ARTIFICIAL MODIFICATIONS OF TOXINES WITH SPECIAL REFERENCE TO IMMUNITY.

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Whatever may be the final form of the theories of Ehrlich as to the constitution of the bacterial toxines and as to the essential nature of immunity there can be no doubt of the stimulus they have given and are likely to give to inquiries relating to these subjects. As the present paper is intended to give a preliminary account of some researches bearing on the reactions of certain toxines it may be useful to recall the main features of the theories in question so far as they relate to the toxines investigated. The latter were all members of the group, of which the poisons formed by the bacillus of diphtheria are usually taken as the type, and it will be remembered that it was these diphtheria toxines on the investigation of which Ehrlich first based his theories. His fundamental experiments were, briefly, as follows. In default of obtaining the poisons in a pure form and weighing them, the standard amount of diphtheria toxine to which all other quantities are referred is the amount of any impure sample which contains sufficient to kill a guinea-pig of 250 grammes in four days. This is the minimal lethal dose ("M.L.D."). Ehrlich taking one unit of an anti-diphtheritic serum which he made his *standard* serum found how much of any particular toxine could be mixed with this unit *in vitro* so that when the mixture was injected into a guinea-pig no illness took place. Now supposing we had such a mixture of a simple poison like strychnine with its antidote, if such a thing existed, and if we added to this mixture one M.L.D. of strychnine and injected the whole into an animal we would expect it to die. Ehrlich found that on performing

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precisely such an experiment with diphtheria toxine and antitoxine the animal did not die, in fact, in the case of the specimens of toxine investigated by him, before death would take place, there had to be added to the neutral mixture of toxine and antitoxine as much of the toxine as would by itself have killed many animals. The explanation which he gives of this phenomenon is that the crude toxine is not a simple chemical body but a mixture containing a series of bodies which on the one hand have different degrees of affinity for the antitoxine and on the other have different degrees of toxicity for an animal. Thus if the antitoxine in the original mixture were partly saturated by bodies which had great affinity for it and also great toxicity and partly by bodies which had less affinity and also less toxicity, on fresh toxine being added to such a mixture the less avid, less toxic bodies would be displaced by part of the fresh very avid, very toxic bodies. The last would however be prevented having a toxic action on being injected into an animal by being already saturated by the antitoxine. The free toxine added to the neutral mixture would thus after this rearrangement of its constituents had taken place be robbed of part of its toxic power and an amount greater than a M.L.D. would have to be added before there was enough of the less poisonous bodies to cause death. This is putting Ehrlich's results in a very crude way but it is an attempt to reduce them to their simplest terms. What takes place is probably very complicated as bodies having very different affinities and toxic powers exist in the same crude toxine. The bodies having the greatest toxic power associated with the greatest affinity for antitoxine Ehrlich would call the toxines proper; the other bodies of less toxic power even if these had greater affinity for antitoxine he would call toxoids. This differentiation between the combining and the toxic capacities of toxines and toxoids is an essential part of the theory. In it is involved the supposed method by which such poisons as those of diphtheria and tetanus act on the animal body. According to this view susceptibility to one of these poisons depends on the capacity of certain cells to combine with the same group in the toxine or toxoid as the antitoxine combines with *in vitro*. This group Ehrlich names the "haptophorous" or combining group. When by this means the toxine is anchored in the cell then the other group which he names the "toxophorous" begins to exert its poisonous action on the other constituents of the cell. That part of the cell which anchors the toxine is compared to the affinities which occur in the "side-chains" of many complex organic substances whose constitution is known. What then

is the antitoxine found in the serum of an animal immunised against such a disease as diphtheria? Weigert[1] has elaborated a view which accounts for the formation of such a substance on the lines of Ehrlich's theory. It has been shown that the cells of the normal brain of an animal susceptible to tetanus manifest a certain capacity of neutralising tetanus toxine. This would be due to the appropriate side-chains present combining with the toxine. These side-chains which are probably specific for each toxine which attacks the brain must have some normal function in the metabolism of the cell. Now the process of active immunisation against such a disease as diphtheria consists in administering to an animal progressively increasing doses of the toxine. The first administration of the toxine saturates certain of these side-chains and thus robs the cell of their ordinary function. The great capacities possessed by the tissue cells generally to make good a damage provided the latter have not exceeded a certain degree comes into play and the cells give rise to new side-chains. As injection follows injection more and more side-chains are saturated, and more and more are produced till what is in reality a hypertrophy of these elements occurs so that now far more are produced than the cell has any need for. They thus become a waste product and are cast off into the lymph and blood and are the antitoxic elements which can be obtained from the latter. Such in brief outline is Ehrlich's theory in so far as it deals with the diphtheria group of poisons.

This group includes not only the poisons of diphtheria and of tetanus but also certain poisons of non-bacterial origin such as ricin, abrin, the snake poisons, scorpion poison, etc. All of these present certain common features and it is convenient to class them together. The following experiments deal chiefly with the tetanus poisons and also to a small degree with three other members of the group. The object of the research was to investigate a few of the properties of certain artificial modifications of the poisons which are probably to be classed as toxoids. Toxoids, as well as toxines, are produced by bacteria in ordinary fluid media but they also are gradually formed from the latter by the action of such agents as heat, light, oxygen, etc. As will be seen they can also be readily and quickly produced artificially by certain chemical agents.

