Partial Purification and Evidence for Multiple Molecular Forms of the Scrapie Agent†

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ABSTRACT: A procedure for the partial purification of the scrapie agent from mouse spleen was developed based on its sedimentation profile. Differential centrifugation and detergent treatment with sodium deoxycholate yielded a fraction designated "P_5" which was enriched for scrapie infectivity ~20-fold with respect to cellular protein. The P_5 fraction was devoid of cellular membranes but heavily contaminated with ribosomes as judged by electron microscopy. On centrifugation of the fraction P_5 to near equilibrium in a sucrose gradient scrapie infectivity was distributed over a range of densities from 1.08 to 1.30 g/cm^3. Parallel rate-zonal analysis showed that the infectivity was distributed over a range of particle sizes with $s_{20,w}$ values from ~40 S to >500 S. Incubation of P_5 at 37 or 80 °C, under conditions that disrupt ribosomes, dramatically altered the rate-zonal gradient profile of the agent. Under these conditions, the agent sedimented as particles with $s_{20,w} > 500$ S. The apparent heterogeneity of the scrapie agent with respect to both size and density and its ability to shift from one form to another suggest that the agent may contain hydrophobic domains on its surface.

Because the assay for the scrapie agent is so unusually slow, development of a rational strategy for purifying the agent seemed of utmost importance. Although the choice of purification techniques is empirical, differential centrifugation is generally found to be quite effective in the initial stages of isolating most biological macromolecular structures. Thus, preparatory to developing a purification format utilizing differential centrifugation, we determined the sedimentation characteristics of the scrapie agent in fixed-angle rotors as described in the preceding communication (Prusiner et al., 1977; 1978). From these observations a scheme for partially purifying the scrapie agent from spleen was developed using differential centrifugation in combination with detergent treatment. The purification procedure yields approximately 20-fold enrichment of the agent with respect to total cellular protein. As predicted from the sedimentation profile data, the partially purified preparations of scrapie agent contained many ribosomal structures. Electron microscopy of the preparations confirmed the presence of ribosomes and the virtual absence of organized membranous structures.

Sucrose gradient centrifugation was employed to separate the scrapie agent from contaminating ribosomes. After partial purification and storage at −70 °C, the agent exhibited heterogeneity with respect to both size and density. Heat treatment at 37 or 80 °C increased the apparent size of the agent suggesting aggregation. The data are consistent with the possibility that the scrapie agent contains nonpolar regions or hydrophobic patches on its surface. Indeed, the physical behavior of the scrapie agent appears similar to that observed for hydrophobic proteins which are capable of aggregation and dissociation as well as binding lipids.

Experimental Procedures

Materials. All materials were of the purest grades commercially available. Sodium deoxycholate was obtained from Sigma Chemical Co.; lysolecithin was obtained from Koch-Light Ltd. Yeast RNA and calf thymus DNA were purchased from P-L Biochemicals and recrystallized bovine albumin was obtained from Pentex-Miles Laboratories. Bovine pancreatic ribonuclease (RAF) was obtained from Worthington Biochemicals.

Inoculation of Mice and Assay of Scrapie Agent Infectivity. These procedures were performed as previously described (Prusiner et al., 1978).

Assay of Biochemical Markers. RNA was measured by the procedure of Schneider (1957) using orcinol reagent. DNA was measured using the fluorescent dye 3,5-diaminobenzoic acid with an Aminco-Bowman spectrofluorometer (Kissane & Robins, 1958). Commercial preparations of yeast RNA and calf thymus 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. (1951); bovine serum albumin was used as standard. Absorption at 260 and 280 nm was measured in a Gilford spectrophotometer 252, equipped with a rapid sampling device having a 1-cm pathlength. Sucrose densities were determined by refractometry, using a Bausch & Lomb Abbé refractometer.

Preparation of Samples for Electron Microscopy. Samples for electron microscopy were centrifuged at 50 000 rpm for 90 min in an SW50.1 rotor adapted to accommodate 0.8 mL in cellulose nitrate tubes. The sedimented samples were fixed with Karnovsky's solution, followed by OsO_4. The fixed samples were then dehydrated by using graded alcohol solutions and embedded in Epon resin. The sections were stained with uranyl acetate and lead citrate (Baringer & Prusiner, 1978). Specimens were examined in a Phillips EM 300 at 75 kV.

