ADSORPTION OF TETANUS TOXIN BY BRAIN TISSUE

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(With 1 Figure in the Text)

INTRODUCTION

It was demonstrated by Wassermann & Takaki (1898) that when tetanus toxin was mixed with an emulsion of guinea-pig brain, its toxicity was reduced or abolished. Injection of brain emulsion gave some protection to mice injected with tetanus toxin 24 hr. later. They also noticed that the opalescent fluid obtained after centrifugation of brain emulsion in saline had no detoxicating effect, that cerebrospinal fluid was without effect, and that spinal cord was less effective than brain tissue. Emulsion of liver, spleen, bone marrow and normal serum from the same animal had no detoxicating effect.

These observations were confirmed by a number of other workers. It was further shown (Marie, 1898) that the cerebral cortex was more active than other parts of the brain, and that the brain of mammals was more effective than that of tetanus-resistant animals such as the hen, frog and turtle (Metchnikoff, 1898). Danysz (1899) showed that the degree of detoxication was affected by the type of diluent in which the brain was emulsified, and also that the toxin could be washed out of the tissue after adsorption. He also demonstrated that after heating for 20 min. at 100° C. guinea-pig brain would still reduce the toxicity of toxin and that, indeed, heating appeared to enhance the effect.

It had been noted previously that animals dying of tetanus had no free toxin in the central nervous system, but that toxin could be obtained by grinding up their brain tissue in saline (Tizzoni & Cattani, 1891).

Landsteiner & Botteri (1906) suggested that an alcoholic extract of brain would detoxicate tetanus toxin, but Marie & Tiffeneau (1908) were unable to produce any detoxicating effect with lipoids extracted from brain tissue. Raynaud & Wright (1953) have shown that proportionately very large doses of tetanus toxoid injected intravenously have an antagonistic or blocking action on tetanus toxin subsequently injected subcutaneously in mice. The blocking effect did not occur when diphtheria toxoid was used.

In all experiments quoted the reduction in potency of tetanus preparations has been demonstrated by estimating the reduction in the number of LD_{100} doses/ml. of the preparation. The toxicity of a toxin preparation can be altered, however, by natural toxoiding; in addition, measurements of toxicity cannot be made with any great precision.

The following work was undertaken to amplify further the observations already made and to determine whether the loss of potency was due to toxoiding; all tests

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were made by estimating the serum-combining power of the toxin preparations. This method takes into account the presence of toxoid in a toxic filtrate provided that a suitably *avid* antitoxin is used in the titrations (Barr, Glenny & Stevens, 1954).

MATERIALS AND METHODS

Brain and other tissues were removed from animals within 2 hr. of death. Brain from both rabbits and horses was used and the animals from which tissue was removed had not been actively immunized against tetanus.

Tetanus toxin and preparations of different tissues were mixed in large centrifuge tubes closed with rubber bungs and the mixtures incubated at 37° C. in a water-bath, and shaken at intervals. Incubation was carried out for $2\frac{1}{2}$ hr. except where otherwise stated. As a control, toxin without added tissue was treated in the same way. Mixtures were then centrifuged at 2500 r.p.m. for 10 min., after which the supernatant was withdrawn and the volume measured in graduated cylinders of a convenient size. Small samples were centrifuged in graduated tubes.

The tetanus toxins used were routine autolysate toxins prepared by lysis of a 2-day growth of *Clostridium tetani* by a slight modification of Raynaud's method using molar sodium chloride (Raynaud, 1951).

The *potency* of the toxins before and after adsorption with different tissues was calculated by estimating, from a number of tests, the volume of toxin which when mixed with 0·1 unit of laboratory standard antitoxin would kill a mouse in 72–96 hr., that is, the L+/10 dose. The total unit-equivalents of toxin, in terms of one unit of antitoxin in the material, were then calculated from the total volume of toxic material.

RESULTS

Reduction in potency of tetanus toxin after mixing with fresh rabbit brain

Two samples of fresh rabbit brain tissue were: (*a*) finely chopped with a scalpel, and (*b*) mashed with a pestle and mortar, and then exposed to the action of tetanus toxin for different periods of time at 37° C. The potency of the toxin was estimated before and after treatment, and the unit-equivalents of toxin adsorbed per gram of tissue calculated.

