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Properties of the Scrapie Agent-Endomembrane Complex from Hamster Brain

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Subcellular fractionation of scrapie-infected hamster brain indicated the association of the scrapie agent with a component of the endomembrane system. Characterization by equilibrium density gradient centrifugation, electron microscopy, and marker enzymes suggested a primary association with rough and smooth endoplasmic reticulum and a possible incorporation into the plasma membrane. DNA polymerase activity demonstrated a direct correlation with regions of scrapie activity from the gradient fractions. A scrapie-related product was detected after [^{3}H]TMP incorporation and analysis on 2.2% polyacrylamide gels. Analysis of nucleic acid species extracted from subcellular fractions resulted in a greater quantity from healthy brain; however, no qualitative distinctions were detected.

The transmissible agent of scrapie disease of sheep has unusual properties of thermal stability (8) and a small radiation target size of 2×10^5 daltons (2). Similar characteristics have also been reported for "viroid" agents producing infection of plants (5, 18-20). No nucleic acid species analogous to the low-molecular-weight pathogenic RNA, characterized as the causal agent of viroid diseases (21), has been isolated from scrapie-infected tissue or any other animal system (16). Nevertheless, high titers of biologically active scrapie agent can be demonstrated in membrane preparations (17). The association of the citrus exocortis viroid with a component of the endomembrane system (J. S. Semancik, D. Tsuruda, L. Zaner, J. L. M. C. Geelen, and L. G. Weathers, Virology, in press) further extended the comparative features of these diseases. The characterization of the scrapie agent-membrane complex from hamster brain, employing procedures comparable to those utilized in viroid characterization, is the subject of this report.

MATERIALS AND METHODS

Scrapie agents. The scrapie agent used in these studies originated from the Chandler strain of mouse-adapted scrapie as described previously (11). Fractionation experiments were performed on pools (5 g each) of fresh brain tissue representing the agent's 4th and 5th passages in outbred (LVG:LAK) Syrian hamsters with intermittent passages in inbred hamsters (LHC/LAK), both obtained from the Lakeview Hamster Colony, Newfield, N.J. Additional pools, containing 50 g each of fresh brain from the 6th scrapie passage or from normal animals, were used for fractionation followed by nucleic acid extraction.

Extraction and subcellular fractionation procedure. Hamsters, 4 months old, either uninoculated or showing early signs of disease, were killed with chloroform, and their brains were rapidly removed and placed on wet ice. Healthy or scrapie-infected brains (2.5 g) were quartered and immediately homogenized on ice (Polytron blender, 15 s, no. 4 setting) in 10 to 15 ml of a medium composed of 0.4 M sucrose, 0.01 M tricine (pH 7.5), 0.002 M $MgCl_2$, 0.02 M KCl, 0.1% bovine serum albumin, 0.001 M dithiothreitol, and 0.04 M mercaptoethanol. The extracts were pooled and centrifuged at $250 \times g$ for 10 min, and the pellet was discarded. The 250-g supernatant was sequentially centrifuged at $1,000 \times g$ (fraction 1 pellet) and $13,000 \times g$ (fraction 2 pellet) in a Sorvall RC-2B for 10 min each. The $13,000 \times g$ supernatant was then centrifuged at $80,000 \times g$ for 30 min (fraction 3 pellet) in a Beckman SW27 rotor. Finally, the supernatant was centrifuged at $100,000 \times g$ for 2 h (fraction 4 pellet) in a Beckman 35 rotor. The high-speed supernatant constituted fraction 5. All pellets were suspended by three strokes in a Vitro Teflon homogenizer in a medium containing 15% (wt/wt) sucrose, 0.01 M tricine (pH 7.5), 0.002 M $MgCl_2$, 0.02 M KCl, and 0.001 M dithiothreitol.