Sucrose Gradient Centrifugation. Partially purified suspensions (P_5) containing the scrapie agent were stored at −70

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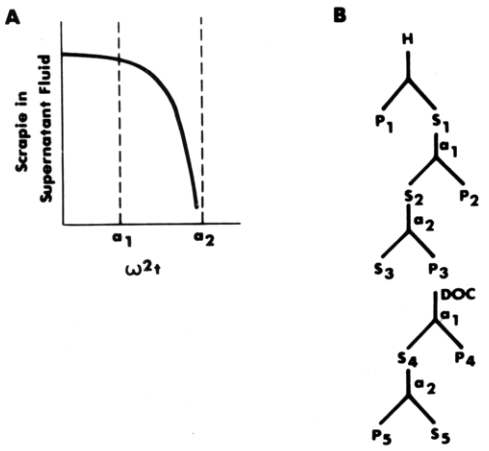

FIGURE 1: Strategy for purification of the scrapie agent. A series of centrifugations in fixed-angle rotors was used to define the optimal conditions for isolation of the agent.

°C. Before use they were rapidly thawed in a 37 °C bath and held at 4 °C prior to sonication for 15 s with a Bronwill Scientific Biosonik sonicator. For near-equilibrium centrifugation, a Buchler conical gradient maker was used to construct the gradients; each gradient had a total volume of 13.2 mL. A sample (0.6 mL) was layered on a 20–65% (w/w) linear sucrose gradient buffered with 20 mM Tris-HCl, pH 7.4. The gradients were centrifuged at 41 000 rpm for 19 h in an L2-65B Spinco ultracentrifuge, equipped with SW41 rotor.

For the rate-zonal gradient centrifugation studies, gradients were constructed by layering in order 3.6 mL each of 65%, 30%, 25%, 20%, and 15% sucrose in 0.02 mM Tris-HCl, pH 7.4. They were left standing overnight at 4 °C before use. Samples (0.45 mL) were floated on the resultant 15–30% (w/w) linear sucrose gradients over 65% (w/w) sucrose cushions. Centrifugation in an SW27.1 rotor at 27 000 rpm was for 3.5 h. All gradients were separated into approximately 20 equal fractions from the top, using a Buchler Auto Densi-Flow apparatus.

$s_{20,w}$ values for particles sedimenting in rate-zonal sucrose gradients were estimated as described by McEwen (1967). Gradients were calibrated with viruses of known $s_{20,w}$ values: SV40, 240 S; bacteriophage λ, 420S; bacteriophage T4, 900S. ^{13}S- SV40, ^{32}P- λ and ^{3}H-labeled T4 were gifts from G. Stark, R. Fisher, and J. Hosoda, respectively. Calibration gradients were buffered with 0.02 M Tris-HCl, pH 7.4, 0.1 M NaCl.

Results

Partial Purification of the Scrapie Agent. In a typical preparation, spleens were removed from 96 mice inoculated intracerebrally with 10^6 ID_{50} units of mouse-adapted scrapie 42 days prior to sacrifice. The spleens were washed in an ice-cold buffer consisting of 250 mM sucrose and 20 mM Tris-HCl, pH 7.4. Peritoneal fat was removed by dissection and the spleens (15 g) were minced in the buffer. A 20% (w/v) homogenate was prepared using a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle (w/v). All procedures were performed at 4 °C. The homogenate was centrifuged in a Sorvall SS34 rotor at 1000 rpm for 10 min and the supernatant fluid removed. The pellet was rehomogenized in additional buffer and the resultant suspension centrifuged at 1000 rpm for 10 min. The rehomogenization procedure was repeated twice. The supernatant fractions from the four 1000 rpm centrifugations were combined and adjusted to a 10% (w/v) concentration. This suspension, shown as S_1 in Figure 1, was centrifuged at 4000 rpm for 30 min in the SS34 rotor ($\omega^2 t = 3.2 \times 10^8$ rad^2/s). The pellet (P_2) from this centrifugation was resuspended in buffer and centrifuged again. The combined supernatant fractions (S_2) were centrifuged at 50 000 rpm for 90 min in a 60Ti rotor ($\omega^2 t = 1.5 \times 10^{11}$ rad^2/s). The resultant pellet (P_3) was covered with buffer, stored overnight at 4 °C, dispersed with a homogenizer, and adjusted to 5 mg of protein/mL. An aliquot of 10% (w/v) sodium deoxycholate (DOC) was added slowly with intermittent vortex mixing to a final concentration of 0.5% (w/v). The DOC-treated suspension was centrifuged at 4000 rpm for 30

