The Fixation of Tetanus Toxin by Frog Brain

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SUMMARY

The apparent non-fixation of tetanus toxin by frog brain emulsion has been re-examined. Frog brain emulsion does fix tetanus toxin, but only with 1/2000th the capacity of mammalian brain emulsion. This low toxin-fixing capacity may be connected with the observation that the ganglioside in frog brain, unlike that in mammalian brain, is extractable with aqueous solvents.

INTRODUCTION

Rowson (1961) showed that when tetanus toxin at a concentration of 1000 mouse LD 50 doses/ml. was mixed with frog brain emulsion at a concentration of 250 mg./ml. there was no measurable fixation of toxin. We have now re-investigated this problem and have found that frog brain does indeed fix tetanus toxin, but that its toxin-fixing capacity is very low as compared with that of mammalian brain. A partial explanation for this low toxin-fixing capacity of frog brain has been found.

METHODS

Frog nervous tissue. Live *Rana temporaria* were obtained at intervals as required from Cornwall or Ireland. They were of both sexes and weighed between 15 and 30 g. They were kept at 4° on moist grass. The frogs were decapitated with large scissors and the brain and spinal cord dissected out, placed in chilled tubes and preserved at -20° when not required immediately. About 20% of the 'brains' consisted of spinal cord. The total yield of nervous tissue from each animal averaged about 100 mg. fresh wt.

Tetanus toxin. The material TD 464 D as described by van Heyningen (1959*b*) was used. It contained about 10 million mouse LD 50 doses/mg., or 25 million LD 50 doses/mg. protein.

Mammalian ganglioside. A partially purified beef-brain ganglioside was used (van Heyningen & Miller, 1961).

Sialic acid. This was assayed by the difference in the resorcinol values (Svennerholm, 1957) before and after hydrolysis with 8 N-HCl for 30 min. at 100° (Long & Staples, 1959).

RESULTS

Fixation of 2 LD 50, 10 LD 50 and 20 L+ units of tetanus toxin. Determinations were made, on various species of animal, of the least amount of brain tissue that would fix 10 mouse LD 50 doses and 20 L+ units (500,000 LD 50) of tetanus toxin, by the biological assay methods of van Heyningen (1959*a*). The results are shown in Table 1. It was not possible in these tests to use brain tissue at a higher concen-

tration than 250 mg./10 LD₅₀ because the brain suspensions were diluted with an equal volume of test toxin solution, and 0.5 ml. of this mixture injected into the mice. In the case of frog brain, although 250 mg. did not protect mice against the lethal effects of 10 LD₅₀ toxin, it appeared to bring about a slight delay in the onset of toxic symptoms. We therefore decided to test the toxin-fixing capacity of frog brain with 2 LD₅₀ instead of 10 LD₅₀, in the manner referred to above. In each of two experiments 94 mg. fresh wt. of frog brain fixed 2 LD₅₀ of tetanus toxin, and in one experiment 38 mg. frog spinal cord fixed 2 LD₅₀ of toxin. Thus, if it be assumed that 0.05 mg. guinea-pig brain can fix 2 LD₅₀ toxin (since 0.25 mg. fix 10 LD₅₀; Table 1), it appears that guinea-pig brain has about 2000 times the tetanus-toxin-fixing capacity of frog brain.

Table 1. *Tetanus-toxin-fixing capacity of brain tissue from various species*

	mg. fresh wt. of brain fixing	
	10 LD ₅₀ doses toxin	20 L + units (0.5 million LD ₅₀ doses toxin)
Guinea-pig	0.25	11
Chicken	0.40	50
Beef	2.50	100
Frog	250 mg. fail	250 mg. fail

Table 2. *Non-diffusible sialic acid contents of guinea-pig and frog nervous tissue (mean of five estimations in each case)*

	μ mole/g. fresh wt.
Guinea-pig cerebral cortex	0.61
Frog brain	0.27
Frog spinal cord	0.26

Ganglioside content of frog brain. Since it had been shown previously that ganglioside was responsible for the fixation of tetanus toxin by mammalian brain (van Heyningen, 1959c; van Heyningen & Miller, 1961), we investigated the ganglioside content of frog brain. The fresh frog brain was extracted with 19 volumes of chloroform + methanol (2 + 1 by vol.) and the ganglioside transferred from this extract into 0.1 M-KCl in water by the method of Folch, Lees & Sloane Stanley (1957). The aqueous phase was dialysed against distilled water for 18 hr. at 4° and sialic acid determined in suitable samples. It was assumed that these values reflected the ganglioside values. The results in Table 2 show that the relatively low toxin-fixing capacity of frog nervous tissue was not due to a lack of ganglioside.

Toxin-fixation by frog-brain ganglioside. Crude preparations of frog-brain ganglioside (0.17 μ mole sialic acid/mg.; compare 0.8 μ mole sialic acid/mg. purified beef-brain ganglioside) were made by the methods described for beef-brain ganglioside by van Heyningen & Miller (1961). On thin layer chromatography the frog ganglioside behaved like purified beef-brain ganglioside. The toxin-fixing capacity of crude

frog-brain ganglioside was measured by the analytical ultracentrifugal method of van Heyningen & Miller (1961); 1 mg. of the crude ganglioside preparation fixed 1 mg. tetanus toxin (compare 4 mg. toxin fixed/mg. purified beef-brain ganglioside). Thus the toxin-fixing capacity of frog-brain ganglioside appears to be similar to that of mammalian brain ganglioside.

