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FIXATION OF TETANUS TOXIN BY SUBCELLULAR FRACTIONS OF BRAIN

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TETANUS toxin is fixed by the grey matter of nervous tissue (WASSERMANN and TAKAKI, 1898; FULTHORPE, 1956; VAN HEYNINGEN, 1959*a*). JANOFF (1964) has recently reported evidence of selective fixation of the toxin by subcellular fractions of rabbit brain homogenate spun down between 800 g × 10 min and 17,000 g × 20 min (mitochondria and lysosomes) and between 17,000 g × 10 min and 100,000 g × 60 min (microsomes). The present work confirms that toxin-fixing capacity can be recovered in the fractions sedimenting in these ranges, but the data suggest that it is not the mitochondria or microsomes that are responsible for the fixation.

MATERIALS AND METHODS

Subcellular fractionation. In most of this work, guinea pig brain homogenate was prepared and fractionated by the method of GRAY and WHITTAKER (1962; except that the P_1 fraction was not washed). In this case the P_2 (crude mitochondria) fraction was obtained by spinning the supernatant of the nuclear fraction at 17,000 g for 55 min (Table 1; P_2 *i*). In addition, some data have been included from experiments where a P_2 fraction was obtained by spinning at 12,000 g for 20 min (Table 1; P_2 *ii*). In the first case, the supernatant of the P_2 fraction was a relatively pure preparation of microsomes while in the second case it was more contaminated with mitochondria and other particles (Table 1; S_2 *i* and S_2 *ii*).

The fractionation procedure was on each occasion followed by electron microscopic examination of the fractions in negative staining (HORNE and WHITTAKER, 1962).

Tetanus toxin was the preparation TD464D kindly supplied by Dr. R. O. THOMSON of the Wellcome Research Laboratories, Beckenham, Kent; it contained 40 per cent of protein of which 75 per cent was toxin (see VAN HEYNINGEN, 1959*b*). Dilutions of the toxin were made in 0·1 M-phosphate buffer, pH 7·0, containing 0·2% gelatin.

Toxin-fixing capacity was measured by finding the least volume of suspension of nervous tissue that fixed either 10 LD_{50} units or 20 L+ doses (about 0·5 million LD_{50} units) of toxin (VAN HEYNINGEN, 1959*a*). The volume of tissue suspension fixing 10 LD_{50} units of toxin is about one fortieth of that fixing 20 L+ doses, but since the toxin-fixing capacities of subcellular fractions are expressed in this paper relative to that of the original brain homogenate these differences are, for the present purposes, irrelevant.

The percentage recovery of toxin-fixing capacity from the original brain homogenate in the subcellular fractions is calculated by taking into account the volumes of the suspensions of fractions and of the original homogenate fixing 10 LD_{50} units or 20 L+ doses of toxin and the total volumes of these suspensions. Toxin-fixing capacity per unit dry weight is calculated as the reciprocal of the mg dry weight in the volume of suspension fixing 10 LD_{50} units or 20 L+ doses of toxin. Relative toxin-fixing capacity is obtained by dividing the values for toxin-fixing capacity per unit dry weight by the values for the toxin-fixing capacity of the original homogenate. Dry weights of suspensions were determined by drying samples at 105° for 12 hr after dialysis against running tap water for 48 hr, taking into account any changes in volume during dialysis.

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RESULTS

Subcellular fractions of guinea pig brain were prepared and their toxin-fixing capacities per unit volume and per unit dry weight determined.

Relative toxin-fixing capacity. The data in Table 1 show that toxin-fixing capacity tends to be concentrated in the crude mitochondrial fractions (P_2 *i* and P_2 *ii*), and in the myelin (*A*) and nerve-ending particle (synaptosome, *B*) fractions (prepared from

TABLE 1.—DISTRIBUTION OF TETANUS TOXIN-FIXING CAPACITY IN SUBCELLULAR FRACTIONS OF BRAIN

Fraction	Centrifugation	Description	Toxin-fixing capacity*	
			% Recovery	Relative to *H*†
			From *H*:	
H		Whole homogenate	100	1
P_1	1,100 g × 11 min	Nuclei, cell debris and myelin	23 ± 6 (8)	0·54 ± 0·17 (8)
P_2 *i*	1,100 g × 11 min–17,000 g × 55 min	Mitochondria, synaptosomes, large microsomes and myelin fragments	40 ± 16 (5)	1·16 ± 0·46 (5)
S_2 *i*	above 17,000 g × 55 min	Microsomes	6·3 ± 3·0 (6)	0·32 ± 0·07 (4)
			From P_2 *i*:	
A	Subfractionation of P_2 *i* on density gradient	Myelin	25 ± 15 (4)	1·21 ± 0·49 (6)
B		Synaptosomes	39 ± 15 (4)	1·72 ± 0·70 (7)
C		Mitochondria	6·0 ± 4·9 (5)	0·70 ± 0·56 (6)
			From *H*:	
P_2 *ii*	1,100 g × 11 min–12,000 g × 20 min	Mitochondria, large synaptosomes and myelin	30 ± 15 (3)	1·80 ± 0·67 (3)
S_2 *ii*	above 12,000 g × 20 min	Microsomes and small synaptosomes	40 ± 27 (4)	0·85 ± 0·26 (3)

* ± Standard deviation; the figures in brackets denote the number of experiments.

