Gel Electrophoresis and Glass Permeation Chromatography of the Hamster Scrapie Agent after Enzymatic Digestion and Detergent Extraction†

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ABSTRACT: Purification of the scrapie agent has been limited by the existence of the agent in multiple molecular forms [Prusiner, S. B., Hadlow, W. J., Garfin, D. E., Cochran, S. P., Baringer, J. R., Race, R. E., & Eklund, C. M. (1978) *Biochemistry 17*, 4993–4999]. Gel electrophoresis and glass permeation chromatography were used to assess the effectiveness of enzyme digestion and detergent extraction in reducing the scrapie agent into its smallest form. Scrapie infectivity in a partially purified fraction P_3 was unaltered by digestion with phospholipases A_2 and C, DNases I and II, RNases A and T_1, micrococcal nuclease, proteinase K, trypsin, Pronase, and chymotrypsin, as well as rattlesnake and cobra venoms. These digestions were performed at 4 and 37 °C for periods of time ranging from 1 to 16 h. The protease digestions were performed in the presence of 0.2% sodium dodecyl sarcosinate (sarkosyl) or 1.0% sodium deoxycholate. Electrophoresis of the scrapie agent into gels containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) at room temperature resulted in loss of >95% of agent infectivity. More than 95% of the recoverable infectivity was found in the uppermost portion of composite 2.5% polyacrylamide–0.5% agarose gels. Entry of the agent into $NaDodSO_4$ gels was not facilitated by prior sodium cholate extraction and precipitation with $(NH_4)_2SO_4$ or exposure to high concentrations of $NaDodSO_4$ up to 1%. The marked heterogeneity of the scrapie agent with respect to size was also observed with controlled-pore glass permeation chromatography in the presence of 0.5% $NaDodSO_4$. Much of the agent eluted in the void volume of the column, indicating a M_r of $>2 \times 10^6$. In contrast to the studies employing $NaDodSO_4$, sarkosyl gel electrophoresis at 4 °C permitted recovery of most of the agent applied to 0.6% agarose gels. Prolonged digestion of P_3 preparations by micrococcal nuclease, followed by proteinase K, permitted more than 30% of the agent to migrate ahead of nucleic acids of $<2 \times 10^6$ daltons. The remainder of the agent migrates with polymers of $>2 \times 10^6$ daltons and is not associated with any stainable protein or nucleic acid. In the eluates from the upper regions of the gel, specific infectivity calculations indicate the agent has been purified 100–1000-fold with respect to protein and DNA.

Attempts to purify the scrapie agent have been complicated by the hydrophobic nature of the agent and the slow bioassays involving animal titrations (Prusiner et al., 1978a, 1979). With the development of a new assay for the scrapie agent based on incubation period measurements, detailed studies on the stability of the agent to chemical reagents including numerous detergents were possible as described in the preceding paper (Prusiner et al., 1980a). These studies provide a foundation for the investigations reported here. Because the agent appears to bind a variety of cellular elements, presumably as a consequence of its hydrophobicity, we examined several experimental protocols designed to dissociate the agent into its smallest or monomeric form. Many studies on membrane-bound hydrophobic enzymes indicate that finding conditions for disaggregation of these proteins into their smallest biologically active form can be quite difficult (Kagawa, 1974; Helenius & Simons, 1975; Wasserman, 1974; Newby & Chrambach, 1979; Storm et al., 1976; Umbreit & Strominger, 1973; Egan et al., 1976; Wallach & Winzler, 1974). Most of these studies have employed detergents in combination with sedimentation and/or gel electrophoresis. The molecular sieving properties of polyacrylamide and agarose gels permit ready assessment of the extent of disaggregation after a given set of treatments (Wasserman, 1974; Newby & Chrambach, 1979).

With a knowledge of the stability of the scrapie agent in numerous detergents (Prusiner et al., 1980a), we began investigating the biophysical behavior of the agent in the presence of a few of these detergents. Gel electrophoresis and glass permeation chromatography were used to evaluate the extent to which the agent had been dissociated into its smallest form. While none of the procedures examined reduced all of the agent into a small form, a combination of hydrolytic enzyme digestion and detergent extraction was partially effective. Excellent recoveries of the agent were obtained in eluates from electrophoretic gels containing sodium dodecyl sarcosinate (sarkosyl).[1] Entry of the agent into these gels during electrophoresis at 4 °C was facilitated by prior digestion with micrococcal nuclease and proteinase K. This sequence of procedures forms the basis for a purification protocol which yielded a preparation highly enriched for scrapie infectivity.

Materials and Methods

Materials. Sodium dodecyl sulfate ($NaDodSO_4$) was obtained from BDH Chemicals, sodium deoxycholate (DOC) was from Schwarz/Mann Biochemicals, Triton X-100, sodium cholate, and sodium dodecyl sarcosinate (sarkosyl) were from Sigma Chemical Co., and agarose was from Marine Colloids. Ultrapure, enzyme-grade ammonium sulfate was purchased from Schwarz/Mann. Controlled-pore glass beads (CPG-700), Trizma base [tris(hydroxymethyl)aminomethane], Coomassie brillant blue R, ethidium bromide, micrococcal nuclease, phospholipases A_2 and C, snake venoms, and trypsin were obtained from Sigma. Proteinase K was obtained from

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DOC, sodium deoxycholate; TX-100, Triton X-100; sarkosyl, sodium dodecyl sarcosinate; CPG, controlled-pore glass.

