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Physical Properties of the Creutzfeldt-Jakob Disease Agent

T. K. SKLAVIADIS, L. MANUELIDIS,* AND E. E. MANUELIDIS

Section of Neuropathology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

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In this report, we present the first physical characterization of the Creutzfeld-Jakob disease agent. Preparations with high yields of infectivity (assayed infectious units) were obtained by a novel, gentle procedure in which initially sedimenting Gp34 ("prion" protein) was disaggregated by a variety of criteria with no subsequent loss of infectivity. Studies with this preparation indicate that most of the Creutzfeldt-Jakob disease agent has both a viruslike size and density. In velocity sedimentation and isopycnic sucrose gradients, infectivity comigrated with nucleic acid-protein complexes of appreciable size.

Scrapie and Creutzfeldt-Jakob disease (CJD) are manifest as progressive, slow neurological diseases with a negligible inflammatory response. These diseases are caused by infectious agents that are poorly characterized in terms of their physical properties and their constituent molecules (26). Despite recent intense investigations of scrapie and CJD, no agent-specific (nonhost) proteins and nucleic acids have been identified. The search for agent-specific molecules has overshadowed efforts to reassess the basic physical parameters of these infectious agents. Although a great deal of information can be gleaned from older studies of scrapie, many agent characteristics have been assessed in crude brain homogenates or very impure and complex brain fractions, with highly discordant results (see Discussion). A few recent studies have attempted to assess specific agent parameters in more-purified preparations, but these fractions which were precipitated and sometimes associated with very low yields of starting infectivity (assayed infectious units) may also reflect inaccurate estimates of size and density characteristic for most of the infectious agent. Such studies also indicate highly divergent size values of 4S (22) to 10,000S (39) for the scrapie infectious agent. Almost all of the physical studies have used scrapie, typically the 263K strain, which yields about $10^{9.6}$ units of the infectious agent per g of brain homogenate after a relatively short incubation period of 70 to 90 days (19).

In the present studies, we attempted to systematically investigate the parameters of agent size and density by comparing both crude and more-purified brain fractions from experimental CJD. Many isolates of human CJD have been transmitted to rodents in our laboratory (see references 26 and 27 for reviews). In this study, we used one isolate that was very stable with respect to incubation period, titer, and pathological effects (23, 28) and was serially carried in hamsters for >18 passages. Except for one homogenate filtration study indicating that the CJD agent is >100 nm (13), these human agents have never been physically characterized. Although the titers of the CJD infectious agent are lower than that of 263K scrapie (10^8 to $10^{8.3}$ infectious units/g of brain homogenate) and the incubation period is longer (110 to 135 days), the study of CJD offers independent, comparative, and medically relevant data for this general class of agents. Here we present novel dissociation conditions of reasonably purified infectious fractions that show that the size of the agent is greater than that of a monomeric protein. Furthermore, a tight peak of infectivity was resolved at a density of nucleic acid-protein complexes greater than the density of most soluble proteins. Together, these data indicate that these agents are likely to contain an appreciable nucleic acid component. Thus, they may be more similar to conventional viruses (protein-nucleic acid structures) (27) than to postulated infectious proteins or "prions" (37).

MATERIALS AND METHODS

The procedures used to partially purify the infectious agent from brains are straightforward, simple, and rapid and have been described in detail previously (28, 29), but briefly they are as follows. Frozen brains were homogenized in the presence of 10 to 15 volumes of 1 or 10% (wt/vol) sarcosyl in 25 mM Tris chloride (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride. A 25,000 × *g*, 30-min supernatant was collected and centrifuged at 215,000 × *g* for 2.5 h. This crude pellet was designated p215. When homogenates were made in 1% sarcosyl, a membrane-rich (white opalescent) layer was formed just above a tight, small, brown pellet, and this membrane-rich fraction was used for comparative sedimentation velocity gradient studies (see Fig. 3). With 10% sarcosyl, the p215 crude pellet did not separate into layers and was extracted twice with 10% NaCl as previously described (29) to generate the standard p215salt preparation. Alternatively, the crude p215 pellet was washed thoroughly with 25 mM Tris chloride (pH 6.8) (500 μl/g of starting brain tissue) and centrifuged at 15,000 × *g* for 30 min at 20°C; the supernatant was discarded, and the procedure was repeated. This low-salt-extracted pellet was designated p215-6.8. In six separate experiments, the recovery of infectious units in all of the above-described p215 pellet fractions was typically ~20% of the infectious units contained in starting brain homogenates; bioassays (vide infra) from several independent experiments showed negligible infectious unit losses in the p215 high- and low-salt supernatants (≤5%). For disaggregation, p215-6.8 pellets were suspended in 1% sarcosyl in 25 mM Tris chloride (pH 8.9) (330 μl/g equivalent of starting brain tissue) and bath sonicated at room temperature for 15 min. Residual aggregated material was pelleted at 15,000 × *g* for 30 min. This procedure was repeated three to five times on the residual aggregated material, and the supernatants (designated s215-8.9) from each step were combined for gradient analysis.

For velocity sedimentation studies, the crude p215 membrane-rich fraction was analyzed on 9.2-ml linear 10 to 30% (wt/vol) sucrose gradients (in 25 mM Tris chloride [pH

* Corresponding author.

7.6]–0.1% sarcosyl) over a 1-ml 87% sucrose cushion; these gradients were loaded with 0.8 g equivalents of initial brain tissue. Gradients were spun at 210,000 × *g* for 6 h at 20°C in an SW41 Ti rotor, and fractions (~550 μl each) were collected from the bottom by tube puncture. Similarly, for analysis of more-purified p215 fractions (p215-6.8 and s215-8.9), samples were loaded on a 3.6-ml linear 10 to 30% sucrose gradient on a 240-μl 87% sucrose cushion and spun in an SW60 Ti rotor at 210,000 × *g* for 8 h. Aggregated p215-6.8 pellets were mechanically suspended and bath sonicated for analysis in gradients containing 25 mM Tris chloride (pH 7.4)–0.05% sarcosyl. Disaggregated s215-8.9 supernatants were analyzed in sucrose gradients containing 25 mM Tris chloride (pH 8.6)–0.05% sarcosyl. Fractions (~250 μl each) were collected by bottom puncture. For isopycnic analysis, 2.6-ml linear gradients of 30 to 87% sucrose over a 0.7-ml 87% sucrose cushion were used, and gradients were spun in an SW60 Ti rotor. We assumed that since the agent could be small, it would be necessary to use long spin times to assure that infectivity would reach its equilibrium density. Thus, gradients were centrifuged at 210,000 × *g* for 41 h at 20°C; collection was from the bottom (~250 μl per fraction). Especially in density gradients, each tube of the SW60 Ti rotor should not be loaded with >0.3 g equivalents of starting brain tissue, because resolution of fraction components is decreased. λ *Hin*dIII restriction fragments were isotopically end labeled by standard methods with the Klenow fragment and were included on parallel control gradients. These fractions were ethanol precipitated and analyzed on standard agarose gels by autoradiography. Sucrose concentrations were determined by refractive index measurements, and S values were calculated by standard methods (32) and corresponded to known values empirically determined in parallel gradients containing [^{32}P]DNA size markers.

Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting techniques for detection of Gp34 and other proteins were as previously described (29, 48). Colloidal gold (Janssen) for detection of proteins was used as recommended by the manufacturer. Marker protein lanes were stained on blots with India ink (29). For analysis of proteins in gradient fractions, samples were dialyzed and lyophilized after removal of samples for bioassay. Densitometric scans of proteins stained on Western blots (immunoblots) were quantitated with a Vax graphics workstation set up as previously described (24). Peak areas were determined by both integration and best Gaussian fit; the two determinations showed essentially identical results (data not shown).

The bioassay of infectious material is based on incubation time, and dilutions yielded incubation times of 145 to 220 days, which are in the optimal linear range of this assay (28). Briefly, six hamsters were inoculated with 50 μl of appropriately diluted material of each preparation or fraction and observed daily for early clinical and terminal signs of disease. The days-of-incubation value for each animal was converted to infectious units on the basis of least-squares linear fit (r = 1.00 for clinical signs; r = 0.99 for terminal disease) of previously published serial dilution points (28). A difference in infectivity of 1 order of magnitude is reflected by an ~29 day difference in incubation time in this CJD model, and daily observation minimized errors in the assay. In general, these results are expressed here as infectious units per starting gram of brain tissue for comparative purposes; the number of infectious units in pooled fractions was divided by the number of fractions pooled to yield the average infectious units per fraction. Brains from animals infected with a very stable strain of the CJD agent (23, 28) serially propagated in this laboratory for >18 passages in LVG hamsters were used for all of the experiments shown here; homogenates of this material reproducibly yielded 10^8 to $10^{8.3}$ infectious units per g of starting brain homogenate.

RESULTS

Our basic strategy was to analyze crude as well as more-purified CJD brain fractions to delineate parameters that could alter the apparent physical characteristics of the agent. Such studies should increase our understanding of the wide discrepancies reported in the scrapie literature. The enormous range of sizes attributed to the CJD or scrapie agent is probably a consequence of particular artifacts of the various techniques used and of the propensity of these agents to aggregate and associate with membrane structures. In addition, many determinations have placed undue emphasis on very small fractions of infectivity persisting after various treatments.

Very mild treatments were used for purification and extraction of infectious units so that we could approach the true physical characteristics of these agents with some confidence. In all of the cases described below in which infectious units were assayed, the conditions chosen were first analyzed in at least three independent preparations to determine reproducibility before bioassay. In these studies, the position of Gp34, a host membrane glycoprotein that has been associated with scrapie and CJD infectivity, was also assessed in Western blots. Gp34 has been shown to be the precursor of a 27- to 29-kilodalton (kDa) proteolytic product (also known as PrP or prion protein) by direct analysis (29, 48) and by sequence studies (35); it is a normal host protein that is highly conserved in evolution (3, 21, 45). More-purified infectious fractions were also analyzed for minor proteins by staining Western blots with colloidal gold, sometimes with intensification, as described elsewhere (L. Manuelidis, T. Sklaviadis, and E. E. Manuelidis, *in* L. Court, ed., 2nd Symposium on Non-conventional Viruses of the Central Nervous System, in press).

A novel procedure for mild disaggregation of infectivity in more-purified preparations. It was essential to study more-purified fractions in which complex lipid-rich membranes would not interfere with accurate determination of the S value of the agent. We have shown that a p215salt fraction yields reasonably high recoveries of the infectious agent and relatively little contamination with proteins other than Gp34 (26, 29, 48; Manuelidis et al., in press). These protein analyses have been confirmed in scrapie (15). By electron microscopy, this aggregated preparation showed essentially no intact membranes, and scrapie-associated fibrils were also not significantly present before treatment with proteinase K (26; Manuelidis et al., in press). The problem with this fraction is that it appears to be aggregated by two criteria: electron microscopy and sedimentation characteristics. Both Gp34 and the infectious agent quantitatively sedimented at 13,000 × *g* for 30 min after they were initially pelleted at 215,000 × *g* for 2.5 h.

It was essential to disaggregate this fraction to obtain reliable estimates of the size and density of the infectious agent or, alternatively, to derive an equally infectious and reasonably purified nonaggregated fraction. One approach we tested for disaggregation was the use of detergents, and we chose several that presumably would not affect infectious agent titers or integrity. We analyzed the sedimentation of

Gp34 in these detergents (Nonidet P-40, Triton X-100, Nikkol, SB3-14, and deoxycholate) at concentrations above their critical micelle concentrations in suspensions containing ~0.02 μg of protein and ~10^6 infectious units of the agent. Even with mild bath sonication, none of these detergents reproducibly dissociated Gp34; most (>75%) of this marker readily sedimented after treatment in a visible pellet at 13,000 × *g* for 30 min, as monitored by Western blotting (data not shown). (In some experiments with deoxycholate, dissociation of up to 50% of the starting Gp34 was achieved.) These detergent supernatants were therefore not tested further in lengthy bioassays of infectivity.

Since high-salt extraction conditions might irreversibly aggregate both the infectious agent and Gp34 (28; Manuelidis et al., in press), we also attempted to purify the crude p215 fraction by repeated extraction with low-salt buffers at approximately neutral pH. Although this procedure led to substantial purification of the p215 fraction with respect to other proteins so that it resembled the purity of the p215salt fraction on Western blots (Fig. 1, lanes 2 and 3), Gp34 and the infectious agent were still aggregated, i.e., they sedimented at 13,000 × *g* in 30 min. By electron microscopy, this material resembled the previously described p215salt fraction with aggregates of detergent-protein micelles and additional fluffy or granular material that probably represents disrupted Gp34 (26). Two preparations of the p215 fraction washed with low-salt buffer (p215-6.8) were assayed for infectivity. About 95% of the input infectious units of the crude p215 fraction were recovered in the pellets, and ~5% of the infectious units was lost in the supernatant.

We reasoned that if we could make Gp34 more soluble, we might also release the infectious agent into the supernatant; i.e., either insoluble Gp34 trapped the infectious agent or the agent and Gp34 shared similar physical or chemical characteristics. We therefore exploited known characteristics of Gp34 in order to dissociate it. Isoelectric focusing gels show multiple spots of most of Gp34 at a pI of 6.5 to 8.3 (48). Thus, Gp34 should be insoluble at a neutral pH, near its isoelectric point. However, at a more elevated pH, this protein should be released into the supernatant. We tested pH buffers from pH 8.3 to pH 9.0 and initially used the following three criteria for disaggregation of more-purified p215-6.8 and p215salt fractions under these more alkaline conditions: (i) lack of sedimentation at 20,000 × *g* for 30 min, (ii) increased proteinase K sensitivity of the released Gp34 (based on the concept that it would be more accessible to the enzyme), and (iii) an apparent increase in infectivity of the supernatant. The last criterion is based on the assumption that a more dispersed viral preparation should more effectively seed the recipient organism than one that is cohesively aggregated. We found that treatment of these pellets at pH 8.9 met all of these criteria.

