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Equilibrium Density Gradient Centrifugation of the Scrapie Agent in Nycodenz

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SUMMARY

Plasma membrane-enriched preparations from scrapie-infected and healthy hamster brains were detergent-extracted, then separated by equilibrium density centrifugation in continuous Nycodenz® gradients. The highest level of infectivity was always associated with the insoluble residue which sedimented through 40% Nycodenz. The degree of aggregation in these insoluble complexes varied depending upon treatment. Centrifugation in gradients containing 2 M- to 8 M-urea resulted in the formation of large insoluble aggregates which seemed to retain a high level of infectivity when measured by the method of incubation interval assay. However, measurement of infectivity in these same samples by endpoint titration of tenfold dilutions resulted in values a thousand times lower. These observations reinforce previous findings that scrapie infectivity exists as a macromolecular complex and, furthermore, they emphasize the necessity for using non-denaturing conditions for purification of the scrapie agent.

INTRODUCTION

Scrapie infectivity from mouse (Millson *et al.*, 1971) or hamster (Semancik *et al.*, 1976) brain has been found to associate principally with membrane-containing subcellular fractions. We have recently reported a method of purification of membrane vesicles from scrapie-infected brain and retina using rate-zonal sedimentation of sonicated plasma membrane preparations in continuous Nycodenz® gradients (Marsh *et al.*, 1984). Separation of detergent-extracted vesicles by equilibrium density centrifugation in CsCl gradients resulted in a peak of infectivity at a density of 1·280 g/ml (Marsh *et al.*, 1984), a finding similar to that from centrifugation in Cs_2SO_4 (R. F. Marsh, unpublished results). These results suggest that the scrapie agent is a complex of macromolecules rather than a free protein, lipid, nucleic acid or nucleoprotein.

Centrifugation in high-salt medium has distinct disadvantages when attempting to characterize complexes. In addition to the chaotropic effect of the salt, the high ionic strength may disrupt non-covalent protein–protein, protein–lipid or protein–nucleic acid associations. For this reason, we have expanded our studies on equilibrium centrifugation of scrapie infectivity to include Nycodenz, a non-ionic iodinated medium with low osmolarity.

METHODS

Agent and bioassay. These studies used a source of scrapie serially passaged in outbred hamsters after adaptation from the Chandler strain of mouse scrapie as previously described (Kimberlin & Marsh, 1975). Infectivity was measured by intracerebral inoculation of weanling male outbred hamsters purchased from Harlan Sprague Dawley Inc. (Indianapolis, Ind., U.S.A.) with infectivity titres calculated for all samples by the method of incubation interval assay (Prusiner *et al.*, 1981), and for some samples by the Spearman Kärber method (Dougherty, 1964) after injection of serial tenfold dilutions.

Starting material. Samples used for equilibrium density centrifugation in Nycodenz were prepared from plasma membrane-enriched hamster brain homogenates from scrapie-affected or 12-week-old healthy age-matched controls. These homogenates were sonicated, then separated by rate-zonal sedimentation in continuous Nycodenz gradients yielding a fraction of predominantly large membrane vesicles (Fraction D). The methods and the characterization of this fraction are described in detail in Marsh *et al.* (1984).

Detergent extraction and buffers. The membrane vesicle preparation Fraction D, with a protein concentration of 340 μg/ml and titre of $10^{8 \cdot 2}$ LD_{50}/ml, was dialysed and sonicated before extraction. Two non-ionic detergents, Triton X-100 (Sigma) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio] 1-propane sulphonate; Polysciences, Warrington, Pa., U.S.A.), or the ionic detergent *N*-lauroylsarcosine (Sigma) were used at a 1% (w/v) concentration with incubation at 4 °C for 1 h. Samples were then clarified at 1500 *g* for 30 min before fractionation. Some samples were treated with 50 μg/ml proteinase K at room temperature for 30 min followed by the addition of 0·1 mM-phenylmethylsulphonyl fluoride (PMSF) before detergent extraction. Three salt concentrations were used for extraction and to dissolve the Nycodenz gradient medium: hypotonic (2 mM-$MgCl_2$), isotonic (100 mM-NaCl, 10 mM-$MgCl_2$), and hypertonic (2 M-NaCl, 10 mM-$MgCl_2$). Each solution also contained 10 mM-Tris and 1 mM-dithiothreitol, and was adjusted to pH 7·8 using glacial acetic acid. Two mM-EDTA replaced $MgCl_2$ in samples and gradients containing sarcosine.

