STUDIES OF PPLO INFECTION

II. The Neurotoxin of Mycoplasma Neurolyticum

By LEWIS THOMAS, M.D., FERNANDO ALEU,* M.D., MARK W. BITENSKY,‡ M.D., MORTON DAVIDSON,§ M.D., and BERTRAM GESNER,‡ M.D.

(From the Departments of Medicine, Neurology, and Neuropathology, New York University School of Medicine, and Bellevue Hospital Center, New York)

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Mycoplasma neurolyticum was identified as the cause of "rolling disease" in mice by Sabin (1) and Findlay et al. (2) in 1938. These investigators encountered the agent in the course of intracerebral passages of mouse brain homogenates in mice. The neurological syndrome was bizarre and unique, characterized by the abrupt onset of continual rolling movements to one side or the other, persisting for several hours and terminating in generalized convulsive seizures and death. Rolling was produced by intravenous injections of broth cultures of the mycoplasma, and culture filtrates were shown to contain a thermolabile exotoxin with the same property (3). Histologic study of the brains of mice surviving for 18 hr revealed areas of cystic degeneration in the cerebellar poles, which were considered to be the basis for the neurologic manifestations. Recently, Tully (4) succeeded in cultivating Sabin's original strain of mycoplasma (designated strain A) from lyophilized samples stored since 1943, and reexamined its properties. In confirmation of Sabin's observations, he noted that the exotoxin was readily obtained in broth filtrates of young cultures, but disappeared from cultures incubated 48 hr or longer; the washed organisms elicited the same neurologic syndrome as the exotoxin; young mice and rats were much more susceptible than mature animals; the toxicity of both exotoxin and suspensions of whole mycoplasmas was eliminated by heating at relatively low temperatures.

The present study is concerned with these and other properties of *M. neurolyticum*, in mice and rats. It will be shown that the exotoxin becomes fixed to tissue receptors within a few minutes after injection; rapid inactivation by binding to a sedimentable component of rat brain is demonstrable in vitro; evidence will be presented indicating that this inactivation is mediated by brain ganglioside. Neuropathologic studies of the brain lesions suggest that the action of toxin is primarily directed against membranes regulating the permeability of the brain to fluid.

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Material and Methods

Strains A and KSA of *M. neurolyticum* were supplied by Dr. Joseph Tully, of the National Institutes of Health. Strain A was received as the 21st passage in Difco serum fraction PPLO broth; after several passages in this laboratory, in PPLO broth as modified by Chanock, Hayflick, and Barile (5). Several liters representing the 30th through 33rd passage were distributed in 10-ml aliquots and stored frozen at −70°C. Subcultures of these were used for all experiments to be described. This procedure was used because of earlier observations indicating loss of toxicity after numerous subcultures of this organism. The KSA strain was the 147th passage when received, and produced no demonstrable toxin; this strain was employed for antibody protection experiments to be described below.

Crude preparations of exotoxin were made by inoculating PPLO broth with a 3% inoculum of frozen stock culture, incubating 18 hr or until turbid growth appeared, then making a new 2% inoculum from this and incubating approximately 20 hr. Empirically, it appeared that the most active toxins were obtained from cultures in which the pH had dropped to 7.0 to 6.8 at the time of harvesting. The broth was centrifuged at 13,000 *g* for 30 min, at 4°C, and the supernate decanted. Mice weighing 10 to 12 g were injected with 0.5 ml of this material, by tail vein. For titrations of toxin, dilutions were made in Ringer's solution. Rats, weighing 150 to 200 g, were injected with 2 ml of toxin by tail vein. The toxin remained active after lyophilization, and most of the studies reported below were performed with lyophilized stocks of known potency.

Anti-mycoplasma antibody was prepared in rabbits by injecting a suspension of 10-fold concentrated mycoplasmas, incorporated in Freund's adjuvant, subcutaneously. Antisera for protection experiments were obtained 2 wk after the 4th weekly injection of antigen. The presence and specificity of antibody was determined by Clyde's growth-inhibition method (6).

RESULTS

Description of Rolling Disease

The onset of neurologic signs usually occurred between 30 and 60 min after intravenous injection of the toxin derived from whole broth. The earliest signs were spasmodic hyperextension of the head and raising of one forepaw, followed within a few minutes by the beginning of rolling. The animals rolled on the long axis of the body, intermittently at first and then continually, with occasional intervening periods in which they leaped or ran rapidly about the cage. Rolling persisted for 1 or 2 hr, after which they became immobile and comatose. Some died in this condition, while others developed repeated convulsive seizures, with rigid hyperextension of the hind limbs terminally, and then died. Most of the mice died within less than 4 hr after the onset of rolling, but approximately 5% of each group survived in a comatose condition for 1 or 2 days.