The crude toxines of the tetanus and diphtheria bacilli which were used consisted of filtered bouillon cultures of these organisms—the diphtheria in ordinary bouillon, the tetanus in dextrose bouillon. No

[1] Lubarsch and Ostertag, *Ergebnisse der allgemeinen Pathologie*. Vierter Jahrgang (1897), p. 107.

attempt was made to get the toxine in a more concentrated form, as it is difficult in the course of the necessary manipulations to avoid the formation of much toxoid. During the research the toxic filtrates were kept in the dark in a cool place and were protected from atmospheric influences by being covered by a layer of toluol. Notwithstanding these precautions in the course of months it was found that a marked loss of toxicity took place in some instances. This however did not affect the results as these were of a relative and qualitative nature.

When any quantitative results were aimed at, the M.L.D. of a fresh toxine was determined and the latter used for the experiments. The accounts of the experiments have not been burdened with the details of the dilutions of the toxines often necessary to ensure accuracy in the measurement of the small quantities of fluid involved but such dilutions had constantly to be practised. In fact such a research as the present is very much hampered by the requirements of accuracy on the one hand and by the fact that only a limited volume of fluid can be injected into a guinea-pig on the other. In inoculating the experimental animals this was invariably done hypodermically over the abdomen or sternum. In the experiments with tetanus toxines several different toxic filtrates were employed. These are referred to by the letters A, B, G, E and F.

I. *Action of Acids on Tetanus Toxine.*

In their classical paper on diphtheria toxine (*Ann. de l'Inst. Pasteur*, III. 273) Roux and Yersin in stating that lactic and tartaric acids cause a loss of its toxicity add, that, if the acid be subsequently neutralised, the toxine recovers a great measure of its activity. This has always appeared to me a very remarkable fact and I resolved to look into the reaction further. An accident to a quantity of diphtheria toxine prepared for this object led me to confine the first observations to a tetanus toxine which it had been intended to use for finding if the reaction were a general one of the group of toxines in which both are as has been said usually placed. This was very fortunate as the definite functional effects occurring in tetanus enable the slightest response to the poison to be recognised,—an advantage not possessed by the diphtheria toxine, in experimenting with which it is often difficult to assign a true value to a slight local inflammation at the point of inoculation.

The first experiment was as follows:

Experiment 1. (*a*) ·25 c.c. of the toxic bouillon A was exposed to the action of ·25 c.c. of lactic acid of about half-normal strength for quarter of an hour and the mixture then injected into a guinea-pig of 270 g. Result: Death in 36 hrs.

(*b*) The same amount of toxine similarly exposed but at the end of the time named the acid was neutralized with sodium carbonate and the mixture injected into a g.p. of 252 g.[1] Result: Death in the same time.

(*c*) A control animal of 260 g. received ·25 c.c. of the toxine and died in the same time.

As dilution of the acid by the fluid of the toxine had to be allowed for, the effective strength of the former was about a quarter normal. In the time allowed for its action it evidently had no appreciable effect on the toxine.

Experiment 2. (*a*) 1 c.c. of the same toxine as in Exp. 1 was exposed to ·5 c.c. of normal lactic acid for $2\frac{3}{4}$ hrs. At the end of that time a quantity of the mixture containing ·46 c.c. of the toxine was injected into a g.p. of 468 g. No illness resulted.

(*b*) At the expiry of the time named some of the same mixture was rendered slightly alkaline with sodium carbonate and an amount containing ·47 c.c. of the toxine was injected into a g.p. of 470 g. Result: Death in $3\frac{1}{2}$ days.

(*c*) A control g.p. of 365 g. received ·36 c.c. of the simple toxine and died of acute tetanus in 36 hrs.

Here the effective strength of the acid was one-third normal and the exposure was much longer than in the former experiment. It will be noted that the general result is that which Roux and Yersin state to be the case with diphtheria toxine under similar circumstances, namely, that lactic acid is capable of destroying the toxine but that a certain amount of the poisonous effect returns if the acid be neutralised with alkali. That it is only a certain part of the toxic power that returns is indicated by the fact that while the control animal died in 36 hrs. the animal which died from the effects of the "regenerated" toxine, as it were, did not die for $3\frac{1}{2}$ days. As animals of suitable weight were not available at the time it was considered advisable to regulate the dose to the body-weight in the way that it will be noted was done. Another experiment along the same lines need not be detailed; it entirely confirmed that given.

While the results of these experiments indicated that it was the acid part of the lactic acid molecule that acted on the toxine it was considered advisable, seeing that this body also contains a hydroxyl

[1] The abbreviation "g." stands for gramme or grammes, "g.p." for guinea-pig."

group, to test the action of an acid hydrogen atom in such a simple acid as hydrochloric acid. The latter as compared with lactic acid has of course greater avidity and one would expect its action to be more powerful if the reaction depended on acidity.

Experiment 3. (*a*) ·3 c.c. of the same tetanus toxine was exposed to ·15 c.c. normal hydrochloric acid for 20 min. and then injected into a g.p. of 307 g. Result: On the third day there were some spasms but these passed off and the animal completely recovered.

(*b*) ·28 c.c. of the toxine exposed to ·14 c.c. of the same acid for the same time; then the acid was neutralised with normal sodium carbonate and injected into a g.p. of 285 g. Result: Death from tetanus on the 6th day.

Experiment 4. The following experiment was performed with a different toxine (M.L.D. for g.p. of 250 g. about ·0006 c.c.).