The p215-6.8 pellet could be completely extracted into the supernatant at pH 8.9 with no visible residual pellet. The p215salt pellets showed a small residual pellet with centrifugation at 20,000 × *g* for 30 min after treatment at pH 8.9 and centrifugation, and this residual pellet generally contained 10 to 15% of the starting Gp34. The less effective dispersion of the p215salt fraction may be due to the salt aggregation artifacts noted above. Remarkably, after dissociation at pH 8.9, Gp34 in the supernatant was completely digested by proteinase K (Fig. 1, lanes 5 and 11; in lane 11, the band denoted by the open triangle is proteinase K, not Gp34). In contrast, parallel incubations of the aggregated p215 fractions (not pH 8.9 treated) yielded appreciable quantities of lower-molecular-weight proteinase K-resistant Gp34 bands

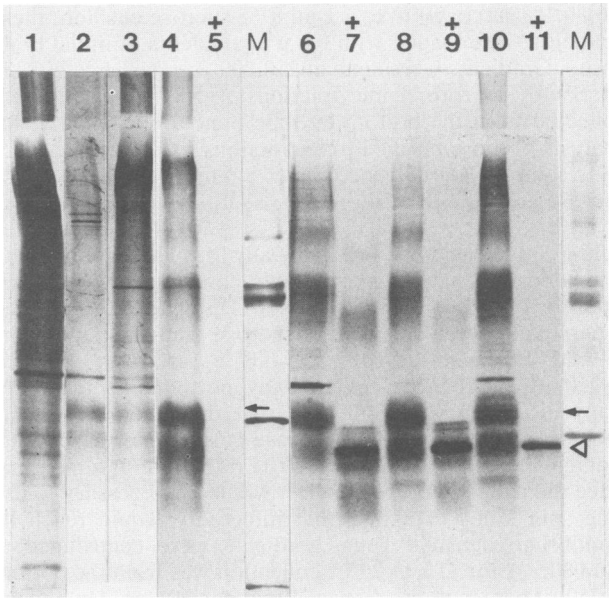


FIG. 1. (Lanes 1 to 3) Relative purification of p215 proteins with extraction, stained with colloidal gold in Western blots. Lanes: 1, crude p215 pellet; 2, p215-6.8; 3, p215salt. Note that both extracted pellets, which retained >95% of the crude p215 infectious units, show a marked decrease in contaminating proteins. Gp34 is indicated by the arrows; colloidal gold (not silver intensified) stains heavily sialated Gp34 with disproportionately low intensity. (Lanes 4 to 11) Western blots of more-purified preparations stained with anti-PrP antibody (a generous gift of R. Kasczak) after incubation without or with (+) proteinase K; Lanes: 4, disaggregated s215-8.9; 5, parallel sample digested with proteinase K (0.025 g equivalent in 12.5 μl containing 5 μg of proteinase per ml and incubated for 2 h at 37°C). No Gp34 or its lower-molecular weight prion protein products are seen. A more extensive repeat experiment (identical digestion conditions, with the proteinase K band detectable probably because of a slightly increased concentration of antibodies [the open triangle points to undigested proteinase K]) is shown in lanes 6 to 11. Lanes: 6, aggregated crude p215; 7, aggregated crude p215 treated with proteinase K showing roughly equivalent amounts of starting Gp34 as proteolytic products at ~27 kDa; 8, aggregated p215-6.8; 9, aggregated p215-6.8 digested with proteinase K showing some residual Gp34 products; 10, disaggregated s215-8.9 showing Gp34; 11, disaggregated s215-8.9 showing no detectable Gp34 or its proteolytic products after treatment with proteinase K (only the proteinase K band is visible). M lanes show protein markers at 13, 29, 55, 58, 98, and 29-55 kDa, respectively. Higher-molecular-weight aggregates (dimers and trimers) are also apparent in lanes 6, 8 and 10. Controls with proteinase K alone confirmed that the resistant band identified above was proteinase K, and experiments with aggregated and pH 8.9 dissociated p215salt preparations were comparable (data not shown).

(Fig. 1, lanes 7 and 9). These resistant bands (of prion protein) have been considered by some to represent the infectious forms of these agents (37, 42).

Since Gp34 in this pH 8.9 preparation is no longer "resistant" to proteolysis, it is clearly more soluble and accessible than in the starting aggregated pellet; it also does not fulfill the criterion of proteolytic resistance previously used for the infectious agent (42). It should be noted that aggregated preparations rich in Gp34 or its derivative proteolytic products, when treated with sodium dodecyl sulfate, also showed no resistance to proteinase K, as did our pH 8.9 dissociated supernatants. However, these sodium dodecyl sulfate-treated fractions were not infectious (41). In contrast, infectious agent assays showed that our pH 8.9 preparations

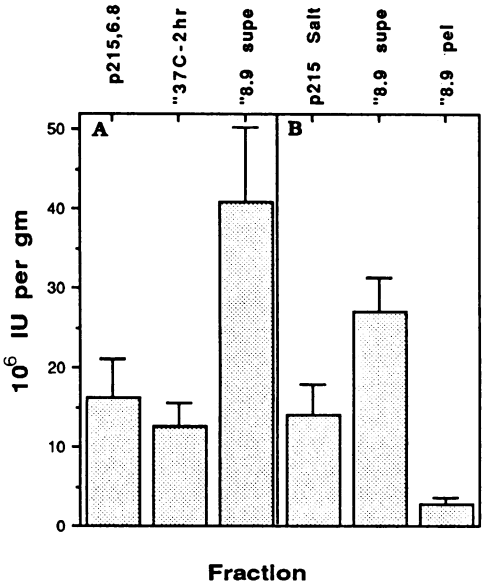


FIG. 2. Apparent increase in infectivity of p215 extracted pellets after disaggregation at pH 8.9. (A) Infectivity of a p215 pellet extracted in low salt (pH 6.8) and at 37°C for 2 h with equivalent titers; the supernatant from a parallel pellet disaggregated at pH 8.9 (no residual pellet) showed increased infectivity. (B) p215salt preparation, supernatant following pH 8.9 dissociation, and residual pellet after treatment at pH 8.9. Note the apparent increase in infectivity (supernatant plus pellet) after pH 8.9 treatment. The standard errors of the means are shown.

contained significant infectivity. Furthermore, they reproducibly contained more apparent infectious agent than their aggregated counterparts (Fig. 2). Thus, a greater-than-twofold increase in infectivity was seen in the pH 8.9 dissociated supernatant compared with the p215-6.8 extracted pellet from which it was derived (Fig. 2A). A similar approximately twofold increase in infectivity was also detected in the pH 8.9 dissociated p215salt fraction (when the residual pellet is considered; Fig. 2B). Although we generally take a difference of >0.5 order of magnitude in the bioassay to be significant, a Student's *t* test of these data showed significant differences ($P < 0.005$ to $P < 0.05$) when either days of incubation or infectious units were analyzed; equivalent starting gram amounts of each preparation were inoculated for these bioassays. Additionally, the reproducibility of increased infectivity in two different pH 8.9 dissociated preparations suggests that this observation was consistent.

