Brit. J. Pharmacol. (1966), **26**, 740–747.

SUBCELLULAR FIXATION OF TETANUS TOXIN IN SUSCEPTIBLE AND RESISTANT SPECIES

A. A. PATEL AND S. SRINIVASA RAO

BY

From the Department of Immunology, Haffkine Institute, Bombay 12

(*Received January* 7, 1966)

It was shown in the previous paper (Patel & Rao, 1966) that tetanus toxin brings about marked lowering of ATP formation in the brain tissue of the susceptible rat but not of the resistant pigeon. To understand more clearly the biochemical lesion leading to paralysis produced by tetanus toxin it is necessary to know the site of its fixation.

The receptor for tetanus toxin in nervous tissue was identified by Van Heyningen (1959) as the gangliosides. These are acidic, acid-sensitive, nondialysable, water-soluble complex glycolipids, containing fatty acid (stearic acid), Sphingosine, hexose (glucose and galactose), hexosamine (N-acetyl galactosamine), and occur largely in the central nervous system and primarily in the grey matter. Van Heyningen (1959) carried out the fixation studies with tetanus toxin and ganglioside preparations using ultracentrifuge. As ganglioside is water-soluble, the biological and chemical assays could not be carried out by centrifuging at low gravity (5,600 *g*), at which speed ganglioside is not sedimentable.

It is known that calcium ions render the gangliosides insoluble. Ganglioside has a high binding capacity for calcium, but calcium does not affect the fixation of toxin by ganglioside (Van Heyningen, 1963). In our studies we have used insoluble gangliosides, prepared by adding $CaCl_2$ to soluble crude gangliosides. Fixation studies with tetanus toxin was carried out using this insoluble preparation.

METHODS

Preparation of different tissues and their subfractions were mixed with known amounts of tetanus toxin, and the mixtures were incubated for 2 hr at 37° C. As a control, toxin without added tissue was treated in the same way. Mixtures were then centrifuged, after which the supernatant was withdrawn. Potency of the centrifugates, before and after adsorption with different tissues and their subfractions was calculated in terms of L+ value. The amount of toxin adsorbed was then obtained by difference between control and experimental values.

Tetanus toxin. Tetanus toxin was the same as used in the previous article (Patel & Rao, 1966). It had a toxicity of 100,000 mouse MLD per ml. and combining value (L+) of 12.5 per ml.

Preparation of homogenates. Albino rats (100 g body wt.) were killed by a blow on the head and adult pigeons (400–450 g body wt.) were killed by bleeding through the carotid artery. The brain and liver tissues of rats and pigeons were then removed and weighed. These were homogenized separately using a glass homogenizer in ice cold 0.32 M sucrose for brain tissue and 0.25 M sucrose for liver. A medium/tissue (v/w) ratio of 10 was used.

Preparation of subcellular fractions. The fractionation of the 0.32 M sucrose homogenates was carried out in No. 40 rotor of a Spinco model L preparative ultracentrifuge. Fractions from brain

homogenates were prepared at 0–3° C as described by Nyman and Whittaker (1963). Sequential centrifugation of brain homogenates at 1,000 *g* (11 min), 17,000 *g* (60 min), and 96,500 *g* (60 min) yielded respectively P1, containing nuclei, cell debris and large myelin fragments; P2, mitoohondria, myelin fragments and nerve endings; P3, microsomes; and S, soluble components. The subfractions were suspended in a minimum volume of 0.32 M sucrose and used for further experiments. Three g rat or pigeon brain tissue and subfractions prepared from it were used throughout the experiments. Liver homogenates were prepared from 3 g liver in 0.25 M sucrose.

Determinations of gangliosides. Gangliosides were determined according to the method of Wolfe (1961) by estimating the yield of *N*-acetyl neuraminic acid. Weighed specimens (80–100 mg) were held at 0° C in homogenizer tubes of 150 mm×12 mm provided with pestles ground smoothly to fit. To this was added 19 v/w chloroform-methanol mixture (2:1 v/v) also at 0° C. The tissue was ground in the solvent and the mixture left for 3 min. Particulate fractions prepared as described above were also extracted similarly by chloroform-methanol mixture.