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Merck and blue dextran was from Pharmacia. Acrylamide, bis(acrylamide), Temed, and ammonium persulfate were purchased from Bio-Rad Laboratories. The remaining chemicals were of the highest purity commercially available.

Assays. Assays for RNA, DNA, and protein were performed as previously described (Prusiner et al., 1978a). Titers of scrapie infectivity were estimated from the length of the incubation period in female hamsters as previously described (Prusiner et al., 1980a).

Partial Purification of the Scrapie Agent. The purification procedure used to obtain fraction P_3 by a series of differential centrifugations and extraction with DOC has been previously described (Prusiner et al., 1980a).

Gel Electrophoresis. Vertical slab gels 14 cm wide, 10 cm high, and 1.5 or 3.0 mm thick with five sample wells were prepared by using an SE 500 apparatus obtained from Hoefer Scientific Instruments. The 0.6% agarose gels as well as the buffer in the upper and lower chambers contained 60 mM Tris–acetate, pH 8.3, 1 mM EDTA, and either 0.1% $NaDodSO_4$, 0.2% DOC, or 0.2% sarkosyl. A 15% polyacrylamide gel plug was placed at the bottom of the agarose gels containing sarkosyl. Electrophoresis with $NaDodSO_4$ or a combination of Triton X-100 and DOC was performed at room temperature with composite or agarose gels at a constant voltage of 120 V. Electrophoresis with sarkosyl was performed at 4 °C for 4.5 h at a constant voltage of 90 V after slowly increasing the voltage during the preceding hour. The electrophoresis was started at 10 V for 5 min and then 20 V for 5 min and maintained at 30 V for the next 45–50 min while the bromphenol blue dye migrated 1.5 cm from the origin.

Upon termination of the electrophoresis, lanes to be assayed for infectivity were cut into sections of 2 cm or less and eluted into 4 mL of buffer containing either 20 mM Tris–HCl, pH 7.4, or 20 mM Tris–acetate, pH 8.3. The gels were homogenized at 4 °C by using a Polytron homogenizer equipped with a PT20ST generator for 15 s at full speed. The pulverized gel suspended in buffer was placed on a gyrotory shaker at 4 °C for 15 h. The suspension was clarified by centrifugation at 3300*g* for 30 min at 4 °C. Fifty microliters of supernatant fluid was then injected intracerebrally into hamsters either undiluted or diluted 10-fold for measurement of the scrapie incubation period.

Lanes to be stained for nucleic acids and proteins were immersed in ethidium bromide solution (1 μg/mL) for 30 min, rinsed with water, and photographed with Polaroid Type 55 film. The ethidium bromide stained gels were illuminated from below with an ultraviolet light source (excitation maximum 302 nm), and the emission was filtered with a Kodak Wratten filter (No. 9) (Aaij & Borst, 1972). Protein was visualized in the gels by staining with Coomassie brilliant blue R (Chrambach et al., 1967).

Electron Microscopy. Negative staining of samples with uranyl formate was performed as previously described (Prusiner et al., 1980a).

Controlled-Pore Glass Permeation Chromatography. A glass column (1.5 × 90 cm) filled with controlled-pore glass beads (CPG-700) with a nominal pore size of 700 Å was equilibrated with 60 mM Tris–acetate, pH 8.3, containing 0.5% $NaDodSO_4$. Immediately prior to chromatography, the samples (1 mL) were equilibrated with the column buffer by dialysis at room temperature for 1.5 h with three changes of buffer (1:500 v/v) using a rapid dialyzer apparatus (Englander & Crowe, 1965). The column was eluted at room temperature by pumping buffer upward at a rate of 1.4 mL/min. The absorbance at 254 and 280 nm was monitored simultaneously by using an LKB Uvicord III detector. The effluent from the detector was collected in 4.5-mL fractions. Five fractions were combined prior to bioassay of the scrapie agent.

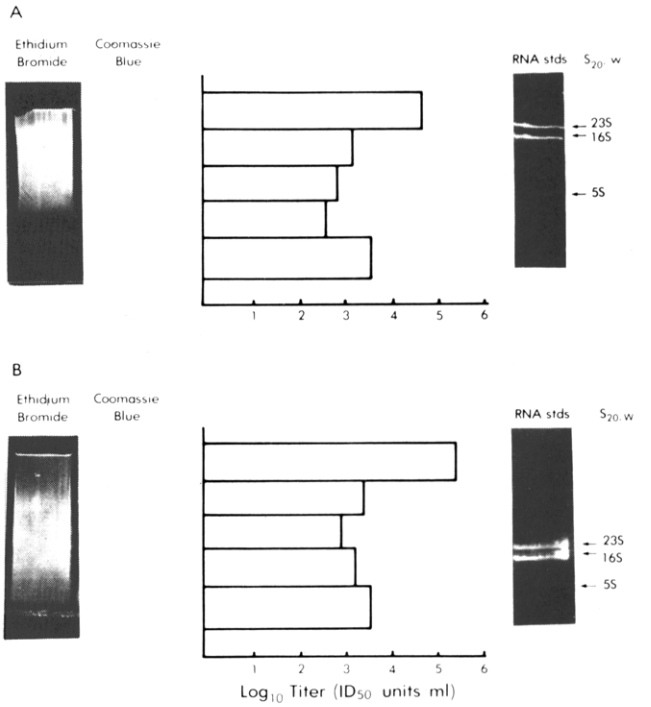

FIGURE 1: Triton X-100–DOC gel electrophoresis of the scrapie agent in fraction P_3. 40-μL samples containing 4% TX-100 and 2% DOC were loaded onto gels (1.5 mm thick) containing 0.5% TX-100 and 0.2% DOC and electrophoresed at room temperature at 120 V for 1 h. Lanes assayed for scrapie infectivity were divided as shown, pulverized with a Polytron homogenizer, and eluted into buffer. The clarified eluate was then inoculated into hamsters. Parallel lanes were stained with ethidium bromide, followed by Coomassie blue. (A) 2.5% polyacrylamide and 0.5% agarose; (B) 0.6% agarose.