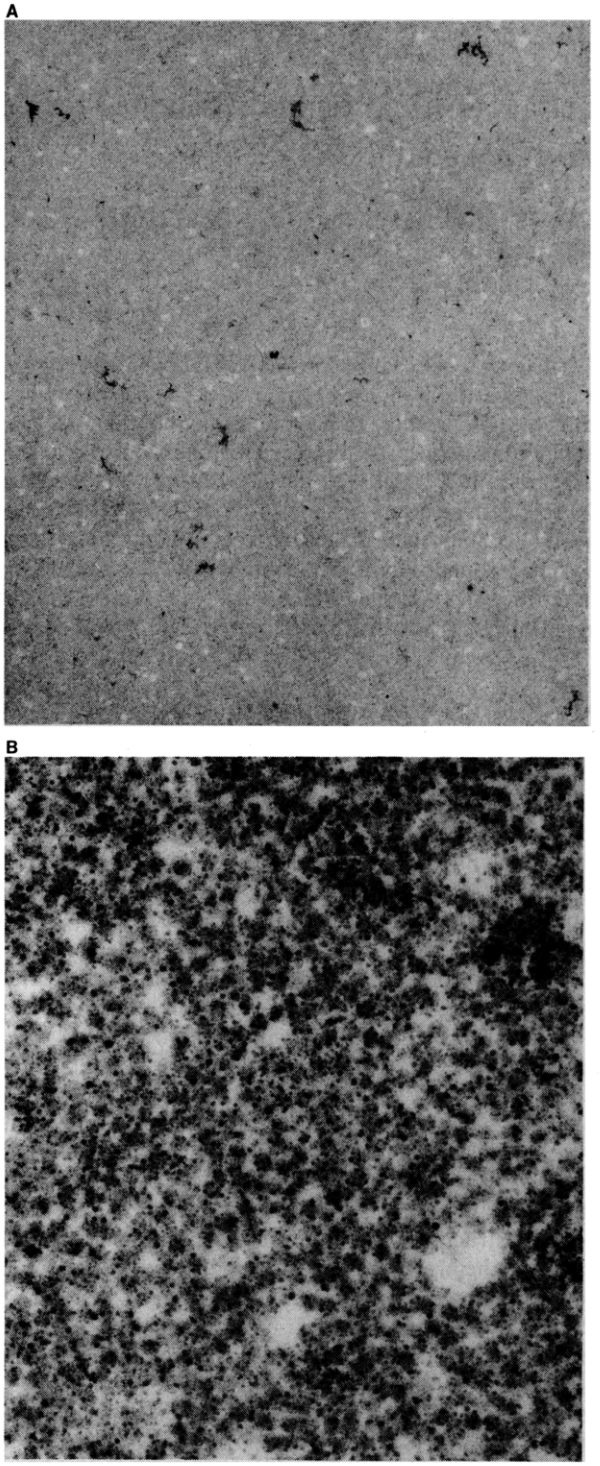

FIGURE 2: Transmission electron micrographs of the DOC-treated pellet fraction P_5 enriched for scrapie agent infectivity. See text for details of preparation. (Plate A) Magnification 10 280×; (B) 98 800×.

TABLE I: Partial Purification of the Scrapie Agent—Protein, RNA, and DNA Content in Fractions.

fraction	vol (mL)	protein total (mg)	protein %	RNA total (mg)	RNA %	DNA total (mg)	DNA %
homogenate	106	3456	100	303	100	467	100
S_1	240	1560	45	176	58	16	3.4
P_1	40	1756	51	158	52	472	101
S_2	300	1290	37	173	57	9	2
P_2	12	274	8	148	49	13	2.7
S_3	280	840	24	30	10	6	1.2
P_3	60	346	10	127	42	4	0.9
S_4	60	307	9	124	41	4	0.9
P_4	2	5	1	1	<1	0.3	<0.1
S_5	50	147	4	30	10	2	0.5
I_5	7	32	0.5	9	3	0.9	0.2
P_5	6	82	2.3	55	18	0.9	0.2

TABLE II: Partial Purification of the Scrapie Agent—Infectivity Titrations.

fraction	prep A vol (mL)	prep A log ID_{50}/mL	prep A % yield	prep A sp infect. log ID_{50}/mg of protein)	prep B vol (mL)	prep B log ID_{50}/mL	prep B % yield	prep B sp infect. (log ID_{50}/mg of protein)
homogenate	106	7.2	100	5.7	90	6.0	100	4.7
S_1	240	6.7	127	5.9	186	5.7	89	4.8
P_1	40	7.0	24	5.3	27	6.7 or >	130	5.4 or >
S_2	300	6.2	30	6.6				
S_3	280	4.4	0.40	3.9				
P_3	60	7.7	179	6.6	171	5.7	79	5.3
P_4	2	5.9	0.06	5.3				
S_5	50	3.2	0.01	2.8				
I_5	7	6.7	2.0	6.2				
P_5	6	8.0	38	6.9	16	6.9	110	6.1

min in the SS34 rotor and the resultant supernatant (S_4) was centrifuged at 50 000 rpm for 90 min in the 60Ti rotor. The final supernatant fraction (S_5) was carefully removed with a pipet. The fluffy opacified interface (I_5) and the firm pellet (P_5) were recovered separately. The pellet was resuspended in sucrose-Tris buffer using a Potter-Elvehjem homogenizer and typically had a protein concentration of 5–8 mg/mL. The suspension of P_5 was stored at −70 °C.