In summary, we developed a procedure that substantially dissociates the agent in more purified, reasonably infectious fractions and operationally defined minimal criteria for solubilizing or disaggregating CJD infectious material that can be used with some degree of confidence in studies of the size and density of the agent. However, we still cannot completely exclude either the presence of dimers or multimers of the infectious agent or complete dissociation of noninfectious proteins from the infectious agent.

Sedimentation characteristics of membrane-enriched, aggregated, and disaggregated p215 purified fractions. To appreciate and understand various preparative and aggregation artifacts, sucrose sedimentation velocity gradients were loaded with (i) a crude membrane fraction, (ii) a more-purified, mechanically dispersed p215-6.8 preparation, and (iii) p215-6.8 pellets gently dissociated at pH 8.9 (s215-8.9). The crude membrane fraction has a high complexity of proteins (Fig. 1, lane 1), comparable to those of other similarly isolated crude p215 fractions previously depicted (28), and contains membrane structures, as shown by electron microscopy (data not shown), in contrast to more-purified p215 preparations. The CJD infectious agent is highly enriched in membrane fractions gently prepared from brain tissue (25). Crude CJD membrane-rich fractions are therefore useful for comparison with results obtained with crude scrapie homogenates and with lipid-rich preparations. Comparable 10 to 30% rate zonal sucrose gradients were used for infectious material bioassays. These gradients accurately resolved smaller (lower-S-value) species but not larger species.

Figure 3A shows the sedimentation profile of a crude p215 membrane fraction derived from a 1% sarcosyl homogenate analyzed on a 10 to 30% sucrose gradient. In this membrane-rich preparation, ~95% of the Gp34 (solid circles) formed a tight band near the top of the gradient, essentially comigrating with the 120-base-pair (bp) λ *Hin*dIII restriction fragment (Fig. 3B; darker exposures of fractions 15 to 20 are not shown). A minor proportion of Gp34 (6%) migrated further into the gradient. The distribution of infectious material on this gradient was determined in two bioassays. In the first, the entire gradient was analyzed by pooling adjacent fractions (Fig. 3A, white bars). All (100%) of the gradient input infectivity was recovered, amounting to 25% of the infectious units in the initial whole-brain homogenate; 91% of the agent ($10^{7.5}$ infectious units) was found at the top of the gradient. A second bioassay of selected individual fractions (Fig. 3A, shaded bars) confirmed that infectious material generally overlies the two peaks of Gp34. Virtually all of the other contaminating proteins detected with colloidal gold in this crude membrane preparation (similar to those depicted in Fig. 1, lane 1) also migrated in this region (data not shown).

Notably, in the above-described gradient analysis, infectious material and the major Gp34 peak were collected in a tight band distributed over only three fractions, similar to each of the discrete λ *Hin*dIII restriction fragments (Fig. 3B). This is in sharp contrast to previous results obtained with non-detergent-treated membrane-enriched fractions, in which multiple infectivity peaks were spread throughout a sucrose density gradient (47). It is likely that in our crude membrane preparation, the detergent concentration (1% sarcosyl) was sufficient to dissociate a number of less tightly bound heterogeneous components from membranes, yielding a more homogeneous population of membranes which still tightly bind most of the infectious agent. Analysis of this crude p215 preparation alone suggests that the agent has a discrete size with a very small S value (0.5S to 6S). However, the association of most of the infectious material with abundant low-density, very lipid-rich brain-derived membranes could also be responsible for the very small apparent S value of the agent, and the minor peak of infectious material at ~55S could potentially represent agent that is more completely dissociated from membranes. We therefore analyzed the apparent size of the agent in more-purified fractions, in which membranes were thoroughly disrupted.

Despite mechanical dispersion, all of the Gp34 in the aggregated p215-6.8 fraction pelleted to the bottom of the tube in sedimentation velocity studies (Fig. 4A is representative). Bioassay of pooled fractions from the gradient shown in Fig. 4A indicated that essentially all (>99%) of the infectious material was also contained in this sucrose pellet. Seven percent of the input infectious units was recovered