The time of onset of the syndrome, and the time fo death, were closely related to the dose of toxin administered (Table I). With concentrated toxin, prepared by reconstituting lyophilized toxin in distilled water half the volume of the original broth, rolling began within 5 to 10 min and death occurred in less than 30 min. Some of these animals developed acute pulmonary edema simultaneously with rolling, and died with bloody froth at their nostrils within the next minute or so. With smaller doses, representing dilutions of 1 to 8 or 1 to 16

of the original broth, the onset of rolling was usually delayed for as long as 2 hr, with death several hours later.

The end point in titrations of toxin was always very sharp, as though an all-or-none reaction was involved. A near-threshold dose sufficient to cause rolling in 1 mouse was almost always effective in all mice tested, while a slightly smaller amount failed to cause rolling in any. The point is illustrated in the titration shown in Table I.

TABLE I

Titration of M. neurolyticum Toxin in Mice

Dose of toxin*	Time of onset of rolling‡	Time of death‡	Incidence of death§
mg	*min*	*min*	
10	5, 5, 10, 10, 30	10, 10, 15, 60, 120	5/5
8	5, 5, 30, 30, 60	20, 30, 60, 120, 120	5/5
6	20, 30, 30, 40, 60	60, 90, 90, 120, 180	5/5
4	40, 60, 75, 90, 90	120, 120, 180, 180, 180	5/5
2	40, 60, 90, 120, 120	120, 120, 240, 240, 240	5/5
1	– – – – –	– – – – –	0/5

* Lyophilized toxin from supernatant broth, reconstituted in distilled water in a concentration of 20 mg per ml.

‡ Numbers refer to time after injection of toxin when individual mice were found rolling or dead. Absence of rolling disease indicated by –.

§ Numerator: No. dead; denominator: No. injected.

In general, mice which survived without symptoms for 3 hr after an injection of toxin remained entirely well. No instances of delayed or chronic disease were observed with doses less than the lethal ones; attempts to produce such disease by daily injections of sublethal doses were unsuccessful.

Rats.—The latent interval between intravenous injection and the onset of neurologic signs was similar to that in mice, but actual rolling was infrequently observed. The animals usually became weak and prostrated, made grossly ataxic attempts at circling and leaping, then developed clonic convulsive movements of the extremities and became comatose. Death occurred in most cases 3 to 4 hr after onset. The minimum lethal dose (M.L.D.) of lyophilized toxin was approximately the same as for mice, on a body weight basis. Prolonged survival with continuing neurologic signs occurred more frequently in rats, and a group of animals surviving for 1 day or longer was obtained for some of the neuropathologic studies described below.

Pathology of the Brain

Focal lesions of spongiform degeneration, with small cystlike cavities, were seen in the brains of mice and rats surviving for 8 hr or longer after intravenous toxin. The lesions were encountered in many regions of the cerebrum and cere-

bellum, but were most prominent in the deep layers of the frontoparietal cortex and underlying white matter, and in the molecular layer of the cerebellum.

Cortex.—The lesions consisted of extensive, well demarcated areas of sponginess, associated with the accumulation of many vesicles, closely apposed to each other and measuring 20 to 40 μ in diameter. Between the vesicles were thin, delicate septae in which glial nuclei were frequently found. The relationship of astrocytes to the accumulations of vesicles was a consistent one, and in some instances these cells appeared to be the source of vacuolization. There was no consistent relationship between capillaries and vesicles, although occasionally large vacuoles surrounding a capillary were seen.

Photographs illustrating the vesicular lesion are shown in Figs. 1 and 2.

The meninges and superficial cortical layers were intact, and did not contain vesicles. The neurons appeared normal, and the Nissl substance was regularly distributed. There was some tortuosity of axones, with focal swelling and ballooning revealed by Bielchowsky and Romanes stains, but their integrity was maintained. The microglia and other cells appeared normal. No vascular lesions or hemorrhages were seen.

White Matter.—Vacuolization was observed within the white matter, but the areas were less clearly defined. When present, they were usually in immediate proximity to similar changes in the cortex and appeared to represent extensions of the same lesion. Damage to myelin sheaths in these areas was indicated by patchy zones of pallor in sections stained for myelin (Figs. 3 and 4). Sudanophilia was not demonstrable in these regions. No evidence of axonal degeneration was seen in association with the myelin damage.

