Journal of Neurochemistry, 1971, Vol. 18, pp. 1751 to 1759. Pergamon Press. Printed in Great Britain.

THE BINDING OF BOTULINUM TOXIN TO MEMBRANE LIPIDS: SPHINGOLIPIDS, STEROIDS AND FATTY ACIDS

L. L. SIMPSON[1] and M. M. RAPPORT

Division of Neuroscience, New York State Psychiatric Institute and Departments of Psychiatry and Biochemistry, Columbia University, College of Physicians and Surgeons, New York, N. Y. 10032

(*Received* 8 *January* 1971. *Accepted* 8 *March* 1971)

Abstract—A number of lipids known to be constituents of nerve-ending membranes were tested for their ability to inactivate botulinum toxin. Inactivation of the toxin by a lipid was taken as presumptive evidence that the lipid might be the *in vivo* receptor for the toxin. Several sphingolipids (sphingosine, galactosylceramide, glucosylceramide, lactosylceramide, cytolipin K and cytolipin R), steroids (cholesterol and deoxycholic acid) and fatty acids (palmitic acid, stearic acid, prostaglandin E_1) did not affect the potency of botulinum toxin, and thus were discounted as potential toxin receptors. However, the gangliosides did inactivate botulinum toxin rapidly (in less than 5 min), within a temperature range of 2°–40°C, and at ionic strengths of 0·05–0·40. Inactivation diminished as pH fell below 6. The activity of gangliosides in suppressing the potency of botulinum toxin was a function of the number of sialic acid residues in the lipid. Thus, the data suggest that a molecule containing sialic acid may be the receptor for the toxin.

THE RECEPTOR *in vivo* for botulinum toxin has not been identified. Histological (ZACKS, METZGER, SMITH and BLUMBERG, 1962) as well as electrophysiological (BROOKS, 1956) data suggest that the receptor for the toxin must be in the presynaptic membrane or ground substance of the synaptic interspace at peripheral cholinergic synapses. These data are consistent with the proposed mechanism of action for botulinum toxin, namely, suppression of the release of acetylcholine (LAMANNA and CARR, 1967; SIMPSON, 1971*b*).

The amount of nerve-ending material at peripheral cholinergic synapses is too small to be useful in preliminary studies on the isolation of botulinum toxin receptor. A more appropriate approach is the study of the interaction between botulinum toxin and known constituents of the membrane. Although protein components of most membranes are poorly characterized, many lipid components have been identified and in some instances have been synthesized chemically. The availability of both naturally-occurring and synthetic lipids facilitates their use in the study of receptor properties. In our work, we have examined the interaction between botulinum toxin and a number of membrane lipids or their precursors, with particular reference to recent publications describing both qualitatively and quantitatively the lipid composition of nerve-ending membranes (EICHBERG, WHITTAKER and DAWSON, 1964; LAPETINA, SOTO and DE ROBERTIS, 1968; SEMINARIO, HREN and GOMEZ, 1964; WIEGANDT, 1967).

MATERIALS AND METHODS

Materials. Cholesterol and deoxycholic acid were purchased from Sigma Chemical (St. Louis, Mo.); palmitic acid and stearic acid were purchased from Nutritional Biochemical Corp. (Cleveland,

[1] Present address: Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032.

Ohio); sphingosine was purchased from Miles Laboratories (Elkhart, Ind.). Prostaglandin E_1 was a gift from Upjohn Co. (Kalamazoo, Mich.).

Gangliosides G_{D1b}, G_{M2}, asialo-G_{M1} and asialo-G_{M2} were a gift of Drs. Robert Ledeen and Robert Yu (Saul Korey Department of Neurology, Albert Einstein College of Medicine). These materials were chromatographically pure. Gangliosides G_{T1}, G_{D1a} and G_{M1} were obtained from Supelco, Inc. (Bellefonte, Pa.). These materials were almost homogeneous (more than 90 per cent) on TLC. All weights of gangliosides specified in the text were based on resorcinol analysis and calculated contents of sialic acid. Phosphorus contents of such preparations were less than 0·1 per cent. Terminology for gangliosides is that proposed by SVENNERHOLM (1963).

Galactosylceramide (RADIN, BROWN and LAVIN, 1956), lactosylceramide (RAPPORT, SCHNEIDER and GRAF, 1962), cytolipin K (RAPPORT, GRAF and SCHNEIDER, 1964) and cytolipin R (RAPPORT, SCHNEIDER and GRAF, 1967) were isolated by methods previously described. Glucosylceramide was obtained from bovine spleen.