The chloroform-methanol extracts were subjected to solvent partition. The procedure followed was based on those of Folch, Lees & Sloane-Stanley (1957) and Long & Staples (1959). The sucrose in the particulate fractions was then removed by dialysis and the neuraminic acid content was determined in the nondialysable material.

For estimation of neuraminic acid, measured quantities of the dialysed extracts of the tissues and their subfractions were evaporated to dryness. *N*-acetyl neuraminic acid was determined with thiobarbituric acid reaction method of Warren (1959) and modified by Balakrishnan & McIlwain (1961).

Gangliosides. Crude bovine ganglioside was purchased from SIGMA chemical company. This was water soluble giving an opalescent solution.

RESULTS

Adsorption of tetanus toxin by tissue homogenates

Incubation of the brain and liver homogenates of rats and pigeons with tetanus toxin showed (Table 1) that there was no significant difference between brain tissue of the rat and brain tissue of the pigeon in its ability to fix the toxin. The pigeon brain homogenates had only 20% less adsorption capacity than the rat brain homogenates under the conditions of the experiment. However, homogenates prepared from liver tissue of the rat and the pigeon did not adsorb toxin.

TABLE 1

FIXATION OF TETANUS TOXIN BY HOMOGENATES OF BRAIN AND LIVER OF RATS AND PIGEONS

	Potency of toxin		
Homogenate	Added L+	Recovered L+	% Reduction in toxicity
Rat brain	25	2·5	90
Pigeon brain	25	6·2	74
Rat liver	25	25	—
Pigeon liver	25	25	—

Adsorption of tetanus toxin on the subcellular fractions

Since brain homogenates adsorb tetanus toxin, it was of interest to see which of the subfractions of the brain tissue has this property. Adsorption of tetanus toxin was studied by preparing subfractions from rat and pigeon brain tissue as described in the

Methods. The subfractions were suspended in 1.0 ml. of 0.32 M sucrose and incubated with toxin. They were then centrifuged at the gravity used for the preparation of subfractions, and the toxicity of the supernatant was determined. The results obtained (Table 2) showed that the mitochondrial fraction (P2), prepared from rat and pigeon brain had the highest capacity for adsorption of the toxin. The supernatant (S) after removing the particulate fractions did not inactivate tetanus toxin.

TABLE 2

ADSORPTION OF TETANUS TOXIN ON THE SUBFRACTIONS OBTAINED FROM RAT AND PIGEON BRAIN TISSUES

Tissue	Subfraction	Centrifugal force	Potency of toxin		
			Added L+	Recovered L+	% Reduction in toxicity
Rat brain	P1, Nuclear	1,000 *g* (11 min)	25	12·5	50
	P2, Mitochondrial	17,000 *g* (60 min)	25	4·1	83
	P3, Microsomal	96,500 *g* (60 min)	25	16·6	33
	S, Supernatant	—	25	25	—
Pigeon brain	P1, Nuclear	1,000 *g* (11 min)	25	12·5	50
	P2, Mitochondrial	17,000 *g* (60 min)	25	8·3	66
	P3, Microsomal	96,500 *g* (60 min)	25	16·6	33
	S, Supernatant	—	25	25	—

Demonstration of binding of tetanus toxin with gangliosides

Van Heyningen (1959) demonstrated the combination of toxin and ganglioside in an analytical ultracentrifuge. The peaks of the ganglioside-toxin complexes of greater mol wt (200,000) than ganglioside alone were observed. In our experiments, we have precipitated the gangliosides as the calcium complex in presence of the toxin.

For each experiment, 10 mg ganglioside was dissolved in 2.0 ml. saline and was mixed with 2.0 ml. tetanus toxin and then 1.0 ml. 10% (w/v) $CaCl_2$. In the first experiment, ganglioside plus toxin mixture was incubated for 2 hr at 37° C. After the incubation, 1.0 ml. of $CaCl_2$ was added. In the next experiment, ganglioside and $CaCl_2$ mixture was incubated for 15 min, then toxin (2.0 ml.) was added, and incubated for 2 hr at 37° C. After the incubation, both the mixtures were centrifuged at 5,000 *g* for 15 min. The sediment and the supernatant were collected and assayed for L+ value. Three controls, ganglioside + toxin + saline, toxin + $CaCl_2$ + saline, and toxin + saline were also incubated at the same time for 2 hr at 37° C. In the toxin + $CaCl_2$ + saline mixture, a little precipitate of calcium phosphate appeared after the addition of $CaCl_2$ due to the presence of phosphates in the toxin. These were removed by centrifugation and the supernatant was tested for L+ value. The results of the experiments are described in Table 3.

