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Molecular Properties, Partial Purification, and Assay by Incubation Period Measurements of the Hamster Scrapie Agent†

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ABSTRACT: The scrapie agent causes a progressive degeneration of the central nervous system of animals after a prolonged incubation period. Measurements of incubation period length, defined as the time from inoculation to the onset of clinical signs of neurological dysfunction, were related to the titer of the agent and the dilution of the inoculated sample. Equations defining the relationship provide a new assay for the agent requiring fewer animals than end point titrations. By use of this incubation period assay, the scrapie agent from hamster brain was found to have an $s_{20,w}$ of <300 S but >30 S assuming $\rho_p = 1.2$ g/cm^3. A partially purified fraction P_3 was obtained by differential centrifugation and sodium deoxycholate extraction. When P_3 was extracted with phenol, virtually no infectivity was found in the aqueous phase even after examining such variables as pH, salt concentration, and predigestion of samples with proteinase K. Nonionic and nondenaturing, anionic detergents did not inactivate the scrapie agent; in contrast, denaturing detergents inactivated the agent. Sodium dodecyl sulfate ($NaDodSO_4$) inactivated greater than 90% of the agent at a $NaDodSO_4$ to protein ratio of 1.8 g/g. Inactivation by $NaDodSO_4$ appears to be a cooperative process. Addition of a nonionic detergent to form mixed micelles with $NaDodSO_4$ prevented inactivation of the agent by $NaDodSO_4$. Weak chaotropic ions do not inactivate the scrapie agent while strong chaotropic ions like SCN^- and Cl_3CCOO^- destroy infectivity at concentrations of 0.2 M. These data provide evidence in support of a protein component within the scrapie agent which is essential for maintenance of infectivity. Thus, it is unlikely that the scrapie agent is composed only of a "naked" nucleic acid as is the case for the plant viroids.

The scrapie agent causes a progressive deterioration of the central nervous system (CNS) of infected animals after a prolonged incubation period during which the animals exhibit no signs of neurological dysfunction (Sigurdsson, 1954; Eklund et al., 1967). The CNS degeneration is characterized pathologically by neuronal vacuolation and astroglial proliferation. Most striking is the lack of any sign of inflammation in an infectious process which has devastated the CNS. Two similar disorders in humans, kuru and Creutzfeldt-Jakob disease (CJD), have been identified (Gajdusek, 1977). The causative agents of scrapie, kuru, and CJD all appear to have many unusual biological properties. The unusual properties of the scrapie agent seem, in large part, to be a consequence of its small size and apparent hydrophobicity (Alper et al., 1966; Prusiner et al., 1978c, 1979).

To date, the scrapie agent has eluded isolation and identification. This appears to be due mainly to the lack of rapid assay for the agent and the hydrophobic nature of the agent itself.

In this communication, we describe an assay for the scrapie agent in hamsters based on the length of the incubation period and the development of equations relating titer, dilution, and incubation period. This assay substantially reduces the number of animals needed for quantitating the agent in a given sample when compared to the end point titration method. Using this new assay procedure, we have been able to develop a partial purification protocol and to define some biochemical and

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biophysical properties of the agent which should provide a foundation for further purification. Because isolation of hydrophobic macromolecules remains largely an empirical process involving the use of detergents, chaotropic salts, and organic solvents, we examined the stability of the scrapie agent to a wide variety of these chemical reagents. With the bioassay as our only means of detection and assay, it is necessary to identify conditons for dissociation of the agent from nonessential molecules with retention of infectivity. The empiricism involved in developing purification procedures for hydrophobic macromolecules is underscored by the experience of others attempting to isolate membrane-bound enzymes (Helenius & Simons, 1975; Kagawa, 1978).

Our studies show that the scrapie agent in a partially purified fraction P_3 was stable in a wide variety of nondenaturing detergents and weak chaotropic ions. The agent was labile in denaturing detergents such as sodium dodecyl sulfate ($NaDodSO_4$)[1] and in solutions of strong chaotropic ions such as sodium thiocyanate and sodium trichloroacetate. The inactivation of the agent by $NaDodSO_4$ was prevented by forming mixed micelles composed of nonionic detergents and $NaDodSO_4$. The agent was also labile in phenol but was both stable and precipitable in ethanol. These properties of the scrapie agent from hamster brain are consistent with our earlier suggestion that the agent is a small hydrophobic particle displaying an unusual degree of heterogeneity. In many respects the scrapie agent appears to mimic the behavior of hydrophobic membrane proteins.

Materials and Methods

Materials. $NaDodSO_4$ and lithium dodecyl sulfate ($LiDodSO_4$) were purchased from BDH Chemicals, sodium deoxycholate (DOC) was from Schwarz/Mann Biochemicals, Ammonyx LO was from Onyx Chemicals, sulfobetaines (Zwittergents) were from Calbiochem, and the other detergents were from Sigma Chemical Co. All of these detergents were used without further purification. Proteinase K was purchased from Merck and RNases were from Worthington. Bovine serum albumin (BSA) was crystalline fraction V obtained from Miles Laboratories (Pentex). Trizma base [tris(hydroxymethyl)aminomethane] was purchased from Sigma. The remaining chemicals were of the highest purity commerically available.

Source of the Scrapie Agent. Hamster-adapted scrapie agent was obtained from Dr. Richard Marsh (Marsh & Kimberlin, 1975). The agent was in its sixth passage in an LHC/LAK inbred hamster. The brain was homogenized in 320 mM sucrose, and the homogenate was clarified by centrifugation at 1000*g* for 10 min. Random-bred LVG/LAK female weanling hamsters obtained from Charles River Laboratories were inoculated intracerebrally with 50 μL of a 10^{-1} dilution. Fifty to sixty days after inoculation, these hamsters were sacrificed after developing clinical signs of scrapie. Homogenates containing the agent were prepared from these animals, and the agent was passaged again prior to use in the studies described here. The titer of the final inoculum (eighth hamster passage) was 10^{10} ID_{50} units/g of brain tissue as determined by end point titration using the method of Spearman & Kärber (Dougherty, 1964). Details of the dilution procedure and clinical signs of scrapie are described under Results.