In Table I, the protein, RNA, and DNA values are recorded for each fraction from the purification procedure. As shown, the procedure removes greater than 95% of the total protein and DNA, but only 80% of the RNA. Since sedimentation profiles in fixed-angle rotors for RNA, which is primarily ribosomal RNA, are similar to those for the scrapie agent, this result was expected (Prusiner et al., 1977, 1978; deDuve, 1971; Bonanou-Tzedaki & Arnstein, 1972). No apparent differences were found in the protein, DNA, and RNA contents of preparations from infected mice compared to those from uninfected controls.

In Table II, the scrapie infectivity data for two typical preparations are given. In the case of preparation A, approximately a 16-fold enrichment of the agent in P_5 with respect to protein was observed, while preparation B showed a 25-fold enrichment. In the final pellet of preparation A, 38% of the total scrapie agent was recovered and, in preparation B 110% was recovered.

Electron Microscopy of Fraction P_5. Electron microscopy of the P_5 fraction demonstrated the virtual absence of organized membranous structures (Figure 2). Many small, round, electron-dense structures 20 nm in diameter were seen on a background of amorphous material. These dense bodies were of similar size and shape as monoribosomes (Bonanou-Tzedaki & Arnstein, 1972). The findings were expected since the sedimentation profiles on which the purification was based showed that the scrapie agent sediments with polyribosomes (Prusiner et al., 1977, 1978). The amorphous background structures are presumably phospholipid–protein complexes which were not solubilized by DOC treatment. They are reminiscent of the hydrophobic mitochondrial proteins which bind phospholipids and are not readily solubilized by detergent treatment (Kagawa & Racker, 1971).

Electron microscopy of the interface material (I_5) did show membranous structures. As noted in Table II, 2.0% of the total scrapie agent infectivity in the DOC-treated suspension (P_3) was recovered in the I_5 fraction.

Properties of the Agent in P_5. Titrations of scrapie infectivity require that serial dilutions be made of the sample being assayed. Many investigators have added fetal calf serum to the diluent to stabilize the agent at high dilution, but data supporting such a requirement are lacking. Diluents containing 10% (v/v) fetal calf serum, 7.5% (w/v) recrystallized BSA, and 0.5% (w/v) horse cytochrome *c* were compared. No differences in the titer of the scrapie agent in the final pellet from preparation A could be discerned.

The stability of the P_5 fraction to chemical treatments and elevated temperatures is shown in Table III. The addition of a second detergent lysolecithin did not alter the titer of scrapie infectivity substantially, nor did the addition of KCl, EDTA, puromycin, or RNase, all of which have been shown to facilitate the degradation of ribosomal structures (Adelman et al., 1974). Elevation of the temperature to 37 or 80 °C for 30 min also did not change the titer of the agent substantially. Trip-

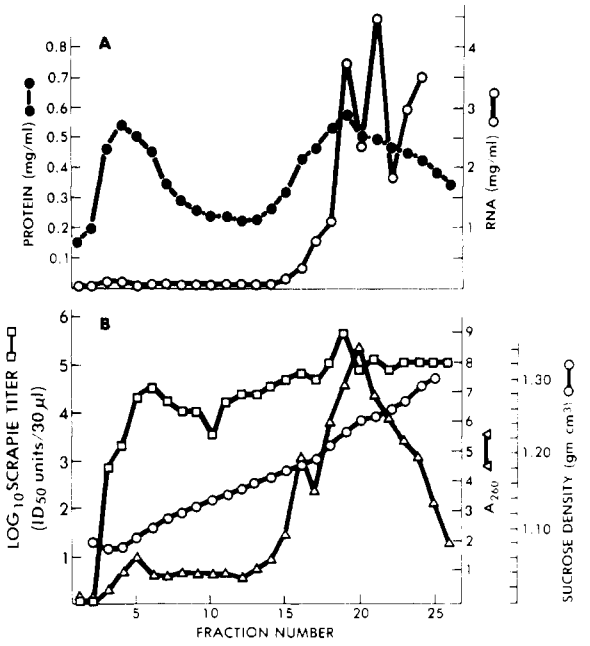

FIGURE 3: Near-equilibrium sucrose density gradient centrifugation of fraction P_5 enriched for the scrapie agent. A 20–65% (w/w) linear sucrose gradient was centrifuged for 19 h at 41 000 rpm in an SW41 rotor. Fractions were collected from the top (left) of the gradient beginning with fraction 1; the last fraction in each gradient represents the pellet. (A) Protein (●) and RNA (○); (B) A_{260} (△), sucrose densities (○), and scrapie agent infectivity (□).

