

- Hall, C. L., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem.* 254, 2023-2031.
- Hauge, J. G., Crane, F. L., & Beinert, H. (1956) *J. Biol. Chem.* 219, 727-733.
- Ikeda, Y., & Tanaka, K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 41, 1192.
- Massey, V. (1960) *Biochim. Biophys. Acta* 37, 310-314.
- Massey, V., & Swoboda, B. E. P. (1963) *Biochem. Z.* 338, 474-484.
- Massey, V., & Palmer, G. (1966) *Biochemistry* 5, 3181-3189.
- Massey, V., & Hemmerich, P. (1978) *Biochemistry* 17, 9-17.
- Mayhew, S. G., & Massey, V. (1973) *Biochim. Biophys. Acta* 315, 181-190.
- McKean, M. C., Frerman, F. E., & Mielke, D. M. (1979) *J. Biol. Chem.* 254, 2730-2735.
- Mizzer, J. P., & Thorpe, C. (1981) *Biochemistry* 20, 4965-4970.
- Moore, S., & Stein, W. H. (1960) *Methods Enzymol.* 6, 819-831.
- Noda, C., Rhead, W. J., & Tanaka, K. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 2646-2650.
- Reinsch, J. W., Feinberg, B. A., & McFarland, J. T. (1980) *Biochem. Biophys. Res. Commun.* 94, 1409-1416.
- Ruzicka, F. J., & Beinert, H. (1977) *J. Biol. Chem.* 252, 8440-8445.
- Spencer, R. L., & Wold, F. (1969) *Anal. Biochem.* 32, 185-190.
- Steenkamp, D. J., & Gallup, M. (1978) *J. Biol. Chem.* 253, 4086-4089.
- Swingle, S. M., & Tiselius, A. (1951) *Biochem. J.* 48, 171-174.
- Thorpe, C. (1981) *Methods Enzymol.* 71, 366-374.
- Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry* 18, 331-337.
- Weber, K., & Osborn, M. (1969) *J. Biol. Chem.* 244, 4406-4412.
- Whitfield, C. D., & Mayhew, S. G. (1974) *J. Biol. Chem.* 249, 2811-2815.

Further Purification and Characterization of Scrapie Prions[†]

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ABSTRACT: The scrapie agent is prototypic of a novel class of small infectious pathogens called prions. Prolonged bioassays as well as the hydrophobicity of the agent have complicated its purification. A purification protocol for the agent in hamster brain has been developed, which results in preparations enriched for the agent between 100- and 1000-fold with respect to protein. The protocol includes Triton X-100/sodium deoxycholate extraction, poly(ethylene glycol) precipitation, nuclease and protease digestion, cholate/sodium lauryl sarcosinate extraction, (NH₄)₂SO₄ precipitation, Triton X-100/sodium dodecyl sulfate extraction, and sedimentation through a discontinuous sucrose gradient. The highest degree of purification was found in a fraction from the 25% and 60% sucrose interface near the bottom of the gradient. Examination by electron microscopy of rotary-shadowed samples from the gradient interface which contained >10^{9.5} ID₅₀ units/mL of the scrapie agent revealed aggregates composed of amorphous

material and numerous flattened rods measuring 25 nm in diameter by 100-200 nm in length. Radioiodination of the interface fraction has identified a protein that is unique to preparations from scrapie-infected brains. The apparent molecular weight of the protein is between 27 000 and 30 000. The unique protein was also radiolabeled with [¹⁴C]diethyl pyrocarbonate. Although the properties of the scrapie prion and this unique protein must be sufficiently similar to allow copurification, further experimental data will be needed to establish whether or not this protein is required for infectivity. In contrast to the protein radiolabeling studies, 5'-end labeling with [γ-³²P]ATP of nucleic acid molecules in the purified fraction failed to identify a unique nucleic acid. The results of these radiolabeling studies are consistent with earlier observations showing that prions are inactivated by procedures that modify proteins but are resistant to inactivation by procedures that modify nucleic acids.

The scrapie agent causes a degenerative disorder of the nervous system of sheep and goats many months or even years after inoculation or exposure. Its novel properties have prompted introduction of the term "prions" (Prusiner, 1982). The scrapie prion is prototypic of a unique class of small infectious pathogens. Similar pathogens probably also cause

two human diseases, kuru and Creutzfeldt-Jakob disease (Gajdusek, 1977; Hadlow et al., 1980).