Density gradient centrifugation. Sucrose solutions were prepared in the resuspension buffer. Samples (4 to 6 ml) were layered over a linear (25 to 40%, wt/wt) sucrose gradient over 4 ml of 50% (wt/wt) and 2 ml of 60% (wt/wt) sucrose and then centrifuged for 21 h at 25,000 rpm in a Beckman SW27 rotor at 4 C. Fractions (1 to 2 ml) were collected by needle-puncturing the bottom of the tube, and the density of the consecutive samples was determined with an Abbé refractometer. Aliquots of these samples were employed for the enzyme assay, analytical analysis, and scrapie bioassay.

Analytical and enzymatic determination. RNA, DNA, and protein were quantitated by the orcinol, diphenylamine, and Folin tests, respectively (22). The plasma membrane (PM) marker enzyme (ATPase) and endoplasmic reticulum (ER) marker enzyme (reduced nicotinamide adenine dinucleotide [NADH] cytochrome *c* reductase) were determined by the method of Hodges and Leonard (9). DNA and RNA polymerase activities were monitored by [^{3}H]TMP and [^{3}H]UMP (J. L. M. C. Geelen, L. G. Weathers, and J. S. Semancik, Virology, in press) incorporation.

Nucleic acid extraction and gel electrophoresis. Nucleic acid preparations were extracted and partitioned as reported previously (17) and analyzed on 5 and 2.2% gels with 0.5% agarose (3). Gels were scanned at 260 nm in a Beckman Acta II spectrophotometer.

Bioassay of scrapie infectivity. Coded fractions were tested for scrapie infectivity by intracerebral inoculation (0.05 ml) of weanling outbred hamsters. Four animals were inoculated with each dilution, beginning at 10^{-5}, and end points were calculated at the end of 15 weeks by the method of Spearman and Kärber (7).

Electron microscopy. Gradient samples of subcellular fractions were pelleted and immediately placed in 2% glutaraldehyde in 0.01 M potassium phosphate buffer, pH 7.2. After a 2-h fixation, the pellets were rinsed well with buffer and postfixed in 1% buffered osmium tetroxide for 90 min. After several buffer and water rinses, specimens were dehydrated through an acetone series and embedded in Epon (14). Thin sections were stained with uranyl acetate and lead citrate. Specimens were viewed and photographed with a Hitachi HU-11 microscope.

RESULTS

Subcellular fractionation of hamster brain. A differential centrifugation sequence similar to that employed for detecting the association of the citrus exocortis viroid with a component of the endomembrane system (Semancik et al., in press) was utilized to process polytron homogenized brain tissue. After the 250 × *g* (10 min) crude pellet was discarded, the series of fractions was taken, as presented in Table 1, and assayed for scrapie activity (SA). The SA detected in the three fractions collected between 250 and 80,000 × *g*, containing the bulk of the endomembrane system, was usually 1 log greater than the final high-speed pellet or supernatant. When subjected to equilibrium centrifugation in a 25 to 60% (wt/wt) sucrose density gradient, characteristic light-scattering patterns were observed in the three fractions, indicating the presence of a PM-like zone (37%) in the 1,000 × *g* pellet, a heavy mitochondria zone (39%) in the 13,000 × *g* pellet, and a more diffuse ER-like zone at lower densities (30 to 34%) in the 80,000 × *g* pellet. The latter two fractions also displayed more faint zones in the PM region. Since the levels of SA in the three fractions were comparable, yet the 13,000 × *g* pellet was greatly enriched in mitochondria, we chose to focus on the characterization of the scrapie-associated component in the 1,000 and 80,000 × *g* pellets, thus using the most distinct subcellular membrane fractions not contaminated with excessive levels of mitochondria.

TABLE 1. *Association of SA with subcellular fraction from infected hamster brain.*

Pellets	Total recoverable SA[a]		
	Expt 1	Expt 2	Expt 3
(1) 1,000 × *g*, 10 min	$10^{9.25}$	$10^{8.75}$	10^{9}
(2) 13,000 × *g*, 10 min	10^{9}	$10^{8.75}$	10^{9}
(3) 80,000 × *g*, 30 min	$10^{9.25}$	$10^{8.75}$	10^{9}
(4) 100,000 × *g*, 2 h	$10^{8.75}$	$10^{8.25}$	$10^{8.75}$
(5) Supernatant	$10^{7.75}$	$10^{8.25}$	10^{8}

[a] Mean lethal dose per total volume of the resuspended pellet.