It was found that more toxin was adsorbed as the time of incubation increased, and that mashed brain took up toxin to a constant level very quickly (Table 1).

In this experiment it would appear that mashed brain adsorbs most toxin per gram of tissue. It was not appreciated at that time, however, that the relative proportion of adsorbent and adsorbate affected the equilibrium concentration. In the tests with mashed brain the weight of brain was considerably less than in the first three tests: the two sets of results in Table 1 are therefore not strictly comparable in this respect.

Two finely chopped rabbit brains were mixed thoroughly and divided into three portions having weight-ratios of about 1:2:4. The same volume of a toxin was added to each sample and the material treated in the usual way. It was found that the

Table 1. *The reduction of potency of tetanus autolysate toxin* (5·0 *ml.*) *after exposure to adsorbing action of fresh rabbit brain. The influence of time and the degree of division of the tissue on the quantity of toxin adsorbed*

State of tissue	Time of adsorption (hr.)	Weight of tissue (g.)	Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Added	Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Recovered	Total unit-equivalents of toxin adsorbed	Unit-equivalents of toxin adsorbed per gram of tissue
Chopped finely with scalpel	½	1·4928	0·018	0·06	19·45	13·70
	1	1·2981	0·018	0·05	17·80	13·71
	2½	1·1370	0·018	0·095	22·55	19·75
Mashed with pestle and mortar	½	0·9146	0·018	0·095	22·55	24·66
	1	0·9575	0·018	0·11	23·25	24·30
	2½	0·7365	0·018	0·11	23·25	31·43
Potency of toxin controls	(*a*) Heated at 37° C. for 2½ hr. 0·018 ml. ≡ 0·1 unit antitoxin (*b*) Unheated 0·018 ml. ≡ 0·1 unit antitoxin				5·0 ml. contains 27·75 unit-equivalents of toxin	

Note. For the purpose of calculating the total unit-equivalents of toxin adsorbed it is assumed that the toxin concentration of the supernatant after centrifugation of the adsorbing brain tissue will be the same as the concentration of toxin in the fluid retained in the interstices of the chopped tissue. The toxin lost can therefore be calculated from the difference in '*potency*' of the toxin before and after adsorption.

total unit-equivalents of toxin adsorbed increased as the quantity of adsorbent increased. The unit-equivalents of toxin adsorbed per gram of tissue, however, were reduced as the quantity of adsorbent increased (Table 2).

Table 2. *Reduction in potency of tetanus autolysate toxin* (10·0 *ml.*) *by adsorption with samples of chopped rabbit brain of different weight*

Weight of tissue (g.)	Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Added	Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Recovered	Total unit-equivalents of toxin adsorbed	Unit-equivalents of toxin adsorbed/g. of tissue
9·9125	0·007	0·026	104·4	10·4
4·1666	0·007	0·0135	68·8	16·5
1·9338	0·007	0·010	42·9	21·6

Effect of physical agents on the adsorptive capacity of rabbit brain for tetanus toxin

Finely chopped rabbit brain was treated with ethanol or acetone or both, dried on filter-paper and ground up; other samples were steamed at 100° C. for 30 min. and some material was steamed and then treated with ethanol or acetone. These different forms of treatment caused no significant alteration in the adsorptive capacity of the brain tissue. The quantity of toxin adsorbed per gram of brain (estimated on the weight of tissue before dehydration) was about the same for each sample (Table 3).