In rats and mice which survived for 2 days or longer after the onset of rolling, extensive lesions of necrosis involving all tissue elements were seen in the cerebral hemispheres and cerebellum (Fig. 5). These were bilateral and roughy symmetrical, resembling large areas of ischemic necrosis, with infiltration at the periphery by small numbers of mononuclear cells. Here, as in the earlier, acute lesions, the blood vessels appeared intact.

Sections of kidney, liver, lung, spleen, adrenal, and heart were normal in all animals examined, including several with severe brain lesions which survived for a period of 6 days.

Electron Microscopy.—The areas in which vesicles were seen by light microscopy were found to contain greatly distended astrocytes, with large quantities of intracellular fluid. The swollen cells were large enough to impinge on neighboring structures, causing distortion and compression of myelin sheaths. A detailed account of the ultrastructural changes induced by *M. neurolyticum* toxin is presented in a paper to follow (7).

The neuropathologic observations suggest the possibility that the toxin acts primarily on the membranes of astrocytes, causing damage to components which govern the permeability of this system of cells. This possibility will be

raised again in a later section, in considering the in vitro binding of toxin by a sedimentable component of brain.

Effect of Toxin on the "Blood-brain Barrier"

According to current views, the blood-brain barrier is, at least in part, dependent on the integrity of the astrocytes, since the brain contains virtually no extracellular fluid space (8). In view of the histologic evidence of increased permeability of these cells (7), it was of interest to examine the passage of inperitoneally injected trypan blue into the brains of mice treated with toxin.

Three groups of 8 mice each were used. The first received intravenous toxin, in a dose sufficient to produce rolling with 1½ to 2 hr and death within 5 to 6 hr in all animals. The second received inactivated toxin, heated at 56°C for 30 min. The third received no intravenous injection. All were given 0.1 ml of 0.5% trypan blue solution in saline intraperitoneally just before the injection of toxin, and again at hourly intervals thereafter. The brains of all mice in the group receiving toxin were removed immediately after death, and at the same time, equal numbers of control mice from the groups receiving heated toxin and no injections were killed with ether and their brain removed. At the end of 5 hr, at which time there were 2 surviving mice in the toxin-injected group, both rolling and moribund, the experiment was terminated and all brains removed.

The brains of all toxin-injected mice were stained with trypan blue, with the exception of 1 mouse which died 15 min after the injection of toxin. Some were a dusky grey color, while others were deep blue. None of the control animals, given heat-inactivated toxin or no toxin, showed any trace of staining of the brain.

Similar results were obtained with rats, using intravenous toxin in a dose sufficient to cause severe neurological symptoms within 2 hr. Four rats were given 0.5 ml of 0.5% trypan blue intraperitoneally immediately before toxin, and at hourly intervals thereafter. At the end of 6 hr, all showed deep blue staining throughout the cerebral hemispheres (Fig. 6). The cerebellum and brain stem were also stained, but with somewhat less intensity. Control rats, given heat-inactivated toxin and trypan blue in the same doses, showed no staining of the brain.

Properties of the Toxin

The following experiments were performed with the same lot of lyophilized toxin, stored in sealed containers at −20°C. The material was dissolved in distilled water in a concentration of 20 mg per ml, and the mice were given 0.5 ml by tail vein; this amounted to 5 minimum lethal doses per mouse (see Table I).

The toxin was completely inactivated by heating at 45°C for 15 min, and by

standing at 37°C for 2 hr. It was not affected by freezing at −70°C, nor by 10 cycles of freezing and thawing at this temperature. It retained full activity after lyophilization when stored at −20°C for 4 months.

Trypsin, in a concentration of 0.025 mg per ml in the distilled water used for dissolving the lyophilized toxin, caused complete inactivation within 10 min. The addition of crystalline soy bean inhibitor, in the same concentration, prevented inactivation by trypsin (Table II).

The toxin was not sedimented by centrifugation at 140,000 *g* for 2 hr. It was not removed by pressure dialysis through a membrane admitting molecules of molecular weight less than 30,000.

