PRODUCTION AND BIOLOGICAL CHARACTERISTICS OF AN EXTRACELLULAR NEUROTOXIN FROM *MYCOPLASMA NEUROLYTICUM*

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ABSTRACT

TULLY, JOSEPH G. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Production and biological characteristics of an extracellular neurotoxin from *Mycoplasma neurolyticum*. J. Bacteriol. 88:381-388. 1964.—Toxigenic strains of *Mycoplasma neurolyticum* were found to produce a potent extracellular neurotoxin for mice under a variety of cultural conditions. The observed biological characteristics of this toxin were quite similar to those originally described by Sabin. The toxin was again found to be readily inactivated by prolonged incubation and, when separated from *Mycoplasma* cells by filtration, possessed a greater heat resistance than whole cells. Young mice and rats were generally susceptible to neurotoxin, whereas most other experimental animals were resistant. Filtration results confirmed a minimal reproductive unit of 125 to 200 m μ for two neurotoxic *Mycoplasma* strains, with both cell-free filtrates reproducing the rolling disease syndrome in mice. Twice-washed *Mycoplasma* cells were also observed to be as neurotoxic as filtrates. Plate counting results revealed an intravenous LD₅₀ of approximately 3×10^8 washed cells for young mice.

The recovery and reidentification of Sabin's (Sabin, 1938a, b, 1939b, 1941; Sabin and Johnson, 1940) type A and C mouse *Mycoplasma* (pleuropneumonia-like organisms) from 20-year-old lyophilized cultures were recently reported (Tully and Ruchman, 1964). The original neurotoxic type A strain was shown to be serologically related to the L₅ mouse *Mycoplasma* strain recovered almost simultaneously by British workers (Findlay et al., 1938) and, although the type A strain was lost for many years, the descriptions given by Sabin (1941) and the successful maintenance of the L₅ strain by British investigators made possible the classification of these two strains as *M. neurolyticum* (Edward and Freundt, 1956). Schauwecker (1947), Roth (1951), and

Lemcke (1961) all have reported the isolation of neurotoxic pleuropneumonia-like organisms from brains of mice, but these strains, although exhibiting the virulence characteristics of the original L₅ and type A strains, were not subjected to any detailed serological comparison at the time of initial recovery. However, the KSA strain of Lemcke (1961) was recently found to be immunologically related to type A and L₅ strains of *M. neurolyticum* (Tully and Ruchman, 1964). These investigations also revealed that type A and KSA strains were highly virulent for mice, with intravenous inoculation of whole-cell cultures producing the "rolling disease" syndrome in 1 to 3 hr and death within 3 to 5 hr. In contrast, the L₅ culture was originally found to be of lower virulence than type A, and no evidence was obtained that the British strain produced an in vitro exotoxin. "Rolling disease" of mice was observed only when L₅ cultures were mixed with agar and inoculated intracerebrally (Findlay et al., 1938), and even this ability to induce disease appears to have been lost after prolonged cultivation of the L₅ culture (Edward, 1954). We have not been able to demonstrate any pathogenicity of the PG-28 strain of L₅ for mice (Tully and Ruchman, 1964).

The current report presents some observations on factors affecting the in vitro production of neurotoxin from type A and KSA strains of *M. neurolyticum*, the separation of toxin from whole cells, and several characteristics of the toxic filtrates, washed whole-cell suspensions, and fluid broth cultures of these two *Mycoplasma* strains.

MATERIALS AND METHODS

Mycoplasma strains. The type A strain of *M. neurolyticum* was recovered in this laboratory from a culture dried in Sabin's laboratory in 1943. It has been through more than 60 broth passages since its reisolation. The KSA strain was originally isolated by Lemcke (1961) from a normal

mouse brain, and a subculture of the fourth passage was received from Ruth Wittler, Walter Reed Army Institute of Research.

