ON THE OCCURRENCE OF TOXIC COMPOUNDS OF TETANUS TOXIN AND ANTITOXIN, TETANUS TOXIN AND BRAIN EMULSIONS[1].

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Introduction.

Ehrlich had not long announced his theory of immunity, when Wassermann and Takaki (1898) published their discovery, that the brain matter of the guinea-pig was capable of neutralising tetanus toxin. This was taken as a demonstration of the existence of Ehrlich's receptors in the cells of the normal brain.

Wassermann's strongest argument for the chemical nature of the combination between tetanus toxin and brain matter is its specificity.

A certain parallelism has been demonstrated to exist between the susceptibility and the toxin-neutralising property of the brain of an animal. Thus the cerebral cortex of highly susceptible animals, man and horse (Wassermann) and mouse (Metchnikoff) is strongly antitoxic, the brain of the less susceptible, rabbit, pigeon and fowl (Wassermann and Metchnikoff), has a feebler action, whereas in the case of the highly refractory tortoise and frog, the brain has no neutralising power.

Wassermann and Takaki thought at first, that brain and toxin

[1] This research was carried out in the laboratory of the Serum Department of the Lister Institute. To the Bacteriologist-in-charge of that department, Dr Dean, I am deeply indebted for inspiration and kindly criticism.

combined in the proportions which give the neutral mixture, just as on Ehrlich's theory a neutral mixture of toxin and antitoxin is one in which the combining affinities of both substances are satisfied. Metchnikoff (1898), however, pointed out that it was not necessary to conclude that the toxin was neutralised by the brain emulsion in the same way as by antitoxin. Metchnikoff, Roux and Salimbeni (1896) had shown that leucocytes could destroy cholera toxin, which they ingest while it is still retained within the bodies of the vibrios. Vibrios and toxin together are digested by the phagocytes. On the other hand a corresponding dose of toxin extracted beforehand from the vibrios, kills the test animal rapidly. Metchnikoff therefore argues, that the toxin in Wassermann's experiment may be absorbed physically by the solid particles of brain, and in this state may be taken up and digested by phagocytes.

Besredka (1903), went into the matter more thoroughly and showed that whatever the nature of the reaction, brain matter can combine with very much more toxin than it can neutralise. Besredka saturated the brain matter with a large excess of toxin, and then washed the combination in a centrifuge six times, till the washings only produced slight tetanus in a mouse, in a dose of 20 drops. One drop of the sediment of this brain matter was however sufficient to produce rapidly fatal tetanus in a mouse. The neutral mixture does not therefore represent a saturated chemical compound.

From the above brief outline of the literature of the subject it is evident that the nature of the combination between tetanus toxin and brain substance, is still open to discussion.

The main problems with which we have to deal are whether this combination is specific and whether it belongs to the same order of phenomena as the combination between antitoxin and toxin.

It seemed possible that a minute study of the quantitative combining relations under different conditions between brain substance and antitoxin respectively with tetanus toxin might throw further light upon the nature of their interaction.

The present paper records such a comparative study of the reaction between antitoxin and toxin, with that between brain and toxin.

In comparing a brain emulsion with an antitoxic serum, we are not really comparing a solid with a solution. The brain particles contain water, and, chemically considered, are equivalent to an emulsion of a colloid. Antitoxin is associated with the globulin of serum, and W. B. Hardy (1905) has shown that the transition from solution to precipitate, in the case of globulins, is not to be considered as a change

of state. The colloidal particles in the "solution" contain water and are bounded by a surface, but are too small to cause interference with a beam of light, and are easily held in suspension in water. The particles of the "precipitate" contain less water, and are larger, and therefore are visible, and sink to the bottom of the test tube. The difference between the clear "solution" and the flocculent "precipitate" is one of degree, not of kind.

The actual conditions under which tetanus toxin is absorbed by brain emulsion and antitoxic serum respectively are not therefore so dissimilar as might be supposed at first sight, and as shown by Danysz (1902) and Bordet (1903) some of the phenomena of toxin-antitoxin union suggest that this action is of the nature of absorption rather than that of combination between a strong (Ehrlich) or a weak (Arrhenius and Madsen) acid and base.

The relation of toxin absorbed by brain substance to original toxin concentration.