Results

From earlier studies, the need to find conditions for obtaining the majority of the scrapie agent in its smallest or monomeric form to facilitate purification became apparent (Prusiner et al., 1978a, 1979). Sedimentation studies showed that the smallest form of the agent is ≤40 S and thus suggested that the superior resolution of gel electrophoresis might prove useful. In our initial studies, we employed a combination of Triton X-100 and DOC or $NaDodSO_4$ alone. Triton X-100 and DOC were chosen because the agent in a partially purified fraction P_3 was found to be stable in each of these detergents (Prusiner et al., 1980a) and the combination was found to be effective in the electrophoresis of hydrophobic proteins (Helenius & Simons, 1977). Sodium dodecyl sulfate was chosen because of the results reported by others suggesting that the agent was monomerized by 0.1% $NaDodSO_4$ (Malone et al., 1979).

As shown in Figure 1, dispersion of the P_3 fraction in 4% Triton X-100 and 2% DOC failed to facilitate the entry of the agent into a 0.6% agarose gel. Greater than 99% of the recoverable agent was found at the top of the gel although detectable amounts migrated throughout. Similar results were obtained with a composite gel composed of 2.5% polyacrylamide and 0.5% agarose and with a 7.5% polyacrylamide gel. Less than 10% of the agent applied to these gels and electrophoresed in the presence of Triton X-100 and DOC at room temperature was recovered. These poor recoveries were unexpected in view of the stability of the agent in each of these detergents alone and in combination. Inactivation of the agent

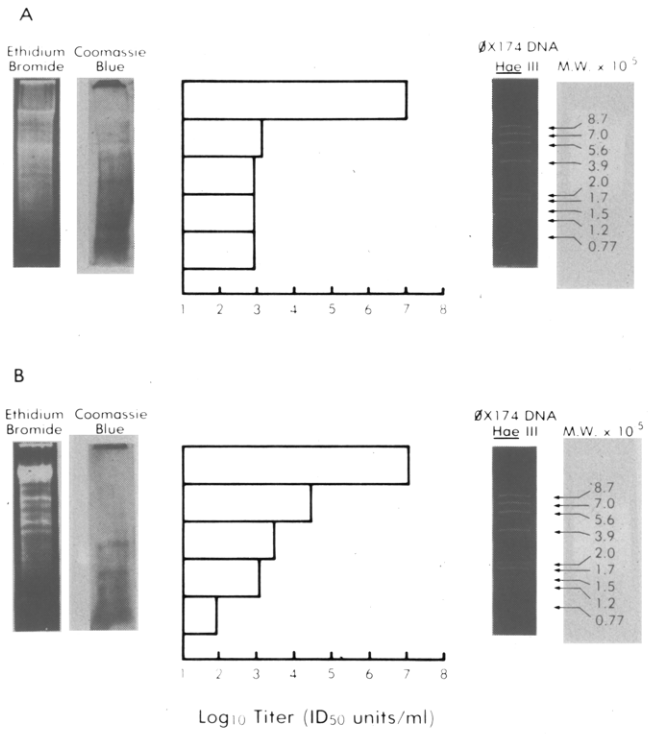

FIGURE 2: $NaDodSO_4$ gel electrophoresis of the scrapie agent in fraction P_3. (A) The sample was dialyzed against 0.5% $NaDodSO_4$ for 1.5 h at room temperature. (B) The sample was extracted with 0.2% sarkosyl and 2% cholate, followed by precipitation with 40% $(NH_4)_2SO_4$. After dialysis at 4 °C to remove the $(NH_4)_2SO_4$, the sample was dialyzed against 0.5% $NaDodSO_4$ for 1.5 h at room temperature. The concentration of $NaDodSO_4$ was increased to 1.0% prior to electrophoresis of both samples. 40 μL of each sample were loaded onto 2.5% polyacrylamide and 0.5% agarose gels (1.5 mm thick) containing 0.1% $NaDodSO_4$ and electrophoresed at a constant voltage of 120 V for 2 h at room temperature. Lanes assayed for scrapie infectivity were treated as described in Figure 1. Parallel lanes were stained with ethidium bromide followed by Coomassie blue. See Table I for further studies.

may be due to extensive delipidation since the combination of Triton X-100 and DOC has been reported to efficiently delipidate bacteriorhodopsin during gel filtration (Huang et al., 1980).

Our initial studies with electrophoresis in the presence of 0.1% $NaDodSO_4$ indicated that >90% of the recoverable agent was also found at the top of 0.6% agarose and composite gels (Prusiner et al., 1980b). In an attempt to facilitate entry of the agent into the gel, $NaDodSO_4$ gel electrophoresis under a variety of other conditions was investigated. These included dispersion of the P_3 fraction in 1% $NaDodSO_4$, and extraction of P_3 with sarkosyl and cholate, followed by precipitation with 40% ammonium sulfate prior to electrophoresis. As shown in Figure 2A, gel electrophoresis after dispersion of the sample in 1% $NaDodSO_4$ did not permit significant entry of the agent into a composite gel of 2.5% polyacrylamide and 0.5% agarose. Prior extraction of agent with a combination of sarkosyl and cholate, followed by $(NH_4)_2SO_4$ precipitation, did not increase the entry of the agent into the gel upon electrophoresis at room temperature (Figure 2B). Less than 10% of the agent applied to the gel could be recovered upon elution in most experiments under a variety of conditions listed in Table I.