(*a*) 1 c.c. tetanus toxine B exposed to ·5 c.c. normal hydrochloric acid for 25 min. and mixture injected in a g.p. of 263 g. Result: No illness.

(*b*) 1 c.c. of same toxine similarly exposed and at the end of the time named the acid neutralised with normal sodium carbonate and the whole injected into a g.p. of 275 g. Result: Death in three days.

These experiments show that, like lactic acid, hydrochloric acid also has the power of destroying a certain amount of the toxicity of the toxine but that when the acid is neutralised a return to some extent of the poisonous property results. There can be little doubt also that the action is due to the acid hydrogen atom in the molecule. Further it will be observed that the time required for the more powerful acid to have a definite action was much shorter than in the case of the weaker lactic acid—a fact which supports the idea that it is the acid *qua* acid that is the cause of the reaction.

These experiments establish the fact that it is possible to hold in abeyance the toxicity of tetanus toxine by treating it with an acid. The phenomenon is a very remarkable one and very difficult to understand. It might be thought that if the poisonous properties of a toxine could be made to return by neutralisation of an acid outside the body that the same neutralisation would take place inside the body in consequence of the fact that the blood, lymph, etc. are alkaline. It may be that the very weak alkalinity of the body fluids may make any such action very slow and thus effect that the body has only a very small amount of active toxine to deal with at a time Unfortunately it was impossible to test the time taken by very weak alkaline fluids to bring out the phenomenon of this "return toxicity," as I may call it, for the amount of fluid would have been such as could not have been injected into a small guinea-pig.

As has been pointed out, according to Ehrlich's theory there are produced by such bacteria as the diphtheria bacillus not only highly poisonous bodies—the toxines, but also bodies of less virulence—the toxoids, and the latter are also developed in the toxines by the action of light, etc. It therefore suggested itself that it might be worth while to enquire whether the bodies which were responsible for the phenomenon of "return toxicity" might not belong to the group of toxoids. As we have seen, one of the features of the latter is that while the toxicity of these bodies may be less than that of the toxine the immunising power possessed by them may be the same. I therefore proceeded to enquire whether the toxines altered by hydrochloric acid possessed any immunising capacity. A preliminary experiment may be given.

Experiment 5. An earlier experiment designed to show the effects of the same amount of acid on different amounts of toxine furnished two guinea-pigs as follows:

(1) This animal had received ·5 c.c. of toxine B which had been exposed to ·5 c.c. of normal HCl for 20 min. Its weight was 290 g. No illness had resulted.

(2) This animal (Wt. 270 g.) had received 1·5 c.c. of toxine B exposed to ·5 c.c. of the same acid for the same time. No illness had resulted.

Eleven days after the first inoculation both received doses of unaltered toxine as follows:

G.P. 1 (Wt. now 352 g.) received ·001 Tox. B (M.L.D. ·0006 c.c.). Result: Death in 96 hrs.

Control g.p. (Wt. 333 g.) received ·001 c.c. Tox. B. Result: Death in 72 hrs.

G.P. 2 (Wt. now 318 g.) received ·0025 c.c. Tox. B. Result: Death in 120 hours.

Control g.p. of 378 g. received ·003 c.c. Tox. B. Result: Death in 48 hrs.

In this experiment the animal which had received the smaller dose of "HCl toxine" (as the toxine modified by HCl may be called) lived a day longer than the control animal, while that which received a larger amount of the modified toxine survived three days longer than its corresponding control animal. This experiment is quoted because it encouraged the search for more convincing proof. A definite immunisation of three animals by repeated doses of the modified toxine was now attempted.

Experiment 6. Three guinea-pigs were taken weighing 210, 251, 277 g. respectively.

1st injection. 5 c.c. Tox. B was exposed for 20 min. to the action of 2 c.c. of normal HCl, then the mixture was diluted to 20 c.c. and 5 c.c. were injected into each animal. Thus each received about 1·25 c.c. of the toxine which had been subjected to an acid of two-fifths normal strength and at the moment of injection

the acid was of one-tenth normal strength. This latter concentration of acid is rather too much for the subcutaneous tissues of a guinea-pig to stand as in all of these animals slight sloughing occurred at the point of inoculation which, however, soon healed. Subsequent experiments showed that it was necessary to avoid using an acid of greater concentration than one-fifteenth normal, *i.e.* in the case of hydrochloric acid, roughly, a quarter per cent.

2nd injection 18 days later. 1 c.c. Tox. B exposed to ·5 c.c. normal HCl for 25 min. then diluted to 15 c.c. and 5 c.c. injected into each animal, *i.e.* allowing for dilution the effective strength of the acid was one-third normal and the strength at the time of injection was one-thirtieth normal: further each animal received one-third of a c.c. of the modified toxine.

3rd injection on the 29th day same as the second.

4th „ „ 39th „ „ „

5th „ „ 49th „ „ „

After the first injection there never was any local reaction beyond a very slight swelling at the point of inoculation.

On the 61st day after the first injection,—

G.P. 1 (Wt. increased from 210 to 449 g.) received *one* M.L.D. Tox. G.

G.P. 2 (Wt. increased from 251 to 474 g.) received *four* M.L.D. Tox. G.

Neither manifested any symptom of tetanus.

On the 72nd day g.p. 3 (Wt. increased from 277 to 520 g.) received 33 M.L.D. Three days later it had a slight spasm of one leg which continued for about a week and then disappeared.