Samples of lyophilized toxin, reconstituted in distilled H_2O, were placed in Sephadex G-200 columns, 45 cm in height, 2.5 cm in width, with resin volume

TABLE II

Inactivation of Toxin by Trypsin

Toxin treatment*	Incidence of death‡
Toxin incubated with trypsin (0.025 mg/ml 10 min)	0/6
Toxin incubated with crystalline soy inhibitor (0.025 mg/ml) and trypsin	6/6
Toxin incubated with soy inhibitor alone	6/6
Toxin alone	6/6

* Each sample of toxin incubated 10 min at 37°C.

‡ As in Table I.

of 200 ml, prepared with phosphate buffer 0.05 M, at pH 6.5. All of the toxin emerged in a protein fraction immediately following the void volume eluate, in a peak between 70 and 80 ml. The finding indicates that the toxin has a molecular weight in excess of 200,000, although the possibility cannot be excluded that it is attached to a protein constituent of this molecular weight derived from the broth.

Importance of Route of Injection.—The toxin was only effective when injected intravenously. Although its neurotoxic properties indicate an action on the central nervous system, it did not cause rolling or death when injected intracerebrally, even when an amount of toxin 20 times the minimum lethal dose was given. The same was true for the intraperitoneal and subcutaneous routes. The results, summarized in Table III, indicate that the toxin affects primarily the blood vessels of the brain, and is either bound or inactivated locally when injected by other routes.

Protection by Antibody; Evidence for Rapid Binding of Toxin

Neutralization of the toxin by rabbit antibody against the A strain was demonstrated by mixing equal volumes of toxin (containing 10 M.L.D.'s per

ml) with antiserum in various dilutions, incubating the mixtures for 10 min at 37°C, and injecting in amounts of 0.5 ml per mouse. Antibody, in dilutions as high as 1 to 40, furnished complete protection. No protection was observed

TABLE III

Effect of Route of Injection on Lethal Action of Toxin

Route of injection*	Dose of toxin	Incidence of death‡
	mg	
Intravenous	10	6/6
	2	6/6
	1	0/6
Intracerebral	40	0/6
	10	0/6
Intraperitoneal	40	1/6
	10	0/6
Subcutaneous	40	0/6
	10	0/6

* Toxin injected in volume of 0.02 ml for intracerebral injections, 0.5 ml for others.
‡ As in Table I.

TABLE IV

Protection of Mice Against Rolling Disease by Antibody

Antibody*	Dilution	Timing	Incidence of death
Anti-A strain	1-5	Before toxin	0/6
	1-20	" "	0/6
	1-40	" "	0/6
	1-80	" "	6/6
	1-2	1 Min after toxin	0/6
	1-2	2 Min " "	0/6
	1-2	3 " " "	6/6
	1-2	4 " " "	6/6
Anti-KSA strain	1-2	Before toxin	6/6
Anti-*M. orale*	1-2	" "	6/6
Anti-*M. gallisepticum*	1-2	" "	6/6
Normal serum	1-2	" "	6/6

* Rabbit antisera used throughout, in dose of 0.5 ml intravenously, in dilutions indicated.

with normal rabbit serum, nor with rabbit antiserum against *Mycoplasma orale* or *Mycoplasma gallisepticum.*

Protection by antiserum in vivo was also demonstrable, but here the timing of the injection of antibody was of great importance. Table IV summarizes the

results of an illustrative experiment. When given immediately before toxin, or within 1 or 2 min after, antiserum was completely protective. But when delayed for as short a time as 3 min after toxin, no protection was demonstrable. The control mice in this experiment did not begin to exhibit neurologic signs until 1 hr or longer after the injection of toxin, indicating that within the 2 min period in which antibody was protective the toxin was still available for neutralization, but after this time the material became bound to receptor sites inaccessible to antibody. Alternatively, the toxin may have accomplished irreversible injury within 3 min after injection. In either case, the results imply a high degree of affinity in vivo between the toxin and its target.

It is of interest that antibody against the nontoxic strain of *M. neurolyticum*, the KSA strain, failed to neutralize the toxin in vitro or in vivo (Table IV). This antiserum was indistinguishable from the anti-A antibody in its capacity to inhibit growth of the A strain on agar plates, suggesting that the toxin is immunologically distinct from the antigenic determinants involved in growth inhibition.

Inactivation of the Toxin by Brain Sedimentable Component

The foregoing experiments suggested that rapid combination occurs in vivo between the toxin and a target receptor in the central nervous system. Accordingly, experiments were designed to learn whether binding of the toxin by brain tissue could be demonstrated in vitro.