Recent studies on the scrapie agent have shown that it contains a protein that is required for infectivity (Prusiner et al., 1981b). These studies were made possible by the development of purification procedures that substantially reduced the amount of cellular protein and lipid contaminating scrapie agent preparations. To date, all attempts to inactivate the agent by hydrolyzing or modifying its hypothetical nucleic acid have been unsuccessful (Prusiner, 1982; Alper et al., 1967, 1978). Studies on the molecular size of the scrapie agent suggest that any nucleic acid contained within the agent would be too small to function as a gene coding for the protein(s)

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of its putative shell (Prusiner, 1982). These properties of the scrapie prion clearly distinguish it from viruses and viroids (Prusiner, 1982).

The purification protocol reported in this paper represents a substantial improvement over an earlier procedure, which created preparations of the agent that could be inactivated by protease digestion and chemical modification with DEP¹ (Prusiner et al., 1981b; McKinley et al., 1981). The earlier procedure yielded a partially purified fraction (E₆) that was increased approximately 100-fold in specific infectivity compared to homogenates. Two major problems plagued that purification procedure: (1) sedimentation of the agent in a microsomal membrane fraction required prolonged ultracentrifugation and thus severely limited the size of preparations; (2) preparative sarkosyl-agarose gel electrophoresis of the agent was slow, tedious, and of limited capacity. Our new purification protocol replaces the ultracentrifugation step with PEG precipitation and the preparative gel electrophoresis with rate-zonal sucrose gradient centrifugation. In addition, the earlier protocol used only anionic detergents while the new scheme employs combinations of nonionic and anionic detergents.

Besides being more rapid and efficient, this new purification procedure has permitted us to identify a unique protein that purified with the scrapie prion (Bolton et al., 1982). Whether this protein is a structural component of the agent or a pathologic product of infection remains to be established.

Materials and Methods

Chemicals and Source of the Scrapie Agent. All chemicals were the highest grades commercially available. Sarkosyl, sodium cholate, DOC, Triton X-100, poly(ethylene glycol) 8000, and micrococcal nuclease were purchased from Sigma. Enzyme grade ammonium sulfate and sucrose were purchased from Schwarz/Mann. Proteinase K was purchased from Merck. NaDodSO₄ was obtained from BDH Chemicals. Na¹²⁵I was obtained from Amersham. *N*-Succinimidyl 3-(4-hydroxy-5-iodophenyl)propionate was purchased from Calbiochem.

A hamster-adapted isolate of the scrapie agent was passaged and prepared as described (Prusiner et al., 1980b).

Assay of the Scrapie Agent. Scrapie agent titers were determined by measuring the incubation time intervals from inoculation to the onset of neurological illness and to death (Prusiner et al., 1982). Weanling female hamsters (LVG/LAK) were purchased from Charles River Laboratories. The animals were inoculated intracerebrally with 50 μ L of the sample following dilution by a factor of 10 or 100 as previously described (Prusiner et al., 1980b).

Chemical Assays. Protein was determined by a modification of the Lowry technique (Peterson, 1977). RNA and DNA were determined by the orcinol and 3,5-diaminobenzoic acid dihydrochloride (DABA) fluorescence procedures, respectively (Schneider, 1957; Kissane & Robins, 1958).

Radioiodination. Fractions containing the scrapie agent were mixed with 10% NaDodSO₄ at room temperature to yield a final concentration of 0.2%. The NaDodSO₄-protein complexes were precipitated upon addition of 0.1 M quinine

hemisulfate in 0.1 N HCl (R. K. Durbin, personal communication). Five volumes of distilled water was added and the suspension centrifuged at low speed for 15 min. The supernatant was discarded and the pellet washed in acetone to remove residual quinine. The final pellet was resuspended in 0.1 volume of 0.1 M sodium borate, pH 8.5, containing 0.1% NaDodSO₄. Radioiodination of 10–50 μ L of this suspension with *N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate was performed on ice for 30–60 min (Bolton & Hunter, 1973). *N*-Succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate was synthesized by using chloramine T (Bolton & Hunter, 1973). The radioiodination reaction was terminated upon addition of 0.5 mL of 0.2 M glycine in 0.1 M sodium borate, pH 7.5. The residual radioiodinated glycine was removed by reprecipitation of the iodinated proteins with NaDodSO₄ and quinine hemisulfate-hydrochloric acid.

[¹⁴C]DEP Labeling. Samples were concentrated by precipitation using NaDodSO₄ and quinine hemisulfate as described for the radioiodination studies. The precipitates were then resuspended in 60 mM sodium phosphate, pH 7.2, containing 0.2% sarkosyl. Ten millimolar [¹⁴C]DEP (53 mCi/mmol) was reacted with each sample for 1 h at room temperature. The samples were then digested with 100 μ g/mL proteinase K for 30 min at room temperature. The digestion was terminated by addition of an equal volume of 2 \times concentrated NaDodSO₄ electrophoresis sample buffer and boiling for 2 min.

