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Physical and Chemical Properties of the Transmissible Mink Encephalopathy Agent

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The size of the transmissible mink encephalopathy (TME) agent is estimated to be less than 50 nm on the basis of its passage through membrane filters. The agent is sensitive to ether, relatively resistant to 10% Formalin, resistant to ultraviolet irradiation, and susceptible to proteolytic digestion with Pronase. Attempts to extract an infectious nucleic acid fraction with hot phenol were unsuccessful. The results of these studies indicate that the TME agent has biochemical properties which are similar to those described for the transmissible agent of scrapie.

Transmissible mink encephalopathy (TME) was first reported in 1965 by Hartsough and Burger (8). The disease is characterized by a long incubation period, progressive neurological dysfunction, and degenerative lesions of the brain. The agent was reported to be filterable (0.5 μm; Seitz), resistant to treatment with diethyl ether, resistant to heating in a boiling-water bath for 15 min, and resistant to treatment with 0.3% Formalin for 12 hr at 37 C (2). Because of this apparent high stability to physical and chemical inactivation, a similarity between the agents of TME and scrapie was noted.

The degree to which the TME agent is resistant to ether, heat, and Formalin was not known, since the initial experiments were performed on crude brain suspensions which were not titrated. The present study was undertaken to characterize further the physiochemical properties of the TME agent and to attempt to examine the extent of the analogy to the scrapie agent.

MATERIALS AND METHODS

The TME agent used in these experiments was originally obtained from a natural case of the disease on a mink ranch near Hayward, Wis., in 1963. The agent had been serially passed four times in mink with storage at −70 C between passages. Homogenates were prepared in Ten Broeck grinders, and the suspending medium, unless stated otherwise, was phosphate-buffered saline (PBS) at *p*H 7.2. Titration of brain suspensions in mink was by intracerebral (ic) or intraperitoneal (ip) inoculation of 0.1 or 1.0 ml of inoculum, respectively. The incubation period was the time in days from inoculation until the animal's locomotor coordination was noticeably impaired. Diagnosis of TME in inoculated mink was based on the presence of a positive clinicopathological syndrome, and the end points were calculated by the method of Reed and Muench (11). Mink were supplied by the University genetics department and were known to be free from diseases of the central nervous system.

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RESULTS

Effect of filtration on infectivity. A 10% TME brain suspension was centrifuged at 2,400 × *g* for 15 min. The supernatant fluid was then serially passed through membrane filters (25 mm in diameter and containing syringe adapters; Millipore Corp., Bedford, Mass.) with average pore diameters of 220, 100, and 50 nm. LD_{50} End points calculated at 300 days after ic inoculation were $10^{6.5}$ (supernatant fluid), $10^{6.0}$ (220 nm), $10^{5.0}$ (100 nm), and $10^{4.5}$ (50 nm). (*See* Table 1.)

Fractionation by gel filtration. A 10% saline suspension of TME brain was centrifuged at 2,000 × *g* for 15 min, and the supernatant fluid was clarified by serial passage through D-5, D-7, and D-9 filter pads (F. R. Hormann & Co., Inc., Milldale, Conn.). A portion of the filtrate was fractionated by gel filtration on Sephadex G-25 fine (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) according to the procedure described by Flodin and Killander (5). The slurry was carefully packed to a height of 42 cm in a glass chromatograph column measuring 2.5 by 50 cm. The flow rate was adjusted to 18 ml/hr, and 5 ml of the TME brain suspension was applied to the column. The eluant was PBS at *p*H 7.1. Serial 2-ml samples were collected and read for optical density on a Beckman model DU spectrophotometer. As can

TABLE 1. *Effect of filtration on infectivity*

Dilution	Supernatant fluid	Avg pore diameter		
		220 nm	100 nm	50 nm
10^{-2}	NT[a]	4/4 (152–163)[b]	2/2 (171–209)	2/2 (182–210)
10^{-3}	NT	4/4 (160–187)	2/2 (178–198)	2/2 (190–213)
10^{-4}	2/2 (196–214)	4/4 (172–198)	1/2 (218–288)	2/2 (234–253)
10^{-5}	2/2 (196–211)	4/4 (191–218)	1/2 (254–280)	0/2
10^{-6}	2/2 (243–262)	2/4 (224–243)	0/2	0/2
10^{-7}	0/2	0/4	0/2	0/2
10^{-8}	0/2	NT	NT	NT
LD_{50}[c]	$10^{6.5}$	$10^{6.0}$	$10^{5.0}$	$10^{4.5}$

[a] Not tested.