Table 3. *The reduction in potency of tetanus autolysate toxin* (10 *ml.*) *by adsorption with rabbit brain previously subjected to various forms of treatment*

Weight of brain before treatment (g.)	Physical treatment	Method of dehydration		Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Added	Potency of toxin (ml. ≡ 0·1 unit of antitoxin) Recovered	Total unit-equivalents of toxin adsorbed	Unit-equivalents of toxin adsorbed per g. of toxin
			A				
7·8658	Chopped finely	Absolute ethanol, 36 hr. R.T.	Dried off on filter-paper and ground to powder	0·005	0·0135	126	16·0
8·2134	Chopped finely	Absolute acetone, 36 hr. R.T.	Dried off on filter-paper and ground to powder	0·005	0·010	100	12·2
10·1272	Chopped finely and steamed for 30 min. at 100° C.	Nil	—	0·005	0·020	150	14·8
			B				
8·2571	Chopped finely and steamed for 30 min. at 100° C.	Absolute ethanol, 4 days R.T.	Dried off on filter-paper and ground to powder	0·0065	0·030	121	14·6
7·3212	Chopped finely and steamed for 30 min. at 100° C.	Absolute acetone, 4 days R.T.	Dried off on filter-paper and ground to powder	0·0065	0·021	106	14·5
8·1427	Chopped finely and steamed for 30 min. at 100° C.	1:1 ethanol-acetone mixture, 4 days R.T.	Dried off on filter-paper and ground to powder	0·0065	0·027	117	14·4

R.T. = room temperature.

Recovery of toxin from rabbit brain

Chopped rabbit brain treated in the usual way with tetanus toxin was washed several times with physiological saline after centrifugation and removal of the supernatant toxin. The material was washed three times in succession for 30 min. periods and the last change of saline was left at 37° C. for 24 hr.

The amount of free fluid retained by the tissue was estimated in each case and the total unit-equivalents of toxin retained (*a*) in the interstices of the tissue, and (*b*) adsorbed on to the tissue, were calculated. It was assumed that at each centrifugation the potency of the supernatant fluid would be the same as that of the free fluid in the tissue. From this experiment it appeared that the toxin present in the *free* fluid was rapidly removed by short periods of contact with saline, and that thereafter toxin diffused slowly out of the tissue. Some samples were kept for periods of several weeks and continued to yield small quantities of toxin into large volumes of saline. It became apparent, however, that in toxin-brain mixtures in saline, when equilibrium was reached, slow destruction of toxin took place even at 4° C. which made it impossible to estimate the total quantity of toxin which could be recovered (Table 4).

Antitoxin combining power of rabbit brain-tetanus mixtures

Two samples of chopped rabbit brain were treated with tetanus toxin in the usual way. The brain-toxin deposit after centrifugation was treated with tetanus antitoxin of known potency, the mixture was incubated at 37° C. for an hour. Rabbit brain not previously treated with toxin was also treated with antitoxin at the same time as a control.

The mixture was centrifuged and the supernatant titrated for antitoxin content. The total units of antitoxin and unit-equivalents of toxin lost were estimated from the volumes of recovered fluid and the potency of the recovered material.

Table 4. *The recovery of toxin from chopped rabbit brain* (7·5576 *g.*) *exposed to* 10·0 *ml. of tetanus autolysate toxin, by repeated washing with physiological saline* (10·0 *ml.*) *at* 37° C.

Fluid added to chopped brain	Time of contact of fluid with tissue	Vol. of fluid recovered by centrifugation (ml.)	Potency of fluid recovered (ml. ≡ 0·1 unit of antitoxin)	Unit-equivalents of toxin recovered in supernatant	Theoretical unit-equivalents of toxin retained as free fluid	Total unit-equivalents of toxin retained by tissue
Tetanus toxin	2½ hr.	8·4	0·014	59·9	11·5	121·9
Saline						
1st washing	30 min.	9·0	0·055	16·4	4·7	105·5
2nd washing	30 min.	10·0	0·25	4·0	1·0	101·5
3rd washing	30 min.	10·0	0·38	2·6	< 1·0	98·9
4th washing	24 hr.	10·0	0·045	22·2	5·8	76·7

Potency of toxin control before adsorption: 0·0055 ml. ≡ 0·1 unit of antitoxin, 10·0 ml. contains 182 unit-equivalents of toxin.

For the purpose of the estimations shown in col. 6 it has been assumed that fluid retained in the interstices of the brain tissue after centrifugation of the brain-toxin and brain-saline mixture will have the same concentration of toxin as the supernatant recovered on each occasion.