Distribution of ganglioside in frog brain. The ganglioside in mammalian brain cannot be extracted with aqueous solvents (see Folch, Arsove & Meath, 1951; van Heyningen, 1959*c*). However, we found that the ganglioside of frog brain is readily extractable with water, 0.1 M-phosphate buffer (pH 7) and frog Ringer solution. Frog brain was homogenized in 5 volumes of each of these solvents for 20 min. and centrifuged at 5000 *g* for 1 hr. The aqueous supernatant fluid was dialysed against three changes each of 5 l. distilled water during 24 hr. and the non-diffusible sialic acid determined. The results are shown in Table 3. It appears that all the ganglioside in the frog brain was extractable with aqueous solvents.

Table 3. *Non-diffusible sialic acid in aqueous extracts and residues of frog brain*

	μ mole sialic acid/g. fresh wt.								
	Water			0.1 M-phosphate buffer (pH 7)				'Frog Ringer'	
Extract:	0.27,	0.33,	0.17,	0.27,	0.25	0.17,	0.27,	0.09*,	0.10*
Residue:	0,	0,	0.2,	0,	0	0,	0,	0*,	0*

* These values obtained in July, remainder in April/May.

Tetanus-toxin-fixation at low toxin concentration. In the experiments on the fixation of tetanus toxin by frog brain, the frog ganglioside would have been in solution and would not have been sedimented in the centrifugal field (5000 *g*) used. The experiment with 10 LD₅₀ of tetanus toxin was therefore repeated, but this time the frog brain was centrifuged at 198,000 *g* (50,000 rev./min. in the Spinco L 50) for 2 hr. Again it was observed that 250 mg. frog brain did not fix 10 LD₅₀ of toxin, although all the ganglioside was recovered from the sedimented residue. However, we had reason to believe that isolated ganglioside (as distinct from ganglioside *in situ* in the brain) would have a very low toxin-fixing capacity in dilute toxin solutions (20 LD₅₀/ml.) although it has a very high toxin-fixing capacity in concentrated toxin solutions (namely, 4 mg. toxin fixed/mg. ganglioside at 5 mg. toxin/ml., or 100 million LD₅₀ fixed/mg. ganglioside at 125 million LD₅₀ toxin/ml.; van Heyningen & Miller, 1961).

It had previously been observed (van Heyningen, 1959*a*) that at low toxin concentrations the toxin receptor activity of the 'protagon' fraction of mammalian brain (i.e. crude water-insoluble ganglioside + cerebroside complex) was not greater than that of whole brain, whereas at high toxin concentration it was much greater. This was confirmed in an experiment in which beef-brain ganglioside at 0.5 mg./20 LD₅₀/ml. 0.1 M-phosphate buffer (pH 7), was centrifuged at 198,000 *g* for 2 hr. Less than 20 LD₅₀ toxin/ml. were centrifuged down, whereas the same concentration of ganglioside in the presence of 125 million LD₅₀ toxin/ml. would have centrifuged 50 million LD₅₀ toxin/ml. (van Heyningen & Miller, 1961).

DISCUSSION

The susceptibility of the frog to tetanus toxin increases with temperature; at 26° the frog is about 3000 times as resistant as the mouse (Rowson, 1961). It is not known what the relative susceptibilities of these two species would be at the same temperature, but a very rough extrapolation of Rowson's data (the temperature effect curve flattens out above 23°) suggests that if it were possible to maintain frogs at 37° the increase in susceptibility over that at 26° would be less than tenfold. We have shown that mammalian (guinea-pig) brain has about 2000 times the toxin-fixing capacity of frog brain, and perhaps the greater resistance of the frog to tetanus toxin is due to the smaller capacity of its nervous tissue to fix the toxin. This low toxin-fixing capacity of frog brain is not due to a lack of ganglioside, or to the inability of the isolated ganglioside to fix toxin under the same conditions as does mammalian brain ganglioside. It appears to be connected with our observation that frog-brain ganglioside, unlike mammalian brain ganglioside, is extractable with aqueous solvents. In the test system for measuring tetanus toxin-fixation by brain tissue, the frog-brain ganglioside is therefore in solution and in a different physical state from that in emulsified mammalian brain (or in the intact frog brain?). At very high toxin concentrations (125 million LD 50/ml.) free ganglioside in solution has a far greater toxin-fixing capacity than brain emulsion (namely, 100 million LD 50/mg. ganglioside, as compared with 5000 LD 50/mg. fresh brain tissue; van Heyningen, 1959*a*; van Heyningen & Miller, 1961); but at low toxin concentration (20 LD 50/ml.) mammalian brain emulsion has a greater toxin-fixing capacity than free ganglioside in solution (namely, 40 LD 50/mg. fresh brain tissue, as compared with < 40 LD 50/mg. free ganglioside). This anomalous behaviour has been observed before, when it was suggested that it might be due to slight differences in the curves of the adsorption isotherms for brain emulsion and isolated receptor at low concentrations of adsorbate (van Heyningen, 1959*a*).

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