PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 513–516 (1978)

Effect of Different Gradient Solutions on the Buoyant Density of Scrapie Infectivity (40236)

PAUL BROWN,[1] ERNEST M. GREEN,[2] AND D. CARLETON GAJDUSEK[1,3]

[1]*National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20014 and* [2]*New York City Public Health Research Institute, Otisville, New York 10963*

Previous attempts to characterize and purify scrapie virus have often included density gradient banding of infected mouse brain preparations, in which the density of maximum scrapie infectivity has varied from 1.14 g/ml in potassium tartrate to 1.35 g/ml in cesium chloride (1–6). In this study, we have simultaneously banded a single preparation of scrapie-infected mouse brain in three different gradients: cesium chloride, sucrose, and metrizamide, an iodinated benzamido-derivative of glucose, and have shown these solutions to have very little effect on the density of partially purified scrapie virus, with maximum infectivity confined to the region of 1.17–1.22 g/ml.

Materials and methods. Scrapie virus. Fourth mouse passage of a strain of scrapie isolated in mice by us in 1962 from an Illinois Suffolk ewe (C506) was used in the study. This is designated as the "American strain" of scrapie, in distinction to the Chandler, or Compton, strain isolated in England from a sheep and subsequently passaged in goats and mice, which has been used in all earlier studies both in England and America.

Preparation of scrapie mouse brain. Brains from 30 symptomatic NIH Swiss albino mice, inoculated 5 months earlier, were minced by hand through a fine-meshed sieve and diluted to a 10% suspension (130 ml) in phosphate-buffered saline (PBS) pH 7.4. This suspension was subjected to 900 psi nitrogen for 20 min at room temperature in a Parr Bomb, and the resulting creamy liquid centrifuged for 30 min at 3500*g*. The supernatant fluid (100 ml) was passed successively through 450 nm and 220 nm Millipore filters that had been prewetted with a 10% albumin solution to reduce nonspecific adsorption, and the clear filtrate (100 ml) then centrifuged at 152,000*g* for 17 hr at 4°. The pellet was resuspended in 20 ml, or one-fifth the input volume, of 0.01 *M* Tris buffer pH 7.4, sonicated in an ice bath for 1 min at 7.5 amperes and frozen at −70° until use the next day.

Density gradients. Sucrose was made up as a saturated solution (1.34 g/ml) and stored at 4° until diluted for the test. Cesium chloride was made up as a 1.60 g/ml solution and also stored at 4° until used. Metrizamide was freshly prepared on the day of the test, as it is unstable on storage. Tris buffer was used as the diluent for each gradient material.

New cellulose nitrate tubes (13 ml) were treated for 1 min with 1% silicone (Siliclad), air dried, and bacterially sterilized by ultraviolet irradiation before use. The three stock gradient materials were diluted to the following densities: sucrose, 1.34, 1.30, 1.25, 1.20, 1.15, and 1.10 g/ml; cesium chloride and metrizamide, 1.40, 1.30, 1.20, and 1.10 g/ml. Duplicate tubes for each gradient were prepared by successively adding 2 ml vol (sucrose) or 3 ml vol (cesium chloride and metrizamide) of each dilution to the tubes, to which were then added 0.5 ml vol of the pelleted and resuspended mouse brain preparation, and the tubes topped with Tris. The tubes were placed in an SW 40 rotor and centrifuged for 16 hr at 152,000*g* at 4° in a Beckman L2-65B ultracentrifuge. Sixteen 0.75 ml fractions were collected on ice from the bottom of each tube in a Beckman Universal Fractionator, and 0.2 ml aliquots immediately frozen at −70° for subsequent assay of infectivity. The remaining volumes were used for density determinations, protein assay, and examination by electron microscopy.

Density determinations. Refractive indices were measured on a Bausch & Lomb refractometer and densities calculated from standard conversion tables. For metrizamide a table was constructed using the equation $P = 3.350\,r - 3.462$ (7).

Protein measurements. The ultraviolet light

[3] Send all correspondence.

0037-9727/78/1584-0513$01.00/0

absorption of each fraction was measured at wavelengths of 260, 280, and 320 nm. Colorimetric assays were also made using the Amido-Schwarz method for protein (8). Readings were made in a Beckman DU-2 spectrophotometer, and standard conversion curves were constructed using crystalline bovine albumin.