The results showed that gangliosides when precipitated with $CaCl_2$ could bind about 75% of toxin from solution under the conditions of the experiments. The soluble ganglioside + toxin + saline mixture did not inactivate tetanus toxin when tested *in vivo*. The toxin bound to ganglioside and precipitated with calcium was also released *in vivo*. These experiments showed that tetanus toxin is probably loosely bound with the gangliosides, since it is dissociable *in vivo*.

TABLE 3

EFFECT OF CALCIUM ON THE ABILITY OF CRUDE GANGLIOSIDES TO BIND TOXIN

Experimental	Potency of toxin: Added L+	Potency of toxin: Recovered L+
Toxin + saline (control)	16·6	16·6
Toxin + $CaCl_2$ + saline (control)	16·6	16·6
Ganglioside + toxin + saline (control)	16·6	16·6
(Ganglioside + toxin) incubated for 2 hr. 37° C + $CaCl_2$, centrifuged	16·6	
A. Sediment		12·5
B. Supernatant		2·5
(Ganglioside + $CaCl_2$) incubated for 15 min. + toxin, incubated for 2 hr. 37° C	16·6	
A. Sediment		12·5
B. Supernatant		2·5

Subcellular distribution of gangliosides

The amount of gangliosides in the whole brain homogenates of rats and pigeons and their subcellular distribution was next studied. Preparations of homogenates and subfractions as well as their extraction with chloroform-methanol mixture were carried out as described in the Methods. The amount of gangliosides extracted was then estimated (see Methods section). It will be seen from Table 4 that the ganglioside content of pigeon brain homogenate is only 20% less than that of rat brain homogenate. The highest amount of ganglioside is present in the mitochondrial fraction, sedimented at 17,000 *g* (60 min). The supernatant obtained after removing the subfractions contained negligible quantity of gangliosides. The amount and the subcellular distribution pattern of gangliosides in the homogenates and the subfractions resemble the pattern of adsorption of tetanus toxin on these homogenates and subfractions.

TABLE 4

AMOUNT OF GANGLIOSIDES IN THE WHOLE BRAIN HOMOGENATES OF RATS AND PIGEONS AND THEIR SUBCELLULAR DISTRIBUTION

Tissue	Fraction	Ganglioside content (μmoles of neuraminic acid/g. of tissue)	% Fixation of toxin
Pigeon brain	Whole homogenate	1·10	74
Rat brain	Whole homogenate	1·37	90
	Nuclear, P1	0·44	50
	Mitochondrial, P2	0·75	83
	Microsomal, P3	0·11	33
	Supernatant, S	0·07	—

DISCUSSION

Our results show that gangliosides when precipitated with calcium could bind about 75% of toxin from solution under the conditions of the experiments. The ganglioside-toxin mixture did not inactivate tetanus toxin. This suggests that tetanus toxin is not chemically bound with the gangliosides but merely gets adsorbed or is loosely bound. Fulthorpe (1956) also found that toxin which was adsorbed on the brain tissue could be recovered by washing with physiological saline.

Since tetanus toxin acts in a very high dilution, it has been repeatedly suggested that it may be itself an enzyme or may block the activity of an essential enzyme. The possible substrate for the enzyme may then be the ganglioside which is found in the nervous tissue. One would therefore expect a change in the gangliosides content of brain after injection of tetanus toxin. We removed the brain tissue from paralysed rats, 48 hr after the subcutaneous injection of two lethal doses of tetanus toxin. Ganglioside was then estimated as described in the Methods section. We obtained 1.30 μmoles of nondialysable neuraminic acid per g tissue. This amount of ganglioside was not significantly different from the normal values. These results confirm the findings of Van Heyningen & Miller (1961) who were unable to detect hexosamine, sialic acid or sphingosine residues in the dialysate after dialysis of the ganglioside and toxin mixture. Their results and ours show that ganglioside is not degraded after it is bound with tetanus toxin.