Characterization of membrane components of subcellular fractions. Subcellular fractions of the 250 to 1,000 × *g* and 13,000 to 80,000 × *g* pellet, designated H-1 and H-3, respectively, were prepared from Polytron-homogenized, healthy hamster brains. After equilibrium sedimentation in a 25 to 60% (wt/wt) sucrose gradient, samples were collected and analyzed for density (Fig. 1A) protein concentration (Fig. 1B), NADH cytochrome *c* reductase activity (Fig. 1C) as a mitochondria and ER marker, ATPase activity (Fig. 1D) as a PM marker, and [^{3}H]TMP incorporation into an acid-insoluble product as an indication of DNA polymerase activity (Fig. 1E). Since these experiments were designed to localize the fractions in the gradient enriched in either ER or PM, the marker enzyme activities are presented as values relative to other gradient samples. The [^{3}H]UMP incorporated across gradient samples was insufficient to serve as a marker for RNA polymerase activity.

The high level of cytochrome *c* reductase activity (Fig. 1C) in sample 4 (~42%) from both the H-1 and H-3 preparations indicates that residual mitochondria, as well as aggregated debris, collect on the high-density (~55%) cushion. The second peak of cytochrome *c* reductase activity at ~37% in the H-1 gradient could represent either larger sheets of rough ER, since Mg^{2+} is present in the gradient, or mitochondrial membrane fragments. The area of reductase activity at 30 to 35% in the H-3 gradient is compatible with the density properties of smooth ER.

The prominent ATPase activity (Fig. 1D) at 37% in the H-1 gradient, indicating the proba-