Electron microscopy. Samples of each fraction were adsorbed for 5 min onto Formvar-coated 300-mesh grids, after which the grids were rinsed in distilled water and then stained with 2% phospho-tungstic acid pH 7.2. The grids were immediately examined under a Philips 201 electron microscope.

Infectivity assays. Aliquots of each fraction were diluted in 10-fold steps with PBS pH 7.4, and 0.03 ml was inoculated ic into 12–15 g weanling Swiss albino mice, using eight mice per dilution. Mice were observed for a period of 1 year, but mortality calculations were based on an 8-month observation period, since it was found by neuropathological screening that nonscrapie deaths vastly outnumbered scrapie deaths during the last 4 months.

Brains from all dead mice were examined to confirm the diagnosis of scrapie. Several coded ventral and caudal whole brain sections from each mouse were stained with hematoxylin and eosin, and the presence of spongiform change was noted. When present, vacuolation was found to be almost invariably widespread and intense, and mice with questionable or no vacuolation were excluded from LD_{50} titer calculations. The resulting titers are therefore somewhat lower, but more uniform, than histologically unconfirmed crude LD_{50} titers.

Results. The original 130 ml of 10% brain suspension titered $10^{6.2}LD_{50}$/0.03 ml, and contained a total of $10^{9.8}LD_{50}$ of scrapie activity and 754 mg protein. Final yields obtained for the regions of peak infectivity in each gradient were $10^{8.1}LD_{50}$ of scrapie and 58 mg protein in cesium chloride fractions 7–11; $10^{8.5}LD_{50}$ of scrapie and 64 mg protein in sucrose fractions 7–12; and $10^{8.6}LD_{50}$ of scrapie and 47 mg protein in metrizamide fractions 9–12. These peak regions contained 94–96% of the total gradient infectivity, and 2–6% of the infectivity present in the original brain suspension.

In Fig. 1 the gradients are presented in a composite arrangement showing fraction densities, infectivity, protein concentration, and a semiquantitative estimate of membrane content. Although some scrapie activity was detected in the lightest density fractions of all gradients, no activity occurred above a density of 1.33 g/ml in cesium chloride, and approximately 1.30 g/ml in sucrose and metrizamide. The major area of infectivity ranged from 1.17 to 1.27 g/ml, with peak values of 1.22 g/ml in cesium, 1.21 g/ml in sucrose, and 1.17 g/ml in metrizamide. Titers of the peak fractions were lowest in cesium chloride, intermediate in sucrose, and highest in metrizamide.

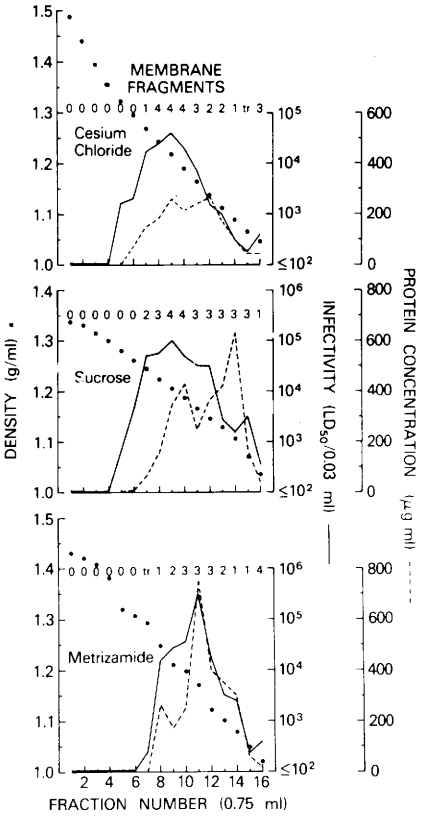

FIG. 1. Cesium chloride, sucrose, and metrizamide gradients of partially purified scrapie mouse brain showing density, infectivity, protein concentration, and membrane content of each gradient fraction.

In each gradient, two protein peaks were seen, of which one was associated with maximum infectivity. The content of membrane fragments was also maximal in the region of greatest infectivity, with an abrupt appearance at a density of about 1.25 g/ml, and

tapering gradually toward the lighter end of the gradients, except in cesium chloride and metrizamide, where the lightest fraction contained considerable floating membraneous material. Electron microscopy revealed the peak infectivity regions to contain a heterogeneous collection of membrane fragments and vesicles varying from 30 to 300 nm in size.

This concentration of membraneous particles and protein in the region of highest infectivity was further confirmed by the results of ultraviolet light spectrophotometry (Fig. 2). Not only was absorption at 260 and 280 nm highest in the peak infectivity fractions, but maximum absorption was similarly observed at 320 nm, indicating that light scatter from particulate matter was most intense in these same fractions.