Possible mode of action of tetanus toxin

It is believed by Nachmansohn (1958) that nerve transmission is triggered by the release of acetylcholine from the bound form at the synapse, which causes the permeability change in the cell membrane, permitting influx of Na^+ and efflux of K^+ ; thus creating an effective potential gradient for the travel of nerve impulse. The free acetylcholine is rapidly hydrolysed by acetylcholinesterase, which is present in very close proximity to the receptor in the nerve membrane. The resynthesis of acetylcholine as well as the active transport of Na^+ outside and K^+ inward requires energy in the form of ATP. So it is to be expected that tetanus toxin may act by interference with energy-yielding biochemical reactions vital for nerve and muscle metabolism. We have actually found low levels of ATP in brain and muscle of rats injected with two lethal doses of tetanus toxin, as reported in the previous paper (Patel & Rao, 1966). The fact that paralysis is observed, not immediately after injection of tetanus toxin, but after a certain incubation period, extending over two to three days, indicates that it takes time for ATP levels to come down to the levels necessary to cause paralysis.

Two forms of tetanus are well recognized. In local tetanus produced in experimental animals by injecting sublethal dose intramuscularly, spasticity (rigidity) is confined to the muscles in the region of the injection, while in general tetanus, produced by injecting a lethal dose intravenously or intracerebrally, there is widespread spasticity and death (Laurence & Webster, 1963). In local tetanus, the action of toxin is confined to a few motor neurones in the cord by virtue of the limiting factor of neural transport. The local tetanus may therefore be due to low ATP levels in the muscle, which is brought about by the activation of ATPase of myofibrils as reported in our previous paper (Patel & Rao, 1966). General tetanus may be caused by the low levels of ATP in the brain and possibly in the neurones because of the action of toxin in reducing the formation of energy-rich phosphates by the mitochondria as reported earlier by us.

It was of interest to see whether intramuscular injections of ATP can overcome the local paralysis produced in rats by injecting intramuscularly sublethal doses of tetanus toxin. A group of six rats were injected with a sublethal dose (3/4 lethal dose) and after 24 hr a series of ATP injections were given intramuscularly at the same site. These rats also developed paralysis about 72 hr after the toxin injection along with the controls

not given ATP injections. The failure of the injected ATP to prevent the paralysis may be due to its immediate hydrolysis by ATPase in the muscle cells.

Role of ganglioside

The receptor for tetanus toxin has been identified as ganglioside (Van Heyningen, 1959), which has the property of adsorption of the toxin and concentrating it from the surrounding fluid. It is also likely that it is responsible for the centripetal transport of the toxin in the nerve trunks, and then to its site of action in the spinal cord and brain. The fact that tetanus toxin is not destroyed after it is bound with the receptor in the nervous tissue supports this claim.

Tetanus toxin has been obtained from animals dying of tetanus by grinding up their brain tissue in saline (Fulthorpe, 1956). This was repeated by us and confirmed. The overall concentration of ganglioside in nerve trunks is very small (Folch-Pi & LeBaron, 1957) and the capacity of nerve trunks to fix toxin is low compared with that of brain (Fulthorpe, 1956). However, a significant concentration of ganglioside may be present within the axon. On the other hand, if the suggestion by Wright (1955) is accepted that the transport of toxin takes place in the space between the nerve fibres themselves, and that this is actuated by pressure following movement of limbs, then the importance of localization of ganglioside may be more in the brain and spinal cord rather than peripherally.