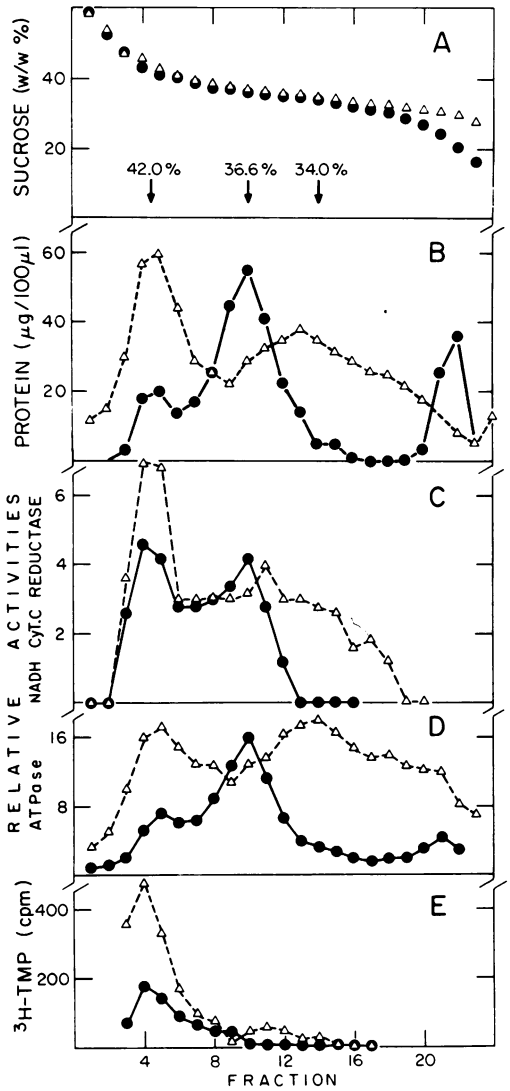


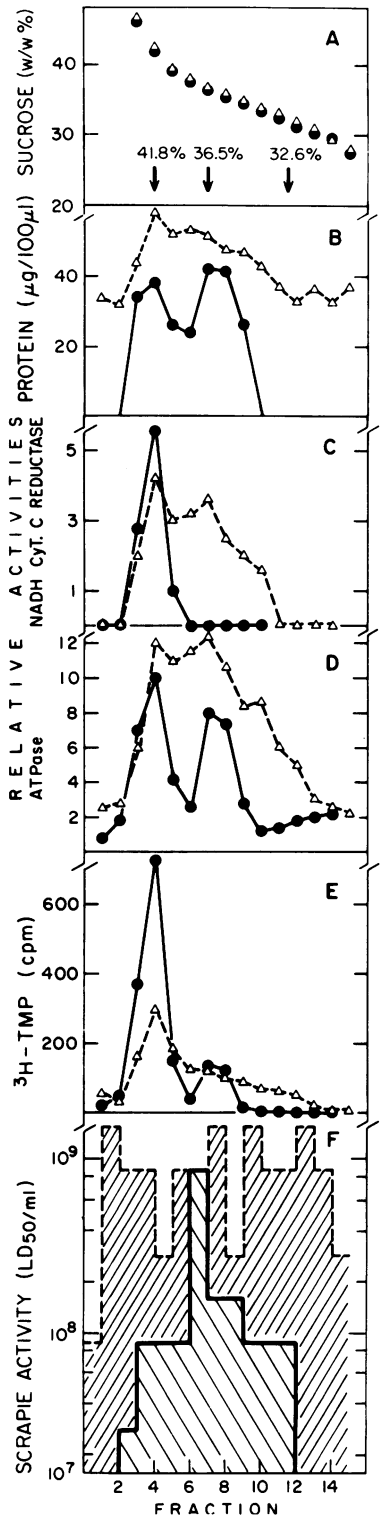
FIG. 1. *Distribution of protein (B), NADH cytochrome c reductase (C), ATPase (D), and [³H]TMP incorporated (E) by gradient (A) samples after equilibrium sedimentation (21 h at 25,000 rpm) of a 1,000 × g, 10-min (H-1) pellet (——) and an 80,000 × g, 30-min (H-3) pellet (-----) from healthy hamster brain.*

ble sedimentation position of PM, demonstrates that the 250 to 1,000 × *g* pellet comprises a PM-rich fraction. This suggests that the PM-rich zone of the H-1 preparation might display an equilibrium density comparable with a portion of the rough ER (Fig. 1C). Other regions of ATPase activity at 42% and the disperse 30 to 35% region probably correspond to mitochondria-rich and smooth ER-rich regions, demonstrating nonspecific phosphatase activities. Incorporation of [³H]TMP is principally confined to the mitochondria region (Fig. 1E) and is in good agreement with the density indicated by the cytochrome *c* reductase and ATPase levels.

The equilibrium sedimentations of the 250 to 1,000 × *g* pellet (S-1) and 13,000 to 80,000 × *g* pellet (S-3) from scrapie-infected brains present similar distributions of subcellular components (Fig. 2). Mitochondria and aggregated debris are indicated at about 42%, a PM-rich region is indicated at about 36.5% as noted by the high ATPase activity (Fig. 2D), and an ER-rich region is indicated at about 30 to 35% as noted by the diffuse cytochrome *c* reductase activity (Fig. 2C). The [³H]TMP incorporation profile of the S-1 preparation is distinct from the healthy profile, with a minor peak of activity localized at about 36 to 37%, corresponding to the PM-rich region described by the ATPase activity in Fig. 2D. This putative DNA polymerase activity might result from mitochondrial fragments; however, no corresponding cytochrome *c* reductase activity was detected (Fig. 2C).

The distribution of SA in the S-1 preparation demonstrates coincidence with the peak ATPase and [³H]TMP activity at 37%, suggesting the possible association of a PM-like constituent with DNA polymerase activity. A similar region of SA is observed in the S-3 preparation; however, significant levels of SA are also displayed in the 42% and 30 to 35% regions. Therefore, the DNA polymerase activity does not totally represent scrapie-specific activity. Since the S-3 pellet did not resuspend with homogeneous consistency, the activity at 42% may reflect aggregated debris. Nevertheless, the membranes sedimenting at 30 to 35% appear to be well dispersed and presumably represent a smooth ER-rich region. Since it is difficult to envision such a high concentration of PM-like membranes at 30 to 35% after equilibrium sedimentation for 21 h, the data suggest an association of the scrapie agent with smooth ER at 30 to 35%. It might follow then that the SA at the center of the gradient (37%) could be related to the presence of rough ER.