On the same day a control animal to No. 3 (Wt. 550 g.) received 33 M.L.D. It died of tetanus in less than sixteen hours.

During the whole process of immunisation no symptom of tetanus was manifested in any of the animals. There was thus no doubt that while hydrochloric acid could completely hold in abeyance the poisonous properties of tetanus toxine it did not at the same time entirely remove the immunising action of the latter. The interpretation of this experiment will be discussed later. Meantime, it may be noted regarding it that immunisation here was at least commenced with a toxine containing potentially poisonous action, as the following fact demonstrated.

Experiment 7. About the time when the first injection of Exp. 6 was performed 1 c.c. of the same toxine used in the latter experiment was exposed to ·5 c.c. of normal HCl for 20 min., the acid was then neutralised with sodium carbonate and the whole injected into a g.p. of 275 g. Death resulted in 2 days. Here the animal received ·25 c.c. less toxine than the guinea-pigs in the immunisation experiment.

In the action of HCl on tetanus toxine there comes a time when the phenomenon of "return toxicity" ceases to be capable of manifestation, as the following experiment shows.

Experiment 8. The general scheme of this experiment was to expose 1 c.c. of toxine G to 1 c.c. of normal HCl for varying times, then to neutralise the acid with normal sodium hydrate and inject the whole into the experimental animals.

Exposure to HCl	No. of Guinea-pig	Weight in grammes	Result
1¾ hrs.	1	325	Death in 24 hrs.
2½ ,,	2	265	No illness
4½ ,,	3	302	,,
6½ ,,	4	310	,,

Such being the case it became interesting to enquire whether with complete disappearance of the "return toxicity" the power of immunisation was also lost. The next experiment throws light on this point.

Experiment 9. The scheme was to immunise three guinea-pigs by means of a toxine whose toxicity was so modified by HCl as to be beyond recovery by neutralisation by alkali. That there might be no doubt on this point the poison was exposed for four hours at room temperature to the action of the acid. The weights of the animals at the beginning were 258, 183, 208 g. respectively.

1st injection: 5 c.c. toxine G was exposed to 5 c.c. normal HCl for the time named. The mixture was then rendered very slightly alkaline with normal NaOH and diluted. Of the dilute liquid g.p. 1 received a portion containing two-thirds of a c.c. of the original toxine, g.p. 2 one c.c. toxine, and g.p. 3 one and two-thirds of a c.c. toxine. (This injection was in reality a part of another experiment.)

2nd injection: on the 8th day following, each g.p. received 1 c.c. of the toxine which had been similarly exposed to the acid which had been then similarly neutralised.

This latter procedure was repeated on the 14th, 30th, 35th, and 39th days.

On the 44th day the animals were injected with unaltered toxine G as follows: No. 1 (Wt. now 330 g.) received five minimal lethal doses; No. 2 (Wt. now 317 g.) received one M.L.D.; No. 3 (Wt. now 361 g.) received eight M.L.D. In no case did any symptom of tetanus appear.

A control g.p. of 339 g. which received eight M.L.D. died of acute tetanus in 48 hrs.

This showed that from a virulent toxine a modification could be easily obtained having no toxic properties but which still retained immunising power. The amount of immunity obtained by these methods may, to judge from the tests applied, not have been very great in degree. The last experiment detailed had however shown that immunity could be developed without any inconvenience to the animal employed, for the neutralisation of the acid did away with the risk of causing pathological effects by its strength. This method of immunisation by neutralised "HCl toxine" was therefore taken advantage of for investigating on a larger scale the extent of the protection effected

by modified toxine. The ordinary method of immunisation by gradually increasing doses of the immunising agent was also departed from. The disadvantage of the latter procedure is of course that, in increasing the dose, the investigator has no guide as to what increase in any particular stage it is safe to make. Though this danger was not present when one was working with toxines whose toxicity was entirely destroyed it was considered that many interesting results might be obtained by using all through the immunisation process exactly the same doses of the same toxine.

Experiment 10. Twenty guinea-pigs were taken all of them rather more than half grown so that the body weight might not increase at a very great rate during the immunisation process. The weights in g. were as follows: 543, 555, 580, 575, 505, 452, 533, 425, 545, 555, 450, 447, 505, 510, 710, 450, 517, 713, 465, 546. The toxine used was G, the M.L.D. of which at this time was for a g.p. of 250 grs. about ·03 c.c. Each inoculation consisted in the animal receiving hypodermically one-third of a c.c. of the toxine which had been exposed to normal HCl for four hours at the room temperature, the acid at the end of that time being neutralised by sodium hydrate.

All the twenty animals were inoculated on the 1st, 5th, 8th, and 11th days of the experiment. Ten of them received further injections on the 14th, 17th, 21st and 24th days after its commencement. In no case during the immunisation process were any symptoms of tetanus manifested.

There thus resulted two lots of animals one of which had received at nearly equal intervals of time four injections of equal amounts of the same toxine, the second of which had received eight similar injections at similar intervals. The degree of immunity thus obtained could be now enquired into, and especially it could be noted how the two groups differed in the amount of immunity possessed by each. With regard to each group two enquiries were conducted, firstly, What was now for each the fatal dose of tetanus toxine? Secondly, What amount of antitoxic action did the serum of each possess? It was found that the number of animals immunised was too small to enable complete answers to these questions to be given, but nevertheless the results were of an important character.