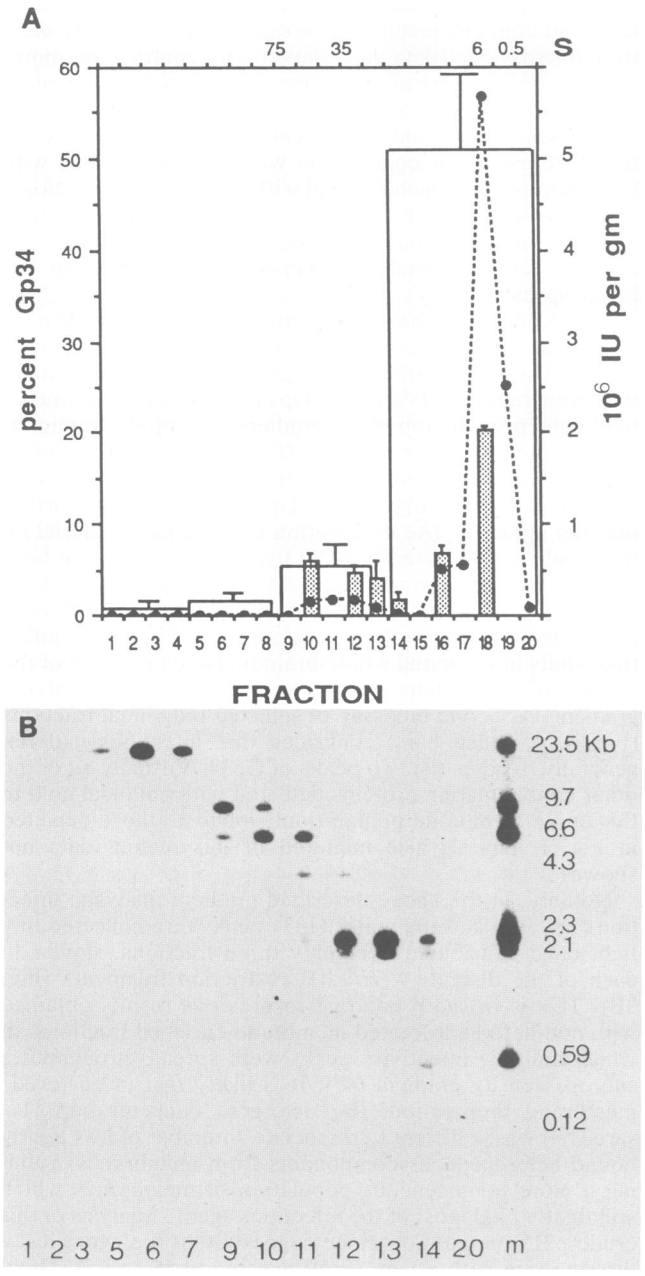


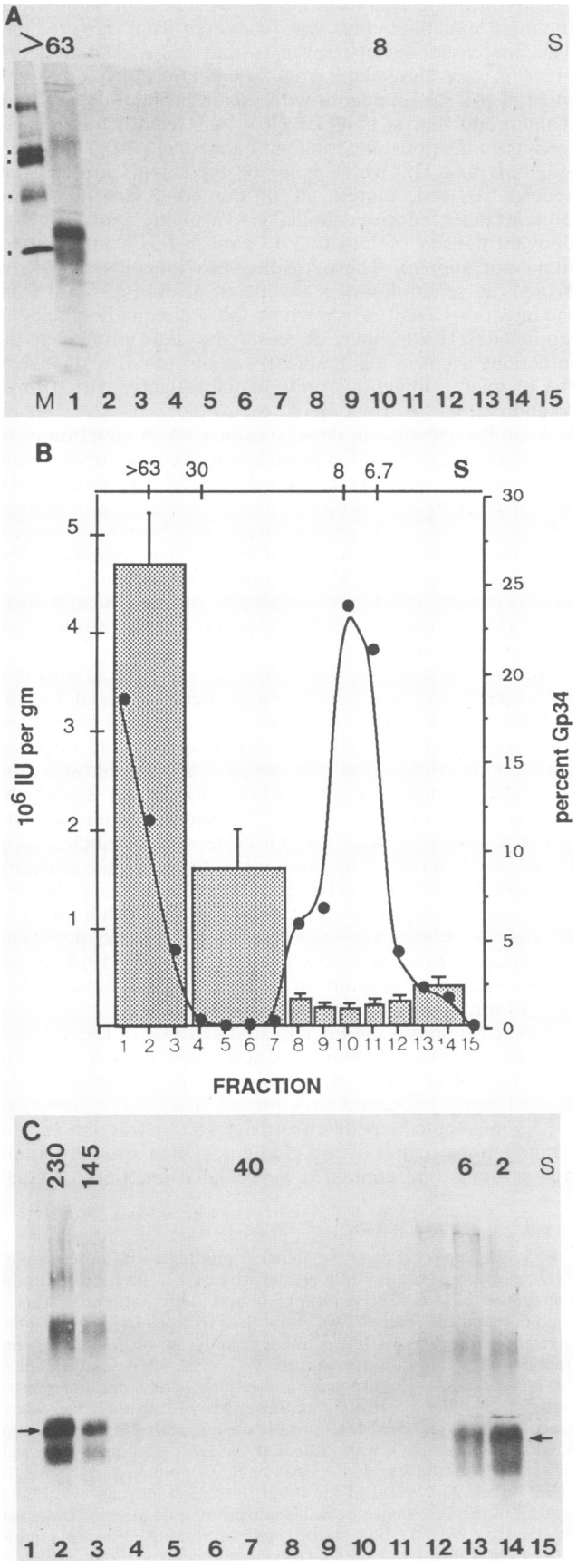
FIG. 3. Sedimentation of crude-membrane-rich p215 and nucleic acid markers. (A) Crude p215 assayed for Gp34 (●) and inoculated into hamsters for bioassay. In the first bioassay, fractions were pooled (open bars); the average infectious units per single fraction and the standard error of the mean are depicted. Most of the infectious units were present in pooled fractions 14 to 20, which contained Gp34, as well as most of the other proteins detectable with gold in this preparation. A second bioassay of individual fractions (shaded bars) confirmed these results. In this and subsequent figures, recovered infectious material is expressed per gram of starting brain tissue. (B) Parallel gradient loaded with a λ *Hin*dIII fragment (end labeled with ^{32}P; autoradiograph). Each size fragment was collected in a reasonably tight band (as was Gp34 above) and confirmed the calculated S value. The lane numbers correspond to fraction designations. Lane m contained molecular size markers.

from this gradient, and losses were most likely due to pelleting and incomplete recovery of the pellet from the bottom wall of the tube during collection by puncture. The high S value obtained with this fraction probably reflects both aggregation and disruption of lipid-rich membranes. The S value was no longer artificially small, as in the p215 crude membrane fraction, but was probably artifactually large as a result of aggregation. Both of these artifacts can be compared to the sucrose sedimentation characteristics of the scrapie agent, which migrates only at both extremely small and extremely large S values in partially purified preparations (38). The artificially small scrapie agent size in synthetic liposome-scrapie agent preparations (12) may also be compared to the apparent small S value of CJD infectivity depicted in our membrane-rich preparation.

In contrast to aggregated preparations, Gp34 was detected in two different regions in gradients loaded with pH 8.9 dissociated supernatants. In the gradient shown in Fig. 4B, 36% of the infectious units loaded on the gradient was recovered in the fractions. This represents a reasonably high recovery, since (i) few carrier molecules were present in these more-purified preparations (i.e., nonspecific losses were likely to be associated with the collection tubing and centrifuge tube walls) and (ii) the bioassay is not completely precise. Indeed, this CJD agent recovery compares favorably with recoveries of 40 to 60% in conventional viral preparations similarly sedimented in sucrose in which labeled nucleic acids were monitored (52). Fractions 8 to 15 were individually assayed, because this is where most (65%) of the Gp34 was found on the gradient (Fig. 4B), and presumably this is where the infectious agent should comigrate (based on the crude-membrane results described above). However, 88% of the recovered infectious units (70% in fractions 1 to 3 and 21% in fractions 4 to 7) was well resolved from this major low-S Gp34 peak seen in fractions 10 to 11. Thus, a significant proportion of Gp34 was dissociated from the infectious agent at pH 8.9. The S value of this dissociated Gp34 (6.7-8S) is consistent with the S values of many soluble monomeric or dimeric proteins (6, 9) and further indicates that our dissociation conditions were reasonably effective. Most of the infectious agent was found at the bottom of the gradient with an S value of ≥63, but unlike the gradient from aggregated material, the infectious agent was not pelleted. The above-described gradient defines the lowest limit of size for the infectious agent.

Further studies were directed toward an analysis of the upper limits of size of the infectious agent in these preparations. For this purpose, the sedimentation times were decreased to resolve the larger elements at the bottom of the gradient. Since the high-S fractions associated with Gp34 were highly infectious, we reasonably assume comigration of the infectious agent with this more minor peak of Gp34 in the following analyses. With a 2-h spin, Gp34 did not penetrate the sucrose cushion, and the Gp34 associated with infectious material had an S value of ≥150 (Fig. 4C). Notably, an identical pattern of separation of Gp34 into two widely separated bands was also observed in this gradient. Although the low-S (noninfectious) peak barely entered the gradient, it had a value of ≤6S. Further studies using sedimentation for 1 h even more effectively resolved the infectious Gp34 peak migrating at a high S value. Most of this peak was found in three adjacent fractions, with a mean value of 375S, and an additional more minor peak at 188S was also resolved (data not shown).