In Figure 3, the precipitation of the scrapie agent by increasing concentrations of $(NH_4)_2SO_4$ in the presence of 0.2% sarkosyl and 2% cholate is depicted. The ammonium sulfate suspensions were clarified by centrifugation at 10000*g* for 30 min, and the supernatant fluid was removed by aspiration. As shown, 35% $(NH_4)_2SO_4$ causes precipitation of more than 95%

Table I: $NaDodSO_4$ Gel Electrophoresis of the Scrapie Agent: Extraction of the Agent with Varying Concentrations of Detergent and Precipitation with Ammonium Sulfate

(A) Recoveries of Agent after Electrophoresis

sample applied to gel (log ID_{50} units)	recovery [log ID_{50} units (%)] in electrophoretic gel system[a]			
	A	B	C	D
I[b] 7.5	6.0 (3.1)	5.6 (1.3)	5.9 (2.5)	7.0 (31)
II[c] 7.8	6.6 (6.4)	6.5 (5.1)	7.1 (20)	6.0 (1.6)

(B) Distribution of Recoverable Agent in Electrophoretic Gels

	region of gel	% recoverable infectivity			
		A	B	C	D
I[b]	1 (top)	95	96	99	99
	2	4.7	2.4	0.04	0.01
	3	0.19	1.2	0.05	<0.01
	4	0.15	0.60	0.05	<0.01
	5	0.10	0.48	0.02	<0.01
II[c]	1 (top)	98	99	99	99
	2	0.08	0.08	0.20	<0.01
	3	1.6	0.16	0.03	0.02
	4	0.12	<0.01	0.01	<0.01
	5	<0.01	<0.01	<0.01	<0.01

[a] All gels were composed of 2.5% polyacrylamide and 0.5% agarose. The electrophoresis was performed at room temperature. Samples loaded into gel A contained 0.1% $NaDodSO_4$, gel B contained 0.5% $NaDodSO_4$, and gels C and D contained 1% $NaDodSO_4$. All gels as well as the electrophoresis buffers contained 0.1% $NaDodSO_4$. [b] 0.1% $NaDodSO_4$ was added to samples loaded on gels A and C. The samples were then rapidly dialyzed against 0.1% $NaDodSO_4$ for 1.5 h at room temperature prior to electrophoresis—three changes of buffer [1:500 (v/v)]. Samples loaded on gels B and D received 0.5% $NaDodSO_4$ and were dialyzed against 0.5% $NaDodSO_4$. The concentration of $NaDodSO_4$ was increased to 1% prior to electrophoresis for samples loaded onto gels C and D. [c] Samples contained $NaDodSO_4$ concentrations as described above. However, these samples were derived from fraction P_3 by extraction with 0.2% sarkosyl and 2% cholate, followed by $(NH_4)_2SO_4$ precipitation at 40% saturation at 4 °C. The pellet was collected after centrifugation at 10000*g* for 30 min and dialyzed overnight against five changes of buffer at 4 °C. Samples were then dialyzed against $NaDodSO_4$ as described above.

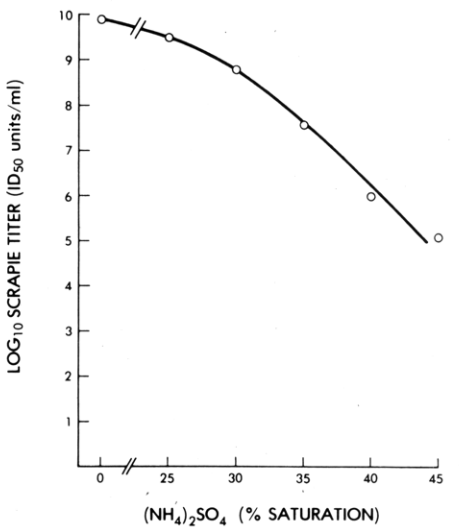

FIGURE 3: Precipitation of the scrapie agent by ammonium sulfate. The mean scrapie titer of four to eight determinations in the supernatant fluid is plotted as a function of the ammonium sulfate saturation. Fraction S_2 containing 0.2% sarkosyl and 2% cholate was precipitated by addition of solid $(NH_4)_2SO_4$. After centrifugation at 10000*g* for 30 min at 4 °C, supernatant fluids were separated from pellets by aspiration.

of the scrapie agent while 40% removes more than 99.9% of the agent in the sarkosyl–cholate extract after centrifugation.