† $\frac{\text{Toxin-fixing capacity per unit dry weight of fraction}}{\text{Toxin-fixing capacity per unit dry weight of } H}$

P_2 *i* by density gradient centifugation). Since the relatively homogeneous mitochondrial fraction (*C*) of the density gradient has a low toxin-fixing capacity, the higher capacity of the crude mitochondrial fractions (P_2 *i* and P_2 *ii*) could be due to their content of synaptosomes. There also appears to be some concentration of toxin-fixing capacity in the myelin (*A*) but whether this is due to myelin itself or to contamination of the fraction with synaptosomes is not clear. The crude nuclear fraction (P_1), which contains a relatively large amount of myelin, has a low toxin-fixing capacity. (It has long been known that the toxin-fixing capacity of white matter is low; WASSERMANN and TAKAKI, 1898.)

It is evident that the toxin-fixing capacity of microsomes (S_2 *i*) is low; the greater toxin fixation by S_2 *ii* as compared with S_2 *i* is presumably due to some synaptosomes (and myelin?) which are not spun down from the supernatant (S_2 *ii*) during the shorter period of centrifugation at a lower speed.

Recovery of toxin-fixing capacity. The total amount of toxin-fixing capacity recovered in P_1, P_2 *i* and S_2 *i* is only about 70 per cent of that in the original homogenate; similarly, the total amount recovered in *A*, *B* and *C* is only about 70 per cent of that in P_2 *i*. There is no apparent reason fo ascribing this loss to the preparation of any

particular fraction. In the relatively pure preparations of microsomes (S_2 *i*) and mitochondria (*C*) there is a low yield of toxin-fixing capacity; in the relatively pure preparations of myelin (*A*) and synaptosomes (*B*) there is a high yield of toxin-fixing capacity; when the microsomal and mitochondrial fractions contain synaptosomes (S_2 *ii* and P_2 *i*, P_2 *ii*) the yield of toxin-fixing capacity is high.

DISCUSSION

The present indication that tetanus toxin may be fixed by synaptosomes suggests that in the intact animal the toxin would be fixed at synaptic regions in the central nervous system. If there were a relation between the fixation of tetanus toxin by nervous tissue and its action, these findings would be relevant to the demonstration by Brooks, Curtis and Eccles (1957) that the toxin suppresses synaptic inhibition. However, there is at present no evidence whether the fixation of tetanus toxin is, or is not, essential for its toxic action (see Discussion in van Heyningen, 1959*c*).

The fixation of the toxin by nervous tissue is due to ganglioside (van Heyningen, 1959*c*; van Heyningen and Miller, 1961). However, recent experiments have shown that the toxin-fixing capacity of ganglioside can be greatly increased under certain conditions by complexing it with cerebroside or sphingomyelin—substances which do not themselves fix toxin (van Heyningen and Mellanby, in preparation). Since there is an optimal proportion of ganglioside for fixation by these complexes, and since it is not yet known how other substances in nervous tissue may affect toxin fixation by ganglioside, no attempt has been made to correlate the toxin-fixing capacity of the subcellular fractions with their absolute ganglioside content.

The data on the recovery of toxin-fixing capacity in the different fractions can be compared with the qualitative results obtained by Janoff (1964). He observed selective adsorption of toxin by mitochondrial and microsomal fractions. In his procedure, these fractions roughly correspond with the P_2 *ii* and S_2 *ii* fractions in the present work (see Table 1) both of which have a relatively high yield of toxin-fixing capacity. The present analysis of toxin-fixing capacity reveals that despite the high yield in the microsomal (S_2 *ii*) fraction its toxin-fixing capacity per unit dry weight is low. The further fractionation of a crude mitochondrial preparation (P_2 *i*) shows that the high toxin-fixing capacity of such fractions (P_2 *i* and P_2 *ii*) is unlikely to be due to mitochondria.

SUMMARY

Assays of the capacity of subcellular fractions of guinea pig brain to fix tetanus toxin showed that the toxin was bound more by fractions with a high content of synaptosomes (and nearly as well by a myelin fraction) than by relatively pure mitochondrial or microsomal fractions.

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