Table 5. *Neutralization of antitoxin by chopped rabbit brain previously exposed to tetanus autolysate toxin*

Weight of tissue (g.)	Vol. of toxin added (ml.)	Total unit-equivalents of toxin: Added	Total unit-equivalents of toxin: Lost	Vol. of antitoxin added (ml.)	Units of antitoxin added	Vol. of antitoxin recovered (ml.)	Units of antitoxin: Recovered	Units of antitoxin: Lost	Estimation of toxin retained as free fluid after first adsorption
					A				
8·2640	10·0	142·9	75·8	10·0	180	8·7	95·7	84·3	15·7
					B				
8·9245	20·0	425·6	176·4	10·0	260	10·0	100·0	160·0	58·4

Potency of toxins before adsorption: A, 0·007 ml. = 0·1 unit of antitoxin; B, 0·0047 ml. = 0·1 unit of antitoxin.

The total unit-equivalents of toxin lost in col. 4 has been calculated from the volume of supernatant recovered by centrifugation and the potency of that material. The estimation of toxin retained in the tissue as free fluid is shown in col. 10, and has been calculated from the volume of fluid lost on centrifugation, it is assumed that the concentration of toxin in the material will be the same as that in the supernatant.

An estimate was made of the toxin retained in the tissues as free fluid. It was found that the amount of antitoxin lost far exceeded the equivalent of that amount of toxin retained as free fluid, and was very close to the estimate of total unit-equivalents of toxin retained by the tissue (i.e. 'toxin lost', Table 5).

The control brain had no effect on the concentration of antitoxin in units/ml. with which it was treated though it did, however, retain 1·0 ml. of antitoxin as free fluid.

Two similar experiments were carried out in which the toxin-brain mixture was washed once with physiological saline prior to the addition of the antitoxin. This

had the effect of reducing the theoretical estimate of *free* toxin retained in the tissues to less than 1·0 unit-equivalent. In these two experiments the units of antitoxin neutralized approached very closely the calculated total unit-equivalents of toxin retained in the tissues after washing ('toxin lost, antitoxin lost', Table 6).

Table 6. *The neutralization of antitoxin* (10·0 *ml.* ≡ 210 *units*) *by chopped rabbit brain previously exposed to tetanus autolysate toxin* (10 *ml.*) *and washed in physiological saline* (20 *ml.*) *before the addition of the antitoxin*

Weight of brain (g.)	Total unit-equivalents of toxin added	Total unit-equivalents of toxin recovered in		Total unit-equivalents of toxin lost	Units of antitoxin lost	Estimate of free toxin retained as fluid in the interstices of the brain after washing
		Toxin-brain supernatant	Saline washings			
			A			
9·2762	153·8	59·3	5·26	89·2	80·0	< 1·0
			B			
8·9765	153·8	59·3	7·4	87·1	85·0	< 1·0

Potency of toxin used for both adsorptions: 0·0065 ml. ≡ 0·1 unit of antitoxin. 10·0 ml. contains 153·8 unit-equivalents of toxin.

The estimations of toxin and antitoxin recovered has been made from the volumes of fluid recovered by centrifugation and the potency of the materials as in Table 5.

These experiments suggest that toxin is not destroyed or chemically combined by contact with brain tissue since the addition of antitoxin to the brain-toxin mixture results in the neutralization of an amount of antitoxin about equivalent to the estimated quantity of toxin retained by that tissue. The objection may be raised that estimation of the amount of antitoxin neutralized in these tests may be erroneous as a result of the Danysz effect (Danysz, 1902). This will be discussed at a later stage.

Effect of time on the adsorptive capacity of rabbit brain

Samples of rabbit brain were kept at room temperature on the bench in beakers with watch-glass covers and then tested for their power to reduce the potency of tetanus toxin. The results obtained were variable; some samples gave results comparable with fresh tissue but others were very much less effective.

Adsorption of tetanus toxin by other tissues

Samples of chopped rabbit brain and other tissues were treated with tetanus toxin in the usual way. It was found that the potency of the toxin was not affected by contact with thigh muscle, liver, kidney, heart muscle, spleen, thigh bone or sciatic nerve. These materials were used in quantities of similar weight to that of the brain tissue, with the exception of the sciatic nerve, when the quantity of tissue available was rather small and the slight reduction in potency obtained was within the limits of error of the test of potency.