[b] Number of mink developing TME/number inoculated. Numbers in parentheses represent mean length of incubation, mean time of death or sacrifice in days.

[c] The ic LD_{50}/0.1 ml calculated at 300 days.

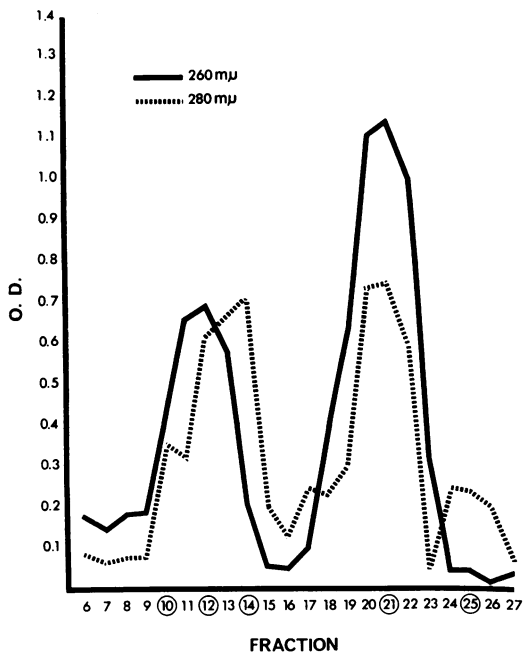

FIG. 1. *Optical density (OD) of fractions of a TME brain suspension collected after gel filtration of Sephadex G-25 fine. Circled fractions were inoculated ic into mink to assay for infectivity.*

be seen in Fig. 1, there were two peaks of ultraviolet (UV) adsorbance at 260 nm and three at 280 nm. Five fractions were each inoculated ic into two mink. The result of this bioassay indicated that no fraction had a titer of over $10^{1.0}$ (ic LD_{50}/0.1 ml). The unfractionated TME brain filtrate contained a titer of $10^{5.0}$ (ic LD_{50}/0.1 ml).

Resistance to 10% formalin. A cerebral hemisphere from a TME brain was diced into tissue fragments approximately 0.5 to 1.0 mm square and was placed into 100 volumes of 10% Formalin buffered with $CaCO_3$ to *p*H 7.5. The container was sealed and allowed to stand at room temperature. Periodically, samples of tissue were removed from the container, washed, homogenized in Ten Broeck grinders, and suspended in PBS. Titration was by the ip route with the LD_{50} end points calculated at 400 days after inoculation (Table 2). After 4 months of exposure to Formalin, the tissue had a titer of $10^{4.8}$ (ip LD_{50}/1.0 ml); at 11 months, $10^{3.8}$; and at 20 months, $10^{3.2}$. The other cerebral hemisphere, which had been frozen for 20 months at −70 C, contained $10^{6.5}$ ip LD_{50}/1.0 ml. It appears that, although the TME agent is quite resistant to the effect of 10% Formalin, it is capable of being slowly inactivated by prolonged exposure.

Sensitivity to ether. A 220-nm membrane filtrate of a saline suspension of TME brain was shaken with two volumes of diethyl ether and allowed to incubate at 4 C for 18 hr. The filtrate was exposed both as an undiluted 10^{-1} suspension and after 10-fold dilutions had been made for inoculation. The results (Table 3) show a 1.5 to 1.8 log reduction in titer indicating that the TME agent is sensitive to ether.