In a preliminary trial, various quantities of a crude 10% saline homogenate of rat brain were added to a solution of lyophilized toxin, the mixtures incubated for 10 min at 37°C, and then centrifuged at 13,000 *g* to remove the sedimentable brain fraction. It was found that 0.5 ml of toxin was completely inactivated by the addition of 0.025 ml of brain homogenate (Table V).

The supernatant fraction of brain homogenate obtained by centrifugation at 13,000 *g* for 30 min, had no inactivating effect on the toxin. The active material was most abundant in sedimentable fractions prepared by Aldridge's (9) modification of the Hogeboom–Schneider (10) technique for the isolation of mitochondria. An illustrative experiment follows:

Two rat brains, weighing 3.5 g, were homogenized in 35 ml 0.3 M sucrose, using a Potter homogenizer with a Teflon pestle. The suspension was centrifuged at 1000 *g* for 10 min, and the supernatant fluid then centrifuged at 10,000 *g* for 15 min. This sediment was taken up to the original volume in 0.3 M sucrose, homogenized again, and recentrifuged at 10,000 *g* for 15 min. This process was repeated twice, and the final suspension then tested for its effect on toxin. To aliquots of 4 ml of toxin were added 0.8, 0.4, 0.2, and 0.1 amounts of brain suspension, the mixtures rotated in an incubator at 37°C for 10 min, and then centrifuged at 13,000 *g* for 15 min. The supernatant toxin samples were compared for activity with toxin to which the same volumes of 0.3 M sucrose were

added, with the same periods of incubation and rotation. Six mice were tested with each sample, in doses of 0.5 ml each. The toxin used was a lyophilized stock reconstituted in distilled water in a concentration of 50 mg per ml; this preparation contained 5 minimum lethal doses in 0.5 ml. To eliminate the

TABLE V

Inactivation of Toxin by Brain Extracts

Tissue extract*	Amount of brain extract added to 0.5 ml toxin‡	Incidence of death	Protection by extract
Brain, 1000 *g*, supernate	0.1 ml	0/6	+
	0.05 "	0/6	+
	0.025 "	0/6	+
	0.01 "	6/6	0
Brain, 10,000 *g* sediment, (resuspended as 10% suspension)	0.1 "	0/6	+
	0.05 "	0/6	+
	0.025 "	0/6	+
	0.01 "	6/6	0
Brain, 10,000 *g* supernatant	0.1 "	6/6	0
	0.05 "	6/6	0
Brain, 10,000 *g* sediment, treated with 0.0005 M K periodate	0.1 "	6/6	0
	0.05 "	6/6	0
Brain, 10,000 *g* sediment, heated 80°C 30 min	0.05 "	0/6	+
	0.025 "	0/6	+
	0.01 "	6/6	0
Brain, 10,000 *g* sediment, treated with trypsin	0.05 "	0/6	+
	0.025 "	0/6	+
	0.01 "	6/6	0
Brain ganglioside	0.25 mg	0/6	+
Liver, 10,000 *g* sediment	0.1 ml	6/6	0
	0.05 "	6/6	0
Kidney, 10,000 *g* sediment	0.1 "	4/6	±
	0.05 "	6/6	0
Kidney, 10,000 *g* sediment, heated 70°C 30 min	0.1 "	6/6	0
	0.05 "	6/6	0

* All tissues derived from rat except for ganglioside, which was prepared from beef brain.

‡ Each mixture of tissue extract and toxin incubated 37°C 10 min before injection into mice. Toxin dose per mouse = 0.5 ml, containing 5 M.L.D.'s.

possibility of lethal reactions to residual thromboplastic material following exposure of toxin to brain sediment, heparin was added to all preparations of toxin in a concentration of 0.05 mg per ml (11). By itself, heparin had no effect on the activity of the toxin.

The results are shown in Table V. The sedimentable brain fraction caused complete inactivation when amounts as low as 0.025 ml were combined with 0.5 ml toxin. No inactivation occurred with the supernatant brain extract obtained by centrifugation at 10,000 *g*.

No toxin was demonstrable in the brain sediments after incubation with toxin, when these were resuspended in Ringer's solution containing heparin and injected into mice. It thus appears that if the inactivation of toxin by the brain component involves adsorption of the toxin, the process is not reversible.

The factor responsible for inactivation of toxin was remarkably thermostable, withstanding heating at 80°C for 1 hr. It was not affected by treatment with trypsin. Exposure of the washed brain sediment to 0.0005 M K periodate for 10 min at 37°C resulted in complete loss of activity. These properties are summarized in Table V. The material remained active after lyophilization, and could be stored as dry powder for several weeks without change in activity. However, it was observed on several occasions that suspensions of the fraction in saline or Ringer's solution lost their inactivating property after incubation at 37°C for 1 hr. The possibility that enzymes may be present in these preparations which are capable of destroying the inactivating factor is currently under study.