Cultivation. The medium used for production of neurotoxin and maintenance of *Mycoplasma* strains consisted of 7 parts PPLO broth (Difco), 1 part 25% fresh yeast extract, and 2 parts sterile horse serum (Cappel Laboratories, West Chester, Pa.) (Chanock, Hayflick, and Barile, 1962a; Chanock et al., 1962b; Taylor-Robinson et al., 1963). Antibiotics and other bacterial or fungal inhibitors were normally omitted. Maintenance cultures were passed every 2 to 3 days and, when fully toxic cultures were desired, 2 or 3 daily passages were made in the same medium, supplemented with 0.5% sterile glucose. Agar plates (60 by 15 mm) were prepared by adding 1.5% agar to the horse serum-yeast extract-broth medium. All cultures were incubated aerobically at 37 C. Viability of whole-cell *Mycoplasma* broth cultures and dilutions was estimated by plating 0.25 ml to agar plates and grading growth from confluent (4+) to any microscopically discernible colonies (1+).

Plate count procedures. Broth cultures (24-hr) were centrifuged at 10,000 rev/min ($16,300 \times g$) for 1 hr, and sedimented cells were washed twice in 100 ml of phosphate-buffered saline (pH 7.2). The final washed sediment was resuspended in the same buffer and adjusted to 60% light transmittance at 450 m μ in a Coleman Junior spectrophotometer. Tenfold dilutions of *Mycoplasma* suspension were made in broth media, and 0.01 ml of appropriate dilutions was removed with micropipettes; the tips were dried with gauze; and agar plates were inoculated. The inoculum was confined to a marked area of approximately 20 by 25 mm in the center of the plate. After the inoculum had dried, the plates were sealed with cellophane tape to prevent media dehydration and were incubated aerobically for 5 days at 37 C. Colony counts were made with a microscope having a 10 $\times$ objective and 5 $\times$ eye-pieces.

Toxicity tests. Detection of neurotoxin in whole-cell fluid cultures, filtrates, and washed-cell suspensions was accomplished by inoculation of 0.5-ml amounts of a 24-hr culture into the tail vein of 8- to 10-g National Institutes of Health general-purpose female mice. Controls received 0.5 ml of uninoculated broth by the same route. Hourly incidences of neurological signs, paralysis, and deaths were recorded for 8 hr. An occasional mouse receiving a terminal dilution would demonstrate obvious central nervous system signs

and survive 24 hr, but death usually occurred within 48 hr. Final recordings of mortality were made at 48 hr.

RESULTS

Neurotoxin vs. cultural conditions. Although *M. neurolyticum* strains have been shown to grow better under aerobic than anaerobic conditions (Freundt, 1958), attempts were made to detect differences in neurotoxin production under various atmospheric environments. The type A strain was passed three times at 24-hr intervals in horse serum-glucose broth, one series being maintained aerobically while the other was incubated in a sealed desiccator where the atmosphere had been evacuated and flushed three times with a 95% nitrogen-5% CO₂ gas mixture. A portion of the final fluid culture of each series was centrifuged at 15,000 rev/min ($27,000 \times g$) for 1 hr to remove large cell structures, the supernatant was passed successively through Millipore filters of 450 and 100 m μ porosity, and dilutions were made in uninoculated broth. The results (Table 1) showed only slight variation in amounts of neurotoxin produced by whole-cell cultures or cell-free filtrates under aerobic or anaerobic conditions.

Since many *Mycoplasma* strains will also grow in a Beef Heart Infusion-yeast extract broth supplemented with 1% serum fraction (Difco), the activity of type A whole cells and filtrates recovered from cultures maintained in a reduced serum environment was determined. This medium consisted of 9 parts PPLO broth (Difco), 1 part of 25% yeast extract, 1% serum fraction (Difco), 500 units/ml of penicillin, and 0.5 mg/ml each of amphotericin B and thallium acetate. The type A strain was passed approximately 30 times in this medium without evidence of adaptation. However, D. L. Madden of this Laboratory successfully adapted the type A strain by passing it in serum fraction broth containing 0.5% maltose, and has maintained it through more than 40 subsequent passages. Tests for neurotoxin were performed at the 41st passage and indicated (Table 1) that, although the culture did not flourish as well in a lower serum concentration, there was no loss or decrease in neurotoxicity.