Evidence for the PM-rich 1,000 × *g* pellet and ER-rich 80,000 × *g* pellet, as well as the cosedimentation of PM and rough ER membrane, is provided by electron microscope examination of fractions 6 through 10 from the S-1 and S-3 gradients (Fig. 3). It can be observed that this ATPase-rich region of the S-1 contains large PM-like vesicles (Fig. 3A). Nevertheless, even though no cytochrome *c* reductase activity was detected in those samples, a trace amount of rough ER-like vesicles could be observed. The presence of mitochondrial fragments might explain the [³H]TMP incorporation into this frac-



tion. Conversely, examination of the S-3 preparation (Fig. 3B) indicates smaller vesicles, some identifiable as rough ER, with a minor quantity of large PM-like vesicles.

Assuming that the scrapie agent might be comprised, in part, of a nucleic acid component, the detection of the [^{3}H]TMP incorporation coinciding with SA lends credence to the possible involvement of nucleic acid metabolism in the synthesis of a scrapie agent-membrane complex or the pathogenic expression of the scrapie disease. A similar interaction was suggested previously for the pathogenic (viroid) RNA of exocortis disease (Semancik et al., in press). With these considerations, a 13,000 to 80,000 × *g* resuspended pellet was subjected to equilibrium sedimentation in a lighter gradient (~20 to 40%) as indicated in Fig. 4A. In this way, an attempt was made to pellet the heavy scrapie-containing fraction (42%) while achieving a better definition of the two lighter regions, ~37% and 30 to 35% (Fig. 4E). When the gradient samples were assayed for [^{3}H]UMP (Fig. 4C) and [^{3}H]TMP (Fig. 4D) incorporation, distinct profiles emerged, suggesting that contamination by nuclear fragments was probably not prevalent. The distribution of [^{3}H]TMP incorporation coincided with SA, possibly implicating a DNA-related phase in scrapie pathogenesis.

To test for the presence of a scrapie-specific product, gradient fractions from healthy and infected brains were allowed to incubate overnight in [^{3}H]TMP followed by nucleic acid isolation (by phenol extraction). When analyzed by electrophoresis on 2.2% polyacrylamide gels, the incorporation product could be detected principally in high-molecular-weight DNA from both healthy and scrapie-infected preparations (Fig. 5). However, a very low, reproducible profile of incorporation into low-molecular-weight DNA could be detected in both the S-1 and S-3 preparation but not in the comparable fraction from healthy tissue.

Nucleic acid species recovered from subcellular fractions. Quantitative and qualitative determinations of nucleic acid species extracted from subcellular fractions were undertaken. About 50 brains each from healthy and scrapie-infected hamsters were Polytron homogenized

FIG. 2. *Distribution of protein (B), NADH cytochrome c reductase (C), ATPase (D), [^{3}H]TMP incorporation (E), and SA (F) by gradient (A) samples after equilibrium sedimentation (21 h at 25,000 rpm) of a 1,000 × g, 10-min (S-1) pellet (——) and an 80,000 × g, 30-min (S-3) pellet (-----) from scrapie-infected hamster brain.*