Botulinum toxin type A crystals were kindly provided by Dr. E. J. Schantz (U.S. Army Biolabs, Fort Detrick, Maryland).

Analytical. The presence or absence of binding of botulinum toxin to a specific lipid was detected by measuring the potency of the toxin after its incubation with the lipid. Each lipid was diluted into a solution of chloroform–methanol (2:1, v/v) prior to use. At the time of testing, a sample of the lipid was placed in a test tube and the organic solvent was removed in a stream of N_2. The lipid was redissolved by adding 0·05 ml of absolute ethanol followed by 0·95 ml of 0·154 M-NaCl, unless otherwise indicated. All solutions were water clear. Samples of toxin (0·01 ml) were added. Control tubes were identically prepared, except for omission of lipid.

Potency of the toxin was assayed *in vivo* by the acute toxicity test on mice and *in vitro* on the isolated, neuromuscular preparation of the rat. Acute toxicity tests were performed as described elsewhere (BOROFF and FLECK, 1966). The volume for injection was 0·1 ml of the original 1·0 ml of incubation solution. Neuromuscular preparations (phrenic nerve–diaphram) were dissected, bathed and used as previously reported (SIMPSON and TAPP, 1967; SIMPSON, in press). The entire 1·0 ml of incubation mixture was added to the nerve–muscle bath.

RESULTS

Sphingolipids and botulinum toxin. A group of lipids of increasing structural complexity were tested for their ability to bind to botulinum toxin. Sphingosine, galactosylceramide, glucosylceramide, lactosylceramide, cytolipin K or cytolipin R, each at 5·0 μg/ml, was incubated with botulinum toxin type A at 5·0 μg/ml (1 mouse $LD_{50} \simeq 29$ pg of toxin protein). The potency of lipid-incubated toxin was compared to that of untreated toxin by the acute toxicity test on mice. The survival times of mice injected with toxin (N = five mice/group) did not differ from the survival times of mice receiving a toxin-lipid mixture. Negative results were also obtained when tests were conducted on the isolated neuromuscular preparation. Apparently none of these lipids binds to or shields the active site of botulinum toxin.

In contrast to other sphingolipids, the gangliosides did inactivate botulinum toxin. Individual gangliosides (Fig. 1), were incubated for 15 min at 25°C with 5·0 μg of botulinum toxin. The mixture was then added to a 300 ml bath in which a rat phrenic nerve–diaphragm was twitching. The large bath volume served to dilute out any unexpected effects that could be secondary to the pharmacological actions of lipids. The time elapsed between addition of toxin or toxin-lipid mixture and 90 per cent failure of muscle twitch was referred to as paralysis time. The logarithm of paralysis time is a linear function of the logarithm of toxin dose (Fig. 1) (SIMPSON, 1971*a*). The asialo-G_{M2} and asialo-G_{M1}, the ceramide tri- and tetrasaccharide moieties without sialic acid residues, exerted no significant effects on paralysis times. On the other hand, the compounds containing sialic acid were all active. Gangliosides G_{T1}, G_{D1a}, G_{D1b} and G_{M1} (5·0 μg) each caused at least an 80 per cent loss of activity of 5·0 μg of botulinum toxin. Ganglioside G_{M2}, the monosialo-ceramide trisaccaride, was notably less potent.

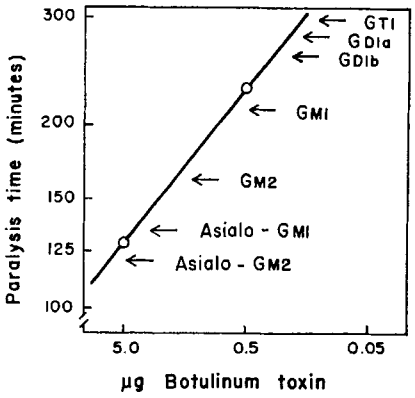

FIG. 1.—Effects of different gangliosides on botulinum toxin-induced paralysis of the isolated phrenic nerve–diaphragm preparation. The straight line plots the dose–response curve obtained by poisoning neuromuscular preparations with various amounts of botulinum toxin (abscissa). Arrows indicate the paralysis times (ordinate) of neuromuscular preparations (five in each group), receiving 5·0 μg of toxin plus 5·0 μg of ganglioside. Increased time until paralysis means decreased toxin potency.