Photosensitivity. A 10^{-2} saline suspension of a 220-nm membrane filtrate of TME brain was distributed as a thin layer (3 ml) in a 60-mm petri dish and exposed to UV irradiation at 253.7 nm. A Champion 15-w germicidal lamp was used as the source of UV. The dose rate was 112 ergs per mm^2 per sec, as measured by a General Electric germicidal UV intensity meter. There was no detectable loss in infectivity after exposure to

TABLE 2. *Effect of 10% Formalin on the TME agent*

Dilution	Length of exposure to 10% Formalin			
	None[a]	4 months	11 months	20 months
10^{-1}	2/2 (180–214)[b]	3/3 (224–251)	3/3 (253–271)	3/3 (250–294)
10^{-2}	2/2 (173–201)	3/3 (233–264)	3/3 (258–292)	3/3 (268–309)
10^{-3}	2/2 (193–223)	3/3 (271–313)	3/3 (287–321)	2/3 (308–339)
10^{-4}	2/2 (211–239)	3/3 (270–307)	1/3 (380–393)	0/3
10^{-5}	2/2 (237–281)	1/3 (323–341)	0/3	NT[c]
10^{-6}	2/2 (271–300)	0/3	NT	NT
10^{-7}	1/2 (294–341)	0/3	NT	NT
LD_{50+}	$10^{6.5}$	$10^{4.8}$	$10^{3.8}$	$10^{3.2}$

[a] Frozen at −70 C for 20 months.
[b] Number of mink developing TME/number inoculated. Numbers in parentheses represent mean length of incubation, mean time of death or sacrifice in days.
[c] Not tested.
[d] The ip LD_{50}/1.0 ml calculated at 400 days.

TABLE 3. *Effect of diethyl ether on the TME agent*

Dilution	Treatment		
	None	Diluted after exposure to ether[a]	Diluted before exposure to ether
10^{-2}	4/4 (152–163)[b]	3/3 (180–190)	3/3 (173–191)
10^{-3}	4/4 (160–187)	2/2 (183–197)[c]	2/3 (193–210)
10^{-4}	4/4 (172–198)	2/3 (223–242)	2/3 (217–231)
10^{-5}	4/4 (191–218)	1/3 (221–228)	0/3
10^{-6}	2/4 (224–243)	0/3	0/3
10^{-7}	0/4	0/3	0/3
LD_{50}[d]	$10^{6.0}$	$10^{4.5}$	$10^{4.2}$

[a] Exposed as a 10^{-1} dilution.
[b] Number of mink developing TME/number inoculated. Numbers in parentheses represent mean length of incubation, mean time of death or sacrifice in days.
[c] One animal died immediately after inoculation and was excluded.
[d] The ic LD_{50}/0.1 ml calculated at 300 days.

doses as high as 1.34×10^5 ergs/mm^2 (Table 4). A dose of 2.3×10^2 ergs/mm^2 reduced the activity of bacteriophage T_4 to 1% when exposed in the TME brain suspension.

Phenol extraction. A 10% suspension of TME brain containing a titer of $10^{5.5}$ (subcutaneous LD_{50}/1.0 ml) was prepared in a buffer solution (0.15 M NaCl, 0.02 M sodium citrate) and lysed with the addition of duponol (sodium lauryl sulfate) to a final concentration of 10%. This lysate was then extracted with hot phenol according to the method described by Wecker (13). The final aqueous phase was precipitated with a double volume of absolute ethyl alcohol containing three drops of 2 M sodium acetate per 2 ml. The precipitate was resuspended in 5 ml of PBS at *p*H 7.2, and 0.1 ml was inoculated ic into each of four mink. These animals remained unaffected for a period of 2 years, at which time the experiment was terminated. Histopathological examination revealed no lesions indicative of encephalopathy.

Enzymatic digestion and phenol extraction. A portion of the TME brain filtrate described in the experiment on gel filtration was exposed to Pronase at a concentration of 1 mg/ml and was incubated for 55.5 hr at 37 C. The filtrate had been stored at −70 C for 37 days between experiments. The digest was centrifuged at 1,000 × *g* for 15 min, and the supernatant fluid was divided into two samples. One was assayed for infectivity by ic inoculation. The other sample was extracted with phenol by the method described previously and the extract was inoculated. The untreated filtrate suspension had a titer of $10^{4.2}$ (ic LD_{50}/0.1 ml). The filtrate suspensions treated with Pronase and Pronase followed by phenol extraction were not infectious after an observation period of 1 year.