The KSA strain of *M. neurolyticum* has been found to possess growth and neurotoxic properties very similar to the type A strain. Cell-free filtrates and whole fluid cultures were almost equally toxic for mice (Table 1).

Additional investigations on the in vitro pro-

TABLE 1. Neurotoxicity of 24-hr whole-cell fluid cultures and cell-free filtrates of *Mycoplasma neurolyticum*

<i>Mycoplasma</i> strain and growth conditions	Broth or filtrate dilution	Whole-cell broth		Cell-free filtrates	
		Mouse toxicity*	Viability†	Mouse toxicity*	Viability†
Type A, aerobic, 20% horse serum, glucose	Undiluted	5/5	4+	5/5	Neg
	1:2	5/5	4+	5/5	—
	1:4	5/5	4+	5/5	—
	1:8	5/5	4+	5/5	—
	1:16	5/5	4+	5/5	—
	1:32	0/5	3+	2/5	—
	Control‡	0/5	Neg	—	—
Type A, aerobic, 20% horse serum, no glucose	Undiluted	5/5	4+	5/5	Neg
	1:2	5/5	4+	5/5	—
	1:4	5/5	4+	5/5	—
	1:8	5/5	3+	5/5	—
	1:16	5/5	3+	5/5	—
	1:32	0/5	3+	2/5	—
	Control	0/5	Neg	—	—
Type A, anaerobic, 20% horse serum, glucose	Undiluted	5/5	4+	5/5	Neg
	1:2	5/5	3+	5/5	—
	1:4	5/5	2+	5/5	—
	1:8	5/5	2+	4/5	—
	1:16	3/5	1+	0/5	—
	1:32	0/5	Neg	0/5	—
Type A, aerobic, 1% serum fraction, glucose	Undiluted	5/5	3+	5/5	Neg
	1:2	5/5	2+	5/5	—
	1:4	5/5	1+	5/5	—
	1:8	5/5	Neg	5/5	—
	1:16	5/5	Neg	5/5	—
	1:32	4/5	Neg	3/5	—
	Control	0/5	Neg	—	—
KSA, aerobic, 20% horse serum, glucose	Undiluted	5/5	4+	5/5	Neg
	1:2	5/5	4+	5/5	—
	1:4	5/5	4+	5/5	—
	1:8	5/5	4+	5/5	—
	1:16	5/5	3+	5/5	—
	1:32	1/5	3+	0/5	—

* Deaths per number inoculated with 0.5 ml intravenously.

† Neg = negative; — = not done.

‡ Uninoculated broth.

duction of neurotoxin by these two *Mycoplasma* strains have indicated that glucose is not essential for neurotoxicity (Table 1) and that the addition of antibiotics or other inhibitors either to the 20% horse serum medium or 1% serum fraction formula had little effect on growth or toxicity of type A or KSA *Mycoplasma* strains.

Sabin (1938b, 1941) had previously shown the loss of neurotoxicity by cultures incubated 48

hr or longer; the present studies (Table 2) have confirmed this observation. Rapidly growing cultures in glucose broth show a rather sharp decrease in activity after 38 hr, but the estimated number of viable cells seems to remain relatively constant during this same period. Cell populations remain viable long after toxic properties are lost. The neurotoxicity of type A cultures grown in the absence of a carbohydrate is pro-

TABLE 2. *Time relationship of neurotoxin production by type A Mycoplasma neurolyticum in horse serum broth cultures*