We have found that the adsorption of tetanus toxin was highest on the brain mitochondrial fraction which is a mixture of mitochondria, myelin fragments and pinched-off nerve endings. The adsorption of the toxin on myelin sheaths seems to be unlikely since recent evidence suggests that gangliosides are situated within the nerve cell bodies and their dendrites and axons whereas myelin sheaths and membranes of nerve cells and their fibres contain mostly the cerebrosides which is not the receptor for tetanus toxin (Svennerholm, 1957). Recent studies on the subcellular localization of gangliosides (Burton, Howard, Baer and Balfour, 1964) indicate that the gangliosides are located in small discrete granules which are distinct from mitochondria. These are synaptic vesicles, which are obtained after disruption of the nerve endings. The adsorption of tetanus toxin takes place, most probably, in the synaptic vesicles which also contain the gangliosides as well as the transmitter substances such as acetylcholine (Burton *et al.*, 1964).

At present nothing is known about the significance of the attachment of toxin to the gangliosides. This attachment does not explain the resistance of the pigeon since there was no significant difference between the brain tissue of rats and pigeons in their ability to fix tetanus toxin. The resistance of the pigeon is also not due to lack of the receptor in the nervous tissue, since there was not much difference in the amount of gangliosides in the brain of rats and pigeons.

To see whether gangliosides stabilize tetanus toxin and protect it from the destructive action of proteolytic enzymes, we studied the effect of trypsin on the ganglioside-toxin mixture *in vitro*. It was found that trypsin destroyed tetanus toxin alone or in combination with the gangliosides *in vitro*. However, conditions *in vivo* may be different, and may be free of the destructive influence of proteolytic enzymes. It was also of interest to see whether gangliosides bind acetylcholine as it does with certain drugs such as strychnine, brucine thebaine, serotonin and a number of related compounds as reported by Van Heyningen (1963), and whether tetanus toxin prevents this binding by competing for its site in the gangliosides. This information would be important because the ganglio-

sides are found in the synaptic vesicles which also contain acetylcholine. Furthermore, Burton *et al.* (1964) have proposed a hypothesis for the role of ganglioside in synaptic vesicles according to which the role of ganglioside is to transport the bound acetylcholine from the synaptic vesicles in the cytoplasm through the presynaptic nerve membrane into the synaptic cleft as " free " acetylcholine. Prevention of binding of acetylcholine and its subsequent release from the ganglioside may result in paralytic symptoms. However, our experiments to verify this showed that bovine brain ganglioside does not bind acetylcholine *in vitro*.

Tetanus toxin is known to lower the acetylcholine content of the intoxicated iris of the rabbit (Ambache, Morgan and Wright, 1948). The decrease in the acetylcholine is not due to the effect of toxin on the cholineacetylase (Stevenson, 1958) and cholinesterase (Stevenson, 1958) enzymes. In the absence of any effect of toxin on these enzyme systems the low acetylcholine may be due to two causes: (1) it is possible that the binding of gangliosides with the toxin in the vesicles might in some way lead to the prevention of liberation of acetylcholine from the bound form resulting in paralytic symptoms or (2) the decreased acetylcholine content may be due to decreased synthesis because of lack of ATP. The low ATP levels in the brain and skeletal muscle tissues in tetanus as reported by us supports the second view.

Gangliosides are only now in the process of chemical definition. However, several indications of their possible physiological functions have appeared. Gangliosides from ox brain restore the respiratory response to electrical stimulation in cerebral-cortex slices in which this response had been abolished by cold pretreatment of the tissue by basic proteins (McIlwain, 1961). Irwin & Trams (1961) have recently demonstrated that gangliosides compete for curare at neuromuscular junction. Though we have not provided a direct evidence for the role of ganglioside in production of paralytic symptoms in tetanus, we have to presume at this stage that in view of its localization in the synaptic vesicles and its ability to adsorb the toxin, it may play the role of concentrating the toxin, which has the property of reducing the ATP levels in brain and muscle.

SUMMARY

1. Tetanus toxin is adsorbed by the brain homogenates of susceptible species such as rat as well as the resistant species such as pigeon. However, liver homogenates from both the species did not adsorb toxin.

2. There was a fairly close correlation between the adsorption of tetanus toxin by subcellular fractions and their ganglioside content. The highest content of gangliosides was present in the mitochondrial fraction.

3. Gangliosides when made insoluble with $CaCl_2$ could bind about 75% of added tetanus toxin. The binding is loose since the toxin is released *in vivo*.