The seven gangliosides tested *in vitro* were also tested *in vivo*. The anti-toxin activities of gangliosides in the acute toxicity test on mice exhibited a rank order similar to the neuromuscular data shown in Fig. 1. Asialo-G_{M2} and asialo-G_{M1} were devoid of anti-toxin activity *in vivo*. Because G_{T1} was the most active ganglioside both *in vivo* and *in vitro*, subsequent tests focussed on this compound.

G_{T1} and botulinum toxin. To determine the optimum conditions for interaction between G_{T1} (1·0 μg) and botulinum toxin (0·5 μg), incubation of the two compounds was conducted under varying conditions of temperature, pH, ionic strength and incubation time (Fig. 2). Unless otherwise indicated, incubations were carried out for 15 min at 25°C in unbuffered 0·154 M-NaCl. Acute toxicity tests were used to assay toxin potency. Under the various conditions examined, the potency of untreated toxin was not markedly altered. Similarly, G_{T1} appeared to reduce toxin potency at all temperatures, ionic strengths and incubation periods examined. This consistency of ganglioside effect contrasted with the effects of phospholipids which also inactivated botulinum toxin (SIMPSON and RAPPORT, 1971*b*). Ganglioside–toxin interaction did appear to be pH dependent (as indicated in Fig. 2B). Optimum conditions for inactivation of toxin by G_{T1} involved incubation for at least 10 min in buffered 0·154 M-NaCl at pH 6–8 and a temperature of 10°C or higher.

Acute toxicity and neuromuscular toxicity tests of the inactivation of botulinum toxin by G_{T1} were run on mixtures incubated for 15 min at 25°C in 0·154 M-NaCl at pH 7 (Fig. 3). Various concentrations of G_{T1} were used to antagonize three concentrations of toxin (5·0, 0·5 and 0·05 μg/ml, or one-tenth of these amounts per mouse) for acute toxicity tests, and one toxin concentration (5·0 μg) for neuromuscular toxicity tests. Both the survival times of mice and the paralysis times of neuromuscular preparations were lengthened as the amount of G_{T1} incubated with the toxin was increased. In acute toxicity tests, all the mice that received 0·1 ml of 1·0-ml mixture containing 0·05 μg of toxin plus 100 μg of G_{T1} survived, and 40 per cent of the mice

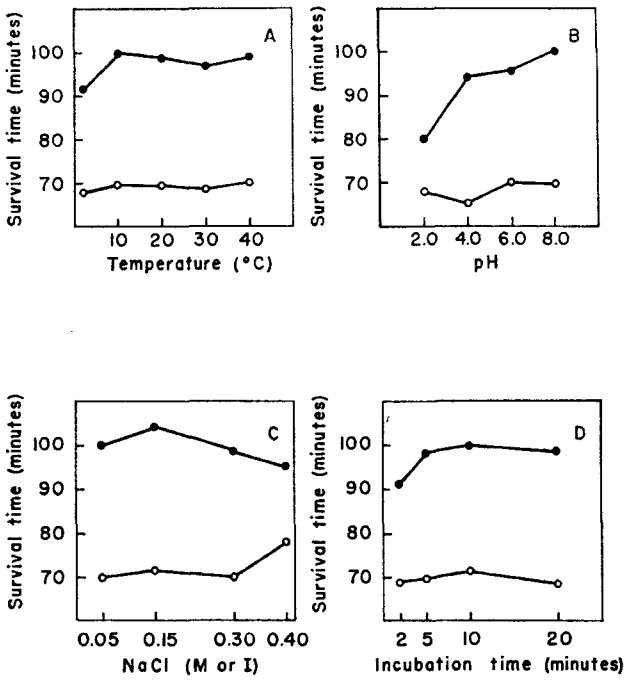

FIG. 2.—Effects of temperature (A), pH (B), ionic strength (C) and length of incubation (D) on the interaction between G_{T1} and botulinum toxin. Open symbols (○) refer to survival times of mice (five mice/group) receiving only botulinum toxin; closed symbols (●) refer to survival times of mice receiving a mixture of toxin and G_{T1}.

that received 0·1 ml of a 1·0-ml mixture containing 0·5 μg of toxin plus 100 μg of G_{T1} survived. Since the lethal dose for the mouse is only slightly more than 29 pg of protein (1 $LD_{50} \simeq 29$), the *in vivo* data indicate that G_{T1} can completely protect against a supra-lethal injection of toxin.