Together, these data indicate that the infectious agent is considerably larger than often considered. At the very least,



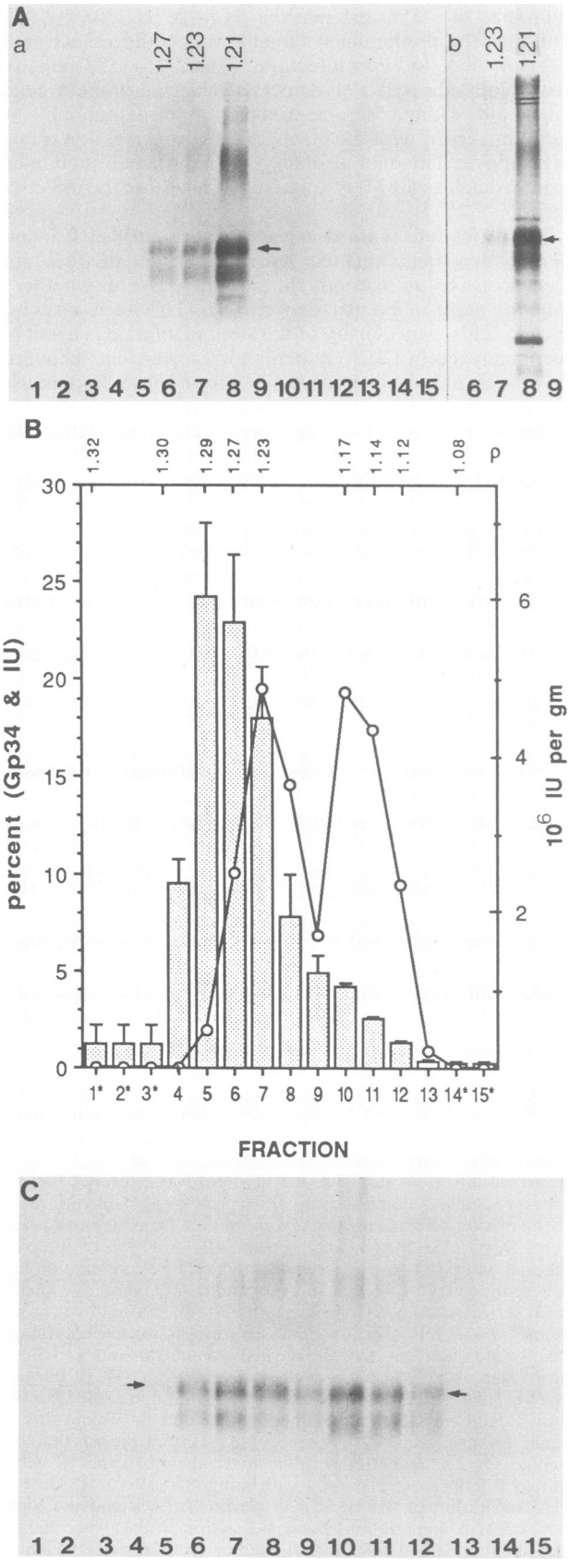
the infectious agent released from membranes has a minimum size of ~55S and may be as large as 150S to 400S. Although the predominant larger forms could reflect multimers of three to eight infectious agents of ~55S, electron microscopy showed well-dispersed material without aggregates and without scrapie-associated fibrils (unpublished data), in accord with the biochemical disaggregation criteria cited above. The data also suggest that a small monomeric protein (such as Gp34) is unlikely to constitute the infectious agent.

Density of infectious material in more-purified fractions. Density determinations can indicate whether the infectious agent consists of monomeric protein only or whether it contains more inherently dense molecular species, such as nucleic acids. The density of the scrapie infectious agent has been analyzed in CsCl gradients (5, 14, 46), but recoveries have been uniformly poor, and two of these studies used enormously complex brain homogenate preparations. Furthermore, known salt aggregation effects could artifactually alter agent density determinations. Since the CJD agent is associated with membranes, incomplete disruption of membranes can yield artifactually low buoyant densities (vide supra). A more gentle method for determination of density under conditions that can yield more reproducible and higher recoveries of infectious agent was also warranted. Isopycnic sucrose gradients have been used with success for analysis of several viruses (11, 16, 17, 49) and were used by us to determine the density of the infectious agent in aggregated and disaggregated p215 purified fractions that did not contain significant contamination with more-intact lipid-rich membranes.

Figure 5A shows the density profile of the aggregated p215-6.8 preparation, stained with antibodies (a) to detect Gp34 or colloidal gold (b) to show the general protein pattern (lanes not shown in panel B were blank). Both Gp34 and minor proteins were found in a narrow band with a mean density of 1.21 g/ml. The infectivity of pooled fractions across the gradient showed that most (94%) of the infectious material recovered was confined to these five fractions; 39% of the input aggregated p215 infectious units was recovered from this gradient.

Identical preparations disaggregated at pH 8.9 showed an even higher density of the infectious agent. The mean

FIG. 4. Sedimentation velocity studies of more-purified CJD agent preparations. (A) Western blot stained with antibodies of fractions 1 to 15 from an aggregated p215-6.8 preparation spun for 8 h to resolve lower-S-value components. All of the Gp34 and recovered infectious material (see the text) was entirely pelleted in fraction 1. Gold staining showed other proteins also only in fraction 1. The marker proteins in lane M were blotted from the same gel and were 29, 43, 55, 58, 68, and 94 kDa. (B) Analysis of infectious material and Gp34 from a disaggregated s215-8.9 preparation similarly sedimented for 8 h in a sucrose gradient. Quantitative analysis of Gp34 by densitometry of a Western blot (●) showed two separate peaks; 70% of the infectious units recovered (shaded bars; means plus the standard errors of the means) was in fractions 1 to 3, with an S value of ≥63, and was well separated from 65% of the Gp34 loaded. The tailing 21% of the infectious material in pooled fractions 4 to 7 may be largely contained in fraction 4, which was not separately assayed. (C) Disaggregated s215-8.9 preparation sedimented for 2 h to better resolve material with higher S values; blotted fractions were stained with antibodies to Gp34. Note the wide separation of the two Gp34 peaks (arrows) and the high S values for Gp34 in fractions 2 and 3. Fractions 4 to 11 were blank by gold staining, and almost all of the minor proteins detected were in fractions 9 to 12 (data not shown).



density of the infectious material was 1.27 g/ml, and 61% of the input infectious units was recovered from this gradient. This increased density suggests that more contaminating proteins were dissociated from the infectious agent by treatment at pH 8.9. In accord with this interpretation, a significant proportion (~55%) of Gp34 was clearly dissociated from the infectious material and banded at 1.14 to 1.17 g/ml (Fig. 5B and C), which is more typical of pure protein species. Indeed, almost all of the contaminating minor proteins detected with colloidal gold also migrated at the low buoyant density of 1.14 to 1.17 g/ml in this same gradient (data not shown). These results also independently confirmed the separation of a significant portion of Gp34 from the infectious agent, as shown by the sedimentation velocity studies described above. A reasonably tight profile for the infectious agent in this gradient was observed, with 65% of the recovered infectious units banding tightly within three fractions (Fig. 5B, fractions 5 to 7).

A similar increase in density occurs when membranes or lipid coats are removed in purification of cores from conventional viruses, such as alphaviruses (17), retroviruses (43, 49), and hepatitis B virus (10, 16). This increase in density reflects an increased proportional contribution of nucleic acids consequent to removal of lipid-rich membranes. The increase in density in CJD agent preparations observed with pH 8.9 treatment is highly analogous, and the mean density of infectivity suggests that these agents contain a significant nucleic acid component.