Time of incubation hr	Mouse toxicity		Viability		pH changes	
	With glucose	Without glucose	With glucose	Without glucose	With glucose	Without glucose
18	5/5*	5/5	4+	4+	6.8	7.0
36	5/5	5/5	4+	4+	5.8	6.8
37	5/5	5/5	4+	4+	—†	—
38	5/5	5/5	4+	4+	5.5	6.8
40	0/5	5/5	4+	4+	—	—
42	1/5	5/5	3+	4+	5.5	6.8
44	1/5	5/5	3+	4+	5.5	6.5
46	2/5	5/5	3+	3+	—	—
48	0/5	4/5	3+	3+	5.3	6.4
60	1/5	0/5	2+	4+	5.3	6.4

* Deaths per number inoculated with 0.5 ml intravenously.

† Not done.

TABLE 3. *Response of mice of various ages to 24-hr horse serum broth cultures of Mycoplasma neurolyticum*

Mycoplasma strain	Age of mice weeks	Weight range g	Mouse toxicity of broth cultures	
			Undiluted	1:8
Type A	3	12-16	5/5*	5/5
	4	16-18	5/5	0/5
	6	22-28	4/4	0/4
	8	25-30	4/4	0/4
	12	26-32	4/4	0/4
KSA	3	12-16	5/5	4/5
	4	16-18	5/5	0/5
	6	22-28	4/4	0/4
	8	25-30	4/4	0/4
	12	26-32	4/4	1/4

* Deaths per number inoculated with 0.5 ml intravenously.

longed beyond 48 hr, but the toxin is inactivated between 48 and 60 hr of incubation, although there again appears to be no diminution in viability. Plate counts were not performed during these periods to confirm these estimates. Changes observed in pH of cultures containing carbohydrate were consistent with the active fermentation of glucose by both type A and KSA strains, but the inactivation of toxin by acid alone does not appear likely from observations recorded in

Table 2. Although neurotoxin from *M. neurolyticum* strains is inactivated by prolonged incubation, we found no loss of activity in 24-hr whole-cell cultures or cell-free filtrates frozen and held 2 months at -70°C .

Neurotoxin vs. animal susceptibility. Random-bred and four strains of inbred mice (8 to 10 g) were all found to be equally susceptible to *Mycoplasma* neurotoxin. Inbred mice of the C₅₇, C₃H, and BALB/c strains reacted somewhat more rapidly to neurotoxin, and most animals receiving undiluted broth cultures died within 1 hr after inoculation. DBA and random-bred mice exhibited the rolling syndrome to a greater degree, and most deaths occurred between 3 and 5 hr after challenge. The response of random-bred mice of various ages to type A and KSA cultures is shown in Table 3. The results clearly demonstrated an increased resistance to neurotoxin in older mice when diluted 24-hr whole-cell *Mycoplasma* cultures were administered intravenously. However, even 3-month-old mice were found to be very susceptible to undiluted cultures.

The susceptibility of other experimental animals to *M. neurolyticum* toxin was determined by inoculating rats, hamsters, guinea pigs, rabbits, and chickens with undiluted 24-hr broth cultures. Young rats (4 to 5 weeks) appeared to be as sensitive to type A and KSA neurotoxin as mice (Table 4), and 1 ml of either culture given intravenously induced neurological signs within 1 hr. Most rats did not display rapid rolling but showed signs of lethargy, slow rotation, loss of movement in hind legs, and a rather severe edema in both fore and hind legs; the majority died within 4 to 5 hr. Older rats, from 6 to 8 weeks of age, exhibited some resistance to a whole culture challenge of 1 ml but succumbed to a 2-ml dose. All deaths recorded in the older rats occurred between 24 and 72 hr.