It seemed probable that the amount of toxin fixed by an emulsion of brain, would be some function of the amount of toxin originally present. This is the case, as may be shown by placing brain matter in the presence of varying quantities of toxin, centrifuging and estimating the amount of toxin remaining free in the supernatant fluid. By subtracting the observed free toxin from the known quantity originally present, we deduce the amount which has been fixed by the brain.

The following notation is used in the account of experiments:

−, =, ≡, denote increasing degrees of severity of local tetanus.

×, general tetanus, the animal certainly about to die within a few hours. Such animals were killed.

+, found dead.

The toxin used was prepared in April 1906. It was the filtrate from a 10 days' growth of tetanus bacilli in beef bouillon, previously freed from sugar by the growth in it of colon bacilli. It was preserved in the dark, at 10° C. under toluol.

This toxin was tested on mice on May 8th and October 22nd 1906, with the result given in Table I. It will be seen that the toxin lost a little in the five months interval.

TABLE I.

Dose per gram weight	Result after days given					
May 8, 1906	1	2	3	4	5	6
·000005	0	–	≅	≅	≅	≅
·00001	0	=	≅	+		
·00002	0	≅	≅	+		
·00004	0	≅	+			
·0001	0	×				
Oct. 22, 1906						
·000015	0	–	=	+		
·00003	0	=	≅	+		
·00006	–	≅	+			
·00012	=	+				

Experiment I. October 15th. Six tubes were charged with increasing doses of toxin; saline was added to make up the volume to 4 c.c. To each tube was added 2 c.c. of a 20 per cent. emulsion of guinea-pig's brain. The mixtures were allowed to stand for 11 hours at 10° C. and then a sample was removed from each tube and centrifuged. The supernatant fluid was tested for toxicity with the result given in Table II. In this table one mouse is selected for each tube, from a series all of which had different doses.

TABLE II. *Experiment I.*

October 16, 1906.

Total toxin	Toxic units lethal for 1 gm. of mouse in each ·024 c.c.	Total brain	Dose of supernatant fluid per gm. weight of mouse	Date and result									Toxic units, found in each ·024 c.c. of supernatant fluid
				17th		18th		19th		20th	21st	22nd	
				M.	E.	M.	E.	M.	E.	M.	M.	M.	
·3 c.c.	20	·4 gm.	·048 c.c.	0	–	=	=	≅	≅	≅	≅	+	$\frac{1}{8}$
·6	40	·4	·024	0	0	=	≅	≅	≅	≅	≅	+	$\frac{1}{4}$
1·1	73	·4	·048	0	=	≅	≅	×					$\frac{1}{2}$
1·6	107	·4	·024	0	–	≅	≅	+					1
2·4	160	·4	·012	0	–	≅	≅	+					2
4	267	·4	·003	0	–	≅	≅	+					8

The relation existing between the toxin found free in solution, and that combined with the brain matter.

In my experiments I have chosen as the unit of volume, not the whole contents of the test tube, but ·024 c.c. because the test mice had a dose of this volume, or some multiple or whole fraction of it, for each gram body weight. The toxic unit, in which I have expressed the

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quantities of toxin, is the amount per gram weight necessary to kill a mouse in 3 days (cf. Table I.). The unit of brain matter is the mass contained in ·024 c.c., that is ·0016 gram.

I have plotted the results in Fig. 1 where the ordinates represent the observed free toxin, and the abscissae the amount of toxin fixed by unit mass of brain matter. In the same figure I have plotted a curve calculated from Arrhenius and Madsen's equation for the reaction between toxin and antitoxin. It will be seen that the reaction between