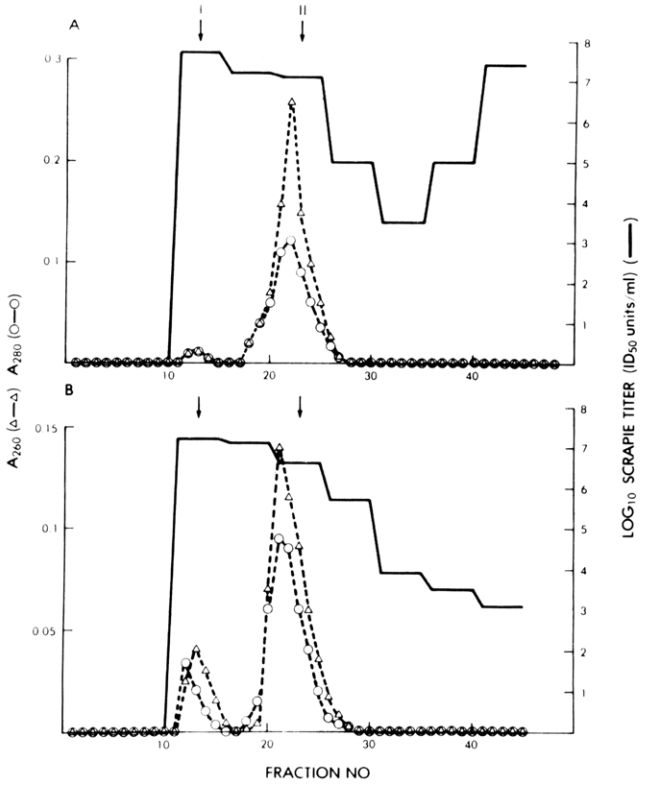

FIGURE 4: Controlled-pore glass bead chromatography of the scrapie agent in fraction P_3. (A) The sample was digested with micrococcal nuclease (50 μg/mL) and phospholipase A_2 (25 μg/mL) for 1 h at 37 °C in the presence of 2 mM $CaCl_2$, followed by RNases A (50 μg/mL) + T_1 (2 units/mL) and proteinase K (100 μg/mL) for an additional 1 h at 37 °C in the presence of 1% DOC. The sample was then dialyzed overnight at 4 °C in the presence of 4% DOC. (B) The sample was extracted with 0.2% sarkosyl and 2.0% cholate at 4 °C, followed by precipitation with 40% $(NH_4)_2SO_4$. After removal of the $(NH_4)_2SO_4$ by dialysis, the resuspended precipitate was digested with RNases A and T_1 and proteinase K as described above for 1 h at 37 °C, followed by dialysis in 4% DOC. The DOC in both samples was exchanged for 0.5% $NaDodSO_4$ by dialysis. The void (V_0) and included volumes (V_t) of column are denoted by arrows I and II, showing the elution of blue dextran (M_r 2 × 10^6) and AMP (M_r 347.2), respectively. Scrapie infectivity was assayed after combining every five fractions.

Virtually complete recovery of the agent was obtained in pellets from sarkosyl–cholate extracts which were subjected to precipitation with 35% or more $(NH_4)_2SO_4$. Pellets obtained from 40% $(NH_4)_2SO_4$ precipitations were dialyzed and loaded onto $NaDodSO_4$–agarose gels for electrophoresis as described above and used for $NaDodSO_4$ glass permeation chromatography depicted below. The scrapie agent was found to be stable in various combinations of sarkosyl and cholate up to 2.0 and 4.0% (w/v), respectively.

In view of the difficulties encountered with detergent disaggregation of the agent described above, we explored a variety of conditions for enzymatic digestion of cellular components in the P_3 fraction. Sequential digestion of samples by phospholipases and nucleases, followed by proteases, did not diminish the infectivity of the agent. Each of these digestions was for 1 h at 37 °C. DNases I and II, RNases A and T_1, and micrococcal nuclease all failed to inactivate the scrapie agent in fraction P_3 even when the samples were concurrently digested with phospholipases A_2 and/or C. Further incubation of the samples with proteinase K also failed to inactivate the agent. That nucleases and proteases under these conditions catalyzed the degradation of nucleic acid polymers and protein complexes, respectively, was shown by analysis of samples after electrophoresis on 0.8% agarose gels. The gels were then

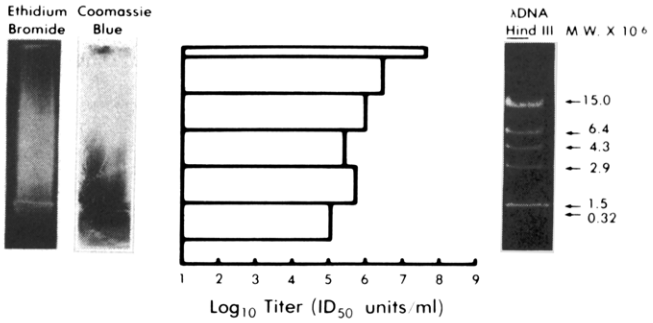

FIGURE 5: Sarkosyl–agarose gel electrophoresis of the scrapie agent in fraction P_3. 60-μL samples containing 1% sarkosyl were loaded onto 0.6% agarose gels (3.0 mm thick) containing 0.2% sarkosyl and electrophoresed at a constant voltage at 4 °C. For the first 5 min, the voltage was 10 V, followed by 5 min at 20 V and 45–50 min at 30 V. The voltage was then maintained at 90 V over the next 4.5 h. The agent was eluted and assayed as described in Figure 1. See Table III for further details.

stained with ethidium bromide for nucleic acids and Coomassie blue for proteins. The activity of phospholipase A_2 was monitored by thin-layer chromatography (Kates, 1972).