Gradients, centrifugation and fractionation. Two types of gradients were used for these studies. The first was an 8 ml preformed gradient of 30 to 60% Nycodenz (Accurate Chemical Corp., Westbury, N.Y., U.S.A.). The second was a 6 ml 10 to 30% or 40% Nycodenz gradient layered onto a 1 ml 50% Nycodenz cushion. The gradients, in Beckman Ultra-Clear® tubes (no. 344059, 12 ml), were centrifuged at 100000 *g* for 16 h at 4 °C in a Beckman SW41 rotor. Centrifuged gradients were carefully examined for visible bands before collection of 1·5 ml fractions from the bottom of the tube and dialysis against isotonic buffer at 4 °C for 48 h.

Protein analysis. All dialysed fractions were analysed for protein concentration using the dye binding method of Bradford (1976), and by protein gel electrophoresis. Samples requiring concentration were dialysed against deionized water, then dried in a Savant Speed Vac Concentrator®. Samples containing 4 to 6 μg of protein were heated to 100 °C for 5 min in 0·3 M-Tris–HCl pH 6·8, 1% SDS, 3 M-urea, 0·5% 2-mercaptoethanol and 1 mM-EDTA, then separated in discontinuous 16% polyacrylamide gels run according to the method of Laemmli (1970). Gels were silver-stained (Wray *et al.*, 1981) after soaking for 24 h in 50% methanol, then in water for 2 h followed by 50% methanol for 1·5 h.

RESULTS

Effect of Nycodenz on the agent and on the bioassay

To test the effect of Nycodenz on the scrapie agent and on the bioassay procedure, a sample of Fraction D prepared from scrapie-infected brain tissue was divided and half treated with Nycodenz to a final concentration of 50% (w/v). The treated and untreated samples were placed at 4 °C for 16 h then dialysed against isotonic buffer for 48 h at 4 °C. Both of these samples were then bioassayed with and without the addition of 20% Nycodenz to the inoculum. No differences were found in the length of incubation periods produced by any of the inocula, indicating that Nycodenz does not have an effect on the agent or on the bioassay procedure which can be detected by the method of incubation interval assay.

30 to 60% gradients

Table 1 shows the distribution of scrapie infectivity in an 8 ml 30 to 60% gradient separating 4 ml of Fraction D before and after extraction with 1% Triton X-100 in isotonic buffer. Detergent extraction resulted in a substantial increase of infectivity recoverable lower in the gradient at a density of 1·28 g/ml. No infectivity was found in either sample at a density higher than 1·30 g/ml.

10 to 40% gradients without urea

Since separation on 30 to 60% gradients indicated that infectivity did not penetrate beyond a density of 1·30 g/ml, gradients were prepared composed of 6 ml of continuous 10 to 40% Nycodenz layered onto a 1 ml 50% cushion. Five ml of sample was applied to each gradient. Bioassay of gradient fractions of Fraction D treated with various detergents in buffers of different ionic strength invariably resulted in the highest infectivity being present in a visible band at the 40/50% interface. The amount of infectivity and protein at the interface varied depending on the buffer used and the presence or absence of detergent in the gradient (Table 2). The appearance of the interface bands varied from very granular in samples extracted in low salt, to quite faint and opalescent in gradients containing sarcosine. Pretreatment of samples with proteinase K before extraction resulted in a diminution or loss of the visible band accompanied by a 95 to 98% loss in protein concentration and, usually, a five- to tenfold drop in infectivity titre.

Table 1. *Separation of scrapie infectivity on 30 to 60% Nycodenz gradients before and after extraction with 1% Triton X-100*

Sample	Fraction	Density (g/ml)	Protein concn. (µg/ml)	$\log_{10}$ LD_{50}/ml*
Before extraction	1	1·340	<1·0	<1·8
	2	1·308	<1·0	<1·8
	3	1·282	<1·0	<1·8
	4	1·238	248	7·8
	5	1·212	340	8·5
After extraction	1	1·352	<1·0	<1·8
	2	1·315	<1·0	<1·8
	3	1·280	86	7·6
	4	1·242	140	8·2
	5	1·210	187	7·0

* As calculated by the method of incubation interval assay.