The sedimentable component of rat liver, prepared in the same manner, had no inactivating effect on toxin. Relatively slight and inconstant inactivation was observed with suspensions of rat lung and kidney sediment, but the property disappeared when these suspensions were heated at 70°C for 30 min.

Inactivation by Ganglioside.—The thermostability of the inactivating factor, and the loss of its effect following treatment with periodate, suggested the possibility that a carbohydrate fraction of brain sediment might be involved. Van Heyningen and his associates have described the selective inactivation of tetanus toxin by gangliosides derived from brain (12), and four different preparations of ganglioside were therefore tested for their effect on *M. neurolyticum* toxin. Three of these were purified ganglioside derived from beef brain; the fourth was a monosialic ganglioside from a human brain with Tay-Sachs disease.[1] All caused inactivation of toxin when added in concentrations of 0.5 mg per ml and incubated for 10 min at 37°C (Table V). It is of interest that the inactivation by ganglioside and by the washed, sedimentable component of rat brain were both temperature-dependent (Table VI). Each was

[1] The authors are indebted to Dr. Irwin Chargaff, Dr. Alan Bernheimer, and Dr. Herbert Kayden for samples of ganglioside preparations.

effective at 37°C, while neither caused inactivation when held with toxin for 1 hr at 0°C.

It is possible that the active material in the sedimentable fraction of rat brain may be its ganglioside, but the point remains speculative at present. On a weight basis, purified ganglioside is more active than the lyophilized brain fraction (0.5 mg as compared with 3 mg per ml), but the difference is less than might be expected if the effect of the brain fraction were entirely due to its content of ganglioside. In this connection, however, it should be noted that Van Heyningen found that the activity of water-soluble ganglioside in binding tetanus toxin was considerably less than that of ganglioside contained in water-insoluble ganglioside-cerebroside-calcium complexes (12).

TABLE VI

Temperature Dependence of Inactivation of Toxin by Brain Extract

Brain extract	Temperature	Incidence of death	Protection
	°C		
12,000 *g* sediment, 0.05 ml in 0.5 ml toxin	37 10 min	0/6	+
	0 1 hr	6/6	0
Ganglioside, 0.25 mg in 0.5 ml toxin	37 10 min	0/6	+
	0 1 hr	6/6	0

DISCUSSION

The following theoretical concept of the mode of action of the exotoxin of *M. neurolyticum* is proposed. The material possesses a high degree of affinity for membrane receptors within the central nervous system, perhaps located in the podocytes of glial cells at their sites of attachment in the capillaries. Interaction between toxin and receptor results in disruption of the normal membrane regulation of fluid transport, and the astrocytes become greatly distended by imbibed fluid. The generalized swelling of the brain, as well as local compression of nerve cells and axones by individual astrocytes, are considered to account for the neurological manifestations and death of the animals.

The evidence for increased permeability of cells throughout the brain derives in part from the vesicular lesions seen by light microscopy, but primarily from the observed changes in electron microscopic sections described in a paper which follows (7). Swelling of glial cells, distended with fluid to such an extent that physical damage to neighboring cells and fibers occurred, was regularly encountered in all regions of the brain in both rats and mice. It is, of course, possible that the phenomenon of rolling may itself be due to some earlier, more primary event; it is conceivable that leakage of substances from the affected

cells may be the cause of conduction disturbances leading to rolling. However, the subsequent, widely disseminated lesions of necrosis in the cerebral hemispheres and cerebellum seem almost certainly to be based on this initial reaction of glial swelling.

The experiments with trypan blue provide further evidence for a generalized disruption of the blood brain barrier, associated with the onset of rolling disease. The deep staining of rat and mouse brains, which occurred when trypan blue was injected intraperitoneally after toxin, may be a direct result of the increased flow of fluid into the astrocytes, or leakage into the extracellular space of the brain. There is little evidence of an extracellular space in normal brain, but an artificial space may be created in these animals by disruption of swollen glial cells.

In view of its thermolability, large molecular size, and inactivation by trypsin, the toxin is probably a protein molecule. Whether or not other nonprotein moieties contribute to its biological properties cannot be determined until more highly purified preparations are obtained.