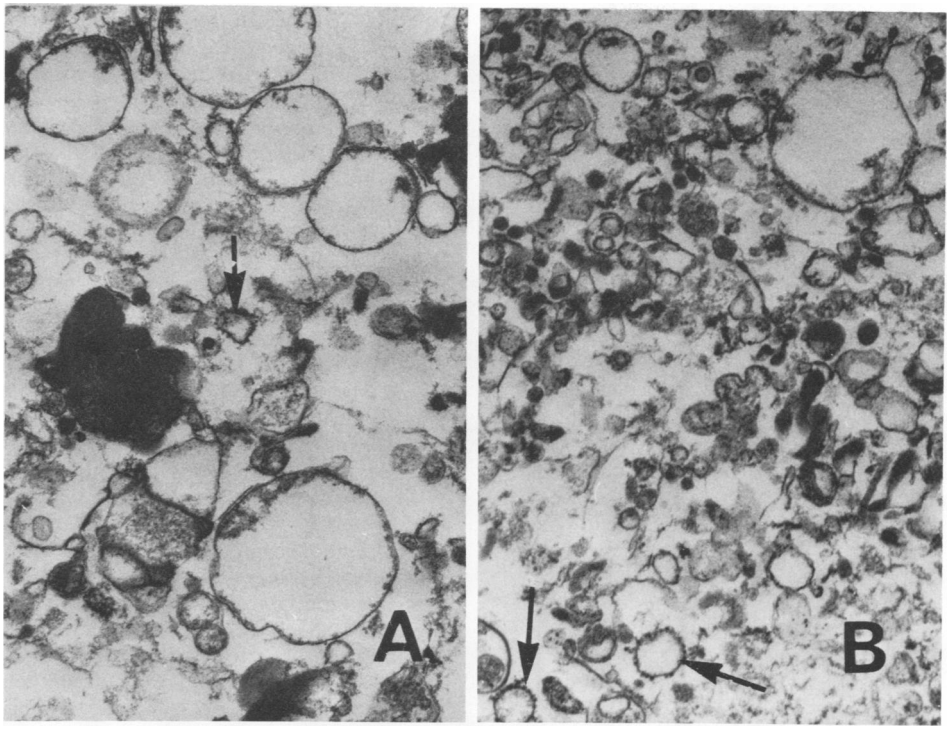


FIG. 3. *Electron micrographs of gradient fractions (7 through 9) of a 1,000 × g, 10-min pellet (A) and of gradient fractions (6 through 10) of an 80,000 × g, 30-min preparation (B) from scrapie-infected hamster brain (see Fig. 2). Arrows indicate rough ER vesicles. ×39,000.*

and fractionated as described above, followed by nucleic acid isolation by phenol extraction. Ethanol-precipitable nucleic acids were partitioned by 2 M LiCl precipitation of ribosomal-like RNA and recovery of high-molecular-weight DNA after addition of 3 volumes of ethanol to the 2 M LiCl supernatant. Determinations of DNA and RNA by diphenylamine and orcinol tests were made on each of the nucleic acid fractions.

A significantly higher level of extractable nucleic acids was detected in all subcellular fractions from healthy, as compared with scrapie-infected, brain tissue. This may reflect the widespread cellular degeneration associated with the scrapie disease process. The distribution of nucleic acid species reflects the distinct quality of the subcellular fractions. The 1,000 and 13,000 × *g* pellets contain relatively high concentrations of DNA (50 and 35%), presumably resulting from nuclei and mitochondria, whereas the 80,000 and 100,000 × *g* pellets are rich (80 and 95%) in RNA, reflecting the presence of membrane-bound and free ribosomes, respectively. When aliquots from healthy and scrapie-infected preparations were analyzed by electrophoresis on 2.2 or 5% polyacrylamide gels, no differences in the quality of the various RNA species were evident.

DISCUSSION

Previous attempts to describe an infectious scrapie nucleic acid species, analogous to the pathogenic (viroid) RNA, have not been successful (16, 23). Renewed interest in the comparative pathology of scrapie disease with viroid infections in plants was stimulated by the finding of a membrane-related phase in exocortis disease (Semancik et al., in press) and the observation of a cytopathic effect on the endomembrane system (Semancik and Vanderwoude, Virology, in press). Therefore, procedures employed in the characterization of the exocortis disease agent have been adapted for use in examining the scrapie agent membrane complex and possibly a putative nucleic acid component of the scrapie agent. These studies have been facilitated by the use of the hamster-scrapie model, providing the highest titers of scrapie infectivity of any known bioassay system (15).