In addition to $NaDodSO_4$ gel electrophoresis, controlled pore glass permeation chromatography in the presence of $NaDodSO_4$ was explored (Collins & Haller, 1973; Frenkel & Blagrove, 1975). Fraction P_3 was prepared by two procedures prior to chromatography. The sample chromatographed in Figure 4A was digested at 37 °C for 1 h with micrococcal nuclease and phospholipase A_2, followed by digestion with RNases A and T_1 and proteinase K for an additional 1 h in the presence of 1% DOC. The samples were then dialyzed overnight at 4 °C in the presence of 4% DOC. The DOC was replaced with 0.5% $NaDodSO_4$ during further dialysis at room temperature. As expected, no scrapie infectivity was detected prior to elution of excluded molecules. The agent did elute coincident with excluded molecules in the void volume (V_0) as well as in all other fractions measured. The agent was found in numerous fractions eluting after AMP (347.2 M_r). Continued elution of the agent after small molecules such as nucleotides had been eluted indicated that the agent was being adsorbed to the column matrix and subsequently desorbed. Extraction of the P_3 fraction with sarkosyl and cholate, followed by 40% $(NH_4)_2SO_4$ precipitation and digestion with RNases and proteinase K, did not alter the chromatographic behavior of the agent on CPG beads (Figure 4B). In the two experiments depicted here, total recoveries of scrapie infectivity of 66 and 41% from the column eluants were obtained (parts A and B, respectively, of Figure 4). The discrete A_{260} and A_{280} peaks eluted from the column indicated that chromatography of most macromolecules in the preparation was occurring without adsorption to the column matrix.

Since previous sedimentation studies indicated that heating partially purified fractions containing the scrapie agent caused it to aggregate with cellular elements (Prusiner et al., 1978a, 1979), we investigated electrophoresis of the scrapie agent at 4 °C (Prusiner et al., 1980b). Electrophoresis with sarkosyl was chosen for these studies since the agent is stable in sarkosyl (Prusiner et al., 1980a) and the detergent is soluble at 4 °C. Fraction P_3 was dispersed in 1% sarkosyl and electrophoresed into 0.6% agarose gels at 4 °C (Figure 5). In contrast to $NaDodSO_4$ gel electrophoresis, the majority of the infectivity applied to sarkosyl gels was recovered in the eluates (see Table III). However, like $NaDodSO_4$ gels, most of the recoverable agent was found in the uppermost region of the gel.

Before combining enzymatic digestion with gel electrophoresis to provide a further purification of the agent, the

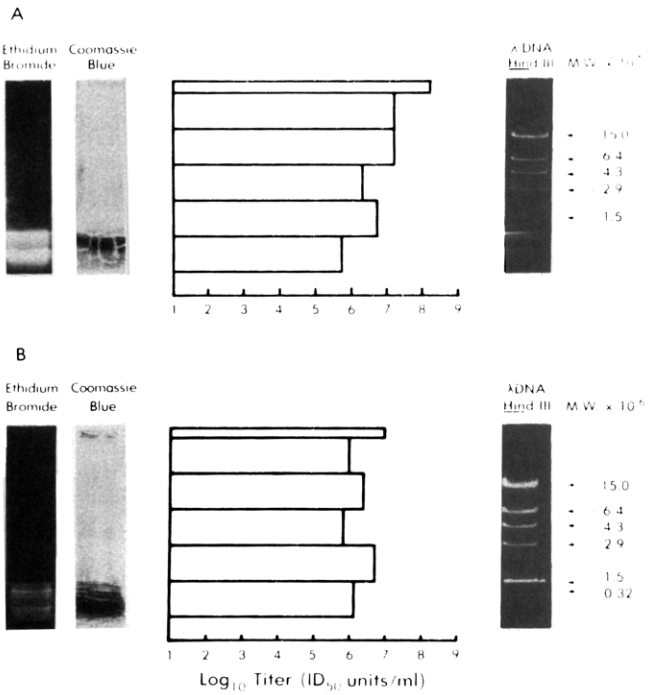

FIGURE 6: Sarkosyl–agarose gel electrophoresis of the scrapie agent in enzyme-digested fraction P_3. Fraction P_3 was digested with micrococcal nuclease (50 μg/mL) at 4 °C in the presence of 2 mM $CaCl_2$, followed by proteinase K (100 μg/mL) at 4 °C in the presence of 0.2% sarkosyl. (A) Digestion with micrococcal nuclease for 1 h was followed by proteinase K digestion for 2 h; (B) digestion with nuclease for 16 h was followed by proteinase digestion for 8 h. The sarkosyl concentration in the sample was increased to 1% prior to electrophoresis. See Figure 5 and Table III for further details.

stability of the scrapie agent at both 4 and 37 °C to nucleases, proteases, phospholipases, and snake venoms was first studied. Scrapie infectivity was unchanged by DNases, RNases, micrococcal nuclease, and S_1 nuclease (50 μg/mL). Detergents were omitted from the nuclease digestions (1 h) because DNases are readily inactivated by low concentrations of $NaDodSO_4$ (Liao, 1975). However, 0.2% sarkosyl was added to the second digestion (2 h) since the catalytic activities of proteinase K and RNases have been reported to be unaltered or enhanced by low concentrations of $NaDodSO_4$ (Hilz et al., 1975; Richards & Wyckoff, 1971). A combination of proteinase K (100 μg/mL) and trypsin (50 μg/mL) was examined because it has been found to be more effective than either protease alone (Rauber et al., 1978). This combination of proteases did not alter the infectivity of the agent. The infectivity of the agent at this stage of purification was also not altered by digestions with Pronase (500 μg/mL) or chymotrypsin (50 μg/mL). Rattlesnake and cobra venoms (500 μg/mL) also failed to inactivate the scrapie agent.

In Table II, the results of a time course study on the stability of the agent to sequential digestion by micrococcal nuclease and proteinase K are given. Micrococcal nuclease digestion for up to 16 h at 4 °C did not alter scrapie titer. Proteinase K digestion for up to 8 h in the presence of 0.2% sarkosyl also did not influence the level of scrapie infectivity.