Data from the neuromuscular toxicity tests also showed that G_{T1} could inactivate large quantities of toxin. However, unlike the whole animal, the isolated neuromuscular preparation cannot metabolize unbound toxin. Consequently, only a minute portion of the toxin need remain active or subsequently be reactivated in order to cause paralysis. For example, botulinum toxin (5·0 μg) by itself paralysed transmission after an average time of 125 min, botulinum toxin (5·0 μg) + G_{T1} (10·0 μg) paralysed transmission after an average time of 400 min. On the basis of the logarithmic dose response correlation (SIMPSON, 1971*a*), it would require less than 0·05 μg of toxin to produce a paralysis time of approximately 400 min.

Since small quantities of G_{T1} were relatively active in diminishing toxin potency, we attempted to inactivate toxin *in vivo* without prior incubation. Two techniques were used. In the first, mice (five mice/group) were injected intravenously with G_{T1} (0·1 ml of a solution of 1 μg/ml), and this injection was followed at different time intervals with botulinum toxin (0·1 ml of solution of 0·05 μg/ml). In the second technique, the order of injections was reversed. In control studies, when G_{T1} and botulinum toxin were incubated together prior to injection, the mean survival time of

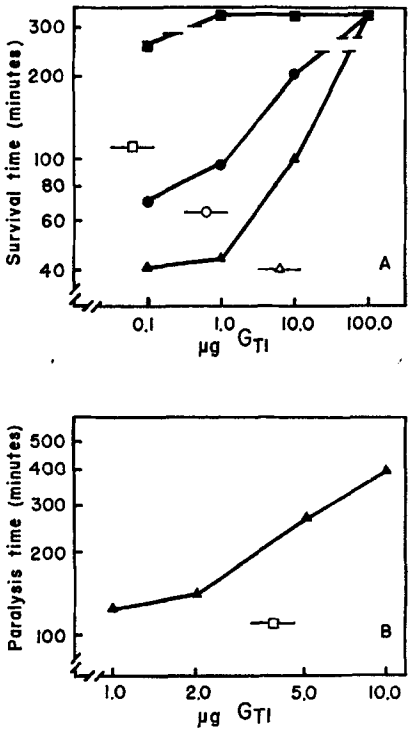

FIG. 3.—Dose-response curves of inactivation of botulinum toxin by G_{T1} both *in vivo* (A) and *in vitro* (B). In both parts of the figure, closed symbols denote: ■—■, 0·05 μg/ml; ●—●, 0·5 μg/ml; ▲—▲, 5·0 μg/ml, of botulinum toxin. The open symbols denote values obtained with comparable amounts of toxin unexposed to G_{T1}.

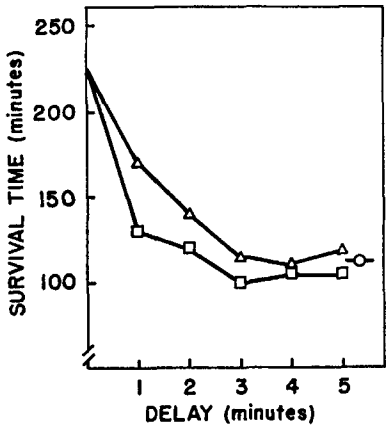

FIG. 4.—Survival times of mice as a function of the interval (delay) between injections of G_{T1} and botulinum toxin. Experimental procedure is given in the text. Symbols denote: △—△, botulinum toxin injected prior to G_{T1}; □—□ G_{T1} injected prior to botulinum toxin; —○—, control value for mice receiving only botulinum toxin.

challenged mice was 225 min; when untreated toxin was injected, the mean survival time was 115 min. G_{T1} did not affect toxicity of botulinum toxin if it was injected prior to the toxin (Fig. 4), even if the interval was as short as 1 min. When injected after the toxin, G_{T1} caused partial inactivation if the time interval was less than 2 min. Factors of dilution as well as possible binding of G_{T1} to formed blood elements may have impeded G_{T1}-toxin interactions in the circulatory system of the mouse. There is no nonspecific binding of botulinum neurotoxin to formed blood elements (SIMPSON, unpublished data).