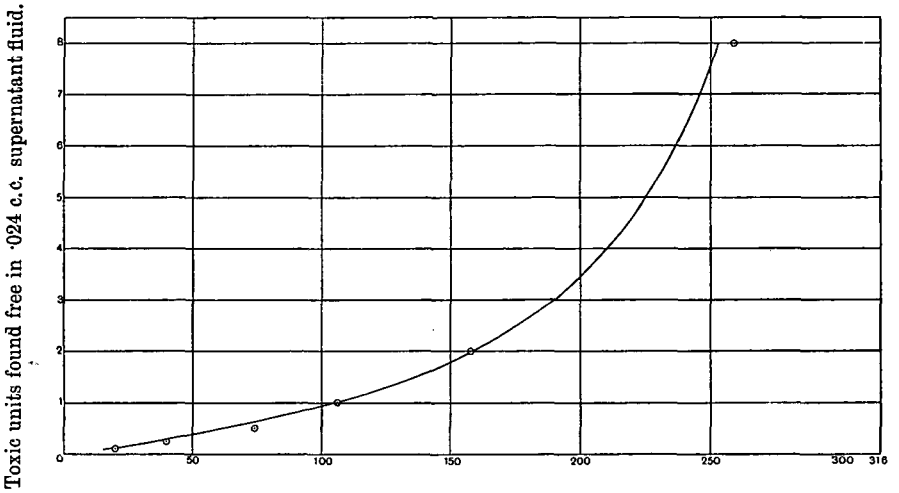

Fig. 1.

toxin and brain may very well follow the same law. The limit of error in the experiment is wide, so that I would not insist further on this agreement, but I hope to follow out this line of inquiry by a more accurate method.

The calculated and observed figures are given below:

Free toxin in ·024 c.c.	Observed combined toxin	Calculated combined toxin
1/8	20	18·6
1/4	40	35
1/2	72	63
1	106	105
2	158	158
8	259	253

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The form of equation used was

$$\left(\frac{\text{Free toxin}}{\text{volume}}\right)\left(\frac{\text{Uncombined brain}}{\text{volume}}\right) = K\,\frac{\text{Combined brain toxin}}{\text{volume}}.$$

The volume being unity may be neglected.

The dissociation constant, K, was found to be 2, and the amount of toxin equivalent to unit mass of brain (·0016 gram) was found to be about 316 toxic units.

Now whether we accept Madsen's or Bordet's view of the reaction between toxin and antitoxin, toxic combinations between the two must exist, comparable to Besredka's "cerveau toxique." These combinations will be formed whenever a toxic mixture of toxin and antitoxin is prepared. Their recognition, however, is difficult, because the presence of free toxin is a necessary condition of their formation. In spite of this they have been isolated in the case of ricin and antiricin. The combinations of ricin and antiricin under favourable circumstances form a precipitate, and Danysz (1902) found that, when ricin was present in excess, the precipitate contained practically all the ricin. He concludes justly that toxin and antitoxin "impregnate each other in variable proportions."

One cannot separate the compound of tetanus toxin with its antitoxin by the centrifuge, but one can remove the free toxin by allowing it to combine with brain matter, and centrifuging. The combined toxin and antitoxin then remain in the solution. In this way I have isolated toxic combinations of tetanus toxin with antitoxin, exactly comparable to Besredka's "cerveau toxique" and the toxic precipitate of Danysz.

In this series of experiments an antitoxin was used of which ·0032 c.c. mixed with 1 c.c. of my toxin gave a slightly toxic mixture. This was tested at the concentration at which the experiments were to be carried out. The amounts given in Table III. were diluted so that the volume in each tube was 8 c.c.; and mice received ·024 c.c. per gram body weight.

To show the combination of antitoxin with an excess of toxin above that contained in the neutral mixture, I chose a dose of antitoxin (·00016 c.c.) which would neutralise ·04 c.c. of toxin.

Test tubes were charged with ascending doses of toxin, beginning in the first tube with the $L+$ dose for the above quantity of antitoxin.

The volume in each tube was made up to 4 c.c. with ·85 per cent.

salt solution. The antitoxin contained in 2 c.c. of the same salt solution was then added, and the mixtures were set at 10° C. for some hours (Ta in the tables). After this time, 2 c.c. of a brain emulsion was added to each tube, and the series set aside again for a time (Tb) which was allowed for the brain matter to combine with the free toxin present. Then a sample from each tube was centrifuged, and the clear fluid tested on a mouse.

TABLE III.

September 3, 1906.

		Result and date						
Toxin	Antitoxin	4	5	6	7	8	9	10
·1 c.c.	·00024 c.c.	0	≡	≡	+			
·1	·00028	0	≡	+				
·1	·00032	0	0	–	–	survived		
		October 4, 1906.						
		5	6	7	8	9	10	11
·7 c.c.	·0016 c.c.	–	+					
·6	·0016	0	≡	+				
·5	·0016	0	=	≡	+			
·4	·0016	0	0	0	0	–	=	

In each experiment a control series was set up, in which no antitoxin was used. The same doses of toxin were diluted to 6 c.c.; and 2 c.c. of the same brain emulsion were added to each tube, immediately after the brain had been added in the antitoxin series. Samples from the control series were centrifuged immediately after the similar samples from the antitoxin series.