When the P_3 fraction was digested with micrococcal nuclease and proteinase K prior to sarkosyl electrophoresis at 4 °C, the entry of the agent into the gel was facilitated. As shown in Figure 6, digestion of P_3 with micrococcal nuclease for 1 h, followed by proteinase K for 2 h, markedly decreased the size of cellular nucleic acids and proteins. Scrapie infectivity was mainly distributed over the upper half of the gel (Table III). Increasing the digestion time to 16 h for micrococcal nuclease and 8 h for proteinase K allowed ~30% of the agent to migrate ahead of DNA restriction fragments of 2×10^6 daltons (Figure 6).

As illustrated in Figure 6, enzymatic digestion prior to gel electrophoresis permitted separation of the majority of the

Table II: Time Course for Nuclease and Protease Digestion of Fraction P_3 Containing the Scrapie Agent from Hamster Brain[a]

sample	time of MN digestion (h)	time of PK digestion (h)	scrapie titer[b] (log ID_{50} units/mL)
1			10.1
2	1		10.6
3	4		10.8
4	8		10.7
5	16		10.5
6		2	9.5
7	1	2	10.6
8	4	2	10.2
9	8	2	10.8
10	16	2	10.3
11		8	10.2
12	1	8	10.5
13	4	8	10.6
14	8	8	11.0
15	16	8	10.3

[a] All digestions were performed at 4 °C. MN = micrococcal nuclease (50 μg/mL) plus 2 mM $CaCl_2$. PK = proteinase K (100 μg/mL). [b] Titer was estimated from incubation period measurements at 10^{-1} dilution of samples.

Table III: Sarkosyl Gel Electrophoresis of the Scrapie Agent: Digestion of the Agent with Micrococcal Nuclease and Proteinase K for Varying Periods of Time

(A) Recoveries of Agent after Electrophoresis

sample applied to gel (log ID_{50} units)	recovery [log ID_{50} units (%)] in electrophoretic gel system[a] A	B	C	D
I[b] 8.2	8.6 (>100)	8.8 (>100)	8.4 (>100)	8.3 (>100)
II[c] 8.4	9.1 (>100)	8.2 (58)	8.3 (75)	7.9 (34)

(B) Distribution of Recoverable Agent in Gels

	region of gel	% recoverable infectivity A	B	C	D
I[b]	1 (top)	81	7.4	63	80
	2	4.1	60	20	8.0
	3	12	9.3	5.2	8.0
	4	0.16	24	7.9	1.0
	5	1.3	0.60	3.1	2.5
	6	1.0	0.01	0.50	0.3
II[c]	1 (top)	96	29	88	49
	2	2.9	11	7.2	4.8
	3	0.50	18	2.2	12
	4	0.20	9.0	0.70	3.0
	5	0.10	18	1.1	24
	6	0.30	14	0.3	6.0

[a] All gels were composed of 0.6% agarose on top of a 15% polyacrylamide plug (region 6). The electrophoresis was performed at 4 °C. Gels A and B were loaded with samples containing 0.2% sarkosyl; gels C and D were loaded with samples containing 1% sarkosyl. [b] Samples loaded onto gels B and D in experiment I were digested with micrococcal nuclease (50 μg/mL) plus 2 mM $CaCl_2$ for 1 h at 4 °C, followed by proteinase K (100 μg/mL) for an additional 2 h at 4 °C in the presence of 0.2% sarkosyl. Samples A and C were not digested. [c] Samples loaded onto gels B and D in experiment II were digested with micrococcal nuclease (50 μg/mL) plus 2 mM $CaCl_2$ for 16 h at 4 °C, followed by proteinase K (100 μg/mL) for an additional 8 h at 4 °C in the presence of 0.2% sarkosyl. Samples A and C were not digested. Data were from Prusiner et al. (1980b).

recoverable agent from cellular nucleic acids and proteins as detected by ethidium bromide and Coomassie blue staining, respectively. This observation has been utilized in the development of a further purification procedure. Preparations of the scrapie agent enriched for infectivity 100–1000-fold with respect to protein and DNA were obtained by elution of the agent from the upper four portions of the gels. Examination of these eluates by electron microscopy did not show any unique particles in scrapie preparations compared to controls when negatively stained with uranyl formate or phosphotungstic acid. Many of the particles observed appear to be fragments of pulverized agarose which were generated by Polytron homogenization of the gel to facilitate elution. The levels of RNA were so low and the blanks were so high due to the pulverized agarose that reliable estimates of these polymers could not be made by conventional spectrophotometric techniques.

Attempts to facilitate the entry of the agent into agarose gels during electrophoresis using nonionic detergents in combination with sarkosyl were without success. Enzyme-digested samples exposed to concentrations of Nonidet P-40, Triton X-100, and Ammonyx LO up to concentrations of 3% (v/v) in the presence of 1% sarkosyl did not alter the entry of the agent observed with sarkosyl alone.

Discussion

The extreme size heterogeneity of the scrapie agent presents unusual difficulties not normally encountered in the purification of conventional viruses. To circumvent this problem, we examined the resistance of the agent to degradative enzymes with the strategy of employing these enzymes to degrade cellular structures while leaving the agent intact. Such strategy has been used in the purification of conventional viruses where the nucleic acid genome is well protected by a protein coat, permitting the use of nucleases to degrade cellular polymers (Rose, 1974). The infectivity of the scrapie agent in fraction P_3 was unaltered by a wide variety of nucleases, proteases, phospholipases, and snake venoms containing glycosidases, esterases, lipases, nucleases, and proteases (Iwanaga & Suzuki, 1979). These findings are consistent with those reported by others on the resistance of the scrapie agent to enzymatic degradation in general (Hunter et al., 1969; Hunter, 1979; Prusiner et al., 1980b). With the development of gel electrophoretic systems permitting virtually complete recovery of the infectivity after electrophoresis, it became feasible to combine an enzymatic digestion sequence with gel electrophoresis. The use of a nondenaturing detergent such as sarkosyl and performance of the electrophoresis at 4 °C both appear to be critical for retention of infectivity.