Neuraminidase and G_{T1} activity. Neuraminidase cleaves the accessible sialic acid residues from gangliosides. Both preliminary data (SIMPSON and RAPPORT, 1971*a*) as well as data reported above indicated that gangliosides required intact sialic acid residues to inactivate botulinum toxin. Accordingly, neuraminidase (source: *Cl. perfringens*) was used to study interaction of G_{T1} with botulinum toxin. Specifically, we attempted to reverse the inactivation of toxin by ganglioside.

Neuraminidase was used in connection with both *in vivo* and *in vitro* tests. In each case, five groups (five mice/group) were used as follows: A) botulinum toxin alone; B) botulinum toxin + G_{T1}; C) botulinum toxin plus neuraminidase; D) neuraminidase-treated G_{T1} plus toxin; and E) an incubated G_{T1}-toxin mixture to which neuraminidase was subsequently added. In all cases incubations were carried out at pH 6 and 37°C in 0·154 M-NaCl (Table 1). G_{T1} prolonged survival times of mice and paralysis time of neuromuscular junctions. Neuraminidase afforded no such protection. G_{T1} that had been exposed to neuraminidase lost anti-toxin activity. This observation confirmed both the necessity for the presence of sialic acid residues for the protective action of ganglioside as well as the activity of neuraminidase in the test system. Finally, G_{T1}-toxin mixtures to which neuraminidase was added were notably more potent (toxic) than untreated G_{T1}-toxin mixtures. Apparently neuraminidase can partially re-activate toxin inactivated by ganglioside.

Other lipids and G_{T1} activity. Auxiliary lipids are important in the interactions

TABLE 1.—EFFECTS OF NEURAMINIDASE ON THE INTERACTION BETWEEN BOTULINUM TOXIN AND GANGLIOSIDE G_{T1}

Drug*	Interval†	Drug	Interval†	Drug	Interval†	Mouse survival time (min ± S.D.)	Synaptic paralysis time (min ± S.D.)
A. Toxin	60					100 ± 9	138 ± 10
B. Toxin	None	G_{T1}	60			181 ± 11	240 ± 14
C. Toxin	None	NANA-ase	60			92 ± 8	128 ± 7
D. G_{T1}	None	NANA-ase	30	Toxin	30	89 ± 8	130 ± 6
E. Toxin	None	G_{T1}	30	NANA-ase	30	110 ± 11	170 ± 12

* For *in vivo* tests (mouse survival) drug concentrations were: toxin, 0·05 μg/ml; G_{T1}, 0·1 μg/ml; NANA-ase, 0·1 mg/ml. For *in vitro* tests (synaptic paralysis), concentrations were: toxin, 5·0 μg/ml; G_{T1}, 5·0 μg/ml; NANA-ase, 0·5 mg/ml.

Temperature was uniformly 37°C.

† Interval refers to time in min between mixing of drugs or use in a toxicity test.

Values represent the mean ± S.D. of five preparations.

Abbreviation: NANA-ase, neuraminidase.

between lipids and antibodies (RAPPORT and GRAF, 1969) and lipids and proteins (VAN HEYNINGEN and MELLANBY, 1968). Three lipids, galactosylceramide, cholesterol and phosphatidylcholine, whose auxiliary functions have been reported in other systems, were tested for their effects on the G_{T1}-botulinum toxin interaction. In a test designed to detect potentiation, a minimal amount of G_{T1} (1·0 μg) was incubated with botulinum toxin (5·0 μg) plus another lipid (3·0 μg). The mixture was tested on isolated neuromuscular preparations. None of the lipids potentiated the anti-toxin activity of G_{T1} (Table 2). Consequently, a test designed to produce a large ganglioside effect

TABLE 2.—EFFECTS OF OTHER LIPIDS ON THE INTERACTION BETWEEN BOTULINUM TOXIN AND GANGLIOSIDE G_{T1}

Amount of G_{T1} (μg)	Lipid	Amount (μg)	Synaptic paralysis time (min ± S.D.)*
1·0	None	—	140 ± 8
1·0	Galactosylceramide	3·0	138 ± 6
1·0	Cholesterol	3·0	148 ± 9
1·0	Phosphatidylcholine	3·0	135 ± 8
5·0	None	—	280 ± 16
5·0	Galactosylceramide	15·0	220 ± 13
5·0	Cholesterol	15·0	180 ± 11
5·0	Phosphatidylcholine	15·0	140 ± 6

* In all cases, five mice/group were used.