Adsorption capacity of dried brain for tetanus and other toxins (rabbit)

The adsorptive capacity of rabbit brain was found to be unaffected by steaming and dehydration with ethanol and acetone (Table 3). Samples of equal weight of dried rabbit brain were placed in contact with tetanus, diphtheria and *oedematiens* α toxins. The potency of the tetanus toxin in this test was reduced by 60 %: the other toxins were unaffected.

A quantity of ethanol-acetone-dried rabbit brain was further subjected to the action of boiling diethyl ether in a Soxhlet apparatus for 8 hr. This resulted in the extraction of a quantity of fatty material. The dried tissue so treated was still capable of adsorbing tetanus toxin to an unimpaired extent, and the fatty material after drying had no action on tetanus toxin.

Toxin adsorbing capacity of dried brain (horse)

Horse brain was steamed for 1 hr. at 100° C. and dehydrated with several changes of ethanol, acetone and diethyl ether. The material was lightly ground to a pale brown powder and 2·0 g. quantities of this material were mixed with different toxins. It was found that the potency of the tetanus toxin used was reduced by 76 %, but that the powder had no effect on the potency of filtrates containing *Clostridium welchii* α, β, ϵ, κ and staphylococcus α toxins. This dried material was stored in a screw-capped amber glass jar and was tested for its ability to adsorb tetanus toxin after 2 years at room temperature. The material retained its specific adsorptive capacity, although the colour had changed to orange.

Recovery of toxin from dried horse brain

A quantity of dried horse brain was used to adsorb tetanus toxin. After centrifugation the residue was washed four times with physiological saline. The first two washings were for 30 min. each at 37° C., the third washing was overnight (18 hr.) at 4° C. and the final washing was continued for 48 hr. at room temperature. The results showed that the first two short periods in contact with saline merely removed the *free* toxin in the tissue spaces, the longer periods, however, permitted adsorbed toxin to diffuse out. The total recovery by washing in this way only amounted to 25 % of the total toxin retained by the tissues after the initial adsorption (Table 7).

Relationship of in vivo *to* in vitro *combining power of tetanus toxin after consecutive adsorption with dried horse brain*

A quantity of tetanus toxin was adsorbed three times consecutively by three successive samples of dried horse brain and the supernatant obtained after each adsorption was tested by *in vivo* and *in vitro* methods. The *in vivo* value declined steadily as was expected, but after the third treatment it was found impossible to obtain flocculation in the region expected to correspond with the *in vivo* value of the last sample (Table 8).

Table 7. *Recovery of toxin from dried brain (horse, 8·0 g.) used to adsorb 80 ml. of tetanus autolysate toxin, by repeated washing with physiological saline*

	Time of contact with tissue	Temperature (° C.)	Vol. of fluid added (ml.)	Vol. of fluid recovered (ml.)	Potency of recovered fluid (ml.≡0·1 unit of antitoxin)	Unit-equivalents of toxin recovered	Unit-equivalents of toxin retained as free fluid	Total unit-equivalents of toxin retained
Tetanus toxin	2½ hr.	37	80	34	0·0033	1020	1380	11,284
Saline								
1st washing	30 min.	37	160	156	0·017	917	295	10,367
2nd washing	30 min.	37	160	160	0·30	48	17	10,319
3rd washing	18 hr.	4	500	500	0·045	1111	111	9,208
4th washing	48 hr.	R.T.	500	500	0·062	806	81	8,402

Potency of toxin before adsorption: 0·00065 ml.≡0·1 unit of antitoxin. 80 ml. contains 12,304 unit-equivalents.