Table 2. *Effect of various detergents and salt concentrations on scrapie infectivity at the 40/50% Nycodenz interface after centrifugation*

Detergent (1%) in sample	Detergent (1%) in gradient	Buffer*	$\log_{10}$ LD_{50}/ml†	Protein concn. (µg/ml)
Triton X-100	None	Hypotonic	7·8	109
		Isotonic	8·0	91
		Hypertonic	8·5	43
Triton X-100	Sarcosine	Isotonic	7·5	28
Triton X-100	CHAPS	Isotonic	8·6	89
Sarcosine	None	Isotonic	8·0	36
		Hypertonic	8·4	27
Sarcosine	Sarcosine	Isotonic	7·8	12
CHAPS	None	Isotonic	8·2	247
CHAPS	CHAPS	Isotonic	8·0	102

* Samples extracted and centrifuged in either hypotonic (2 mM-$MgCl_2$), isotonic (100 mM-NaCl, 10 mM-$MgCl_2$), or hypertonic (2 M-NaCl, 10 mM-$MgCl_2$) buffers. Two mM-EDTA replaced $MgCl_2$ in samples and gradients containing sarcosine.

† As calculated by the method of incubation interval assay.

10 to 30% gradients containing urea

Fractionation of Fraction D after detergent extraction and separation in 6 ml 10 to 30% Nycodenz gradients containing various concentrations of urea resulted in the same finding as before. The highest level of infectivity was at the 30/50% interface. Concentrations of urea above 2 M inactivated infectivity relative to molarity (Table 3). However, there was one apparent discrepancy in this observation. Whereas centrifugation directly into 8 M-urea reduced infectivity a hundredfold at the interface, centrifugation into a linear 2 M to 8 M gradient produced no inactivation. This experiment was repeated two additional times, each resulting in infectivity titres at the 30/50% interface of 10^8 LD_{50}/ml when measured by the method of incubation interval assay.

To examine this observation further, similar samples were sonicated, then centrifuged in an Eppendorf centrifuge 5412 for 1 min (12800 ***g***). The supernatants were carefully removed and the pellets resuspended to 10% (w/v) in isotonic buffer. Each sample was sonicated, then serially diluted tenfold for bioassay. The results were, for the supernatants, $10^{5·6}$ and $10^{6·0}$ LD_{50}/ml by the incubation interval assay method, and $10^{5·3}$ and $10^{5·0}$ LD_{50}/ml by endpoint dilution. For the pellets, the results were $10^{8·2}$ and $10^{8·0}$ LD_{50}/ml by the method of incubation interval assay, and $10^{5·0}$ and $10^{5·0}$ LD_{50}/ml by endpoint dilution.

Table 3. *Effect of centrifugation in various concentrations of urea on detergent-extracted scrapie infectivity at the 30/50% Nycodenz interface*

Detergent (1%) in sample	Concn. of urea in gradient (M)	Buffer*	$\log_{10}$ LD_{50}/ml†	Protein concn. (µg/ml)
Triton X-100	0·5	Hypotonic	8·2	128
	2		8·4	136
	4		7·5	94
	6		6·0	88
Triton X-100	2	Isotonic	8·6	120
	4		7·0	144
	6		5·8	160
	8		6·2	117
Triton X-100	2 to 8	Isotonic	8·0	122
Triton X-100	2	Hypertonic	8·5	51
	4		7·0	27
Sarcosine	2	Isotonic	8·0	34
	4		7·4	38
	6		6·5	47
	8		6·2	56

* Samples extracted and centrifuged in either hypotonic (2 mM-$MgCl_2$), isotonic (100 mM-NaCl, 10 mM-$MgCl_2$), or hypertonic (2 M-NaCl, 10 mM-$MgCl_2$) buffers. Two mM-EDTA replaced $MgCl_2$ in samples extracted with sarcosine.

† As calculated by the method of incubation interval assay.

Gel electrophoresis

In addition to the gradients of scrapie infectivity reported above, comparable gradients of Fraction D prepared from healthy hamster brains were run in parallel in every instance. These gradients were fractionated and dialysed identically to those containing scrapie infectivity. Proteins from all fractions of every gradient were characterized by gel electrophoresis in 16% polyacrylamide gels stained with siliver. No differences were observed in protein profiles of paired fractions from healthy or scrapie-infected gradients. Furthermore, treatment of all interface samples with 50 µg/ml of proteinase K at room temperature for 30 min followed by the addition of 0·1 mM-PMSF before gel electrophoresis showed no differences in the susceptibility of proteins from healthy or scrapie-infected preparations to proteolytic digestion.