TABLE III: Stability of Partial Purified Preparation (P_5) of the Scrapie Agent.

additions	temp of incubation[a] (°C)	scrapie agent infect. (log ID_{50}/0.03 mL)
1.	4	6.3, 5.9, 6.5
2. 0.5 M KCl + 5 mM EDTA	4	6.7
3.	37	5.7
4. lysolecithin (10 mg/mL)	37	6.0
5. 0.5 M KCl + 5 mM EDTA	37	5.8
6. 0.5 M KCl + 5 mM EDTA + 0.5 mM puromycin	37	6.3
7. 0.03 µg of RNase/mg of protein + 5 mM EDTA	37	6.1
8.	80	5.7
9. lysolecithin (10 mg/mL)	80	6.8

[a] Following incubation for 30 min at the temperature indicated, the samples were immediately frozen at −70 °C and stored frozen until titrations could be performed.

licate titrations of P_5 held at 4 °C for 30 min gave values of 6.3, 5.9, and 6.5 log ID_{50} units/0.03 mL and demonstrated clearly the imprecision of the mouse titration assay (Dougherty, 1964).

Sucrose Gradient Centrifugation. The partially purified preparation (P_5) was subjected to sucrose gradient centrifugation. A 0.6-mL sample of P_5 containing $1.0 \times 10^{7.5}$ ID_{50} units of the scrapie agent was layered on the top of a 20–65% (w/w) linear sucrose gradient. The gradient was centrifuged to near equilibrium and upon completion, the gradient was fractioned from the top. As shown in Figure 3, fractions were analyzed for absorption at 260 nm, RNA, protein, and sucrose content, as well as for scrapie infectivity. Two ultraviolet absorbing peaks were found: a small peak at a sucrose density of

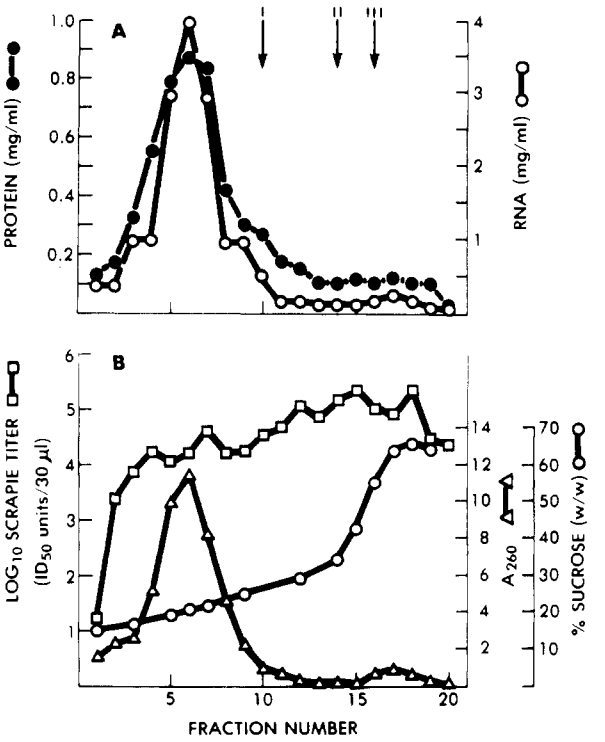

FIGURE 4: Rate-zonal sucrose gradient centrifugation of fraction P_5 enriched for the scrapie agent. A 15–30% (w/w) linear sucrose gradient was centrifuged for 3.5 h at 27 000 rpm in an SW27 rotor. The P_5 fraction was stored at −70 °C and incubated at 4 °C for 30 min prior to centrifugation. (A) Protein (●) and RNA (○). Arrows denote the locations of radiolabelled viruses with known sedimentation coefficients ($s_{20,w}$): (I) SV40, 240S; (II) bacteriophage λ 420S; (III) bacteriophage T_4, 900 S. (B) A_{260} (△), sucrose concentrations (○), and scrapie agent infectivity (□).

1.09 g/cm^3 (fraction 5) and a larger peak at 1.24 g/cm^3 (fraction 20). The RNA profile presumably reflecting ribosomal structures was similar to that for absorption at 260 nm, while two protein peaks of almost equal size were found. In contrast to the distinct peaks observed for these biochemical markers, scrapie infectivity was distributed over the entire gradient ranging in sucrose densities from 1.08 to 1.30 g/cm^3. All of the infectious agent applied to the gradient was recovered (Table IV) and more than 80% of the infectivity in the gradient was found at sucrose densities of 1.21 g/cm^3 and greater.