4. There was no change in the brain gangliosides content in rats injected with tetanus toxin.

5. The mode of action of tetanus toxin in bringing about paralysis of muscles and the role of gangliosides of the nervous system in aiding such action is discussed.

We are grateful to the C.S.I.R., New Delhi, for awarding fellowship to one of us (A. A. P.) and to Dr H. I. Jhala, Director, Haffkine Institute, for providing the laboratory facilities.

REFERENCES

AMBACHE, N., MORGAN, R. S. & WRIGHT, G. P. (1948). Action of tetanus toxin on acetylcholine and cholinesterase content of the rabbit's iris. *Brit. J. exp. Path.*, **29**, 408–418.

BALAKRISHNAN, S. & MCILWAIN, H. (1961). Gangliosides and related substances of isolated cerebral tissues examined in relation to tissue excitability. *Biochem. J.*, **81**, 72–78.

BURTON, R. M., HOWARD, R. E., BAER, S. & BALFOUR, V. M. (1964). Ganglioside and acetylcholine of the CNS. *Biochim. Biophys. Acta.*, **84**, 441–447.

FOLCH-PI, J. & LEBARON, F. N. (1957). Chemical composition of the mammalian nervous system. In *Metabolism of the Nervous System*, ed. RICHTER, D., p. 67. London: Pergamon Press.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. biol. Chem.*, **226**, 497–509.

FULTHORPE, A. J. (1956). Adsorption of tetanus toxin by brain tissue. *J. Hyg. Camb.*, **54**, 315–327.

IRWIN, R. L. & TRAMS, E. G. (1961). The sequestration of d-tubocurarine by ganglioside. *Fed. Proc.*, **20**, 174.

LAURENCE, D. R. & WEBSTER, R. A. (1963). Pathologic physiology, pharmacology and therapeutics of tetanus. *Clin. Pharmacol. Ther.*, **4**, 36–72.

LONG, C. & STAPLES, D. A. (1959). Determination of neuraminic acid in crude brain lipids. *Biochem. J.*, **73**, 385–389.

MCILWAIN, H. (1961). Characterization of naturally occurring materials which restore excitability to isolated cerebral tissues. *Biochem. J.*, **78**, 24–32.

NACHMANSOHN, D. (1958). *Molecular Forces Controlling Ion Movements During Nerve Activity*, ed. BRUCKE, F., p. 26. Symposium on Biochemistry of the Central Nervous System. Proc. 4th Internat. Congress of Biochem., Vienna, 1958. London: Pergamon Press.

NYMAN, M. & WHITTAKER, V. P. (1963). The distribution of adenosine triphosphate in subcellular fractions of brain tissue. *Biochem. J.*, **87**, 248–255.

PATEL, A. A. & RAO, S. S. (1966). Action of tetanus toxin on brain, liver and muscle mitochondria from resistant and susceptible species. *Brit. J. Pharmacol.*, **26**, 730–739.

STEVENSON, J. W. (1958). Bacterial neurotoxins. *Amer. J. med. Sci.*, **235**, 317–336.

SVENNERHOLM, L. (1957). Quantitative estimation of gangliosides in senile human brains. *Acta Soc. Med. Upal.*, **62**, 1–3.

VAN HEYNINGEN, W. E. (1959). Tentative identification of the tetanus toxin receptor in nervous tissue. *J. gen. Microbiol.*, **20**, 310–320.

VAN HEYNINGEN, W. E. & MILLER, P. A. (1961). The fixation of tetanus toxin by ganglioside. *J. gen. Microbiol.*, **24**, 107–118.

VAN HEYNINGEN, W. E. (1963). The fixation of tetanus toxin, strychnine, serotonin and other substances by ganglioside. *J. gen. Microbiol.*, **31**, 375–387.

WARREN, L. (1959). The thiobarbituric acid assay of sialic acid. *J. biol. Chem.*, **234**, 1971–1975.

WOLFE, L. S. (1961). The distribution of gangliosides in subcellular fractions of guinea-pig cerebral cortex. *Biochem. J.*, **79**, 348–355.

WRIGHT, G. P. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridium tetani*. *Pharmacol. Rev.*, **7**, 413–465.