Polyacrylamide Gel Electrophoresis. Radiolabeled proteins were heated to 100 $^{\circ}$ C for 2 min in electrophoresis buffer containing 1.25% NaDodSO₄ and 1.25% β -mercaptoethanol. The proteins were separated by electrophoresis in polyacrylamide gels employing the discontinuous buffer system of Laemmli (1970). Gels were stained in 25% 2-propanol and 10% acetic acid containing 0.03% Coomassie Brilliant Blue R-250 and destained in 10% 2-propanol and 10% acetic acid (Fairbanks et al., 1971). Gels were dried with a Hoefer slab gel dryer. Radioautographs of gels containing ¹²⁵I-labeled proteins were made at room temperature with Kodak XAR-5 film. Gels containing proteins labeled with [¹⁴C]DEP were processed for fluorography, and the radioautograph was exposed at -70° C (Bonner & Laskey, 1974).

Electron Microscopy. Electron microscopy specimen grids, 400-mesh Cohen-Pelco handle type, were filmed with 0.25% Formvar and coated with a thin layer of carbon in an Edwards E206 evaporator. The films were made hydrophilic by subjecting them for ~ 5 s to a glow discharge at 70–80-mtorr (9–11 Pa) air pressure (Williams, 1977). The handles of the grids were prevented from becoming hydrophilic during the discharge by covering them with small pieces of metal. This precaution prevents any liquid drop subsequently applied to the grid surface from spreading to the tips of the forceps that hold the grid. A 5–8- μ L drop containing 1 μ g/mL poly(L-lysine) was placed for 30 s upon each grid. The drop of poly(lysine) solution was drained by touching the edge of the grid with a vacuum-connected aspirator freshly made from a Pasteur pipet flame-drawn to less than 0.5-mm bore diameter (Williams, 1977). The residual liquid on the grid dried in several seconds with the trailing edge of the liquid film showing one to two complete orders of interference colors.

The samples (6 μ L) were applied to the treated films and allowed to absorb for 1 min. The drops were washed away from the grid surface by slowly passing the grid (sample surface down) 10 times across the surface of freshly prepared 0.1 M ammonium acetate. A 5- μ L drop of 1% uranyl acetate was applied to the grid for 15 s, followed by the washing

¹ Abbreviations: DOC, sodium deoxycholate; NaDodSO₄, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate; PEG, poly(ethylene glycol); sarkosyl, sodium lauryl sarcosinate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; GFA, glial fibrillary acidic; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

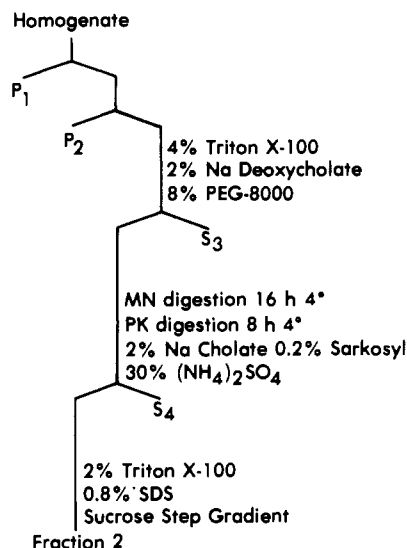


FIGURE 1: Preparative protocol for partial purification of the scrapie agent. MN is micrococcal nuclease and PK is proteinase K. See text for conditions.

procedure described above using 0.01 M ammonium acetate. Any remaining ammonium acetate was removed by the vacuum pipet apparatus. These washing steps effectively removed the detergent after adsorption of the sample.

The dried grids were rotary shadowed with tungsten (Griffith et al., 1971). A 20-mil tungsten wire of 3-cm free length was initially heated with a 31.5-A current, after which the applied voltage was left constant until burnout (about 90 s). This schedule has been found to evaporate ~10 mg of tungsten (Williams, 1977). An oblique angle of 8–10° with a source to sample distance of approximately 7 cm was satisfactory for contrast enhancement. The plate to which the grids were secured inside the evaporator jar rotated at 100 rpm.

The rotary-shadowed grids were examined in a JEOL 100-B electron microscope operated at 60 kV.