Table 8. *The ratio L+ per ml./Lf per ml. of tetanus toxin repeatedly adsorbed with dried brain (horse)*

	In vivo value (unit-equivalents of toxin/ml.)	*In vitro* value (Lf/ml.)	Ratio: L+ per ml./ Lf per ml.
Unadsorbed toxin	154	157	0·98
Toxin after 1st adsorption	77	78	0·99
Toxin after 2nd adsorption	37	34	1·09
Toxin after 3rd adsorption	26	70	0·37

Influence of toxin concentration on the degree of adsorption

High potency tetanus toxin was diluted by stages (*ca.* 100 %) to a final dilution of 1/200. An equal volume of each dilution was added to 0·5 g. quantities of dried horse brain and treated in the usual way. The loss of potency of each sample was calculated as a percentage of the original potency, and the total unit equivalents of toxin adsorbed at each dilution stage was estimated.

It was found that the proportion of toxin removed from the majority of the samples was similar, though somewhat less with the most potent samples. However, the total unit-equivalents of toxin adsorbed increased as the potency of the sample increased (Table 9).

Table 9. *The adsorptive capacity of 0·5 g. quantities of dried brain (horse) for 10·0 ml. samples of tetanus toxin of different potency*

Dilution of toxin used	Potency of toxin (ml.≡0·1 unit of antitoxin)		Reduction of potency (%)	Total unit-equivalents of toxin adsorbed
	Added	Recovered		
1/200	0·135	0·40	66·2	4·9
1/100	0·7	0·21	66·6	9·5
1/50	0·036	0·105	65·8	18·3
1/20	0·014	0·041	65·8	47·0
1/10	0·0065	0·022	70·4	108
1/5	0·0033	0·0125	73·3	220
1/2	0·00135	0·0031	42·9	318
Undiluted	0·00065	0·0012	45·8	704

A formula expressing the relationship between the equilibrium concentration of a solution and the amount of the solution adsorbed on to a suitable surface was expressed as an equation by Freundlich (1926)

$$\frac{x}{m} = aC^n,$$

where x is the amount of a substance adsorbed on to a surface m, C is the final concentration of the substance in solution and a and n are constants for the systems employed.

If x represents the adsorption on to unit quantity of adsorbent then

$$\log x = \log a + n \log C.$$

The formula expresses the fact that increasing quantities of a substance are adsorbed per unit of adsorbing material with increasing concentration of adsorbate. The rate of increase is not directly proportional and depends on the systems employed.

If $\log x$ (unit-equivalents of toxin per gram of brain) is plotted against $\log C$ (equilibrium concentration of toxin after adsorption in unit-equivalents/ml.) the result should be a straight line until such a point is reached that saturation of the adsorbent takes place. When the results obtained in Table 9 were treated in this way, it was found that a curve of this nature resulted (adsorption isotherm, Fig. 1).

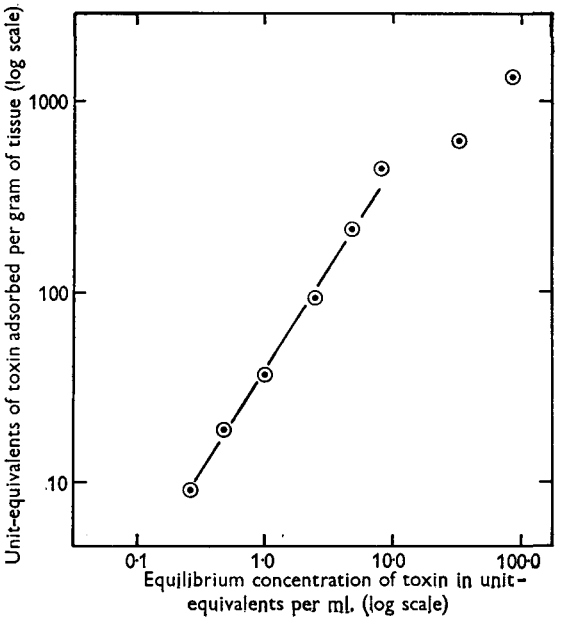

Fig. 1. The adsorption isotherm for tetanus toxin adsorbed on to dried brain (horse).

Effect on the toxin adsorbing capacity of dried horse brain of washing with different fluids prior to adsorption

Quantities of dried horse brain (0·5 g.) were treated with (*a*) physiological saline, (*b*) tetanus toxoid (8 Lf/ml.), (*c*) tetanus toxoid heated at 15 lb. pressure for 30 min., (*d*) and diphtheria toxoid (50 Lf/ml.). The excess fluid was removed by centrifugation and the residues treated with tetanus toxin (8 unit-equivalents/ml.).