Experiment 11. Fatal dose of tetanus toxine for Series 1, *i.e.* those treated with four doses of modified toxine.

The toxine used for the test here was a sample known as E, the M.L.D. of which a number of experiments had shown to be ·09 c.c. for a g.p. of 250 g. It was thus a weak toxine.

On the 14th day after immunisation had been commenced and the 3rd after the last injection g.p. 1 (555 g.) received 66 M.L.D. No illness resulted.

On the 15th day after immunisation commenced, g.p. 2 (580 g.) received 111 M.L.D. Three days later it was markedly tetanic but from this it recovered.

On the 17th day g.p. 3 (575 g.) received 122 M.L.D. It was markedly tetanic on the following day and died 48 hrs. after inoculation.

The fatal dose for this series thus lay between 111 and 122 simple M.L.D.

Experiment 12. Antitoxic properties of serum of Series 1.

The investigation here was unfortunately hampered by the fact that two of the animals died before any use could be made of them, one from (?) tubercle and another from a diarrhoea which had carried off several animals in the laboratory about this time. Thus only two animals were available for obtaining serum. These were killed by arteriotomy, the blood collected in a cylindrical glass and allowed to stand 24 hrs. in a cool place. The serum was then poured off into a Petri's dish and evaporated to dryness over sulphuric acid *in vacuo*. For use, the desired amount was carefully weighed, dissolved in a measured quantity of ·75 % sodium chloride, then ·1 c.c. of toxine E was added (*i.e.* just over one fatal dose), the mixture was allowed to stand half-an-hour at the room temperature, and the whole then injected into the test animal.

The two animals whose serum was used in this experiment were killed during the week succeeding the 4th injection of the modified toxine.

No. of Guinea-pig	Weight in grammes	Amount (in grammes) of serum acting on toxine	Result
1	233	·001	Tetanic 2nd day: Dead 3rd day
2	235	·010	,, 2nd ,, ,, 3rd ,,
3	240	·050	,, 2nd ,, ,, 5th ,,
4	227	·175	,, 4th ,, ,, 7th ,,
5	275	·275	,, 4th ,, ,, 7th ,,
6	260	·498	,, 4th ,, Recovered

The amount of the serum of this series of immune animals necessary to neutralise one lethal dose for a guinea-pig thus lay between 275 and 500 milligrammes. It was unfortunate that a closer approximation could not, for the reasons already given, be arrived at. As it is the condition which ought to be complied with in estimating the strength of a serum, namely, the ascertaining the amount necessary to *completely* neutralise a M.L.D., could not be satisfied. The above experiment however justifies the conclusion that this amount must have been a little over 500 milligrammes. The serum was a very weak one.

Experiment 13. Fatal dose of tetanus toxine for series 2, *i.e.* the animals treated with eight doses of the modified toxine.

The toxine used here was a sample named F and a series of experiments had shown the M.L.D. for a g.p. of 250 g. to be ·0008 c.c.

Seven animals could be devoted to this experiment.

No. of Guinea-pig	Weight in grammes	Number of days between commencement of immunisation and injection of toxine	No. of M.L.D. injected	Result
1	447	31	100	No illness
2	510	32	200	,, ,,
3	600	35	300	,, ,,
4	629	38	400	,, ,,
5	517	39	600	,, ,,
6	465	39	800	Slight stiffness in one leg for a few days. Ultimate complete recovery
7	713	53	1000	Ditto

Thus the number of animals which could be given up to the elucidation of this point was exhausted before the dose of toxine which would be fatal was reached. It will be noted that in the cases of all the animals except the last the times which elapsed between the commencement of immunisation and the injection of toxine were roughly speaking double those given in the corresponding experiment with Series 1. The results of Exp. 12 as compared with those of Exp. 10 are so remarkable that it may be thought that not sufficient care was bestowed on the determination of the M.L.D. of the two toxines used. It may therefore be said that great care was taken in the matter and that, if anything, the error has been rather in making the figure too large than too small.

Experiment 14. Antitoxic action of the serum in Series 2.
The procedure was that pursued in Exp. 11.

No. of Guinea-pig	Weight in grammes	Amount (in grammes) of serum acting on toxine	Result
1	257	·050	No illness
2	274	·025	,, ,,
3	270	·010	,, ,,
4	260	·005	Slight stiffness for a few days,— complete recovery.
5	259	·001	
6	278	·0005	Ditto
7	265	·00025	Ditto

With this serum therefore a M.L.D. was completely neutralised by 10 milligrammes but not completely by five.

Experiment 15. The object of this experiment was to find out, if possible, in how far the immunising capacities of tetanus toxine were affected by the continued action of hydrochloric acid. The method was to take the same amount of toxine as that used in Exp. 10, but instead of exposing it for four hours to expose it only for half-an-hour. If the acid had been now neutralised the animal would

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have run the risk of death from the effect of the return toxicity. The mixture was therefore only diluted to a point at which no evil effects from the acidity would accrue to the animals. Two animals were thus treated and each received four injections of the modified toxine at the same intervals of time as in Exp. 10 and were killed during the week succeeding the fourth injection as in Exp. 12. Amounts of their sera corresponding to the amounts recorded in the latter experiment were tested in the same way with the result that no differences could be observed between the antitoxic properties and those of the sera in Exp. 12. This would indicate that the action of HCl on the immunising capacities of a toxine is very slow as compared with its action on the toxic properties.

Looking now at the results of Experiments 9, 10, 11, 12, 13, we see there are several noteworthy points.