In parallel rate-zonal studies, 0.45 mL of P_5 containing $1.0 \times 10^{8.0}$ ID_{50} units of the scrapie agent was layered on the top of a 15–30% (w/w) linear sucrose gradient. As shown in Figure 4, one major ultraviolet absorbing peak was found corresponding to a $s_{20,w}$ value of 90 S (fraction 6) assuming a particle density of 1.5 g/cm^3. The peak, which is presumably composed of monoribosomes, contained virtually all of the measurable RNA. The infectivity was distributed over the entire gradient corresponding to particles ranging in size from 40 S to greater than 500 S. Approximately 36% of the total infectivity applied to the gradient was recovered after centrifugation. The apparent heterogeneity of the scrapie agent over this range of sizes is consistent with sedimentation profile studies which showed that the scrapie agent in DOC-treated extracts of spleen sedimented within a range of $s_{20,w}$ values from 70 S to 600 S (Prusiner et al., 1977, 1978).

In an attempt to disrupt the ribosomes, which are a major component of the P_5 fraction, samples of P_5 were preincubated with 0.5 M KCl and 5 mM EDTA in the absence or presence of 0.5 mM puromycin (Adelman et al., 1974). Incubations for 30 min were performed at either 4 or 37 °C. As shown in Fig-

TABLE IV: Recovery of Scrapie Agent Infectivity after Sucrose Gradient Centrifugation.

gradient centrifugation conditions	figure in text	scrapie agent applied to gradient (log ID_{50} units)	scrapie agent recovered in gradient fractions (log ID_{50} units)	recovery (%)
1. near-equilibrium	(3)	7.5	7.5	100
2. rate-zonal	(4)	8.0	7.5	36
3. rate-zonal	(5A)	7.8	7.3	29
4. rate-zonal	(5B)	7.0	6.1	12
5. rate-zonal	(5C)	7.6	7.1	40
6. rate-zonal	(6)	8.3	7.6	22

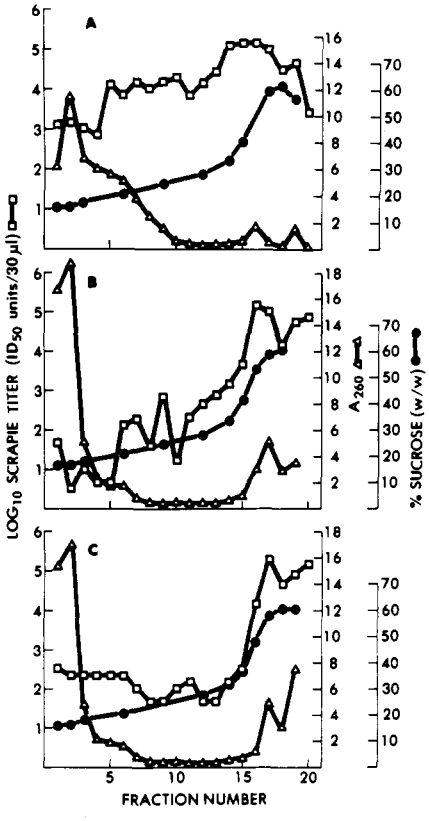

FIGURE 5: Rate-zonal sucrose gradient centrifugation of fraction P_5 enriched for the scrapie agent after incubation with KCl, EDTA, and puromycin. (A) Fraction P_5 was incubated at 4 °C for 30 min in the presence of 0.5 M KCl and 5 mM EDTA. A_{260} (△), sucrose concentrations (●), and scrapie agent infectivity (□) are plotted; (B) P_5 incubated at 37 °C for 30 min with 0.5 M KCl and 5 mM EDTA; (C) P_5 incubated at 37 °C for 30 min with 0.5 M KCl, 5 mM EDTA, and 0.5 mM puromycin. In these three gradients, 0.5 M KCl and 5 mM EDTA were included in the sucrose solutions forming the gradient.

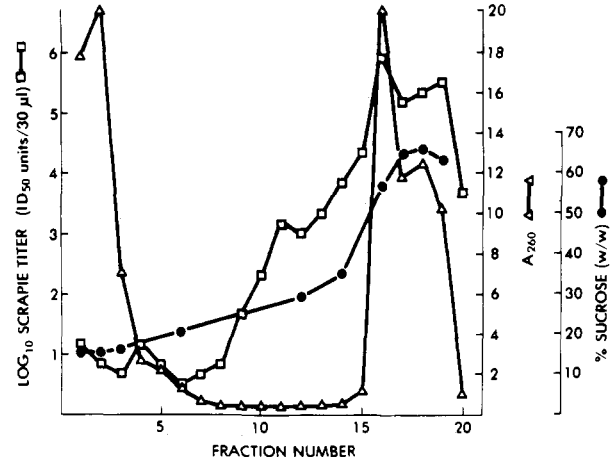

FIGURE 6: Rate-zonal sucrose gradient centrifugation of fraction P_5 enriched for the scrapie agent after incubation at 80 °C for 30 min. A_{260} (△), sucrose concentrations (●), and scrapie agent infectivity (□), are plotted. After heating, the sample was dispersed by sonication in the presence of lysolecithin (10 mg/mL) prior to centrifugation.