Nucleic acid analyses. To verify that nucleic acid migrated with infectivity, fractions from gradients assayed for the infectious agent and protein were also analyzed for nucleic acid. These studies indicated that nucleic acids migrated with the infectious agent, they were of appreciable size, and they were complexed with protein. Figure 6A shows the nucleic acid profile for the s215-8.9 sedimentation velocity gradient shown in Fig. 4B. Discrete sets of nucleic acid bands were detected in fractions 1 to 9 of this gradient. Comigrating nucleic acid bands of appreciable size (>800 bp) were visible in infectious fractions 1 to 3. Gp34-rich fractions 10 and 11, which were not infectious, showed negligible nucleic acid. In comparison, the aggregated p215-6.8 preparations (Fig. 4A) showed all of these nucleic acid species only in fraction 1 (data not shown).

Figure 6B shows the nucleic acid analysis of the s215-8.9 isopycnic gradient. Free nucleic acids were not detectable in this preparation, since there was no silver staining of the highest-density fractions (free nucleic acid with a density of >1.5 g/ml should be pelleted and detected in fraction 1). The noninfectious region of the gradient, which should contain free proteins and contained appreciable amounts of Gp34

FIG. 5. Isopycnic sucrose density gradients of more-purified CJD agent preparations. (A) Western blot of 15 fractions from an aggregated p215-6.8 preparation stained with antibodies (a) or colloidal gold (b). The arrows show the positions of Gp34. Significant infectious material was present only in protein-containing fractions 6 to 8 at a density of 1.21 g/ml. (B) Analysis of a disaggregated s215-8.9 preparation fractionated in a parallel density gradient. A clear peak of infectious material (shaded bars show means plus the standard errors of the means; starred fractions were pooled for bioassay) was collected at 1.27 g/ml and was well separated from the less dense Gp34 peak (○), which banded at 1.14 to 1.17 g/ml. Almost all of the other detectable proteins were also present in this less dense peak visualized by gold staining (data not shown). (C) Western blot stained with antibodies showing separation of Gp34 into two peaks (arrows) and used for the quantitative densitometry determinations shown in panel B.

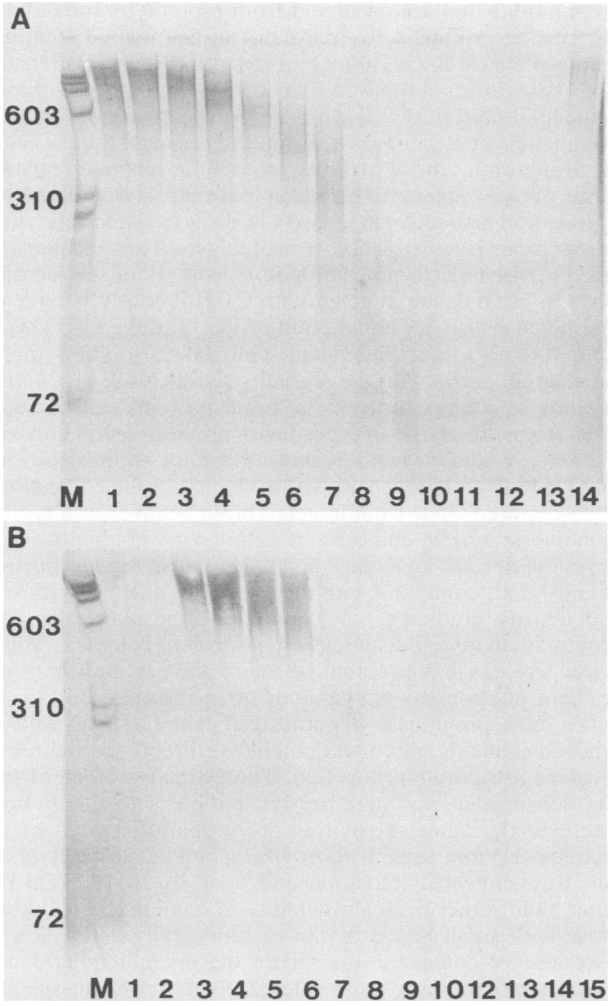


FIG. 6. Nucleic acids in assayed s215-8.9 size and density gradients. A portion of each of 15 gradient fractions (0.1 g equivalent of brain tissue) digested with proteinase K in 2% sodium dodecyl sulfate was extracted with phenol-chloroform and precipitated with glycogen carrier. Glyoxylated nucleic acids were detected by silver staining on a 4% acrylamide gel. A total of 130 ng of $\phi\chi$ *Hae*III-digested markers was loaded in lane M; thus, the 72-bp band contained 1.7 ng, the 310-bp band contained 7.4 ng, and the 603-bp band contained 14.6 ng. (A) Sedimentation velocity gradient showing discrete bands above 800 bp in fractions 1 to 3 that were infectious (Fig. 4B). (B) Isopycnic gradient showing similar-sized nucleic acid migrating in infectious fractions 5 to 6 (ρ = 1.27 g/ml; Fig. 5B) and no detectable nucleic acid in noninfectious Gp34-rich fractions 10 and 11. There was also no detectable free nucleic acid, which should pellet in fraction 1.

(fractions 10 and 11; density, 1.14 g/ml), was also devoid of detectable nucleic acid. However, infectious fractions 5 and 6, the region of predicted nucleic acid-protein complexes, did reveal nucleic acid components; these bands were similar in size to those seen in the infectious sedimentation velocity fractions.

Greater than 95% of the nucleic acids detectable by silver staining were RNA, as evaluated by enzymatic and other treatments, such as Zn hydrolysis (data not shown). About 40% of the nucleic acids in the starting s215-8.9 preparation migrated with the peaks of infectious material, equivalent to ~1 μg of nucleic acid per g of starting brain tissue. Thus, although these fractions were contaminated by non-agent nucleic acid-protein complexes, they were purified ~1,000-fold with respect to starting brain nucleic acid (>1 mg/g of brain) and as such may be of general use for the generation of more-purified fractions with relatively high recoveries of starting infectious units.

DISCUSSION

Using a novel preparation of the CJD infectious agent which minimizes aggregation and membrane association artifacts, we have shown that the size of the infectious agent is significantly larger than that of a monomeric protein and has a density greater than that of most proteins resolved and detected in the same gradient. Although the empirically derived conditions developed for this study may not be perfect, they are superior to those previously used for such analyses in terms of simplicity, reproducibility, and yields of infectious agent. The tight infectious agent peaks and the reproducibly high recoveries suggest that the physical characteristics here described are likely to be a fairly close reflection of the agent itself. The simultaneous determination of density and size in this well-characterized preparation suggests that this general class of agents is largely made up of a protein-nucleic acid complex of appreciable size, potentially falling within the spectrum of known conventional animal viruses. Nucleic acid analyses were consistent with this interpretation.