The test dose per gram weight of mouse was ·024 c.c. in every case.

TABLE IV. *Experiment II.*

August 30, 1906.

Ta = 21 hrs. at 10° C. Tb = 35 mins. at 36° C.

			Date and result							
Toxin	Antitoxin	Brain	31	1	2	3	4	5	6	7
·05 c.c.	·00016 c.c.	·3 gm.	•	–	–	–	–	–	–	–
·1	·00016	·3	•	–	≡	≡	≡	≡	×	
·2	·00016	·3	•	=	≡	+				
			Control Series.							
·05	0	·3	•	0	0	0	–	–	0	0
·1	0	·3	•	0	–	–	–	–	0	0
·2	0	·3	•	–	≡	≡	≡	≡	≡	≡

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TABLE V. *Experiment III.*

September 4, 1906.

Ta = 15½ hrs. at 10° C. Tb = ½ hr. at 36° C. and 2 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 5	6	7	8
·05 c.c.	·00016 c.c.	·2 gm.	0	0	0	0
·07	·00016	·2	0	0	0	–
·1	·00016	·2	0	0	–	≡
·14	·00016	·2	0	–	≡	≡
·2	·00016	·2	0	=	≡	+
		Control Series.				
·05	0	·2	0	0	0	0
·07	0	·2	0	0	0	0
·1	0	·2	0	0	0	0
·14	0	·2	0	0	–	–
·2	0	·2	0	0	–	–

TABLE VI. *Experiment IV.*

September 17, 1906.

Ta = 15 hrs. at 10° C. Tb = 6 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 18	19	20	21	22	23	24
·05 c.c.	·00016 c.c.	·2 gm.	0	0	0	0	0	0	0
·07	·00016	·2	0	–	=	≡	≡	≡	×
·1	·00016	·2	0	0	–	=	=	=	=
·14	·00016	·2	0	=	≡	≡	≡	≡	+
·2	·00016	·2	0	=	≡	≡	≡	+	
			Control Series.						
·05	0	·2	0	0	0	0	0	0	0
·07	0	·2	0	0	0	0	0	0	0
·1	0	·2	0	0	0	0	0	0	0
·14	0	·2	0	0	0	0	0	0	–
·2	0	·2	0	0	–	–	–	–	+

TABLE VII. *Experiment V.*

October 4, 1906.

Ta = 20 hrs. at 10° C. Tb = 5 hrs. at 10° C.

Toxin	Antitoxin	Brain	Date and result 5	6	7	8
1 c.c.	·0016 c.c.	·2 gm.	0	+		
2	·0016	·2	0	+		
4	·0016	·2	≡	+		
		Control Series.				
·5	0	·2	0	=	≡	+
1·5	0	·2	–	≡	+	
3·5	0	·2	=	+		

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TABLE VIII. *Experiment VI.*

June 19, 1906.

	Toxin made up to ·5 c.c.	Antitoxin made up to 2·5 c.c.	Brain made up to 2·5 c.c.	Date and result 20	21	22	23	24	25
Ta = 0	·0625 c.c.	·0001 c.c.	·05 gm.	0	mouse lost				
	·25	·0001	·05	0	≡	≡	+		
Ta = 32′	·0625	·0001	·05	0	0	0	0	0	0
	·25	·0001	·05	−	≡	≡	+		
Ta = 90′	·0625	·0001	·05	0	0	0	0	0	0
	·25	·0001	·05	0	=	+			
Ta = 270′	·0625	·0001	·05	0	0	−	−	=	≡
	·25	·0001	·05	0	+				

TABLE IX. *Experiment VII.*

June 11, 1906.

	Toxin made up to ·5 c.c.	Antitoxin made up to ·5 c.c.	Brain in emulsion in 1 c.c.	Date and result 12	13	14	15	16	17
Ta = 0	·0125 c.c.	·00002 c.c.	·01 gm.	0	0	0	0		
	·025	·00002	·01	0	0	0	0		
	·05	·00002	·01	0	≡	≡	+		
	·1	·00002	·01	=	+				
Ta = 6 hrs.	·0125	·00002	·01	0	0	≡	≡	≡	+
	·025	·00002	·01	0	=	+			
	·05	·00002	·01	0	=	≡	+		
	·1	·00002	·01	−	≡	+			

In all cases the antitoxin had combined with so much toxin, that the fluid in each tube of the antitoxin series was much more toxic than the fluid in the corresponding control tube.

