of course that the temperature is not such as would injure or destroy the toxin-antitoxin content.

If when at a temperature of 40°C. one exposes a neutral toxin-antitoxin mixture to the dissociative action of toxoid, then the following is seen. The amount of toxoid which,

ceteris paribus, is capable of rendering the mixture toxic increases up to the point where flocculation sets in. If the floccules are separated from the fluid and after washing in saline solution are resuspended, then it is possible by the addition of toxoid to render toxic both the floccule suspension and the supernatant. The toxin-antitoxin compound which is bound to the floccules can again pass into the surrounding fluid where it can be demonstrated by means of toxoid.

To what extent this is possible depends on factors which are to be found in the individuality of the given toxins and sera. Time and temperature also play a part, higher temperature and a prolonged period of time for the action of the components on each appear to make the union a firmer one.

In the measurement of a toxoid the Lf value alone does not give a complete picture of its antigenic action, for this method refers only to the binding power of the toxoid, while the other methods the Lb, Lba and Lbp which have been discussed in this work, allow of the determination not only of the binding power but also of the affinity of a toxoid to an antitoxin. The affinity can be expressed thus $R = \frac{Lbp}{Lba}$ The lower the value of R is, then the stronger is the affinity of the toxoid to the antitoxin.

If in a neutral toxin-antitoxin mixture or in mixtures of varying degrees of over-neutralisation, one knows the amount of toxin used in the production of the given mixture, then through dissociation of the compound by means of toxoid one is able to find how much antitoxin per cc. is present in the mixture.

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