The concentration of SA in the subcellular

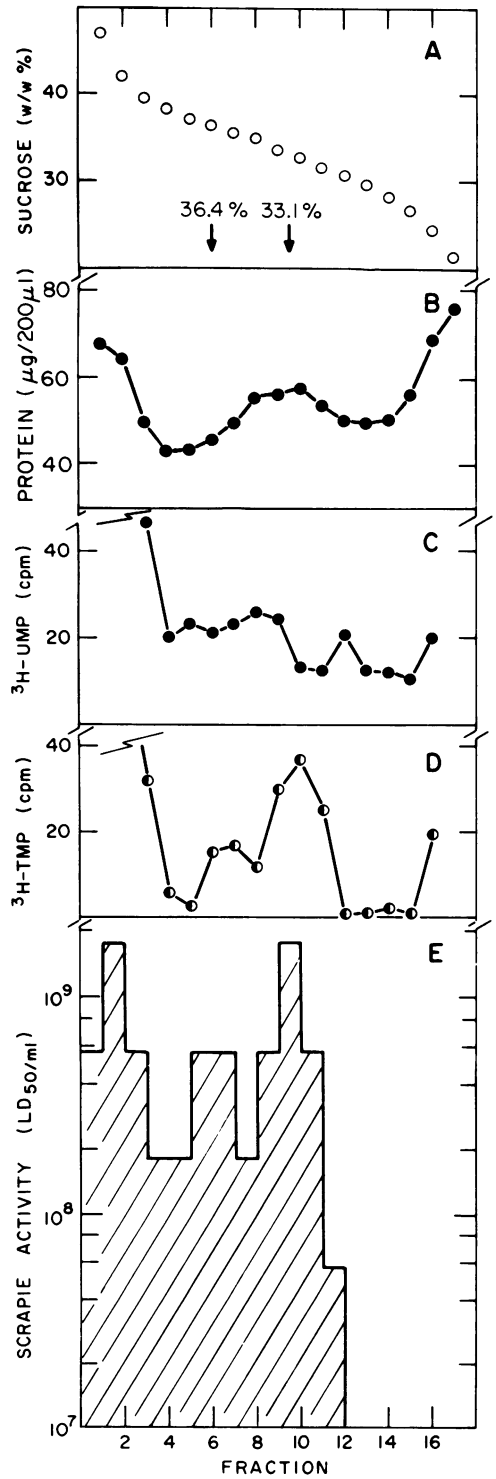


FIG. 4. *Distribution of protein (B), RNA polymerase activity as indicated by [^{3}H]UMP incorporation (C), DNA polymerase activity as indicated by [^{3}H]TMP incorporation (D), and SA (E) by gradient (A) samples after equilibrium sedimentation (21 h at 25,000 rpm) of an 80,000 × g, 30-min (S-3) pellet from scrapie-infected hamster brain.*

fractions pelleted in the 250 to 80,000 × *g* range generally supports the scrapie-membrane hypothesis (8), i.e., the possible association of a small pathogenic nucleic acid, as deduced from the target size of ~10^5 daltons (2), with a membrane component. The primary association with the PM (17) cannot be fully reconciled with the present data. Samples taken from equilibrium density gradients, even after prefractionation of subcellular components, are not sufficiently homogeneous to ascribe SA to a single membrane species. The present data indicate that portions of the rough ER cosediment with the PM-rich regions. Therefore, the preferred explanation of the sedimentation heterogeneity of the scrapie agent in 13,000 to 80,000 × *g* (S-3) pellets suggests an association with rough ER at about a density of 1.17 g/cm^3 and with smooth ER at a density of 1.13 to 1.15 g/cm^3. Alternatively, the density heterogeneity of the SA complex may result from the specialized nature of the brain tissue, since the synaptic vesicles and external synaptic membranes demonstrate characteristically different sedimentation properties (24). Also, the association of the presumptive nucleic acid component in the scrapie-membrane complex may result in an alteration in the density of normal host membranes.