(5·0 μg of G_{T1}; 5·0 μg of toxin; 15 μg of other lipid) was conducted. In this case, the anti-toxin action of G_{T1} was mildly antagonized by galactosylceramide, strongly antagonized by cholesterol and almost completely antagonized by phosphatidylcholine.

Steroids and botulinum toxin. Cholesterol, a prominent constituent of nerve cell membranes, was tested for anti-toxin activity. Both acute toxicity tests in mice (0·5 μg of cholesterol; 0·5 μg of toxin) and neuromuscular toxicity tests (5·0 μg of cholesterol, 5·0 μg of toxin) showed that cholesterol did not alter the survival times of mice or paralysis times of phrenic nerve–diaphragms. Incubation conditions were the same as those used in studies with G_{T1}. In addition, deoxycholate, an acid derivative of cholesterol, was inactive.

Fatty acids and botulinum toxin. Two fatty acids present in complex lipids of nervous tissue, palmitic acid and stearic acid, and one fatty acid released during stimulation of nervous tissue, prostaglandin E_1 (LAITY, 1969; RAMWELL, SHAW and KUCHARSKI, 1965), were tested for anti-toxin activity. None antagonized the *in vivo* (0·5 μg of fatty acid; 0·5 μg of toxin) or *in vitro* (5·0 μg of fatty acid; 5·0 μg of toxin) activity of botulinum toxin.

DISCUSSION

There is no chemical assay for the potency of botulinum toxin. The only test yet proposed, i.e. inhibition of acetylcholinesterase activity (MARSHALL and QUINN, 1967), could not be reproduced (SUMYK and YOCUM, 1968; SIMPSON and MORIMOTO, 1969). Consequently, tests of toxin activity involve bioassay, either on a whole animal or on

an isolated tissue. It is advantageous to use both methods so that non-specific pharmacological actions either *in vivo* or *in vitro* can be detected.

In our studies, we sought to determine whether sphingolipids, steroids or fatty acids that might normally be found in nerve-ending membranes could be the receptor for botulinum toxin. We assumed that any lipid which inactivates the toxin and which does so under physiological conditions of temperature, pH and ionic strength, may be a potential receptor. Most of the lipids tested were inactive, including: the sphingolipids—sphingosine, galactosylceramide, glucosylceramide, lactosylceramide, cytolipin K and cytolipin R, asialo-G_{M1} and asialo G_{M2}; the steroids—cholesterol and deoxycholic acid; and the fatty acids—palmitic acid, stearic acid and prostaglandin E_1. Incubations typically involved a lipid to toxin ratio of 1:1 (w/w). Since botulinum toxin has a molecular weight of nearly 1,000,000 (PUTNAM, LAMANNA and SHARP, 1947), and the largest lipid tested has a molecular weight of little more than 1500, lipid was present in molecular excess with respect to toxin. None of the lipids listed above is likely to be a botulinum toxin receptor.

In contrast to other lipids, the gangliosides are capable of causing inactivation of toxin. This effect is observed both *in vivo* and *in vitro* (Fig. 3) and occurs under physiological conditions (Fig. 2). Prolonged survival of animals or neuromuscular preparations is not attributable to secondary pharmacological effects of gangliosides, because injected ganglioside exhibits little anti-toxin activity unless it has previously been incubated with toxin (Fig. 4). The activity of gangliosides appears to be dependent upon the presence of sialic acid residues, because both asialo-G_{M1} and asialo-G_{M2} are without anti-toxin activity *in vivo* or *in vitro*. The activity of a ganglioside in antagonizing botulinum toxin may be a function of the number of sialic acid residues present (Fig. 1).

Inactivation of botulinum toxin by ganglioside could be a consequence of: (i) specific binding to and inactivation of the reactive site in botulinum toxin, (ii) non-specific binding to other sites and thus non-specific inactivation, or (iii) induction of conformational changes that would cause protein denaturation. Of these three possibilities, denaturation does not seem likely. The fact that neuraminidase can partially restore activity to toxin that has been inactivated by ganglioside (Table 1) shows that the ganglioside—toxin interaction is reversible. It is unlikely that cleavage of sialic acid from ganglioside could reverse denaturation of toxin protein. A more reasonable explanation is that ganglioside binds to botulinum toxin, and loss of toxicity is secondary to this binding. When binding is disrupted, as by neuraminidase, toxin potency is restored. No conclusions can yet be drawn with regard to whether or not binding is to the reactive site of botulinum toxin.