Preparation of Homogenates. Weanling female, random-bred LVG/LAK Syrian hamsters were inoculated intracerebrally with 50 μL of hamster-adapted scrapie agent (10^7 ID_{50} units). The hamsters were sacrificed 60 days after inoculation. Their brains were removed immediately and washed in ice-cold 320 mM sucrose. Homogenization was performed in three 15-s bursts using a Polytron equipped with a PT35K generator. The temperature of the homogenate never exceeded 15 °C. The homogenate from 200 hamster brains was centrifuged at 4 °C in a Beckman JA 14 rotor for 10 min at 250*g*, and the supernatant was collected. The pellet was rehomogenized in sucrose and centrifuged, and the supernatant was added to the initial supernatant to yield a final suspension of 10% (w/v). The combined supernatant fluids from the 250*g* centrifugations (designated S_1) were used for the sedimentation studies as well as the preparation of a partially purified fraction designated P_3.

Sedimentation Analysis in Fixed-Angle Rotors. Analytical differential centrifugation studies were performed in a Spinco L5-65 ultracentrifuge equipped with a 50Ti fixed-angle rotor as previously described (Prusiner et al., 1977, 1978b).

Assay of DNA, RNA, and Protein. RNA was measured by the procedure of Schneider using orcinol reagent (Schneider, 1957). DNA was measured by using the fluorescent dye 3,5-diaminobenzoic acid with an Aminco SPF 500 spectrofluorometer (Kissane & Robins, 1958). Commercial preparations of yeast RNA and calf DNA were treated with phenol and used as standards, assuming $E_{260}^{1\%} = 250$ for RNA and $E_{260}^{1\%} = 200$ for DNA. Protein was determined by the method of Lowry et al.; bovine serum albumin was used as a standard (Lowry et al., 1951). Absorption at 260 and 280 nm was measured in a Gilford Spectrophotometer 252, equipped with a rapid sampling device having a 1-cm path length.

Electron Microscopy. Samples prepared for negative staining were deposited on a formvar-coated, carbon-stabilized grid treated with polylysine ($M_r \sim 2000$; 5 μg/mL) following glow discharge for 5 s (Williams, 1977). After 30 min, the excess sample was removed by touching the edge of the grid with a vacuum aspirator made from a flame-drawn Pasteur pipet. Freshly prepared 1% glutaraldehyde buffered with 20 mM TrisOAc, pH 8.3 was then applied to the grid for 5 min. The grid was then washed in 0.1 M NH_4OAc followed by another wash with 0.01 M NH_4OAc. Excess NH_4OAc was removed by aspiration, and the grids were stained immediately with 1% uranyl formate (R. C. Williams, personal communication) or 2% phosphotungstic acid.

Material for thin sections was sedimented in a 50Ti rotor at 50000 rpm for 90 min. The pellet was fixed in 3% buffered glutaraldehyde and was encased in an agar block prior to osmication (2% buffered OsO_4 for 1 h). Dehydration of the block was performed with a graded acetone series prior to embedding in Epon resin (Spurr, 1969). Silver-gray sections were cut on a Sorvall MT-2 microtome, stained with uranyl acetate and lead citrate, and examined in a Hitachi 11E electron microscope at 75 kV.

Phenol Extractions. Prior to extraction with phenol, a partially purified fraction P_3 was suspended in 30 mM Tris–acetate, pH 8.3, containing 0.5 mM EDTA and digested with proteinase K (30 μg/mL) for 60 min at 27 °C. RNase A (30 μg/mL) was then added and the incubation was continued for an additional 30 min at 27 °C. Upon completion of these digestions, the sample was cooled to 4 °C. Aliquots were extracted with phenol at varying pH values and salt concentrations (Zasloff et al., 1978). The pH of 0.5-mL aliquots was

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DOC, sodium deoxycholate; TX-100, Triton X-100; sarkosyl, sodium dodecyl sarcosinate; $LiDodSO_4$, lithium dodecyl sulfate; PEG, poly(ethylene glycol); NP-40, Nonidet P-40; CTAB, cetyltrimethylammonium bromide.

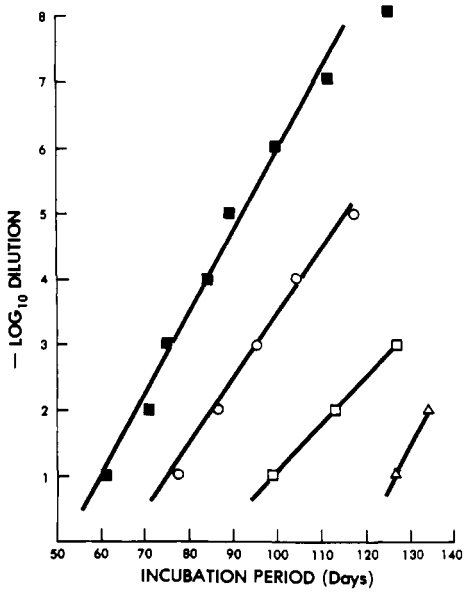

FIGURE 1: Relationship between incubation period and dilution of samples containing the scrapie agent. Samples containing different titers (ID_{50} units/50 μL) of the scrapie agent as determined by end point quantal titration are plotted: $10^{7.8}$ (■), $10^{5.5}$ (○), $10^{3.3}$ (□), and $10^{2.0}$ (△).

adjusted to a desired value with NaOAc to give a final concentration of 50 mM. NaCl was then added to achieve a desired salt concentration. Identical buffers were used to equilibrate the phenol. Equal volumes of cold equilibrated phenol were used to extract the samples at 4 °C. Phase separation was accomplished by centrifugation at 3500*g* for 10 min at 4 °C. The aqueous phase was recovered for determination of scrapie infectivity. Control samples were not extracted or centrifuged. Aliquots of all samples were diluted 10-fold for estimation of titer by measurements of incubation period.

Gradient Centrifugation. Postmitochondrial supernatant fractions (S_2) containing 320 mM sucrose were mixed with detergents at 4 °C and stirred for 30 min. The detergent extract was then centrifuged at 48000*g* for 30 min to remove large particles. Fifteen milliliters of supernatant fluid was layered on a 25% (w/v) sucrose cushion (10 mL). After centrifugation in polycarbonate tubes at 50000 rpm for 120 min in a 50.2 Ti fixed-angle rotor, the tubes were divided into 10 equal fractions (2.5 mL) by collection from the top using a Pasteur pipet.