³²P 5'-End Labeling. Preparations of the scrapie agent were extracted by addition of equal volumes of phenol saturated with 60 mM sodium phosphate, pH 7.2 (Palmiter, 1974). After being mixed, the phases were separated by low-speed centrifugation. Equal volumes of phenol and chloroform/isoamyl alcohol (24:1) were added to the extracted aqueous phase. The phenol/chloroform/isoamyl alcohol extraction was repeated once, and this was followed by two chloroform/isoamyl alcohol extractions. The aqueous phase was made 0.2 M in NaCl, and 2.5 volumes of cold ethanol was added; the precipitate was collected after storage at -20 °C by centrifugation in an Eppendorf microfuge.

Prior to phosphorylation of these phenol/chloroform/isoamyl alcohol extracted samples, calf intestine alkaline phosphatase was added to catalyze the dephosphorylation of the 5' termini. The reaction mixture contained 10 mM Tris-HCl, pH 9.0, and was incubated for 30 min at 65 °C. After the alkaline phosphatase digestion was repeated, the samples were again extracted with phenol/chloroform/isoamyl alcohol as described above. The polynucleotides were phosphorylated in a 100-μL reaction mixture containing 50 mM glycine-NaOH, pH 9.5, 10 mM MgCl₂, 5 mM DTT, and 10 μL containing 100 pmol of [γ-³²P]ATP (3000 Ci/mmol) and 2 units of polynucleotide kinase (Lillehaug & Kleppe, 1975; Maxam & Gilbert, 1977). This reaction mixture was heated at 37 °C for 30 min prior to termination by addition of 100 μL of 4 M ammonium acetate. Samples were ethanol precipitated and resuspended in 10 mM Tris-HCl, pH 7.4, with

Table I: Precipitation of the Scrapie Agent from Detergent Suspensions by Poly(ethylene glycol) 8000

detergents	total scrapie infectivity (ID ₅₀ units)		
	suspension ^a	supernatant ^b	pellet
4.0% Triton X-100	10.7	10.3	10.9
4.0% octyl glucoside	10.7	5.3	9.8
4.0% Brij 35	11.1	8.3	10.5
4.0% sulfobetaine 3-14	9.2	10.0	9.8
0.2% sarkosyl	7.8	4.7	7.6
4.0% DOC	8.2	5.3	7.7
4.0% Triton X-100/2.0% DOC	11.6	9.8	11.5
2.0% Triton X-100/0.8% NaDodSO ₄	9.6	7.6	10.5
2.0% Triton X-100/0.8% NaDodSO ₄ /0.5 M NaCl	10.5	8.2	9.8

^a The suspension contained detergents at designated concentrations. ^b To the detergent extract was added 8% (w/v) poly(ethylene glycol) 8000 at 4 °C as described in the text. Supernatant fractions were separated from pellets after centrifugation at 20 000 rpm in a JA-20 rotor for 30 min.

1 mM EDTA prior to analysis by polyacrylamide gel electrophoresis.

Results

A purification scheme for obtaining highly enriched preparations of scrapie prions is depicted in Figure 1. In a typical preparation, 200 weanling female hamsters (LVG/LAK) were inoculated intracerebrally with ~10⁷ ID₅₀ units of hamster-adapted scrapie agent and were sacrificed 60–65 days later. The brains were removed, and a 10% (w/v) homogenate in 320 mM sucrose was prepared. Details of the homogenization procedure have been published (Prusiner et al., 1980b, 1981b). All operations were performed at 4 °C unless otherwise noted, and only the final concentrations of all added chemicals are given. The homogenate was centrifuged at 2700 rpm for 10 min in a JA-10 rotor. The supernatant fluid (S₁) was decanted and centrifuged again at 4000 rpm for 30 min. To the supernatant fluid (S₂) were added 1 mM EDTA and 1 mM DTT. Triton X-100 and DOC were added at ratios of 4:1 and 2:1 on a detergent:protein (w/w) basis, respectively. To this detergent extract were added 0.03 M Tris-OAc, pH 8.3, 0.1 M KCl, 20% (v/v) glycerol, and 8% (w/v) PEG. The addition of these chemicals was dropwise, and stirring was continued for an additional 30 min. The PEG precipitate was collected by centrifugation at 10 000 rpm in a JA-10 rotor for 30 min. The pellet (P₃) was resuspended in 20 mM Tris-OAc, pH 8.3, containing 0.02% Triton X-100 and 1 mM DTT and adjusted to a protein concentration of 10 mg/mL. This buffer and all subsequent buffers were degassed under vacuum and saturated with argon prior to use.