Hamsters (50 g average) and guinea pigs (240 g average) challenged intracardially with 1 to 2 ml of whole-cell broth culture did not show evidence of neurological toxicity. Two each of five guinea pigs receiving type A and KSA exhibited a short period of ataxia and confusion within 10 min after challenge and appeared to be going into shock. These signs disappeared within another 10 to 15 min, and all survived without sequelae. Rabbits (2.5 kg) and young chicks (11 days old) inoculated with 2 ml of culture by the

TABLE 4. Response of rats to neurotoxin of whole-cell *Mycoplasma neurolyticum* horse serum broth cultures

<i>Mycoplasma</i> strain	Age of rats	Weight range	Toxicity (1 ml iv)
	weeks	g	
Type A	4	50-70	4/4*
	5	100-120	4/4
	6	150-200	3/5
	8	200-250	1/5
KSA	4	50-70	5/5
	5	100-120	5/5
	6	150-200	1/6
	8	200-250	0/6
	8	200-250	2/2†

* Deaths per number inoculated.

† In this case, 2 ml were inoculated.

intravenous route did not show any evidence of neurological involvement.

Filterability. The separation of *Mycoplasma* neurotoxin from viable cells was successfully accomplished by passage of centrifuged broth culture supernatant fluid through Millipore filters with an average pore diameter of 100 mμ (Table 5). The minimal reproductive unit of both strains appears to be in the range of 125 to 200 mμ. Retention of viable cells by 100-mμ filters did not decrease the potent toxicity of active *Mycoplasma* filtrates, and all mice receiving 0.5 ml of the filtrates intravenously displayed typical neurological signs and died within 5 hr.

Thermolability. Whole-cell broth cultures and cell-free filtrates were tested for viability and toxicity after heating to various temperatures. The results (Table 6) show that *Mycoplasma* cells were killed between 30 and 50 min at 45 C, whereas the toxin was not inactivated until this temperature had been maintained for 70 to 90 min. Whole-cell culture viability and filtrate toxicity were both destroyed after 10 to 25 min at 50 C.

Toxicity of washed *Mycoplasma* suspensions. Young broth cultures of type A and KSA were prepared for plate counting procedures as previously outlined. Washed-cell suspensions adjusted to a 60% light transmittance usually gave a viable-cell count between 2×10^9 and 3×10^9 cells per ml. Dilutions of these cell populations tested for toxicity indicated that an intravenous challenge of approximately 2×10^8 to 4×10^8 cells

was required for the induction of the neurological syndrome and death within 4 to 5 hr in 50% of mice inoculated (Table 7). Larger quantities of washed cells shortened the induction period, and mice given 10^9 cells or more usually died within 1 hr. Many of these animals exhibited a hemorrhagic foam around the mouth and nose shortly after death. Since care was exercised not to in-

TABLE 5. Neurotoxicity and viability of filtered horse serum broth cultures of *Mycoplasma neurolyticum*

<i>Mycoplasma</i> strain	Millipore filter type and porosity (mμ)	Mouse toxicity	Viability
Type A	AA, 800 ± 50	5/5*	4+
	HA, 450 ± 20	5/5	4+
	PH, 300 ± 20	5/5	4+
	GS, 220 ± 20	4/4	1+
	VC, 100 ± 8	5/5	Neg†
KSA	AA, 800 ± 50	5/5	4+
	HA, 450 ± 20	5/5	4+
	PH, 300 ± 20	5/5	3+
	GS, 220 ± 20	5/5	1+
	VC, 100 ± 8	5/5	Neg

* Deaths per number inoculated with 0.5 ml intravenously.

† Negative.

TABLE 6. Thermolability of whole-cell horse serum broth cultures and cell-free filtrates of neurotoxic type A *Mycoplasma neurolyticum*

Temp	Time	Whole-cell broth		Cell-free filtrate	
		Mouse toxicity*	Viability†	Mouse toxicity*	Viability†
45	<i>min</i>				
	10	5/5	4+	5/5	Neg
	20	5/5	4+	5/5	—
	30	5/5	2+	5/5	—
	50	5/5	Neg	5/5	—
	60	5/5	Neg	4/5	—
	70	4/5	Neg	2/5	—
	90	0/5	Neg	0/5	—
50	10	5/5	1+	5/5	Neg
	20	0/5	Neg	4/5	—
	25	0/5	Neg	0/5	—
	30	0/5	Neg	0/5	—
	60	0/5	Neg	0/5	—

* Deaths per number inoculated with 0.5 ml intravenously.