ure 5A partial destruction of the ribosomal peak (fraction 5) was achieved during the 4 °C incubation but scrapie infectivity was again spread over most of the gradient. Incubation at 37 °C caused almost complete destruction of the ribosomes and was accompanied by a dramatic change in the scrapie infectivity pattern across the gradient (Figures 5B and 5C). Greater than 90% of the infectivity was recovered in fractions 15–20 at the bottom of these two gradients indicating that the sedimentation coefficients of these infectious particles are greater than 500 S.

A similar shift in pattern of scrapie infectivity was observed after incubation of P_5 at 37 °C for 30 min with ribonuclease and 5 mM EDTA. In this case, greater than 95% of the infectivity was recovered at the bottom of the gradient, again demonstrating the presence of infectious particles with $s_{20,w}$ values greater than 500 S. This gradient pattern for the scrapie agent indicates that KCl is not required for inducing the change in its sedimentation properties.

It is not known whether incubation of P_5 at 37 °C for 30 min alone is sufficient to cause these alterations in the sedimentation characteristics of the agent. Incubation of P_5 at 80 °C for 30 min followed by sonication consisting of three bursts of 15 s each in the presence of 10 mg/mL of lysolecithin did cause a similar shift in the sedimentation pattern of the agent (Figure 6). Greater than 99% of the infectivity was recovered at the bottom of the gradient.

In Table IV, the recoveries of the total scrapie infectivity applied to the seven gradients described above are tabulated. In the majority of the gradients, 30–40% of the infectivity could be accounted for while total recoveries ranged from 12 to 100%.

Discussion

The spleen was chosen for these studies since the titer of the scrapie agent in it reaches a maximum approximately 20–30 days after intracerebral inoculation (Eklund et al., 1967; Kimberlin, 1976). In contrast, more than 100 days of incubation is required to achieve an equivalent titer in brain. The unusual difficulties attendant with fractionation of brain tissue also suggested that purification of the agent from spleen would be advantageous. The absence of histopathological changes in spleen during scrapie infection compared with the extensive spongiform changes seen in brain was an additional reason for choosing the spleen. The major disadvantage of using spleen

is that the final titer in brain after 4–5 months of incubation is usually 10 times greater than the maximal titer in spleen at any time during the course of infection (Eklund et al., 1967; Kimberlin, 1976).

The strategy used to develop an initial purification protocol for the scrapie agent was based on data derived from a series of differential centrifugations using fixed-angle rotors (Prusiner et al., 1977, 1978). By measuring the disappearance of the scrapie agent in the supernatant fractions of samples centrifuged over a wide range of centrifugation speeds and times, it was possible to construct sedimentation profiles for the agent. As predicted from these profiles, a preparation P_5 enriched for scrapie infectivity with respect to total cellular protein was obtained. Numerous ribosomes, but virtually no organized membranous structures, were found by electron microscopy in the P_5 fraction suggesting that cellular membranes may not be required for preservation of infectivity (Hunter, 1972; Kimberlin, 1976).

Although the scrapie agent and ribosomes cosediment, these two entities do not appear to be linked. Heating an extract (S_1) of spleen to 80 °C for 30 min resulted in degradation of the ribosomes, while the sedimentation profile of the agent remained unchanged (Prusiner et al., 1978). Additional evidence demonstrating that the scrapie agent is independent of ribosomal structures is provided by the sucrose-gradient centrifugation studies (Figures 3 and 4). In those studies the ribosomes were recovered either from the top or the bottom of the gradient, depending on the conditions of centrifugation, while the infectivity remained distributed over the entire gradient.

Several attempts have been made to define the buoyant density of the scrapie agent in extracts of murine brain. These preparations were not enriched for infectivity and contained numerous membranous structures. Using a sucrose–NaCl gradient, Siakotos et al. (1976) found multiple peaks of scrapie infectivity distributed over a range of sucrose densities from 1.14 to 1.30 g/cm^3. They suggested that the largest peak of infectivity at 1.19 g/cm^3 was the equilibrium density of the agent. More recently Brown et al. (1978) have found the agent in extracts of brain distributed over a range of sucrose densities from 1.07 to 1.28 g/cm^3 with maximal titers seen over a plateau region with sucrose densities from 1.15 to 1.25 g/cm^3. When metrizamide was used, the agent was found over a range of densities from 1.08 to 1.28 g/cm^3 with a possible peak at 1.17 g/cm^3; with CsCl the agent was found over a range of densities from 1.11 to 1.32 g/cm^3 with maximal titers seen over a plateau region having densities from 1.18 to 1.27 g/cm^3. Earlier studies by Gibbs (1967) showed maximal infectivity in CsCl at densities ranging from 1.29 to 1.37 g/cm^3.