It was found that when tetanus toxin was adsorbed with dried horse brain previously saturated with physiological saline, the potency of the toxin was reduced by 60 %. This reduction in potency is of the same order as that obtained by treating a similar quantity of dried horse brain (0·5 g.) with tetanus toxin of a similar potency without previously saturating the brain with saline (Table 9). When dried horse brain was saturated with tetanus toxoid or heated tetanus toxoid prior to the addition of tetanus toxin, the potency of the toxin added was reduced by 50 %. However, when the dried brain was saturated with diphtheria toxoid (50 Lf/ml.) prior to the addition of tetanus toxin, the potency of the toxin was only reduced by 36 %. This rather surprising effect was again obtained on repeating this test (Table 10).

Table 10. *Absorption capacity of* 0·5 *g. quantities of dried brain* (*horse*) *for tetanus toxin* (20 *ml.*) *after saturation of the tissue with different fluids*

Washing fluid	Potency of tetanus toxin (ml. ≡ 0·1 unit of antitoxin) Added	Recovered	Total unit-equivalents of toxin adsorbed
Physiological saline	0·013	0·031	90
Tetanus toxoid (8 Lf/ml.)	0·013	0·027	80
Heated tetanus toxoid	0·013	0·027	80
Diphtheria toxoid (50 Lf/ml.)	0·013	0·020	54

Absorptive capacity of dried horse brain for tetanus toxoid

A 0·5 g. sample of dried horse brain was treated with 10 ml. of 110 Lf/ml. tetanus toxoid, and the toxoid was recovered by centrifugation and tested in parallel with untreated toxoid by an *in vivo* total combining power method and by flocculation. There was no significant difference between the treated and untreated toxoids by either method: no toxoid appeared to have been adsorbed.

DISCUSSION

The reduction in *potency* (antitoxin combining power) of tetanus toxin by contact with brain has the characteristics of an adsorption phenomenon rather than those of chemical combination.

It is evident (Table 1) that the state of division of the tissue, that is, the surface area of tissue exposed, affects the speed with which toxin is taken up, and also that the relative amounts of adsorbate, and adsorbent determine the total amount of toxin removed, larger quantities of adsorbate remove more total toxin but the efficiency of adsorption becomes less per gram of adsorbent.

Toxin which has been adsorbed on to brain tissue can be recovered by washing with physiological saline. Washing for short periods (30 min.) appears to remove only that toxin which, it has been calculated, has been retained in the tissue as free fluid (Tables 4 and 7), while washing with saline over longer periods results in the recovery of toxin theoretically attached to the tissue. Some samples of brain kept for periods of several weeks continued to yield small amounts of toxin.

When antitoxin was added to toxin-brain mixture, the amount of antitoxin neutralized was about equivalent to the toxin present in the tissue. It is, however, possible that these results are misleading, since multiple combination of excess antitoxin with toxin might occur under these circumstances.

It has been suggested by Pappenheimer, Lundgren & Williams (1940) that multiple combination of diphtheria antitoxin with toxin occurs as complexes from TA 2 to TA 8. Barr *et al.* (1954), using five different diphtheria antitoxins and two preparations of toxin and of toxoid, have shown that multiple combination up to TA 3 was common to three antitoxins and that higher ratios could be obtained with gross excess of antitoxin. Their curves show that where the unitage of antitoxin added per Lf of toxin was between 1·5 and 2·0, the units of antitoxin combined per Lf of toxin would be about 1·25, but when the ratio of antitoxin to toxin was of the order of 2·5, 2·0 units of antitoxin would combine per Lf of toxin.