† Neg = negative; — = not done.

TABLE 7. Neurotoxicity of washed-cell suspensions of *Mycoplasma neurolyticum* grown in horse serum broth

<i>Mycoplasma</i> strain	Dilution	No. of organisms in 0.5-ml challenge	Mouse toxicity
Type A	Undiluted	4.62×10^8 *	4/6†
	1:2	2.31×10^8	2/6
	1:4	1.16×10^8	0/6
	1:8	5.78×10^7	0/6
	1:16	2.88×10^7	0/6
KSA	Undiluted	1.10×10^9 *	6/6
	1:3	3.66×10^8	2/6
	1:5	2.20×10^8	1/6
	1:7	1.57×10^8	1/6
	1:9	1.22×10^8	0/6
	1:11	1.0×10^8	0/6

* Number of organisms based on viable plate count.

† Deaths per number inoculated.

clude air in the intravenous inoculum, it would appear that large numbers of washed cells produced a pulmonary hemorrhage in these animals. Mice receiving fewer than 5×10^7 cells usually showed no evidence of ataxia, neurological involvement, or toxicity, and remained well. However, once signs of neurotoxicity were evident in mice receiving 10^8 or more washed cells, these animals showed continued loss of locomotor control and never survived the challenge.

DISCUSSION

Numerous *Mycoplasma* strains have been isolated from mice, but only *M. neurolyticum* has been afforded a generally recognized taxonomic status to date (Breed, Murray, and Smith, 1957). The classification of Sabin's (Sabin and Johnson, 1940; Sabin, 1941) other *Mycoplasma* strains recovered from the conjunctiva and respiratory tract of mice is still uncertain, as are the agents obtained from mice with infectious catarrh (Nelson, 1937; Edward, 1940) and conjunctivitis (Nelson, 1950). In addition to these unclassified organisms, there is evidence that mice are also capable of harboring *M. pulmonis* (L₃), strains which were originally recovered from lungs of rats (Klieneberger and Steabben, 1937, 1940; Klieneberger and Smiles, 1942; Lemcke, 1961).

Toxigenic strains of *M. neurolyticum* are unique among the Mycoplasmataceae, since they are the only members so far shown to elaborate an exo-

toxin, although an extracellular substance with lytic properties for certain tissue-culture cells has recently been found associated with several human *Mycoplasma* strains (Kraemer et al., 1963; Kraemer, 1964). Sabin (1938a, b, 1941) was the first to demonstrate in vitro and in vivo production of the neurotoxin from murine *Mycoplasma* and to identify it not only as the substance producing the rolling syndrome in mice but also directly involved with a specific neurolytic change in the posterior pole of the cerebellum. Its true exotoxic nature was established by its existence in sterile filtrates, its different heat resistance from that of viable cells, and because it was specifically neutralized by antitoxin. A nontoxic strain of *M. neurolyticum* L₅ (PG-28) has been the only representative serotype available for virulence studies until the recovery of neurotoxic *Mycoplasma* by Lemcke (1961) and the reisolation of Sabin's type A strain (Tully and Ruchman, 1964). The acquisition of these latter cultures afforded an opportunity not only to confirm the earlier observations of Sabin but also to provide some additional information on the biosynthesis and characteristics of *Mycoplasma* neurotoxin.