Results

Assay of the Scrapie Agent. The titer of the scrapie agent was determined either by end point titration or by the incubation period method described below. In all cases, four weanling female hamsters were inoculated intracerebrally (ic) with 50 μL of a given sample at a specified dilution. The diluent was phosphate-buffered saline containing 0.5 unit/mL penicillin, 0.5 μg/mL streptomycin, 2.5 μg/mL amphotericin, and 50 mg/mL BSA. All of the animals showed no sign of neurological dysfunction for more than 6 weeks after inoculation. The onset of clinical scrapie was diagnosed by the presence of at least two of the following signs: generalized tremor, ataxia of gait, difficulty righting themselves from a supine position, and/or head bobbing. With further progression, the ataxia becomes so pronounced that balance is maintained with considerable difficulty. Kyphotic posture, bradykinesia, and weight loss appear 7–15 days after the onset of the illness. Over the next week, the hamsters become unable

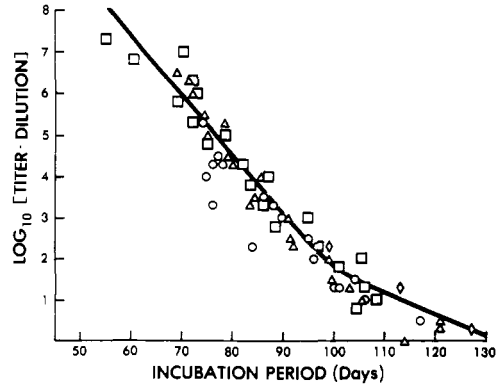

FIGURE 2: Relationship between the incubation period and the inoculated dose (titer × dilution) of the scrapie agent in hamsters. Samples with titers (ID_{50} units/50 μL), as established by end point titration, of $>10^{7.8}$ (□), $10^{7.3}$–$10^{7.8}$ (△), $10^{5.0}$–$10^{6.3}$ (○), and $10^{3.3}$ (◇) are plotted. The two straight lines are described by the equation given in the text for measurement of scrapie titers based on the length of the incubation period.

to maintain an erect posture, lie quietly on their sides and exhibit frantic movements of the extremities when disturbed. Death follows in 3–5 days.

As shown in Figure 1, there is a progressive lengthening of the incubation period as a function of both increasing sample dilution and decreasing sample titer. For example, samples with titers of $10^{7.8}$ ID_{50} units/50 μL of inoculum show a progressive increase in the incubation period as the dilution of the sample increases.

In order to relate the three variables shown in Figure 1, titer, dilution and incubation period, we plotted the log (titer × dilution) as a function of the incubation period as shown in Figure 2. The product of the titer and the dilution is simply the infectious dose of the agent inoculated into the experimental animals. The titers were determined by quantal end point titration according to the method of Spearman and Kärber (Dougherty, 1964). The dilutions were made in 10-fold increments as described above. Incubation period measurements represent the time in days from inoculation to the onset of clinical signs of scrapie. As illustrated in Figure 2, the product of the titer and the dilution is a complex function of the incubation period which can be expressed as the sum of at least two exponentials. Since the scatter of points precludes the advantageous use of curve-fitting techniques, we have chosen to approximate the relationship with two straight lines. The two lines were chosen so as to ensure a good approximation at the shortest and longest incubation periods shown in Figure 2. The straight lines are described by

$$\log \text{ titer} = \begin{cases} 17.27 - \log \text{diln} - 0.1424(x) \text{ for } x \leq 100 \\ 8.27 - \log \text{diln} - 0.0524(x) \text{ for } x > 100 \end{cases} \quad (1)$$

where x is the mean incubation period in days and the titer is expressed in ID_{50} units per milliliter. A computerized record system was developed for calculation of the titers using a Hewlett-Packard 9845 microcomputer.

The validity of the incubation period method for determining scrapie titers as described by eq 1 is supported by several lines of evidence. First, titers of the hamster agent obtained by the incubation period method agree, in general, with those found by end point titration within ±0.5 log ID_{50} unit/mL. Second, no differences between the molecular properties of the agents from hamster and murine sources have been detected by using primarily the incubation period method with the former and end point titration with the latter. Sedimentation profiles, detergent stability studies, and gel electrophoresis behavior