DISCUSSION

These experiments further characterize scrapie infectivity present in a fraction of a sonicated, plasma membrane-enriched preparation from hamster brain which is composed predominantly of large membrane vesicles (Marsh *et al.*, 1984). This fraction (Fraction D) was prepared by rate-zonal centrifugation in 10 to 25% Nycodenz at 100000 g for 90 min. Re-centrifugation of Fraction D for 16 h in 30 to 60% Nycodenz resulted in infectivity remaining at the lower densities at the top of the gradient, indicating that longer centrifugation does not appreciably effect sedimentation. Detergent extraction followed by separation in 30 to 60% gradients reduced the infectivity at the top of the gradient, a finding consistent with the integral association of the agent with a membrane complex. If the scrapie agent were a conventional virus merely associating with cell membrane, sonication and/or detergent extraction would be expected to release infectivity which would then sediment independently of membrane micelles. Measles virus (enveloped) and poliovirus (non-enveloped) have equilibrium densities in Nycodenz of 1·18 and 1·31, respectively (Vanden Berghe, 1983). Scrapie infectivity invariably co-sedimented with the bulk of the membrane residue remaining after detergent extraction.

The most important observation made in these studies was the failure to solubilize a significant amount (> 1%) of scrapie infectivity using different combinations of ionic and non-ionic detergents. This result would not be unexpected for conventional viruses where optimal infectivity is dependent on a complex composed of surface proteins for recognition and binding to susceptible cells, and nucleoproteins for initiation of viral replication. However, this finding does have implications for studies characterizing unconventional viruses which recent speculations have claimed are infectious proteins (Prusiner, 1982), or associated with fibrillar structures (Merz *et al.*, 1981), or are infectious complexes of protein, lipid and nucleic acid associated with cell membrane (Marsh *et al.*, 1984).

The results of these experiments are compatible with the scrapie agent having properties of a fibrillar structure similar to those which make up the cytoskeleton, operationally defined as the insoluble residue remaining after extraction of cell membrane preparations with non-ionic detergents (Sheetz, 1979). Although we have not yet examined the ultrastructure of the insoluble material at the 40/50% Nycodenz interfaces, it is surely enriched for microtubules, intermediate filaments and microfilaments. However, the cytoskeleton is not the only insoluble material associated with cell membrane. Many enzyme complexes and membrane-bound receptors remain insoluble after similar detergent treatment (Helenius & Simons, 1975; Hjelmeland & Chrambach, 1984). Therefore, these studies fail to differentiate the possibility of the scrapie agent being a submembranous cytoskeleton-like structure, or an integral membrane complex.

Protein profiles of all gradient fractions were examined by one-dimensional gel electrophoresis. No differences were seen in proteins present in paired healthy and scrapie-infected fractions. Two studies have reported the presence of 26000 to 30000 molecular weight proteins in purified preparations of scrapie brain which are not detectable in samples from similarly treated healthy hamster brain (Bolton *et al.*, 1982; Diringer *et al.*, 1983). The reason for these conflicting results may reside in the different processing procedures used. Both studies reporting unique scrapie proteins begin by extracting relatively crude brain homogenates and rely on proteolytic digestion to reduce protein concentration in the final preparation, while our studies use physical methods to subfractionate a defined population of scrapie infectivity enriched for membrane vesicles.

Finally, one of the most limiting aspects of scrapie research is the need to measure the transmissible agent by bioassay. Not only is this method time-consuming and expensive, it is inaccurate. Even quantification of infectivity by endpoint dilution results in standard errors of $\pm 0{\cdot}5\ \log_{10}\ LD_{50}$/ml, making it impossible to reliably detect less than a 90% change in titre (R. F. Marsh, unpublished results). The finding that the concentration of scrapie infectivity in an inoculum was directly related to the length of the incubation period in intracerebrally inoculated hamsters (Marsh & Hanson, 1978) has been used to calculate infectivity titres by a quantitative method of incubation interval assay (Prusiner *et al.*, 1981). This method uses far fewer animals and, until recently, has been thought to give values comparable to endpoint dilution. However, it has been reported that the methed of incubation interval assay underestimates the titre of inocula containing detergent (Lax *et al.*, 1983), and the results of the present studies suggest that this method may significantly overestimate the titre of samples containing large aggregates. Classically, viral aggregates cause an underestimation of titre through several particles infecting a single cell. While it is only possible to speculate on why scrapie aggregates produce short incubation periods, the host may be able to dissociate the material into single units capable of infecting individual cells. Alternatively, infection of a single cell with multiple infectious units (e.g. an aggregate) may accelerate the agent's replication.

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