When considering the high specific infectivity of the scrapie agent in the absence of any recognizable agent, one must be extremely cautious in ascribing a specific association to a single component of the endomembrane system. Furthermore, if the agent becomes associated with the endomembrane system at early developmental periods, it may be possible for the agent to retain biological activity during and after the process of membrane differentiation, thus resulting in some SA integrated not only with the ER but also with the PM. A further consideration centers on the association of the SA with some scrapie-directed de novo membrane structure or possible artifacts of subcellular fractionation which cosediments with either PM or ER but is not discernible as a distinct component of the membrane population.

The data are compatible with the hypothesis presented for the pathogenesis of the citrus exocortis viroid (Semancik et al., in press), in which both a nuclear (19) and an endomembrane phase have been postulated for the possi-

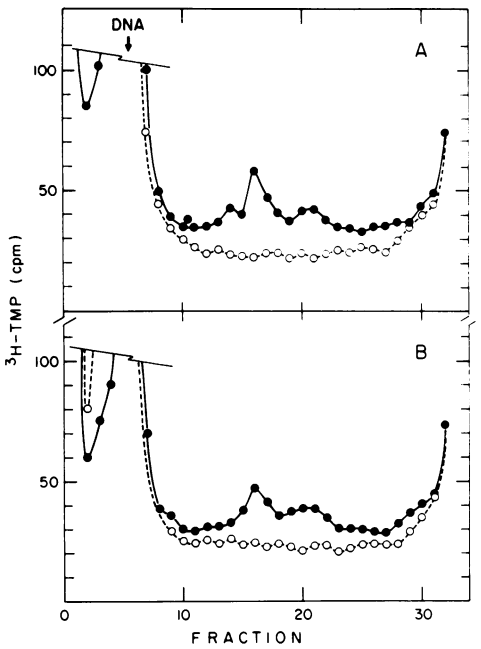


FIG. 5. *Electrophoresis of [^{3}H]TMP incorporation products of subcellular fractions 1,000 × g, 10 min (A) and 80,000 × g, 30 min (B) from healthy (○) and scrapie-infected (●) hamster brain in 2.2% polyacrylamide gels with 0.5% agarose after 4 h and 6 mA/gel. Migration was from left to right, with 3-mm slices treated in Omnifluor-toluene with 3% Protosol for 48 h at 40 C.*

ble site of synthesis and site of accumulation of the pathogenic RNA. The scrapie-membrane complex may reflect a synthetic end point or accumulation of the scrapie agent. Since we do not know the molecular nature of the scrapie agent, much less the template and pathway by which the agent is synthesized, the most cautious interpretation of the present data would be that the scrapie agent appears to be primarily associated with ER-like, and possibly some PM-like, membranes which may represent sites of accumulation of scrapie progeny.

The correlation of [^{3}H]TMP incorporation or DNA polymerase activity with SA can be taken only as a very preliminary indication that this site of accumulation may also represent a site of some synthetic activity of the scrapie agent. A low-molecular-weight DNA species has also been reported in scrapie-infected mouse brain after the intracerebral inoculation of [^{3}H]thymidine (10). These data, together with the dynamics of [^{3}H]thymidine turnover in scrapie-infected mouse brain (1, 12) and the correlation between cell division and scrapie infectivity (4), are compatible with the suggestion of a DNA component in the scrapie agent.

The characteristics of scrapie disease and its transmissible agent present interesting parallels to the plant disease caused by the citrus exocortis viroid. The association of the pathogenic (viroid) RNA of the exocortis disease with the endomembrane system appears to result in a vesicular proliferation or disruption of PM (Semancik and Vanderwoude, in press). Since the overt pathology of scrapie disease also involves aberrations of the PM (13), these cellular phenomena, coupled with the estimates of molecular size and stability, reinforce the possible viroid nature of the scrapie agent (6). Comparative aspects of the two diseases project the possibility of a nucleic acid component in the scrapie agent, as well as a nuclear phase involved in the replication process. The hamster scrapie model presents an excellent system to exploit in the study of membrane-"viroid" interactions, even though a molecular description of the scrapie agent, fundamental to the understanding of the disease, has yet to be established.

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