The results are given in Tables IV., V., VI., VII.

In the last experiment (Exp. V., Table VII.) the amount of toxin in the control series was not the same as that in the antitoxin series, but this amount less by the $L+$ dose.

The long time taken by toxin and antitoxin to enter into combination is well illustrated by this method. In Exp. VI. four series of tubes were prepared, in which the time allowed for the combination of toxin and antitoxin varied from zero to $4\frac{1}{2}$ hours. Table VIII. shows the result. It is only after a considerable time that our toxic compound has become stable enough to resist dissociation by the brain emulsion. Table IX. refers to a similar experiment.

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Dissociation of brain-toxin compound.

Besredka (1903), in the paper already quoted, shows that the brain-toxin combination can be completely dissociated by means of antitoxin, and infers that antitoxin has a greater affinity for toxin than has the brain matter. Even so, both brain and antitoxin may owe their power of combining with toxin to the same side-chain. Organic chemistry is full of instances, where the affinities of a side-chain are altered in degree and not in kind by alterations in the radicle to which they are attached; instance the various chlor-acetic acids.

Besredka's experiment was as follows: To his "cerveau toxique" he added antitoxin and allowed the mixture to stand. The brain matter was then washed in the centrifuge, till the washings were free from antitoxin. It was then tested and found not only to be atoxic, but to have regained completely its protective power. The toxin had all gone over into combination with antitoxin. He used, however, an excess of antitoxin, though he does not state exactly how much.

Experiment VIII.

I repeated the experiment in a modified form, using a minimal quantity of antitoxin. The amount of antitoxin chosen was ·00032 c.c. the $L+$ dose for this quantity being ·1 c.c. of my toxin. ·1 c.c. of toxin was therefore mixed with ·2 gram of brain, in emulsion in 2 c.c. of saline, and the mixture was made up to 4 c.c. In a second tube ·2 c.c. of toxin and ·2 gram of brain were used. The mixtures were left for two hours at 10° C. The toxin remaining in solution in tube (1) would then be nearly nil, and in tube (2) considerably less than the amount required to kill a mouse in a dose of the volume generally used (*vide* Table V., control series).

To each tube was then added ·00032 c.c. of antitoxin, contained in 4 c.c. of saline, and the tubes were left for 8 hours at 10° C.

Now if the antitoxin has lost any of its protective power, it must have taken the toxin from combination of the latter with the brain matter; also there is enough toxin present to neutralise the whole of the antitoxin, so that, if the solution retains any protective power, we know that the brain matter has been able to hold in combination some of the toxin, in spite of the presence of unsaturated antitoxin.

The contents of the two tubes were centrifuged, and the supernatant fluids tested for antitoxin. It was found that unsaturated antitoxin was

present in each. Volumes of 1 c.c. were mixed with different quantities of toxin. Now we started with ·00032 c.c. antitoxin in each tube, and the volume in each tube was 8 c.c. Each cubic centimetre therefore corresponds to ·00004 c.c. of antitoxin, for which the L_0 and $L+$ doses of my toxin are respectively ·01 and ·0125 c.c. The tests are given in Table X. It will be seen that the fluid of tube (1) protected against ·005 c.c. of toxin, therefore that half the antitoxin was unsaturated with toxin, and that the brain matter had been able to retain in combination more than half the toxin which was given to it originally.

We cannot, therefore, argue that the combination of toxin with brain is of a different nature from the combination with antitoxin, on the ground that an excess of antitoxin can take all the toxin from the brain, to which it was previously bound. The toxin is divided between the brain and the antitoxin, and it is only in the presence of excess of the latter that increased mass-action enables it to take over all the toxin.

TABLE X. *Experiment VIII.*

October 4, 1906.

Fluid of tube (1)	Toxin (in 1 c.c.)	Date and result						
		5	6	7	8	9	10	11
1 c.c.	·005 c.c.	0	0	0	0	0	0	0
1	·0075	0	0	0	−	≡	≡	×
1	·01	0	0	=	≡	≡	×	
1	·0125	0	≡	≡	≋	+		
Fluid of tube (2)								
1	·005	0	0	−	≃	=	≃	≡
1	·0075	0	−	=	≋	≡	≡	+
1	·01	0	=	≡	+			
1	·0125	−	≡	+				

Moreover, there is already a large body of evidence showing that dissociation of a toxin-antitoxin combination can occur.