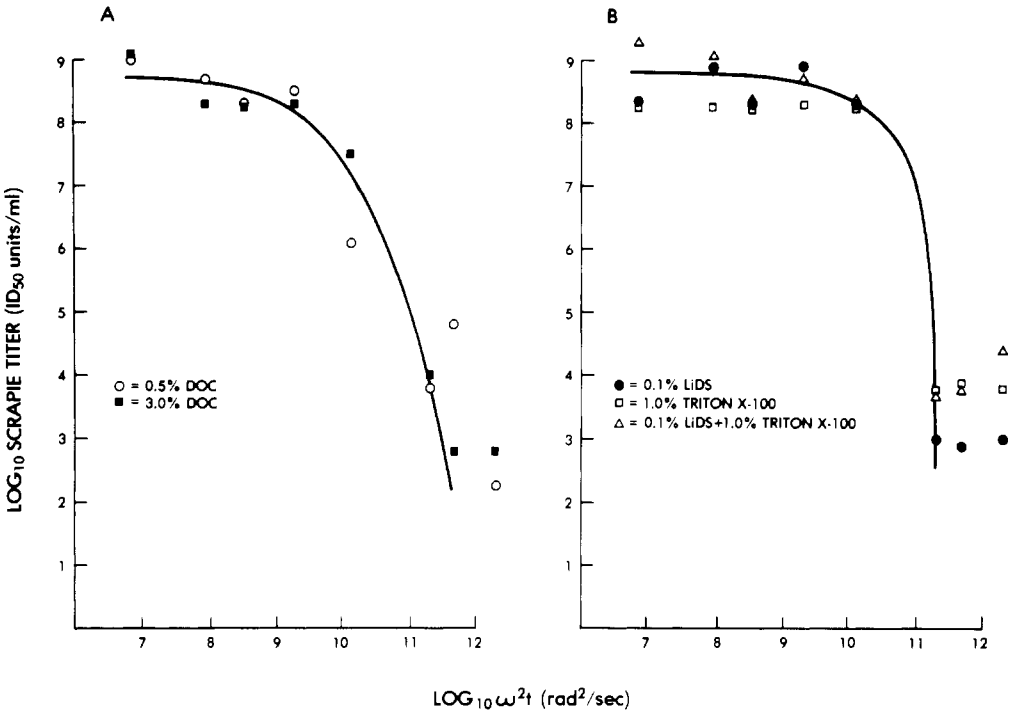

FIGURE 3: Sedimentation profiles of the scrapie agent in detergent extracts of hamster brain. (A) Postnuclear supernatant fraction S_1 was extracted with 0.5% (w/v) DOC (○) and 3% DOC (■); (B) 1% (w/v) Triton X-100 (□), 0.1% $LiDodSO_4$ (●) and a combination of the two detergents (△). Two-milliliter aliquots of the extracts were centrifuged in a 50 Ti fixed-angle rotor and the supernatant fluid was removed. The scrapie titer was estimated in the supernatant fluid for various ω^2t values (rad^2/s) where ω is the angular velocity of the rotor in rad/s and t is the time of centrifugation in s by measuring the length of the incubation period.

of the agent yield the same data whether the assay is performed by the incubation period method or end point titration. Third, a comparison of titers for most samples inoculated at two dilutions 1000-fold apart gives agreement within ±0.5 log ID_{50} unit. Data from 18 samples inoculated ic into hamsters at 10^{-1} and 10^{-4} dilutions are summarized in Table I. As shown, the standard errors of the mean for the samples are <0.6 log ID_{50} unit. The average standard error for the 18 samples is 0.22 log ID_{50} unit/mL by using four animals per sample. End point titrations using even six animals per dilution do not provide better precision.

Sedimentation Analysis in Fixed-Angle Rotors. A postnuclear supernatant fraction S_1 containing the scrapie agent from hamster brain was subjected to centrifugation in fixed-angle rotors for increasing speeds and times. The resultant sedimentation profiles of the agent in detergent-treated extracts are shown in Figure 3. Scrapie infectivity remaining in the supernatant fraction after centrifugation is plotted as a function of ω^2t where ω is the angular velocity of the rotor in rad/s and t is the time of centrifugation in s. At ω^2t values below 10^{10} rad^2/s, the scrapie agent was not sedimented from a postnuclear supernatant fraction. Increasing the centrifugation to an ω^2t value of 10^{11} rad^2/s sedimented virtually all of the scrapie infectivity, i.e., greater than 99.9%. The sedimentation profile of the agent was unchanged by increasing the DOC concentration from 0.5 to 3% (w/v) (Figure 3A). Treatment with the nonionic detergent Triton X-100 (1% w/v), with the denaturing anionic detergent $LiDodSO_4$ (0.1% w/v), or with a combination of the two detergents to form mixed micelles did not alter the sedimentation profile (Figure 3B). These studies, like those in murine spleen and brain, show that the scrapie agent in a postnuclear supernatant fraction sediments as a particle or heterogeneous group of particles with sedimentation coefficients of <300 S but >30 S, assuming a particle density of 1.2 g/cm^3.

Partial Purification of the Scrapie Agent. The strategy used for developing a partial purification protocol for the scrapie agent in hamster brain is similar to that described previously for murine spleen (Prusiner et al., 1978c). The sedimentation profiles for the agent were used to construct a purification protocol employing differential centrifugation. Nuclear (P_1) and mitochondrial (P_2) pellets were discarded and the supernatants S_2 was treated with 0.5% DOC. After the detergent-treated mixture was stirred for 30 min at 4 °C, the agent was sedimented at an ω^2t value of 10^{11} rad^2/s in either a 50.2 Ti fixed-angle rotor or a Ti 15 zonal rotor. The sedimented agent was recovered as a pellet from the bottom of the tubes in the 50.2 Ti rotor or from the external wall of the zonal rotor.

Table I: Comparison of Scrapie Titers Calculated from Incubation Period Measurements at Two Dilutions

	10^{-1} dilution		10^{-4} dilution		
sample	av incubn period ($n = 4$) (days)	log titer (ID_{50} units/mL)	av incubn period ($n = 4$) (days)	log titer (ID_{50} units/mL)	standard error of log titer (±ID_{50} units/mL)
1	64.3	9.0	86.8	8.9	0.05
2	61.5	9.7	86.8	8.9	0.4
3	69.3	8.4	82.0	9.6	0.6
4	70.3	8.3	91.5	8.2	0.05
5	67.8	8.5	87.5	8.8	0.15
6	69.8	8.3	91.8	8.2	0.05
7	67.3	8.7	92.0	8.2	0.25
8	69.0	8.4	92.0	8.2	0.1
9	65.8	8.9	95.0	7.7	0.6
10	65.8	8.9	91.5	8.2	0.35
11	72.5	8.0	97.8	7.3	0.35
12	72.5	8.0	88.3	8.7	0.35
13	67.8	8.6	88.3	8.7	0.05
14	67.0	8.7	88.3	8.7	0.0
15	72.5	8.0	88.5	8.3	0.15
16	64.3	9.1	85.5	9.1	0.0
17	68.5	8.5	88.3	8.7	0.1
18	75.3	7.6	91.7	8.2	0.3

Table II: Partial Purification of the Scrapie Agent from Hamster Brain

fraction	vol (mL)	protein (mg)	recovery (%)	RNA (mg)	recovery (%)	DNA (mg)	recovery (%)	scrapie titer (log ID_{50} units)	recovery (%)
homogenate	1850	29 160		1005		239		12.6	
S_1	1990	19 716	68	616	61	154	64	12.6	100
P_1	320	7 135	25	178	18	52	22	11.4	6
S_2	1640	12 084	41	334	33	105	44	12.1	32
P_2	350	9 900	34	161	16	50	21	12.1	32
S_3	1440	10 560	36	211	21	66	28	11.2	4
P_3	63	552	1.9	105	10	30	12	11.6	10

The sedimented material was resuspended in 20 mM Tris-acetate, pH 8.3. This fraction was designated P_3 and used in the studies described below. Fraction P_3 contained 12% of the DNA, 10% of the RNA, 1.9% of the protein, and 10–90% of the scrapie agent found in the homogenate (Table II). In preparations of P_3, the specific infectivity (ID_{50} units/mg of protein) of the agent was 5–30-fold higher than that of the homogenate as judged by both titration and incubation period measurements.