Additional gradients using normal mouse brain prepared in an identical way as the scrapie-infected brain gave very similar density profiles of protein and membrane distributions. In some of these experiments, the resuspended pellet material was frozen and thawed up to 10 times and resonicated before addition to the gradients. Since a larger amount of protein was detected in gradients of such frozen-thawed brain than in gradients of fresh brain, the possibility was entertained that freeze-thaw cycles might also increase infectivity in scrapie mouse brain. However, in duplicate experiments using scrapie brain material, a 100% increase in the peak protein fraction was not accompanied by any change in scrapie activity in either cesium chloride or sucrose gradients, and the infectivity curves and total yields were almost identical to those shown in Fig. 1.

Discussion. In a number of previous experiments, scrapie-infected mouse brain has been banded in potassium tartrate, sucrose, and cesium chloride density gradients (Table I). Several potassium tartrate gradients have showed maximum infectivity at an average density of 1.14–1.16 g/ml, although chloroform–methanol extraction caused a marked shift in density to 1.30 g/ml (2, 4). In sucrose gradients most of the scrapie activity has been found in a broad intermediate region of the sucrose gradient, between 1.14 and 1.24 g/ml (2, 6), whereas in cesium chloride, maximum titers have occurred in a density range of 1.32–1.37 g/ml (1, 5).

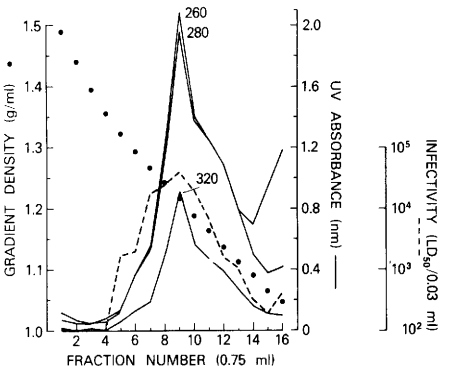

FIG. 2. Relationship between scrapie infectivity and ultraviolet light absorption curves in cesium chloride density gradient.

Although it would thus appear that scrapie infectivity bands at increasingly higher densities in gradients composed of potassium tartrate, sucrose, and cesium chloride, respectively, the different methods used to prepare scrapie-infected mouse brain for application to these gradients and the different speeds and times of centrifugation, make it very difficult to evaluate the effect of the gradient solution itself on scrapie density. Our experiment was designed to eliminate these variables, using a single scrapie preparation, and performing a simultaneous banding in different gradient solutions. The results demonstrate that the gradient solution had but little effect on the density of scrapie, or more accurately, on the density of cellular membrane fragments with which scrapie activity is associated.

Metrizamide was included as a gradient solution because it has been shown to differ markedly from sucrose or cesium chloride in its effect on nucleic acid density (8). This effect has been postulated to be due to its relatively low degree of binding of water molecules, leading to a greater hydration of the nucleic acid molecules in solution. It is possible that the modest downward shift in density observed for scrapie infectivity in our experiment is similarly due to a greater hydration of membrane components in metrizamide than in cesium chloride or sucrose.

Our observation that the maximum infectivity region of each gradient could be identified by the simple expedient of spectrophotometric ultraviolet light absorption at a wavelength of 320 nm, allows immediate fur-

TABLE I. SUMMARY OF PUBLISHED BUOYANT DENSITY EXPERIMENTS ON THE SCRAPIE VIRUS.