Behring and Ransom (1898) demonstrated this in the case of tetanus toxin and antitoxin, by dilution. Using a strong toxin, they prepared a mixture with antitoxin which was nearly neutral. The test mouse had very slight tetanus. This mixture was diluted 10 times, 100, 1000, and 10,000 times and the same volume of each dilution was injected into the test mice. The thousandth dilution caused death in three days, and all the dilutions were more toxic than the original mixture. If this mixture is allowed to stand for 24 or 48 hours before the dilutions are prepared, the effect is not obtained. The toxicity decreases gradually

as the dilution increases. After this time, therefore, the combination has become more stable.

R. Otto and Hans Sachs (1906) have lately confirmed these results.

Carl Bruck (1904), working with Wassermann, showed that if a toxin-antitoxin mixture be injected subcutaneously in the site of a previous injection of adrenalin, its toxicity is greater than in a control animal. He explains the result by stating that the antitoxin is forced to remain at the site of the inoculation, because of the vascular constriction, while the toxin which diffuses more readily, and travels along nerves, is removed from the sphere of influence of the antitoxin. He regards the experiments as a proof that dissociation occurs, and to a greater extent in the adrenalin animal than in the control; but it may be objected that the free toxin, in the absence of a normal circulation, is held at one spot in greater concentration, and is hence able to invade the axis-cylinders in larger quantities than when it is diffused through the body by the blood and lymph streams.

Finally J. A. Craw (1905 and 1906), following up Martin and Cherry's (1898) work with gelatine filters, succeeded in demonstrating the dissociation of a toxin-antitoxin compound in vitro. He used Myatherium lysin and antilysin, and passed neutral mixtures of the two through gelatine filters, under a pressure of 100 atmospheres. The antilysin has the larger molecules, or particles, and is held back by the filter while some of the toxin is forced through. A toxic filtrate is obtained.

The correspondences between brain and antitoxin in their reactions with tetanus toxin are seen to be fairly complete, but there remains the fact, that when we add brain emulsion to toxin, we do not obtain nearly so sharp a neutralisation point as we do on adding antitoxin. Exp. IX. (Table XI.) is an example of this. 1 c.c. of a 2 per cent. dilution of toxin was placed in each of 4 tubes. Different weights of brain, always emulsified in 1 c.c. of saline were added to each tube. Four

TABLE XI. *Experiment IX.*

October 22, 1906.

Toxin	Brain	Weight of guinea-pig	Date and result: 23	24	25	26	27	28	29	30
·02 c.c.	·3 gm.	325	0	0	–	=	≡	≡	≡	≡
·02	·16	330	0	–	=	≡	≡	≡	≡	≡
·02	·08	325	0	=	≡	≡	≡	≡	≡	≡
·02	·04	350	0	=	≡	≡	≡	+		

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guinea-pigs were used as test animals, and each had a dose of ·2 c.c. from a different tube. Though ·04 gram of brain was enough to delay death till the sixth day, ·3 gram was not enough to prevent altogether the onset of tetanus.

I am inclined to believe that this phenomenon is to be explained by the destruction of the brain particles within the tissues. Toxin might thus be set free, in the same way as ricin can be set free from its combination with fibrin by the aid of digestive ferments (Martin Jacoby, 1902).

Conclusions.

(1) The affinity of brain matter for tetanus toxin is specific, as is that of antitoxin.

(2) A solution of pure toxin is easily rendered innocuous by treatment with brain matter; but if a small dose of antitoxin has been added to the toxin some hours beforehand, treatment with brain matter no longer suffices to render the solution atoxic. The free toxin is removed, and we have isolated a toxic compound of toxin and antitoxin.

(3) Both brain-toxin combinations and antitoxin-toxin combinations dissociate with more or less rapidity, unless in the presence of enough free toxin (and free brain or antitoxin) to maintain the state of equilibrium. Consequently to obtain a neutral mixture one adds a large excess of brain or antitoxin beyond the combining equivalent. The dissociation is thus reduced till free toxin is no longer recognisable by our tests.

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