DISCUSSION

A summary of results is presented in Table 5. After serial passage through membrane filters, 1% of the original infectivity present in the supernatant fluid remained in the 50-nm filtrate. Since the filters were not pretreated with proteinaceous material (12), it is possible that the loss of infectivity was due to adsorption and that the majority of the agent exists in a single, free homogeneous particle less than 50 nm in diameter. The loss in infectivity could also be explained by association of the agent with host material of varying particle

TABLE 4. *Effect of UV irradiation on the TME agent*

Dilution	UV treatment			
	None	6.72×10^3 (ergs/mm²)	6.72×10^4 (ergs/mm²)	1.34×10^5 (ergs/mm²)
10^{-2}	4/4 (140–152)[a]	3/3 (148–1161)	3/3 (142–156)	3/3 (137–149)
10^{-3}	4/4 (147–167)	3/3 (165–189)	3/3 (151–169)	3/3 (146–160)
10^{-4}	4/4 (161–177)	3/3 (169–201)	2/3 (169–193)	3/3 (160–173)
10^{-5}	3/4 (193–218)	3/3 (189–211)	3/3 (184–200)	3/3 (179–203)
10^{-6}	1/4 (223–241)	2/3 (213–241)	0/3	1/3 (238–256)
10^{-7}	0/4	0/3	0/3	0/3
LD_{50}[b]	$10^{5.5}$	$10^{6.2}$	$10^{5.5}$	$10^{5.8}$

[a] Number of mink developing TME/number inoculated. Numbers in parentheses represent mean length of incubation, mean time of death or sacrifice in days.

[b] The ic LD_{50}/0.1 ml calculated at 250 days.

TABLE 5. *Summary of results*

Treatment	Loss of infectivity	Recovery of infectivity
	%	%
Filtration		
50-nm membrane	99	1
Sephadex-25	>99.9	>0.01
10% Formalin	99.9	0.1
Ether	95	5
UV irradiation	0	100
Hot phenol (aqueous phase)	100	0
Pronase	100	0

size. The failure to obtain a high-titered fraction by gel filtration would seem to support the latter hypothesis.

The biochemical properties of the TME agent are quite unusual for a virus. The only other agent which has been shown to have similar properties is that of scrapie. The scrapie agent has been reported to have a high resistance to treatment with Formalin (10) and UV irradiation (1). Both the agents of scrapie (4) and TME are sensitive to ether, but not as markedly as are most viruses which contain an essential lipid component. It is possible that these agents are only lipophilic with the lipid being necessary for some degree of stability or infectivity.

There was no detectable infectivity in the aqueous phase of a hot phenol extraction of a TME brain suspension. One possible reason for the failure to extract infectious nucleic acid would be entrapment of the agent at the protein interface. This would be especially important if the agent was associated with cellular protein fractions as has been suggested for scrapie (9). In an attempt to overcome this possibility, the suspension was pretreated with a proteolytic enzyme. The result of this enzymatic treatment was a loss of infectivity. The scrapie agent has been reported to be resistant to mild proteolytic digestion (7); however, it not known whether the agent is susceptible to Pronase, a strong proteolytic enzyme. There have been no reported attempts of phenol extraction of the scrapie agent, although it has been cited as being inactivated by treatment with 90% phenol (6).

The resistance to UV irradiation is remarkable for an agent capable of indefinite serial passage and the ability to increase in titer at any passage level. Several oncogenic viruses have been shown to retain tumorigenicity while losing their infectivity after UV irradiation (3). We do not know what effect irradiation may have on the replication of the TME agent, since tissues from animals inoculated with the UV-treated suspension have not as yet been titrated.

It appears that the TME agent cannot be easily differentiated from the scrapie agent on the basis of their biochemical properties. Since both produce progressive neurological disorders with long incubation periods and comparable neuropathological changes, one must ask whether they are in fact different agents? In the absence of any specific immunological test, the only criteria remaining for separating the two disease agents is species susceptibility.

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