(1) The M.L.D. of toxine G being ·03 c.c. and Series 1 being immunised by in all 1·33 c.c., and Series 2 being immunised by 2·66 c.c. of this toxine, it follows that in Series 1 forty-three M.L.D. produced an immunity equal to between 100 and 110 M.L.D., and in Series 2 eighty-six M.L.D. produced an immunity equal to an amount exceeding 1000 M L.D.

(2) When an animal is immunised by a series of injections of the same amounts of the same toxine and when another animal is immunised by double the number of similar injections the degree of immunity produced in the latter is not double the amount produced in the former in double the time.

To sum up what these experiments show regarding the action of HCl on tetanus toxine we may say that there is a period when the acid weakens but does not destroy the toxicity. This is succeeded by a period when the toxicity is held in abeyance by the acid, but during this time a certain degree of poisonous action can be made to reappear by neutralising the acid by an alkali, and further while the toxicity is in abeyance the power of conferring immunity is present. Finally there is a period when the phenomenon of return toxicity can no longer be elicited, but during the earlier part of which at any rate a very considerable immunising capacity still remains.

The action of hydrochloric acid on other toxines belonging to the same group as the tetanus toxine was investigated though not so thoroughly as in the case of the latter body.

II. *Action of Hydrochloric Acid on Ricin.*

The solution used here was made by taking 20 g. of castor oil seeds, bruising them in a mortar and extracting them for 24 hrs. with 100 c.c. of 10 per cent. sodium chloride. The emulsion was then filtered and a few crystals of thymol were added for purposes of preservation. The M.L.D. of this poisonous fluid was not determined, but one-thirtieth of a c.c. was sufficient to kill a large g.p. in 16 hrs. This toxine was found to be very resistent to the action of the acid as the following experiment will show.

Experiment 16. (*a*) One-thirtieth c.c. ricin exposed to one-thirtieth c.c. HCl of five times normal strength (i.e. actual effective strength of acid was two and a half times normal) for 6 hrs. at room temperature; the mixture was then injected into a g.p. of 250 g. Result: Death in two days.

(*b*) Procedure same as (*a*) but at end of time acid neutralised with normal sodium hydrate and the mixture injected into a g.p. of the same weight. Result: Death in the same time.

(*c*) Procedure same as (*a*) but the mixture kept 3 hrs. at 37 C. and the mixture injected into a g.p. of 243 g. Result: Death in 2 days.

(*d*) Same as (*b*) but the mixture kept 3 hrs. at 37° C. while the acid was acting. G.P. of 263 g. Result: Death in 3 days.

(*e*) Same as (*a*) but mixture kept at 37° C. for 6 hrs. before injection into g.p. of about 280 g. Result: No illness.

(*f*) Same as (*b*) but the mixture kept at 37° C. for 6 hrs. while the acid was acting. G.P. of about 280 g. Result: No illness.

In the case of this poison no evidence was forthcoming of the existence of the phenomenon of return toxicity. Only one immunisation experiment has up till now been done.

Experiment 17. A g.p. of 302 g. received one-thirtieth c.c. of the ricin solution which had been exposed to the same amount of five times normal HCl at 37° C. for 4 hrs. No illness resulted and the same process was repeated on the 5th and 10th days thereafter.

On the 72nd day thereafter the animal received ·15 c.c. of the ricin solution and never showed any symptoms of illness. A control animal of the same weight died in 16 hours.

This experiment is very interesting, for it will be observed that the result of the immunisation was to make the animal resistent to an amount of the toxine larger than the amount which produced the immunity.

III. *Action of Hydrochloric Acid and of Sodium Hydrate on Abrin.*

An abrin extract was prepared by soaking 20 g. of seeds in 100 c.c. of 10 per cent. NaCl for about a week with repeated bruising and then filtering the extract and adding a little thymol. Here one-twelfth of a c.c. was sufficient to kill a large g.p. in 16 hrs. A preliminary experiment showed that if this amount were exposed to an equal quantity of normal HCl at 37° C. for $3\frac{1}{2}$ hrs. the toxicity was destroyed. The result of the next experiment was at first puzzling.

Experiment 18. (*a*) One-twelfth c.c. of abrin was exposed to the action of normal HCl for $3\frac{1}{2}$ hrs. at the room temperature; and being diluted was injected into a g.p. of 320 g. Result: Death in 48 hrs.

(*b*) Procedure the same but the acid neutralised before injection into a g.p. of 311 g. Result: No illness. (It may be explained that in this experiment the acid was not only neutralised but the fluid was rendered slightly alkaline before injection.)

(*c*) Procedure the same as (*a*) but exposure $2\frac{1}{2}$ hrs. at room temperature; injection into g.p. of 316 g. Result: Death in 24 hrs.

(*d*) Procedure the same as in (*b*) exposure same as in (*c*); injection into g.p. of 293 g. Result: Ill next day but recovered.

On consideration the only explanation of this result which appeared feasible was that the recovery of the animals in (*b*) and (*d*) was due to the slight exposure to alkali destroying the toxicity of the abrin which had been little if at all affected by the previous exposure to the acid. This opened up a new field of inquiry.

Experiment 19. One-twelfth c.c. of abrin extract was exposed to the same amount of normal NaOH at the room temperature for 25 min. It was then injected into a g.p. of 335 g. Result: No illness.