In accord with our results suggesting a viruslike size of these agents, no infectious scrapie sonicated membrane or homogenate preparations passed through 27- to 42-nm filters, but significant amounts did pass through 43- to 50-nm filters of the same type (14, 18); in another recent study of sonicated, clarified scrapie agent suspensions, a series of filters with >25-nm pores removed 99% of the scrapie infectious agent (36). One could argue that the 1% of the scrapie infectious agent that passed through a 25-nm filter was significant. However, a similar low amount of infectivity also passed through molecular 100-kDa filters in those studies. Although one might consider that this small amount of filterable material is indicative of a tiny agent size (<100 kDa), it may also reflect imperfections in the filters and may not be significant. Similarly, <1% of the infectious agent on agarose gels was used to calculate a very small S value for the scrapie agent; >99% of the infectious matter did not significantly migrate into the gel in those preparations (22, 40). Using an entirely different approach to disaggregate more-purified preparations of the scrapie infectious agent (sonication and multiple sucrose centrifugations of a proteinase K-digested p215-salt fraction), Diringer and co-workers have found that most of the infectious agent had an apparent size of 100S to 300S (7, 8), a value in accord with the data presented here for the majority of the CJD infectious agent (150S to 400S). The minimum S value of the infectious agent was ~55S, and infectivity comigrated with nucleic acid species (in protein complexes) of appreciable size. Together, these data suggest that the agent size is greater than commonly assumed. It should be noted that preparations of several conventional infectious viruses are less than 40 nm, with S values of ≥60S, and all of these contain significant amounts of nucleic acid. These include, for example, picornaviruses (~28 nm = 140S to 155S; 4.5- to 8-kilobase [kb] genome [11]), parvoviruses (18 to 28 nm; ≈122S; 4- to 5-kb genome [11, 51]), hepatitis B virus cores (27 nm; 63S and 95S; ~3.3-kb genome [1, 16, 50]), and the protein-RNA virusoid complex of hepatitis delta virus (80S to 110S

[calculated from data in reference 44]; 1.68-kb genome [53]). These examples make one reconsider the concept that CJD and related agents may possess the more conventional molecular constituents of viruses.

The density of the CJD agent has not been previously reported. Gradients loaded with crude scrapie homogenate preparations showed a broad density of infectious material at 1.18 to 1.25 g/ml in CsCl (46). However, a scrapie supernatant showed a higher density of infectious material at 1.32 to 1.37 g/ml (14), and a Freon-delipidated preparation similarly yielded a density of 1.33 to 1.34 g/ml in this same medium (5). In all of these CsCl analyses of the scrapie agent, recoveries of infectious agent were poor. In our aggregated CJD preparations, the density of the infectious agent in sucrose was 1.21 g/ml, disaggregation resulted in a clear increase in density to 1.27 g/ml, and recoveries of infectious agent were high. The densities of the CJD infectious agent in both aggregated and disaggregated CJD preparations in sucrose were above the density of the glycosylated membrane protein Gp34 (1.14 to 1.17 g/ml), as well as those of most other detectable proteins released from infectious preparations at pH 8.9. Nucleic acid-protein complexes migrated in the same region of the gradient as the infectious agent, suggesting that the agent contains similar constituents. The single peak of CJD infectivity at 1.27 g/ml is higher than the densities of enveloped retroviruses, which are 1.15 to 1.18 g/ml in CsCl and 1.16 g/ml in sucrose (11). Notably, when membranes are removed from retroviral preparations, the density of the remaining nucleic acid-rich cores is similarly increased to 1.27 g/ml in sucrose (43, 49). For comparison, hepatitis B virus (Dane particle) has a density of 1.24 to 1.27 g/ml (16, 50) and the hepatitis delta virusoid has a density of 1.24 to 1.25 g/ml in CsCl (44). All of these conventional viral values are experimentally close to the density of the CJD agent determined in this study.

It is useful, given the experimental CJD agent density value, to calculate the percent weight of nucleic acid that can be accommodated by this agent. This calculation can be applied to the empirical S values found here, as well as to other theoretical S values, to estimate the percentage of nucleic acid that the agent can accommodate. Since density, ρ, equals weight (wt)/volume, a simultaneous equation can be solved to find the weight/weight ratio of protein to nucleic acid in the agent:

$$wt_P/wt_N = [(\rho_N\rho_P) - (\rho_A\rho_P)]/[(\rho_A\rho_N) - (\rho_N\rho_P)],$$

where A = agent, P = protein, and N = nucleic acids. Substituting 1.17 g/ml (the experimental ρ of Gp34) for ρ_P, 1.27 g/ml for ρ_A, and 1.89 g/ml (the density of RNA in sucrose [31]) for ρ_N, wt_P/wt_N = 3.84/1, and therefore by weight the agent should contain ~20% RNA. Similarly, if the agent contains less dense double-stranded DNA, the proportion of nucleic acid is even higher. In reasonable accord with experimental data for retroviral cores, these calculations predict a total size of 340S for a 70S RNA. These calculations indicate that the CJD agent could contain robust amounts of nucleic acid. Indeed, even an absolutely minimum value of 50S for the agent, empirically the smallest value depicted here, would still accommodate a significant total nucleic acid component of 10S (roughly 1 kb of RNA). We assume in this calculation that the density of Gp34 is fairly representative.

We have previously suggested that the sedimentation characteristics of Gp34 in infected brains are the result of pathological alterations of membranes in vivo (28, 29; Manuelidis et al., in press). Proteolytic products of Gp34 (prion protein) are not substantially seen in CJD infectious agent preparations that are protected from proteolytic breakdown but can be produced by treatment of aggregated fractions with proteinase K (29, 48). On a structural level, aggregated Gp34 also forms unique fibrillar arrays (known as scrapie-associated fibrils; 33, 34) with proteolytic treatment in vitro (Manuelidis et al., in press). From the present studies, these electrophoretic and structural proteolytic enzyme-resistant forms of Gp34 appear to be due at least in part to preparative aggregation artifacts, since Gp34 in disaggregated infectious preparations is not resistant to proteolysis. Thus, proteolytic enzyme resistance is not an inherent property of sedimenting Gp34 in brain tissue infected with CJD. Proteolytic enzyme resistance is also not an inherent property of the CJD (28) or scrapie (4, 20) infectious agent. Our data also show that a substantial portion of the initially sedimenting Gp34 (the putative infectious form of the brain protein) can be separated from infectivity, in accord with previous lectin chromatography results (28) and expression vector studies showing that Gp34 itself is incapable of producing disease (2). Notably, separated Gp34 from infected brain behaves as a typical monomeric protein on both size and density gradients and is devoid of detectable nucleic acids. However, since a portion of Gp34 does comigrate with the infectious agent in both size and density gradients, we cannot exclude a role for this protein in an infectious nucleic acid-protein complex. Minor novel peptides may eventually be detectable in more-purified gradient fractions by antigenic or other means.

We have previously hypothesized that CJD, as well as other long-standing human dementias, directly or indirectly involves retroviral species (27). The estimates of agent size and density depicted here are compatible with this notion. Interestingly, some retroviral core preparations can be infectious (11) and several *Retroviridae* are remarkably resistant to chemical inactivation and heat (1, 10, 30). On the other hand, other protein-nucleic acid complexes, including those with small regulatory or enzymatically active RNAs also deserve consideration. Given the unambiguous densities reported here, a more intense focus on agent-specific nucleic acids seems warranted. The infectious preparations described here may be of value in these analyses.

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