Electron microscopic studies of the P_3 fraction prepared from scrapie-infected and control hamster brains showed no consistent differences. In a preparation of fraction P_3 containing $\sim 10^{9.5}$ ID_{50} units/mL of the scrapie agent, membrane vesicles and amorphous structures were readily visualized by using 2% phosphotungstic acid. Prominent fluffy structures 25–30 nm in diameter resemble those observed by Siakotos et al. (1979). These structures were found with the same frequency in infected and uninfected preparations. Thin sections of the same preparations stained with uranyl acetate and lead citrate showed numerous membranes and ribosomes as well as many prominent electron-dense structures. These dense structures have no discernible membranes and are similar in size, frequency, and distribution to the fluffy structures visualized by negative staining.

In an attempt to diminish the loss of the scrapie agent in nuclear (P_1) and mitochondrial (P_2) pellets, combinations of sarkosyl and cholate were added to homogenates. The concentrations of sarkosyl and cholate were either 0.05 and 0.5% or 0.2 and 2.0%, respectively. The recoveries of the scrapie agent in the postmitochondrial supernantant fraction (S_2) from two separate experiments were unaffected by detergent addition. These recoveries ranged from 50 to 100%. Addition of 0.5% Triton X-100 to homogenates gave similar results.

Precipitation of the Scrapie Agent. In order to concentrate and purify the scrapie agent, we studied the behavior of the agent in various precipitation procedures was studied. In all of the protocols examined, the scrapie agent in fractions S_2 or P_3 was mixed with high concentrations of detergents at 4 °C and then precipitated upon addition of a variety of chemicals. The precipitates were collected by centrifugation at 48000*g* for 20 min. Precipitation of the scrapie agent by poly(ethylene glycol) (PEG M_r 6000), ethanol, streptomycin sulfate, and $ZnCl_2$ was studied by using the postmitochondrial supernatant fraction S_2 to which 4% DOC, 4% Triton X-100, or a mixture of 0.8% $NaDodSO_4$ and 2% Triton X-100 were added. At concentrations of PEG from 6 to 12.5%, more than 95% of the agent could be effectively precipitated from the high concentrations of detergents (Dean & Tanford, 1977). When the 0.5 M NaCl in the PEG solution was replaced by 0.1 M LiBr, 8% PEG was ineffective in precipitating the agent while 12.5% removed more than 99.99% of the agent. Also, 50% as well as 75% ethanol and 3.0% streptomycin sulfate were effective in precipitating the agent at 4 °C. In addition, 2–16 mM $ZnCl_2$, 25–33% ethanol, and 0.75–1.5% streptomycin sulfate were all ineffective as precipitants of the agent from suspensions containing high concentrations of detergents.

The titers of the scrapie agent in supernatant fluids as a function of pH were determined. The titer of the agent at pH 5 in the supernatant fluid was $\sim 10^5$ ID_{50} units lower than the control value at pH 8.3. All of the precipitated agent was recovered in the pellet. The studies suggest the agent has an isoelectric point (p*I*) between 5 and 6. Since the behavior of the agent as a function of pH is similar to that observed with many proteins, the possibility that the agent precipitates at pH 5 due to denaturation of tightly bound cellular proteins must also be considered.

Ammonium sulfate in the presence of mixtures of detergents containing cholate plus sarkosyl or deoxycholate was also effective in precipitation of the scrapie agent. The behavior of the scrapie agent during $(NH_4)_2SO_4$ fractionation in the presence of sodium cholate is described in detail in Prusiner et al. (1980b).

Phenol Extraction of P_3. Our previous studies on the agent from murine spleen indicated that the agent readily aggregates with cellular elements. In an effort to remove nonessential cellular material, we explored a variety of conditions for phenol extraction of the scrapie agent in fraction P_3. Several studies have convincingly shown that organic solvent extraction with phenol inactivates the scrapie agent (Hunter & Millson, 1967; Marsh et al., 1974; Ward et al., 1974); however, variables such as pH, salt concentration, and predigestion with proteases were not examined. The importance of pH and salt concentration in the aqueous and phenol phases has been clearly demonstrated in recent studies using acid–phenol extractions to purify plasmid DNA, small covalently closed circular polymers (Zasloff et al., 1978). Based on these studies, we examined the retention of scrapie infectivity in the aqueous phase as a function of pH, salt, and predigestion with nucleases and proteases. To prevent trapping of the scrapie agent at the interface, we digested samples with RNase A as well as proteinase K prior to phenol extraction. As shown in Table III, virtually no scrapie infectivity was recovered in the aqueous phase over a pH range of 3–8. NaCl concentrations from 0 to 200 mM at pH 4 and 6 were examined, but again virtually no scrapie infectivity was found in the aqueous phase. From these studies, we may conclude that either phenol destroys scrapie infectivity or efficiently removes it from an aqueous environment. Interpretation of the data is complicated by isoelectric precipitation of the agent between pH 5 and pH 6 in an aqueous medium as described above. The possibility that the brief low-speed centrifugation used to accomplish phase separation resulted in sedimentation of the agent in the acidic samples into or through the phenol layer must be entertained. Against this interpretation is the complete loss of scrapie infectivity in the aqueous phase at both acidic and slightly basic pH values.

Effects of Detergents on the Scrapie Agent. In general, since the most effective means of monomerizing hydrophobic

Table III: Phenol Extractions of the Scrapie Agent in Hamster Brain Fraction P_3 Digested with RNase A and Proteinase K

	pH	NaCl (mM)	scrapie titer (log ID_{50} units/mL)[a] control	extract
A:	3.0	75	8.6	<1.3
	4.0	75	7.9	1.3
	4.5	75		2.5
	5.0	75		2.3
	6.0	75	8.2	2.0
	8.0	75	7.6	<1.3
B:	4.0	0	8.1	<1.3
	4.0	75	7.4	<1.3
	4.0	125	8.7	<1.3
	4.0	200	8.0	<1.3
	6.0	0	8.2	<1.3
	6.0	75	7.9	2.5
	6.0	125		2.0
	6.0	200		<1.3

[a] See Materials and Methods for details of the extraction procedure.

particles such as membrane-bound enzymes with retention of biological activity involves the use of detergents, we examined the effect of a variety of detergents on the scrapie agent in fraction P_3. Some detergents more commonly used in the extraction of membrane proteins are listed in Table IV. Triton X-100, Nonidet P-40 (NP-40), Brijs 35 and 56, sulfobetaines 12 and 14, octyl glucoside, and Ammonyx LO did not inactivate the agent. No diminution in titer of the agent was seen at concentrations up to 4–5% w/v or v/v. The protein concentration of P_3 fractions used in these studies varied between 5 and 10 mg/mL. Ammonyx LO is primarily composed of dimethyldodecylamine oxide and is uncharged above pH 7 (Applebury et al., 1974). The sulfobetaines and Ammonyx LO are of particular interest because unlike many other nonionic detergents, they do not absorb at 280 nm.