If multiple combination of antitoxin occurred to any appreciable extent, it would be expected to occur also with that portion of the toxin which remains after washing the brain with physiological saline, since the results obtained in Tables 5 and 6 are very much the same. It is observed, however, that the experiment shown in Table 5A is the only one in which more antitoxin was neutralized than would be expected on a proportional basis (the difference is probably within the limits of error). In Table 5B the ratio of antitoxin neutralized to toxin adsorbed by the brain is of the same order as that found in the two subsequent tests with brain-toxin mixtures washed before the addition of antitoxin (Table 6A and B). However, the two experiments shown in Table 5A and B have ratios of antitoxin added (col. 6) to theoretical toxin retained by the brain (col. 4) of 2·4 and 1·5 respectively. In these two experiments (Table 5) the brain was not washed prior to the addition of antitoxin. It may be possible therefore that excess antitoxin exhibits multiple combination with toxin when that toxin is free in the tissue (that is, not specifically adsorbed). This discrepancy was not observed in the experiments shown in Table 6A and B, although the ratio of antitoxin added (210 units) to toxin retained by the brain (col. 5) was 2·4 in these tests.

If multiple combination were to occur with adsorbed toxin also, it would be expected that the discrepancy between the tests in Tables 5A and 6 would be much greater. It seems extremely probable that some tetanus toxin must combine chemically with some cellular constituent in order to produce the profound physiological effects associated with the disease. It is possible, however, that as in the adsorption of certain dyes on textiles, primary adsorption is followed by chemical combination of a part of the material adsorbed, and that only a small fraction of the toxin attracted to the surface of nerve cells becomes combined in this way.

Consecutive adsorption of tetanus toxin by 1·0 g. quantities of dried horse brain resulted in a progressive fall in the potency of toxin by both *in vivo* and *in vitro* titration (Table 8). The last sample (after three adsorptions), however, failed to flocculate in the region expected. It has been suggested (Pope & Stevens, 1953) that flocculation of diphtheria toxin and antitoxin is a mass effect produced by interaction of a number of antigens and antibodies, the specific toxin-antitoxin

aggregates being carried down by the precipitate produced by the other interacting systems. Presumably adsorption by brain tissue is a specific effect since other toxins which have been tested are not adsorbed, in which case the non-specific antigens would be left in solution at each successive adsorption, until finally they are capable of acting individually at a different level with their respective antibodies, thus producing a discrepancy in the *in vivo/in vitro* ratio.

Samples of dried horse brain saturated with (*a*) physiological saline, (*b*) tetanus toxoid, (*c*) heated tetanus toxoid, (*d*) diphtheria toxoid, when subsequently used to adsorb tetanus toxin, gave rather curious results which were confirmed on repeating the experiments, so that the slight *blocking* effect produced by tetanus toxoid and heated tetanus toxoid is probably significant. It is surprising that diphtheria toxoid should produce an appreciable degree of blocking effect. Such a result was not obtained by Raynaud & Wright (1953) in their *in vivo* tests with mice. This may, however, be a non-specific effect and not relevant to the specific combination within the intact nerve cell, for adsorptive phenomena can be materially affected by the electrolytes with which the adsorbent is washed prior to adsorption of protein substances.

Although the results obtained by these experiments are in several ways unsatisfactory, a number of points have been raised which merit further attention.

SUMMARY

The potency of tetanus toxin as measured by combining power is markedly reduced by mixing with fresh or dried preparations of rabbit brain, and by dried horse brain. A number of other bacterial toxins are not so affected.

Tissues other than brain were not found to have this specific effect.

The state of division of the tissue, the time over which adsorption takes place, and the relative proportions of adsorbate and adsorbent affect the quantity of toxin removed. When the quantity of adsorbent and the volume of adsorbate are constant, the quantity of toxin removed per gram of adsorbent is proportional to the equilibrium concentration of the toxin, but not directly so.

Some of the tetanus toxin adsorbed onto fresh or dried preparations of brain tissue can be recovered by washing with physiological saline.

Brain toxin mixtures, when treated with tetanus antitoxin, neutralized an amount of antitoxin about equivalent to the toxin retained by the tissue.

Successive adsorption of tetanus toxin by brain tissue leads to an alteration in the ratio L+ per ml./Lf per ml. of the toxin.

The adsorption capacity of brain tissue may be reduced by first washing with tetanus toxoid or heated toxoid or with diphtheria toxoid.

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