Although the toxin obviously acts primarily on the central nervous system, and perhaps solely here since no lesions in other organs can be demonstrated, it is of interest that it has no effect when injected directly into the brain. The observation suggests that the neural target for the toxin is only accessible by way of the lumen of the capillaries of the brain. Failure of intracerebrally injected toxin, as well as toxin given by subcutaneous or intraperitoneal routes to cause rolling disease, even with large doses, may perhaps be explained by local binding or inactivation by components of the tissue.

The rapidity with which the toxin becomes attached to its receptor in the living animal is indicated by the experiments dealing with protection by rabbit antibody. Complete protection was provided by antibody given by vein just before the toxin, or within 2 min after, but when the antibody was delayed until 3 min, protection was lost. It is important to note that both the control and antibody-injected animals in this experiment had a symptom-free latent period of at least 1 hr before the onset of rolling. The results suggest that the toxin becomes fully attached within less than 3 min after injection, and then is inaccessible to antibody.

The rapid inactivation of the toxin by exposure to suspensions of the sedimentable component of brain tissue suggests an analogy to the situation described by Van Heyningen with tetanus toxin (12). An important difference, however, is that the neurolyticum toxin becomes completely and irreversibly inactivated, while tetanus toxin retains its activity after adsorption to brain suspensions. As was reported for tetanus toxin, solutions of water-soluble ganglioside inactivate *M. neurolyticum* toxin. The inactivation of the toxin by both ganglioside and crude brain suspensions occurs within a few minutes at 37°C, and not at all at 0°C. It may be that the ganglioside is the active com-

ponent of the crude suspension, and perhaps also the receptor for *M. neurolyticum* toxin in the living animal brain; conceivably, ganglioside may be a substrate for enzymatic action by the toxin. The information available at this time cannot settle the point.

If ganglioside should prove to be a specific, selective receptor for *M. neurolyticum* toxin, a new line of investigation may be opened for common ground between this and another, unrelated mycoplasma with neurotoxic properties. *M. gallisepticum* (the S-6 strain) produces acute neurological manifestations and death within a few hours in turkey poults (as well as cerebral polyarteritis in birds surviving for 2 days or longer) (13). This mycoplasma was shown to adhere to the surface of turkey erythrocytes by binding to a sialic acid receptor at the cell surface (14). It is conceivable that the sialic acid groups of gangliosides may also be concerned in the binding and inactivation of the toxin of *M. neurolyticum*. Further work on the possibility is in progress.

SUMMARY

Rolling disease has been produced and studied in rats and mice, using the exotoxin of the A strain of *Mycoplasma neurolyticum*.

The primary lesion of the brain consists of spongiform degeneration, associated with vesicle formation in the cortex and underlying white matter of the cerebral hemispheres, and in the molecular layer of the cerebellum. The brains of animals surviving 2 days or longer show extensive necrotizing lesions resembling ischemic necrosis, in both cerebral hemispheres.

The brains of rats and mice with rolling disease become deeply stained by intraperitoneally injected trypan blue, indicating early disruption of the blood brain barrier.

The toxin appears to be a thermolabile protein with a molecular weight exceeding 200,000. It is only active when injected by vein, and causes no disease when injected intracerebrally, intraperitoneally or subcutaneously, suggesting the existence of specific receptors within the vascular bed of the central nervous system.

Protection is afforded by rabbit antibody against the toxin, but only when antibody is injected within less than 3 min after intravenous injection of toxin, indicating rapid fixation to receptors in the brain.

The toxin is inactivated by incubation for 10 min at 37°C with suspensions of the sedimentable component of normal brain. The inactivating factor in brain sediment is very thermostable, not affected by trypsin, and eliminated by treatment with periodate. Similar inactivation of toxin is demonstrable with water-soluble gangliosides of brain.

A theoretical concept to explain the action of the toxin is proposed.