Author (Reference)	Preparative (pregradient) methodology	Gradient characteristics	Density of maximum infectivity (g/ml)
Gibbs (1)	10% saline mouse brain susp. 10,000 rpm/30 m. → supernate.	Cesium choride 39,000 rpm/24 hr	1.32–1.37
Hunter and Millson (2)	10% saline mouse brain susp. Extracted with Arcton-13 and Triton-X100. 10,000g/10 m → supernate.	Sucrose 22,500 rpm/18 hr	intermediate (fx #2 of 3)
—	10% saline mouse brain susp. 80°C/30 m. 10,000g/10 m. Extracted with 6M LiCl. 10,000g/10 m → supernate.	K tartrate 22,500 rpm/18 hr	intermediate (fxs #2,3 of 5)
Hunter *et al.* (3)	10% sucrose-mouse brain homogenate. Repeated pelleting at 10,000g and rehomogenization in dist. H_2O. Sonication.	K tartrate 35,000 rpm/16 hr	1.14
—	Same as above, plus chloroform methanol extraction.	K tartrate 35,000 rpm/16 hr	1.30
—	10% saline mouse brain susp. Extracted with Arcton-13. Column electrophoresis in Bio-Gel P-300.	K tartrate 35,000 rpm/16 hr	1.16
Kimberlin *et al.* (4)	Mouse brain homogenized in H_2O. 100,000g/1 hr. Pellet rehomogenized in H_2O, plus lysolecithin. 100,000g/1 hr. Pellet sonicated, plus Triton-X100. 50 nm Millipore filtration. 100,000g/1½ h. Pellet into TRIS.	K tartrate 10,000g/14–16 hr	1.12–1.17
Cho (5)	Mouse brain repeatedly extracted with Freon-113, pelleted at 100,000g, resuspended in saline.	Cesium chloride 243,000g/72 hr	1.33–1.34
Siakotis *et al.* (6)	Sucrose saline mouse brain susp. disrupted under N_2 pressure. 7000g/10 m → supernate.	Sucrose 121,000g/16 hr	1.19–1.29
—	Same as above, plus filtration through 200 nm nucleopore filter and concentration on 30 nm nucleopore filter.	Sucrose 121,000g/12 hr	1.14–1.24
Brown *et al.* (present study)	10% mouse brain susp. disrupted under N_2 pressure. 3500g/30 min. Supernate passed through 450 nm and 220 nm Millipore filters. 152,000g/17 h. Pellet resuspended in TRIS (1/5 vol). Sonicated.	Cesium chloride 152,000g/16 hr	1.22
—	Same as above.	Sucrose 152,000g/16 hr	1.21
—	Same as above.	Metrizamide 152,000g/16 hr	1.17

ther processing of peak infectivity fractions before obtaining results of lengthy titration assay.

Summary. Scrapie-infected mouse brain was passed through a fine mesh sieve, subjected to 900 psi, and centrifuged at 3500*g*. The supernate was passed through 450 nm and 220 nm Millipore filters, and centrifuged at 152,000*g*. The pellet was resuspended to 1/5th the original volume, sonicated, and applied to cesium chloride, sucrose, and metrizamide gradients which were simultaneously centrifuged at 152,000*g* for 16 hr.

Analysis of gradient fractions showed peak infectivity at a density of 1.22 g/ml in cesium chloride, 1.21 g/ml in sucrose, and 1.17 g/ml in metrizamide, with 90–95% total infectivity recovered in a well-defined band bracketing each peak titer fraction. No infectivity was observed at densities higher than 1.33 g/ml in cesium chloride, or 1.30 g/ml in sucrose and metrizamide. In each gradient the area of greatest infectivity coincided with the maximum concentration of cellular membrane vesicles and fragments, and contained between 2 and 6% of the total infective activity present in the original brain suspension. Peak infectivity fractions could be rapidly identified by spectrophotometric scanning at a wavelength of 320 nm.

We conclude that the variability in estimates of the density of scrapie infectivity observed in earlier experiments has resulted from differences in the preparation of starting material, and that density gradients composed of solutions of widely different viscosities and dipole moments do not effect the buoyant density of membrane-associated scrapie virus.

1. Gibbs, C. J., Jr., *in* "Current Topics in Microbiology and Immunology" (J. A. Brody, W. Henle, and H. Koprowski, ed.), Vol 40, p. 44. Springer-Verlag, New York (1967).
2. Hunter, G. D., and Millson, G. C., J. Comp. Pathol. **77,** 301 (1967).
3. Hunter, G. D., Kimberlin, R. H., Millson, G. C., and Gibbons, R. A., J. Comp. Pathol. **81,** 23 (1971).
4. Kimberlin, R. H., Millson, G. C., and Hunter, G. D., J. Comp. Pathol. **81,** 383 (1971).
5. Cho, M. J., Nature (London) **262,** 411 (1976).
6. Siakotos, A. N., Gajdusek, D. C., Gibbs, C. J., Jr., Traub, R. D., and Bucana, C., Virology **70,** 230 (1976).
7. Birnie, G. D., Rickwood, D., and Hell, A., Biochem. Biophys. Acta **331,** 283 (1973).
8. Schaffner, W., and Weissmann, C., Anal. Biochem. **56,** 502 (1973).

Received October 14, 1977. P.S.E.B.M. 1978, Vol. 158.