Treatment of the P_3 fraction with a wide variety of nonionic detergents (Tritons, Tweens, and Brijs) having hydrophile–lipophile balance (HLB) numbers ranging from 11 to 17 did not alter the infectivity of the agent (Griffin, 1949; Umbreit & Strominger, 1973; Egan et al., 1976; Storm et al., 1976). Further studies are needed to determine which, if any, of these detergents can monomerize the agent with retention of infectivity.

Nondenaturing, ionic detergents also did not inactivate the scrapie agent in fraction P_3. Sodium salts of deoxycholic and cholic acids up to concentrations of 5% (w/v) did not alter the infectivity of the scrapie agent (Table V). Likewise, cetyltrimethylammonium bromide (CTAB), a cationic detergent, did not inactivate the agent at concentrations up to 4% (w/v).

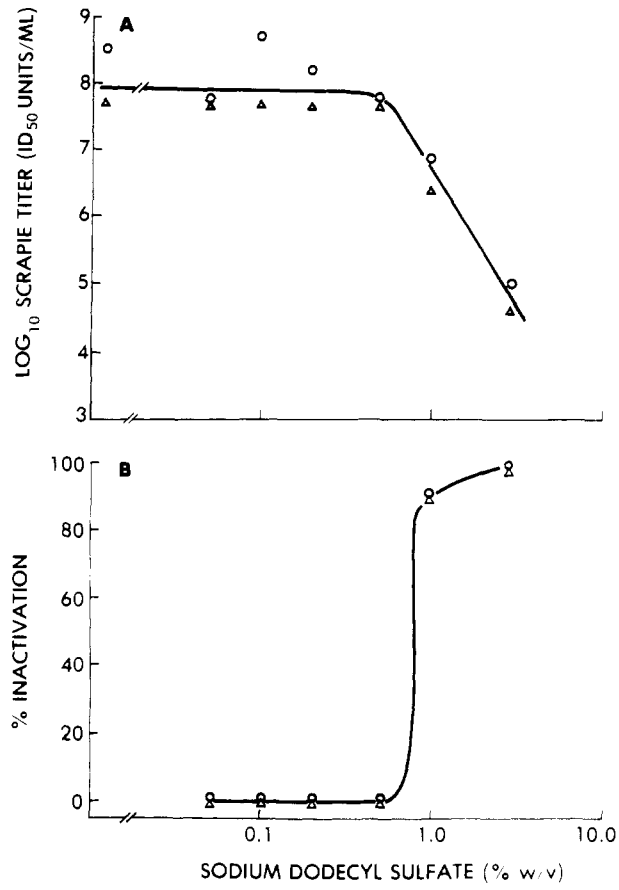

FIGURE 4: Inactivation of the scrapie agent by sodium dodecyl sulfate. $NaDodSO_4$ concentrations of 0.5 and 1.0% correspond to $NaDodSO_4$/protein ratios of 0.88 and 1.75 g/g, respectively. Incubations of $NaDodSO_4$ for 3 h (○) and 24 h (△) with fraction P_3 were performed at 25 °C. Plotted as a function of (A) scrapie titer and of (B) percent inactivation.

In contrast, the denaturing detergent $NaDodSO_4$ inactivated the agent as the concentration approached 1.8 g of detergent/g of protein. Significant decreases in scrapie infectivity were seen at concentrations of 1% (w/v) of $LiDodSO_4$ at 4 °C and of $NaDodSO_4$ at 25 °C (Table VI). The inactivation of the scrapie agent by $NaDodSO_4$ appears to be a highly cooperative process since concentrations of $NaDodSO_4$ below 0.5% (w/v) did not alter the infectivity while 1.0% caused a loss of over 90% (Figure 4). The apparent cooperativity, as well as the concentration range of $NaDodSO_4$ over which inactivation occurs, is similar to the binding of $NaDodSO_4$ to hydrophobic sites on proteins as they unfold during denaturation (Reynolds & Tanford, 1970; Tanford & Reynolds, 1976; Robinson & Tanford, 1975; Steele & Reynolds, 1979). No significant

Table IV: Stability of the Scrapie Agent in Selected Nonionic Detergents

detergent concn [% (w/v)][a]	scrapie titer (log ID_{50} units/mL)[b] Triton X-100	Nonidet P-40	Brij 35	Brij 56	sulfobetaine 3-12	sulfobetaine 3-14	octyl glucoside	Ammonyx LO
0	8.9	8.9	8.5	9.5	9.8	9.8	9.6	8.7
0.05			8.9	8.7				
0.1			9.3	9.6			9.4	9.2
0.5	8.7	9.3	9.5	9.2	10.1	10.5	9.6	9.0
1.0			9.0	9.1	10.0	10.2	9.4	8.8
2.0	9.1	8.8	8.5	8.5	9.1	10.0	9.8	8.2
3.0			10.0	9.0	9.9	9.7		8.8
4.0	9.1	7.9	9.8	9.2	10.3	9.7		
5.0						9.6	9.6	8.0

[a] Hamster brain fraction P_3 in 20 mM Tris-acetate, pH 8.3, was treated with detergents at 4 °C for 3 h. [b] Titer was estimated from incubation periods at 10^{-1} dilution of samples.

Table V: Stability of the Scrapie Agent in Selected Ionic Detergents

detergent concn [% (w/v)][a]	scrapie titer (log ID_{50} units/mL)[b]		
	DOC	sodium cholate	CTAB
0	8.9	8.9	8.5
0.05			8.3
0.1			9.2
0.5	8.2		8.0
1.0		9.0	8.3
1.5	9.0		
2.0			9.1
3.0	8.7	9.3	9.5
4.0			9.7
5.0	9.1	8.8	

[a] Hamster brain fraction P_3 in 20 mM Tris-acetate, pH 8.3, was treated with detergents at 4 °C for 3 h. [b] Titer was estimated from incubation periods at 10^{-1} dilution of samples.