(*b*) Procedure same as (*a*) but the alkali just over-neutralised with normal acid; injection into a g.p. of 283 g. Result: No illness.

It may be deduced from these experiments that in the case of abrin the poison is much more susceptible to the action of an alkali than it is to that of a powerful acid.

IV. *Effects of Acids and Alkalies on Diphtheria Toxine.*

The toxine used here was made by the inoculation of a bouillon prepared from meat which had been allowed to become putrid. Its M.L.D. was at first about ·01 c.c., but before the experiments were completed it had risen to about ·08 c.c.

Experiment 20. (*a*) ·5 c.c. of the toxine was exposed to ·5 c.c. of normal HCl for 25 min. and then injected into a g.p. of 518 g. Result: Death in 24 hrs.

(*b*) Procedure the same but exposure 1 hr. Result: the same.

Evidently if any comparison can be instituted between the action of this acid here and its action as already detailed in the experiments with tetanus toxine the latter is much more susceptible than is the case with diphtheria. The following experiment illustrates this point more forcibly.

Experiment 21. 1 c.c. of the toxine was exposed to the action of 1 c.c. of HCl of twice normal strength (*i.e.* the effective strength was normal), for a period of 4 hrs. A quantity of normal sodium hydrate was added not sufficient to neutralise the acid, in order that the acid originally present might not have an injurious effect on the animal. The whole was then injected into a g.p. of 397 g. Result: Death in 3 days.

(*b*) The same as (*a*); g.p. of 315 g. Result: Death in 2 days.

(*c*) The same as (*a*) but the acid was just over-neutralised and the fluid was thus slightly alkaline; g.p. of 384 g. Result: Death in 3 days.

(*d*) The same as (*c*); g.p. of 440 g. Result: Death in 3 days.

Thus an acid of considerable concentration is requisite for any effect whatever to be produced on this toxine. The only effect manifest in Experiments 19 and 20 is a delay in the fatal issue in cases where the toxine has been exposed longest to the most concentrated acid. The following experiment showed a way in which the toxicity of the poison may however be very easily destroyed.

Experiment 22. This was an earlier experiment performed before the resistance of the toxine to acid was known. It consisted in exposing the toxine to the action of the acid for different times and then just over-neutralising the acid with alkali.

	Amount of toxine in c.c.	Amount of normal HCl in c.c.	Exposure	Weight of guinea-pig in grammes	Result
(*a*)	·5	·5	½ hr.	250	No illness
(*b*)	·5	·5	1 ,,	255	Death 4th day
(*c*)	·5	·5	1½ hrs.	275	,, 24 hrs.
(*d*)	·5	·5	2 ,,	263	,, 24 ,,
(*e*)	·5	·5	4 ,,	335	,, 24 ,,

At first sight these results are perplexing. On consideration however at the time a possible explanation suggested itself. Through a slip, in the case of (*a*), instead of the acid being just over-neutralised, about ·5 c.c. excess of normal sodium hydrate was added and it also happened that as the g.p. was not quite ready for inoculation the mixture stood for a few minutes. The toxine was thus exposed to the action of one-third normal NaOH for a time after having been exposed to the action of the acid. A similar occurrence happened in the case of (*b*) but in a less degree.

It thus appeared possible that the alkali might have a powerful action on the toxine and this point was accordingly investigated.

Experiment 23. ·5 c.c. of the diphtheria toxine was exposed to the action of ·5 c.c. of normal sodium hydrate for different times; the mixture was then almost neutralised and after dilution was injected into the experimental animal.

Guinea-pig	Weight in grammes	Exposure	Result
(*a*)	370	5 min.	Death on the 14th day. (It was doubtful if this was consequent on the inoculation as one or two uninoculated animals in the hutches died at the same time)
(*b*)	400	15 ,,	No illness
(*c*)	405	30 ,,	,, ,,

Experiment 24. Procedure the same as in Exp. 22 but strength of alkali was one-tenth normal.

Guinea-pig	Weight in grammes	Exposure	Result
(*a*)	430	5 min.	Death 48 hrs.
(*b*)	360	15 ,,	No illness
(*c*)	393	30 ,,	,, ,,

There is thus no doubt that diphtheria toxine is very susceptible to the action of sodium hydrate. Here as in the case of abrin it is difficult to compare the action of an acid and an alkali but one can say that while the toxine is not very susceptible to the action of the one it is very susceptible to that of the other. As yet no evidence of the existence of the phenomenon of "return toxicity" has been obtained in the case of diphtheria toxine.

It was natural to enquire whether this modified diphtheria toxine had immunising properties. Up to the present only three animals have been treated with the view of settling this point. The experiment was as follows:

Experiment 25. Two guinea-pigs of 395 and 460 g. were taken and each received ·3 c.c. of the same toxine as above which had been exposed to the action of ·3 c.c. of normal sodium hydrate for 30 min. (the alkali being just under-neutralised with normal HCl before injection). Three days later they received 1 c.c. of the toxine which had been similarly exposed to 1 c.c. of the same NaOH, the subsequent treatment with HCl being the same. They again received 1 c.c. similarly treated on the 5th, 6th, 8th, 11th, 14th, 19th, 24th days after the first injection. On the 26th day one received ·5 c.c. of unmodified toxine,—equal at this time to about 25 M.L.D. and suffered no ill effects.

The action of alkali on diphtheria toxine thus while it destroys the poisonous effects does not entirely remove the capacity of immunisation.