In other studies (VAN HEYNINGEN, 1959), it has been suggested that ganglioside may be the receptor for tetanus toxin. Interestingly enough, both botulinum toxin and tetanus toxin are produced by strains of *Clostridia*. However, there is a difference between the inactivation of tetanus toxin by ganglioside and the inactivation of botulinum toxin by ganglioside. In the former case (VAN HEYNINGEN and MELLANBY, 1968), use of galactocerebroside as an auxiliary lipid (ganglioside–cerebroside, 1:3, w/w) potentiated ganglioside anti-toxin activity. In the latter case (Table 2), galactocerebroside, at the same ratio, antagonized the ganglioside effect. Apparently interaction between ganglioside and tetanus toxin is different from that between ganglioside and botulinum toxin.

Our data suggest that gangliosides should be examined as a potential receptor for botulinum toxin. The presence of sialic acid in glycoproteins requires that these molecules should also be considered as candidates for the role of toxin receptor. Work is currently in progress to determine whether selective shielding of nerve membrane gangliosides will confer resistance to botulinum toxin paralysis.

Acknowledgements—This work was supported by a grant from the National Institute of Mental Health, No. MH 10315-06, and by the Schizophrenia Research Program of the Supreme Council 33° A.A. Scottish Rite, Northern Masonic Jurisdiction.

REFERENCES

Boroff D. A. and Fleck U. (1966) *J. Bact.* **92,** 1580.
Brooks V. B. (1956) *J. Physiol., Lond.* **134,** 264.
Eichberg J., Whittaker V. P. and Dawson R. M. C. (1964) *Biochem. J.* **92,** 91.
Laity J. L. H. (1969) *Br. J. Pharmac. Chemother.* **37,** 698.
Lamanna C. and Carr C. J. (1967) *J. clin. Pharmac. Ther.* **8,** 286.
Lapetina E. G., Soto E. F. and De Robertis E. (1968) *J. Neurochem.* **15,** 437.
Marshall R. and Quinn L. Y. (1967) *J. Bact.* **94,** 812.
Putnam F. W., Lamanna C. and Sharp D. G. (1947) *J. biol. Chem.* **165,** 735.
Radin N. S., Brown J. R. and Lavin F. B. (1956) *J. biol. Chem.* **219,** 977.
Ramwell P. W., Shaw J. E. and Kucharski J. (1965) *Science, N.Y.* **149,** 1390.
Rapport M. M. and Graf L. (1969) *Prog. Allergy* **13,** 273.
Rapport M. M., Graf L. and Schneider H. (1964) *Archs Biochem. Biophys.* **105,** 431.
Rapport M. M., Schneider H. and Graf L. (1962) *J. biol. Chem.* **237,** 1056.
Rapport M. M., Schneider H. and Graf L. (1967) *Biochim. biophys. Acta* **137,** 409.
Seminario L. M., Hren N. and Gomez C. J. (1964) *J. Neurochem.* **11,** 797.
Simpson L. L. (1971*a*) *Neuropharmac.* **10,** 335.
Simpson L. L. (1971*b*) in *Neuropoisons: Their Pathophysiological Actions* (Edited by Simpson L.) p. 303. Plenum Press, New York.
Simpson L. L. and Morimoto H. (1969) *J. Bact.* **97,** 571.
Simpson L. L. and Rapport M. M. (1971*a*) *J. Neurochem.* **18,** 1341.
Simpson L. L. and Rapport M. M. (1971*b*) *J. Neurochem.* **18,** 1761.
Simpson L. L. and Tapp J. T. (1967) *Neuropharmac.* **6,** 485.
Sumyk G. B. and Yocum C. F. (1968) *J. Bact.* **95,** 1970.
Svennerholm L. (1963) *J. Neurochem.* **10,** 613.
van Heyningen W. E. (1959) *J. gen. Microbiol.* **20,** 310.
van Heyningen W. E. and Mellanby J. (1968) *J. gen. Microbiol.* **52,** 447.
Wiegandt H. (1967) *J. Neurochem.* **14,** 671.
Zacks S. I., Metzger J. F., Smith C. W. and Blumberg J. M. (1962) *J. Neuropath. exp. Neurol.* **21** 610.