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EXPLANATION OF PLATE

PLATE 100

FIG. 1. Hematoxylin and eosin–stained section of rat brain illustrating areas of vacuolization in deep cortex and superficial white matter. Most of the vacuoles are closely related to astrocytes. The white matter is pale and has a loose appearance. There is no evidence of inflammation. 20 hr lesion. × 37

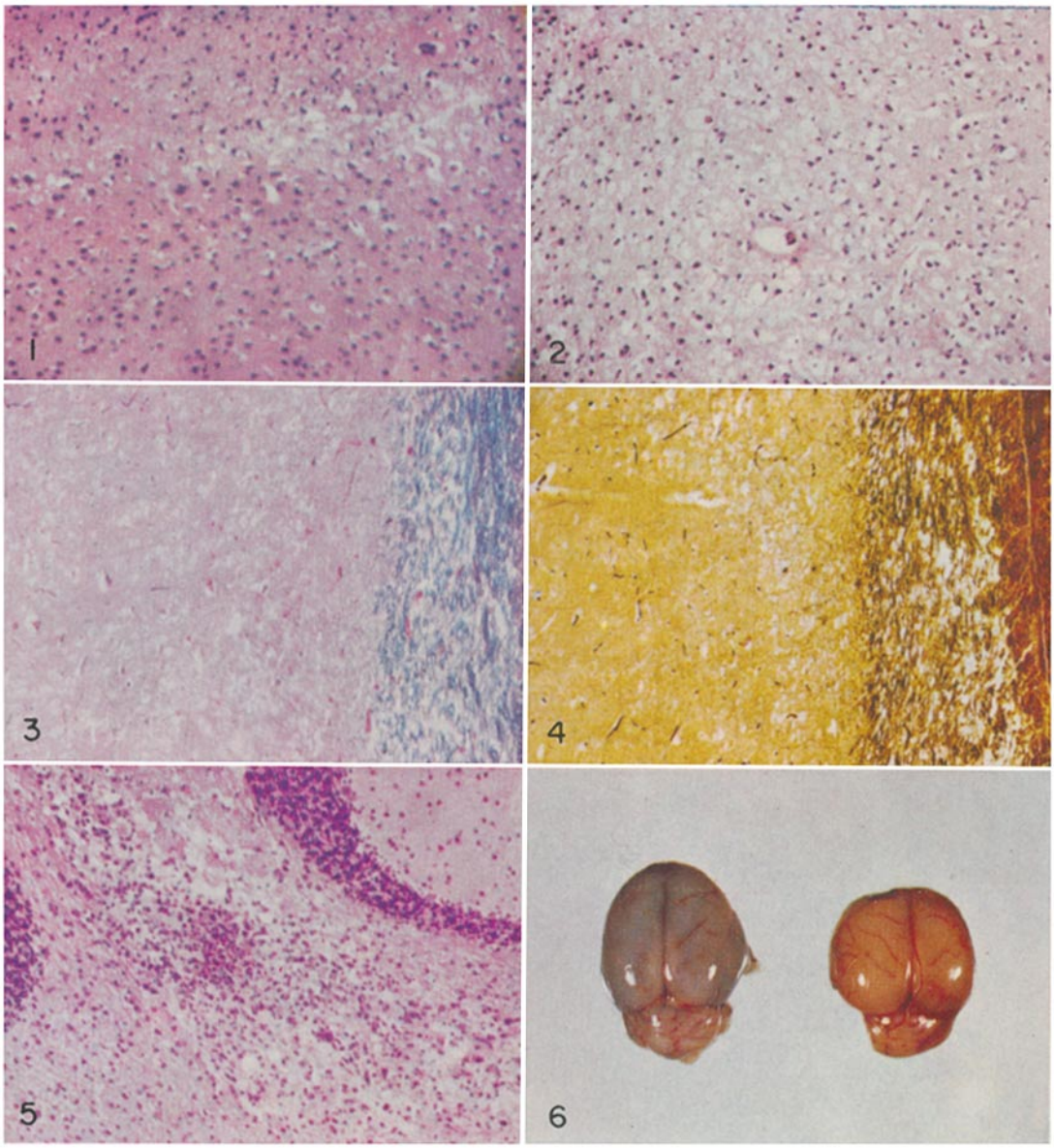
FIG. 2. Hematoxylin and eosin–stained section illustrating another area of vacuolization similar to that shown in Fig. 1. 20 hr lesion. × 37

FIG. 3. Luxol-fast blue with PAS counterstain to demonstrate focal areas of myelin loss in the superficial white matter. 20 hr lesion. × 37

FIG. 4. Bielchowsky preparation from the same region as Fig. 3, illustrating a loose axonal arrangement. 20 hr lesion. × 37

FIG. 5. 2-day-old lesion. Parenchymal destruction of cerebellum associated with a moderate inflammatory cell infiltrate. × 37

FIG. 6. Brains from rats given intraperitoneal injections of trypan blue. The blue brain is from a rat which died 6 hr after an intravenous injection of toxin. The unstained brain is from a control rat given heat-inactivated toxin.



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