Table VI: Comparison of the Effects of Dodecyl Sulfate, Decyl Sulfate, and Dodecyl Sarcosinate on Scrapie Agent Infectivity

detergent [% (w/v)]	detergent/protein[a] (g/g)	scrapie titer (log ID_{50} units/mL)[f] NaDod-SO_4[b]	LiDod-SO_4[c]	detergent/protein[d] (g/g)	scrapie titer (log ID_{50} units/mL)[f] NaDecyl-SO_4[e]	sarkosyl[e]
0		9.6	8.8		9.2	9.4
0.1	0.18	9.9	8.8	0.11	9.7	
0.2	0.35	9.3		0.22		9.5
0.5	0.88	8.9	8.0	0.55	9.0	
1.0	1.75	7.8	7.7	1.09	8.7	9.8
2.0				2.17	7.2	9.1
3.0	5.26	5.2	4.8	3.26	7.0	9.2
5.0				5.43		8.7

[a] Fraction P_3 contained 5.7 mg/mL protein. [b] Samples were incubated with sodium dodecyl sulfate ($NaDodSO_4$) at 25 °C for 3 h. [c] Samples were incubated with lithium dodecyl sulfate (LiDod-SO_4) at 4 °C for 3 h. [d] Fraction P_3 contained 9.2 mg/mL protein. [e] Samples were incubated with sodium decyl sulfate ($NaDecylSO_4$) or sodium dodecyl sarcosinate (sarkosyl) for 24 h at 4 °C. [f] Titer was estimated from incubation periods at 10^{-1} dilution of samples.

difference in the inactivation of the agent was observed upon comparing exposure to $NaDodSO_4$ for 3 and 24 h. Decreasing the hydrophobic chain length from 12 carbons to 10 slightly diminished the effectiveness of the detergent with respect to inactivation of the scrapie agent as shown by the data for sodium decyl sulfate. However, substitution of a sarcosinate moiety for sulfate without changing the hydrophobic dodecyl portion of the detergent renders the molecule ineffective as an inactivator of the scrapie agent. Sodium dodecyl sarcosinate (sarkosyl) did not inactivate the scrapie agent at concentrations as high as 5.0% (w/v).

To determine whether inactivation of the scrapie agent by $NaDodSO_4$ might be prevented by the presence of nonionic detergents, the titer of the agent in fraction P_3 was measured after treatment with detergent mixtures. The agent was stable in concentrations of $NaDodSO_4$ up to 2% in the presence of either NP-40 or Ammonyx LO. Denaturation of proteins by $NaDodSO_4$ is also prevented by addition of nonionic detergents (Converse & Papermaster, 1975; Russell, 1979). Precipitation of $NaDodSO_4$ at 4 °C was not observed upon formation of mixed micelles with NP-40 or Ammonyx LO.

Removal of detergents was accomplished by sedimenting the scrapie agent through a cushion of 25% (w/v) sucrose. To a postmitochondrial supernatant fraction (S_2), 4% DOC, 4% Triton X-100, or a mixture of 0.8% $NaDodSO_4$ and 2% Triton X-100 was added. Virtually all of the detergent and protein was recovered in fluid above the cushion while the agent readily

Table VII: Inactivation of the Scrapie Agent by Chaotropic Ions[a]

concn (M)	scrapie titer (log ID_{50} units/mL)[c] NaTCA[b]	NaDCA	NaMCA	NaOAc	NaSCN	NaCl	NaBr
0	9.6	8.6	9.7	10.2	9.4	9.1	9.1
0.05	9.6	9.3	9.9	9.8	9.9	9.4	9.4
0.2	6.1	10.0	9.5	9.6	7.5	9.9	9.9
0.5	4.4	10.2	9.6	9.8	3.7	9.4	9.4
1.0	<3.2	9.5	9.0	9.7	3.9	9.6	9.6

[a] Aliquots of fraction P_3 in 20 mM Tris-acetate, pH 8.3, were incubated at 4 °C for 3 h with the designated salt. The final pH was always between 7.4 and 8.3. [b] Abbreviations are TCA = Cl_3CCOO^-, DCA = Cl_2CHCOO^-, MCA = $ClCH_2COO^-$, and OAc = CH_3COO^-. [c] Titer was estimated from incubation period measurements of samples diluted 10^{-1}.

entered the sucrose cushion and was primarily found at the bottom of the tube. No significant differences in the sedimentation behavior of the scrapie agent were found among the three detergent conditions described above. The sedimentation of the agent in this sucrose step gradient may provide a means for further purification of the agent and is reminescent of that observed with hydrophobic proteins which readily aggregate upon removal of detergents (Warren et al., 1974).

Effects of Chaotropic Ions on the Scrapie Agent. The scrapie agent is stable in solutions of weak chaotropic ions as shown in Table VII. Cl^-, Br^-, CH_3COO^-, $ClCH_2COO^-$, and Cl_2CHCOO^- did not inactivate the agent at concentrations up to 1.0 M. In contrast, the strong chaotropic ions like SCN^- and Cl_3CCOO^- were potent inactivators of the agent at concentrations of 0.2 M. Similar results were obtained with the potassium salts of these anions.

Discussion

The scrapie agent has eluded isolation and identification of its chemical structure for more than two decades. In large part, three major problems appear responsible for this situation: (1) lack of a rapid assay for the scrapie agent which necessitates the use of animal titrations with prolonged incubation periods, (2) lack of a cell culture system for replication of the agent to high titers, and (3) the apparent hydrophobic nature of the agent which complicates purification.

The biophysical properties of the scrapie agent in hamster brain are clearly similar to those of the agent in murine brain and spleen (Hunter, 1979; Prusiner et al., 1978a; 1979). In all three cases, the agent was stable in nonionic detergents and nondenaturing, ionic detergents. Denaturing, anionic detergents destroyed the infectivity. The agent from hamster brain, like that from murine brain, is readily destroyed and/or removed from the aqueous phase by phenol (Hunter & Millson, 1967; Marsh et al., 1974; Ward et al., 1974). In addition, the sedimentation properties of the scrapie agent in hamster brain are quite similar to those observed for murine spleen and brain in the presence of deoxycholate (Prusiner et al., 1977, 1978b). The inability of the scrapie agent in a partially purified fraction from hamster brain to enter agarose gels upon $NaDodSO_4$ electrophoresis is similar to our findings for fraction P_5 of the scrapie agent in murine spleen (Prusiner et al., 1980a).