In contrast to the penetration of the scrapie agent into $NaDodSO_4$ gels during electrophoresis at room temperature reported by others (Malone et al., 1979), we have been unable to define conditions for entry of the agent into these gels. Increasing the concentration of $NaDodSO_4$ in the sample as well as extraction of samples with sarkosyl and cholate, followed by $(NH_4)_2SO_4$ precipitation, failed to facilitate the entry of the agent into the gels. Similar results were obtained even after digestion of the P_3 fraction with RNases and proteinase K (Prusiner et al., 1980b).

Extraction of the P_3 fraction with sarkosyl and cholate, followed by $(NH_4)_2SO_4$ precipitation, did not alter the behavior of the agent during $NaDodSO_4$ gel electrophoresis or CPG chromatography. Other investigators suggested that $(NH_4)_2SO_4$ precipitation was important for entry of the agent into $NaDodSO_4$ gels even though their procedure did not resolve the agent into a discrete $(NH_4)_2SO_4$ fraction (Malone et al., 1979). A similar lack of resolution has been seen in attempts to fractionate membrane proteins using ammonium sulfate, but the problem can often be rectified by adding cholate (Tzagoloff & Penefsky, 1971). As shown in Figure 3, $(NH_4)_2SO_4$ precipitation in the presence of sarkosyl and cholate resulted in discrete fractionation of the agent.

To determine whether the failure of the scrapie agent to enter $NaDodSO_4$ electrophoretic gels is due to its large size or adsorption to the gel matrix, glass permeation chromatography was investigated. A substantial quantity of the agent was observed to elute in the column void volume, confirming the existence of large forms of the agent of $M_r > 2 \times 10^6$. The agent was also found to elute after AMP, indicating that it was being adsorbed to the column matrix and subsequently desorbed. Chromatography was performed in the presence of 0.5% $NaDodSO_4$ at pH 8.3 since these conditions have been reported to minimize adsorption of protein to the controlled-pore glass (Collins & Haller, 1973; Frenkel & Blagrove, 1975). The interaction of the agent with the glass matrix is presumably due to the hydrophobicity of the agent. Adsorption chromatography of hydrophobic membrane proteins on controlled-pore glass in the absence of $NaDodSO_4$ has been described (Bock et al., 1976).

Our observations showing that prior digestion of the P_3 fraction with micrococcal nuclease and proteinase K allows approximately one-third of the agent in the sample to migrate through a sarkosyl–agarose gel ahead of DNA restriction fragments of $<2 \times 10^6$ daltons are consistent with earlier sedimentation studies (Prusiner et al., 1978b, 1979). Those studies showed that the smallest or monomeric form of the scrapie agent has a sedimentation coefficient of <40 S. Thus, both by electrophoresis and by sedimentation, the scrapie agent is smaller than the smallest known conventional animal viruses. These observations on the small size of the scrapie agent by physical techniques are in accord with earlier studies on the ionizing radiation target size (Alper et al., 1966; Latarjet, 1979). Our results indicate that the resistance of the agent to ionizing radiation is due, at least in part, to the small size of the agent. How much efficient repair mechanisms and aggregated forms of the agent may cause errors in the estimation of the ionizing radiation target size of the scrapie agent remains to be established (Latarjet, 1979).

The molecular mechanisms responsible for size heterogeneity of the scrapie agent remain unknown. Earlier sedimentation studies suggested that this heterogeneity of the agent results from hydrophobic interactions (Prusiner et al., 1978a, 1979). Those studies showed that small forms of the agent disappeared upon heating as the agent aggregated with cellular elements. The gel electrophoresis studies described here show that small forms of the agent can be increased by digestion of the preparation with nucleases and proteases.

The availability of a 100–1000-fold purified preparation of the scrapie agent with respect to protein and DNA should permit a more accurate assessment of the molecular characteristics of the agent once suitable procedures are developed for scaling up such preparations. However, conditions for reducing the agent to its smallest form must still be determined. Defining these conditions is an empirical process involving the examination of such parameters as specific detergents, combinations of detergents, chaotropic salts, pH, and ionic strength (Kagawa, 1974; Helenius & Simons, 1975; Wasserman, 1974; Newby & Chrambach, 1979; Storm et al., 1976; Umbreit & Strominger, 1973; Egan et al., 1976; Wallace & Winzler, 1974). How much the present purification must be extended to obtain a homogeneous preparation is also un-

known. Assuming the molecular weight of the putative scrapie genome is 150 000 (Alper et al., 1966; Latarjet, 1979), then the degree of purification required to obtain a pure preparation can be estimated. These calculations are based on 10^{10} ID_{50} units of the scrapie agent and 3.25 mg of nucleic acid per g of hamster brain. If the particle to infectivity ratio is 100–1000 and thus similar to many other animal viruses, then a 1000–10 000-fold purification will be sufficient. However, if this ratio is 1, then a 10^6-fold purification will be necessary. As the extent of our purification continues to increase, the maximum estimate for the particle to infectivity ratio must decrease.

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