Experiment 26. Antitoxic value of the serum of an animal immunised against diphtheria by toxine modified by alkali. On the 26th day the other g.p. of Exp. 24 was killed and its serum tested in the way detailed under Exp. 11 with the following results.

No. of Guinea-pig	Weight in grammes	Amount of serum acting on toxine	Result
1	225	·050	No illness
2	220	·010	,, ,,
3	225	·005	Death in 4 days

The amount of toxine used for mixing with the quantities of the serum detailed was ·08 c.c. This was just over one M.L.D.

The serum thus exhibited considerable antitoxic power.

V. *Action of Sodium Hydrate on Tetanus Toxine.*

Experiment 27. (*a*) 1 c.c. of tetanus toxine G was exposed to the action of 1 c.c. of normal sodium carbonate for four hours at room temperature; the alkali was then neutralised with normal HCl and injected into a g.p. of 235 g. Result: Death in two days.

(*b*) A second animal, weight 273 g., died in the same time.

(*c*) A third animal (275 g.) similarly treated except that the mixture was kept at a temperature of 37° C. for four hrs. died in 5 days.

(*d*) A fourth animal (213 g.) treated in the same way as (*c*) died in 7 days.

There was evidently a slight action by the alkali on the toxine, but as sodium carbonate is a weak body compared with sodium hydrate further experiments were done with the latter agent.

Experiment 28. (*a*) 1 c.c. toxine F was exposed to the action of 1 c.c. of normal sodium hydrate for 5 min., the alkali was then nearly neutralised with normal acid and the mixture injected into a g.p. of 324 g. Result: No illness.

(*b*) Same as (*a*). Exposure 15 min. Weight of g.p. 312 g. Result: No illness.

(*c*) Same as (*a*). Exposure 30 min. Weight of g.p. 412 g. Result: No illness.

(*d*) Same as (*a*) but the quantity of toxine exposed to the action of deci-normal sodium hydrate for half-an-hour; g.p. of 420 g. Result: Death in 2 days.

(*e*) Same as (*d*). Exposure 1 hr. Weight of g.p. 338 g. Result: No illness.

(*f*) Same as (*d*). Exposure 2 hrs. Weight of g.p. 370 g. Result: No illness.

It is thus evident that just as a strong acid like hydrochloric acid acts more strongly on the toxine than a weak acid like lactic acid, so a strong alkali like sodium hydrate acts more strongly than the weaker sodium carbonate. An immunisation experiment performed with tetanus toxine modified by NaOH was inconclusive.

The general result of these experiments is to show that acids and alkalies have an important effect in modifying the toxicity of certain toxines. That the modifications thus produced are to be classed with the toxoids of Ehrlich there can be little doubt. They are produced from virulent toxines, they show a toxicity less in degree than the original poisons and, like the toxoids naturally produced, they show a capacity of giving rise to immunity. Ehrlich has shown that there is reason to believe that the natural toxoids have a greater tendency to lose their toxicity than they have to lose their immunising power. There is little doubt that the same persistence of the immunising power exists in the artificial toxoids described. In Experiment 6 the whole immunisation was effected by toxine in which the toxicity was held in abeyance, and in Experiments 9 and 10 the toxicity was completely abolished. Experiment 6, in fact, furnishes corroborative proof of Ehrlich's contention that the toxic and the immunising actions of such a toxine as tetanus depend on different factors in it. Meantime the advantage of using non-poisonous toxoids for the production of immunity may be pointed out. The risks of giving **at** any particular stage of the process an overdose are thereby avoided. In the immunisation experiments given, the process has not been pressed in any case very far and yet in the case of tetanus very marked results have been obtained. In this connection it may however be pointed out that it is only within the limits of observation that it can be affirmed that for instance the toxine used in Experiment 10 can be said to have completely lost its toxicity. Certainly 5 c.c. was non-toxic but for quantities greater than this no assurance can be given as it is not safe or practicable to inject much more into a small guinea-pig.

Experiment 10 with its resultant experiments 11 to 14 are of importance because the same dose of the modified toxine was used throughout the whole process of immunisation. The very great rise in the immunity during the second period of the immunisation, though the stimuli remained constant, is in favour of Weigert's theory as to antitoxine production being due to a habit into which certain cells get of producing a certain kind of material.

It may be pointed out that the method of immunisation by means of equal doses of toxine given at equal intervals of time offers great advantages. By its means the progress of immunity can be studied and the immunising capacities of various bodies can be compared.

General conclusions.

(1) Tetanus toxine under the influence of hydrochloric acid loses with comparative readiness its virulently poisonous properties. It does not however so readily lose its capacities of producing immunity and when all trace of toxicity has disappeared the capacity of producing immunity still remains. The less poisonous substances produced in the modified toxine are probably of the nature of toxoids.

(2) Tetanus toxine is also susceptible to the action of alkalies such as sodium hydrate and sodium carbonate, under which it again loses its toxicity.

(3) Ricin is very resistent to the action of hydrochloric acid. There is evidence here also that when the toxicity is destroyed the capacity of producing immunity also remains.

(4) Abrin is also resistent to the action of hydrochloric acid but it is relatively susceptible to that of sodium hydrate.

(5) Diphtheria toxine is very resistent to the action of hydrochloric acid but it is relatively susceptible to the action of sodium hydrate. In the case of toxine which through the latter agent has had its toxicity destroyed there still remains evidence of the capacity of producing immunity.