Our studies with a partially purified preparation (P_5) of the agent from murine spleen showed similar heterogeneity with respect to density. Approximately 80% of the agent was found at sucrose densities from 1.21 to 1.30 g/cm^3, while 20% of the agent was found at densities between 1.08 and 1.20 g/cm^3 (Figure 3). Analysis of the P_5 fraction from spleen indicates that this heterogeneity is not due to the membranous structures which appear to be a constant contaminant in brain extracts containing the agent. In fact, using a purification scheme similar to that shown in Figure 1 for the agent in brain yielded a P_5 fraction containing many membrane fragments and ribosomes (Prusiner, unpublished observations).

Heterogeneity of the scrapie agent with respect to size was also observed. A succession of infectious particles ranging in size from approximately 40 S to greater than 500 S is demonstrated in Figure 4. The monomeric or minimal size of the scrapie agent capable of infection would appear to be 40 S or less. This is smaller than any known animal virus. Larger forms of the scrapie agent probably represent multimers or aggregates, the subunits of which might all be infectious (Prusiner et al., 1977, 1978). This view is consistent with the unusually small ionizing radiation target size reported for the scrapie agent (Alper et al., 1966).

As described in Figures 5 and 6, the scrapie agent can apparently undergo aggregation resulting in particles all having sedimentation coefficients greater than 500 S. Ionic and/or hydrophobic bonding may be responsible for aggregation of the agent. Ionic bonding is a less likely possibility because aggregation occurred in the absence of added salt and also in the presence of EDTA (Figures 5 and 6). The possibility that hydrophobic bonding is responsible for the increased size of the agent would seem more likely since the shift in the size of the agent was in all cases associated with heating to 37 or 80 °C (Figures 5 and 6; Kauzman, 1959; Scheraga et al., 1962; Nemethy & Scheraga, 1962; Tanford, 1973). In general, hydrophobic interactions are increased with increasing temperature while hydrogen bonding is diminished (Kauzman, 1959; Scheraga et al., 1962; Pauling, 1960). Indeed, our data raise the possibility that the scrapie agent may contain molecules, such as a protein and/or nucleic acid, which are capable of extensive hydrophobic interactions. The ability of hydrophobic proteins to aggregate and dissociate, as well as bind lipids, could readily account for the behavior of the scrapie agent in these sucrose gradient centrifugation studies (Wallach & Winzler, 1974; Helenius & Simons, 1975; Spatz & Strittmatter, 1971; Robinson & Tanford, 1975; Scanu, 1972). Hydrophobic interactions stabilizing the secondary structure of nucleic acids are well documented (Herskovits et al., 1961; Levine et al., 1963). It is possible that similar interactions might contribute to the hydrophobicity of the scrapie agent. Juxtaposition of several aliphatic substituents on modified bases (Hall, 1971) could conceivably contribute to this hydrophobicity if they were suitably situated on the surface of the particle. Interestingly, several modified nucleosides with aliphatic substituents exhibit absorption maxima in the region of 240 nm where the inactivation of the scrapie agent by ultraviolet irradiation is the greatest (Alper et al., 1966).

If it can be established that the scrapie agent does contain hydrophobic domains on its surface and the agent is capable of extensive hydrophobic interactions, then several of the mysterious properties currently attributed to it might be explained (Prusiner et al., 1977; Hunter, 1972; Kimberlin, 1976; Gajdusek, 1977). First, the unusual stability of the agent to heating is consistent with such a view since hydrophobic interactions are stabilized at high temperatures (Prusiner et al., 1978; Kimberlin, 1976). Second, the apparent association of the scrapie agent with membrane fractions is understandable since hydrophobic proteins can readily insert themselves into membranes. Third, not unlike experiences with the scrapie agent, many hydrophobic proteins have been found to be exceedingly difficult to purify with retention of activity. Fourth, the scrapie agent like many lipoproteins appears to exhibit minimal antigenicity in contrast to apolipoproteins which are often good antigens (Scanu, 1972; Bjerrum, 1977). Fifth, the apparent discrepancy in the size of the agent as determined by ultrafiltration and by ionizing radiation might be due to the ability of the agent to undergo aggregation and dissociation as a consequence of its hydrophobic surface.

A fascinating picture of the scrapie agent is beginning to emerge. The agent appears to be a particle smaller than any known animal virus and to possess hydrophobic domains on its surface. These domains may explain many of the unusual biological properties associated with the scrapie agent. Whether such agents are more appropriately classified as

hydrophobic viruses or as episomal elements must await elucidation of the molecular structure of these novel entities.

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