The results reported here agree almost fully with the descriptions given by Sabin for the development and properties of type A *Mycoplasma* neurotoxin. Both the type A and KSA strains employed in this study grew well and produced potent toxin under a variety of cultural environments, and each strain has now been through more than 100 broth passages without detectable changes in neurotoxicity. The lability of the neurotoxin was again confirmed by its observed inactivation after 48 to 60 hr in fluid broth cultures, even though viability was retained. Although cultures supplied with a fermentable carbohydrate showed a sharper pH change and toxin was inactivated in a shorter period of time, there appear to be additional factors involved in this loss of neurotoxic activity. Data on the chemical nature of the toxin may help explain these changes. The observed preservation of neurotoxicity by frozen cell suspensions and filtrates should also facilitate the purification and identification of the active material elaborated by these strains.

The susceptibility of other animals to whole-cell fluid cultures of *M. neurolyticum* reported here is not greatly different from that noted by Sabin (1941), who found 4- to 5-week-old mice and 1- to 2-month-old rats resistant. We also

observed an increased resistance of older mice and rats and a general insusceptibility of most other experimental animals to fully toxic cultures of type A and KSA strains. However, the fact that the resistance of older rats could be overcome with increased doses of whole-cell cultures suggests that concentration and purification of the toxin may be necessary to verify its neurotropic properties in other experimental animals. Although arthritis appeared to be an almost constant finding in earlier studies on mice inoculated with type A (Sabin, 1939a), we have not observed this response in animals receiving the revived type A strain. The transient edema in the extremities of young and older rats has been the only sign, other than neurological involvement and pulmonary hemorrhage, noted in challenged animals.

Previous reports on the filterability of viable type A *Mycoplasma* through gradocol membranes (Sabin, 1938b) revealed a minimal reproductive particle of approximately 200 to 300 m μ . Cell-free filtrates produced the typical nervous signs in mice, but neither the brains nor viscera contained a transmissible agent. Subsequent reports of *Mycoplasma* filtration (Elford, 1938; Edward, 1940) indicated an average minimal reproductive unit between 120 and 250 m μ ; these results have received general confirmation in several recent studies (Weibull and Lundin, 1962; Morowitz, Tourtellotte, and Pollack, 1963). The results of our investigation on filtration of type A and KSA strains through Millipore filters are in close agreement with earlier reports. Viable cells from both neurotoxic cultures passed filters with an average pore diameter of 220 m μ but were retained by 100-m μ filters, and the toxin obtained by filtration produced a response similar to that observed with whole-cell broth cultures. Filtration of serum (20%) broth cultures through 100-m μ filters was extremely slow and tedious, but was facilitated somewhat by prior centrifugation of broth and passage of supernatant fluids through 450-m μ filters before final separation.

The thermolability test results of whole *Mycoplasma* cells and exotoxin of type A *M. neurolyticum* were quite similar to the data previously reported for this strain (Sabin, 1938b, 1941), as well as for whole cells of other murine *Mycoplasma* (Edward, 1940). The neurotoxicity of whole-cell broth cultures and cell-free filtrates heated to 45 C clearly show the distinct thermal resistance of toxin over viable cells of type A.

Our attention was also directed to the possibility of endotoxin in *M. neurolyticum*, since an endotoxic lipopolysaccharide has been demonstrated in *M. mycoides* (Villemot, Provost, and Queval, 1962). Washed type A and KSA suspensions were found to be as neurotropic and lethal as cell-free filtrates, and plate counting techniques established an LD₅₀ of approximately 3×10^8 washed *Mycoplasma* cells for mice. Whether washed cells still elaborate exotoxin into the suspending media or whether significant cell lysis occurs with release of endotoxic substances has not been conclusively determined but is under investigation.

These studies, while substantiating much of the earlier work of Sabin, have also demonstrated other unique characteristics of neurotoxic *M. neurolyticum* cultures. Some of these properties may have important implications in establishing the origin of *Mycoplasma*, its relationship to bacterial L forms, and its role in association with bacteria, viruses, and protozoa in mixed infections.

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