The observed similarities between the hamster and murine agents help to validate the use of incubation period measurements for estimation of titer. Even though several investigators have previously reported a relationship between scrapie titer in the inoculum and incubation period in both mouse and hamster, this relationship has not been used in studies on the purification of the agent (Eklund et al., 1963; Kimberlin & Walker, 1977, 1979; Marsh & Hanson, 1977;

Dickinson et al., 1969). All previous studies from our laboratory on the murine agent have used a quantal end point titration assay where titers were calculated by the method of Spearman and Kärber (Dougherty, 1964). In contrast, most of our current work on the hamster agent has been performed by using measurements of incubation period to estimate the titer of agent in a given sample. In fact, the incubation period method may be superior to titration in some respects. We have shown that the scrapie agent is capable of aggregation with cellular elements and possibly itself (Prusiner et al., 1978c; 1979). If aggregation occurs in a titration assay and the agent is not disaggregated during dilution, the actual titer will appear falsely low. Measurements of incubation period using undiluted samples may obviate this problem. Of equal significance is the fact that 5–10 times the number of experiments can be assessed with the same number of hamsters by measuring incubation periods instead of performing end point titrations. While the investigations presented here use primarily measurements of incubation period to estimate titer, the relationship between incubation period and titer must be constantly reassessed as purification proceeds.

The studies reported here clearly show that the scrapie agent is stable in nondenaturing detergents, weak chaotropic ions, and mild organic solvents. In contrast, the agent is inactivated by denaturing anionic detergents, strong chaotropic ions, and harsh organic solvents. Based on our experimental results, the most likely mechanism for inactivation of the agent is that of denaturation of a protein structure critical for maintenance of infectivity. $NaDodSO_4$ and $LiDodSO_4$ inactivate greater than 90% of the agent at a ratio of 1.8 g of detergent/g of protein. At this ratio most proteins undergo denaturation with concomitant saturation of their hydrophobic binding sites (Reynolds & Tanford, 1970; Steele & Reynolds, 1979). Inactivation of the scrapie agent by $NaDodSO_4$ has also been observed in extracts of murine spleen and brain (Hunter, 1979; Millson & Manning, 1979; Prusiner et al., 1980a). Inactivation of the agent by the strong chaotropic ions SCN^- and Cl_3CCOO^- at concentrations below 0.5 M is also consistent with denaturation of a protein structure upon disruption of hydrophobic interactions (Hatefi & Hanstein, 1969, 1974). The efficacy of these low concentrations of SCN^- and Cl_3CCOO^- argue against inactivation through alterations in nucleic acid structure. Breakage of hydrophobic bonds involved in base stacking of nucleotide polymers requires concentrations of strong chaotropic ions of 2–4 M as evidenced by a decrease in the melting point (Hamaguchi & Geiduschek, 1962).

Our studies as well as those of others have shown that the agent is stable in mild organic solvents such as ethanol but readily destroyed by harsh solvents such as phenol. The agent is effectively precipitated by ethanol with most of the infectivity recoverable in the pellet. In contrast, phenol extraction under a wide variety of conditions results in loss of virtually all of the measureable infectivity. Previous studies with neutral phenol extractions of crude homogenates have shown almost complete loss of infectivity from the aqueous phase and recovery of small amounts (<0.1%) from the combined interface and phenol layer (Hunter & Millson, 1967; Marsh et al., 1974; Ward et al., 1974; G. C. Millson, personal communication). Since both the pH and salt concentration greatly influence the extraction of small nucleic acids by phenol, we investigated these parameters systematically (Zasloff et al., 1978). Studies on acid–phenol extraction of circular plasmid DNA molecules have shown that the DNA can be removed from the aqueous layer by elevating the pH or increasing the NaCl concentration (Zasloff et al., 1978). Phenol extraction of the agent after digestion of fraction P_3 by RNase and proteinase K was performed since prior degradation of proteins facilitates the extraction and prevents trapping of molecules in the interface. More than 99.999% of the infectivity was lost from the aqueous phase upon phenol extraction under all conditions. While these phenol extraction studies only demonstrate the efficient removal of scrapie infectivity from the aqueous phase, the results are consistent with the detergent and chaotropic ion studies which suggest the presence of a protein component in the scrapie agent. Phenol is an excellent protein denaturant and presumably inactivates the scrapie agent by this mechanism.

All of the observations described above support our proposal that the scrapie agent is a small particle with hydrophobic domains on its surface and that hydrophobic interactions are probably essential for the maintenance of infectivity (Prusiner et al., 1978c, 1979). The molecular basis of this hydrophobicity remains to be elucidated. but the most likely explanation rests with a protein. Denaturation of the hydrophobic protein presumably occurs when extracts containing the agent are exposed to high concentrations of $NaDodSO_4$, chaotropic ions such as SCN^-, and denaturing organic solvents such as phenol. If the agent were composed only of an unmodified, naked nucleic acid like a viroid, then it should not be labile in $NaDodSO_4$ and phenol but stable in nonionic and nondenaturing, anionic detergents (Diener, 1979; Sanger et al., 1976). While direct proof for the existence of a hydrophobic protein is still lacking, we must continue to entertain the possibility of chemical modifications within a nucleic acid. However, such a nucleic acid must be considerably different from any molecules studied to date, including some tRNAs which are highly modified (Hall, 1971). The hydrophobic nature of the scrapie agent is consistent with recent observations showing that inactivation of the agent by ionizing radiation is dramatically increased by oxygen (Alper et al., 1978) and that some forms of the agent have densities as low as 1.08 g/cm^3 in sucrose, indicating bound lipid (Prusiner et al., 1978c).

Added in Proof

Recent studies show that the profound inactivation of the scrapie agent by thiocyanate ions is reversible upon removal of these ions by dialysis. Addition of nonchaotropic ions such as sulfate also reversed the inactivation caused by thiocyanate ions. These observations may provide an important new approach to the isolation of the scrapie agent.

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