Precipitation of the scrapie agent by PEG from suspensions containing a variety of detergents was explored (Table I). The scrapie agent was readily precipitated with PEG from suspensions containing octyl glucoside, Brij 35, sarkosyl, or DOC. Effective precipitation from suspensions containing Triton X-100 or sulfobetaine 3-14 was not observed; however, addition of DOC or NaDodSO₄ to Triton X-100 suspensions did result in precipitation of the scrapie agent. Since DOC had been employed in earlier purifications, the Triton X-100/DOC combination was chosen for our preparative procedure. Generally 50% or more of the scrapie infectivity was precipitated from a preparative suspension containing Triton X-100/DOC by the addition of 8% PEG as described above. Although concentrations of PEG as low as 2% yielded effective

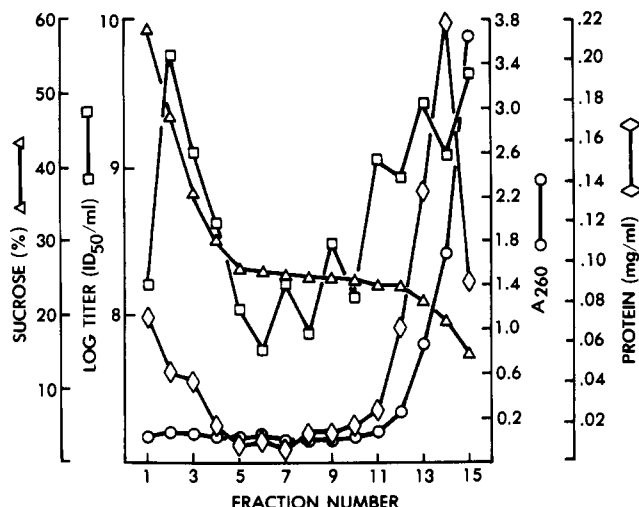


FIGURE 2: Rate-zonal discontinuous sucrose gradient centrifugation for purification of the scrapie agent. Fraction P₄ (4 mL) suspended in 2% (v/v) Triton X-100/0.8% (w/v) NaDodSO₄ was layered onto a sucrose step gradient containing 4 mL of 60% (w/v) sucrose at the bottom and 32 mL of 25% sucrose. The sucrose solutions contained 20 mM Tris-OAc, pH 8.3, 1 mM EDTA, and no detergent. The gradients were centrifuged at 50 000 rpm for 120 min in a vertical VTi50 rotor at 4 °C. The gradients were then fractionated from the bottom. Fractions were stored at -70 °C prior to assay for the scrapie agent (□), sucrose (Δ), protein (◇), and A₂₆₀ (○).

precipitation of the agent on a small scale, preparative batches required a concentration of 8% PEG.

Further purification was achieved by sequential digestions with 12.5 units/mL micrococcal nuclease in the presence of 2 mM CaCl₂ for 16 h at 4 °C, followed by 100 μg/mL proteinase K in the presence of 0.2% sarkosyl and 2 mM EDTA for 8 h at 4 °C. Both digestions were performed with constant stirring. Protease activity was terminated by addition of 0.1 mM PMSF. A solution of sodium cholate was added slowly to give a concentration of 2% (w/v), followed by addition of solid (NH₄)₂SO₄ to 30% saturation (Prusiner et al., 1980b). Stirring of the sample was continued for 30 min prior to centrifugation at 20 000 rpm for 60 min at 4 °C. The pellet (P₄) was resuspended in 20 mM Tris-OAc, pH 8.3, 1 mM EDTA, 1 mM DTT, and 0.2% sarkosyl.

Two percent Triton X-100 and 0.8% NaDodSO₄ were mixed together and then added with stirring to fraction P₄ adjusted to a protein concentration of 0.75 mg/mL. A period of 15 min without agitation preceded layering 4 mL of suspension onto a sucrose gradient. The gradient was formed in 40-mL polyallomer tubes filled with 4 mL of 60% (w/v) and 32 mL of 25% sucrose; the sucrose solutions contained 20 mM Tris-OAc, pH 8.3, and 1 mM EDTA. The tubes were centrifuged at 50 000 rpm in a vertical VTi50 rotor. After 120 min of centrifugation the speed was reduced to 3000 rpm with the brake and the rotor then allowed to coast to a stop. The tubes were fractionated from the bottom after puncture with a needle device. To each fraction was added 0.05% (w/v) sulfobetaine 3-14 prior to freezing in a dry ice-ethanol bath and storage at -70 °C.

The majority of the scrapie agent was found near the bottom of the gradient in fraction 2 at the interface between the 25% and 60% sucrose steps (Figure 2). As much as 50% of the agent was frequently found at the top of the gradient in fractions 14 and 15. Presumably the agent in fraction 2 is associated as aggregates with other hydrophobic molecules, in contrast to the apparently smaller forms in fractions 14 and 15. The formation of these large aggregates probably occurs as the agent sediments into the 25% sucrose step, which

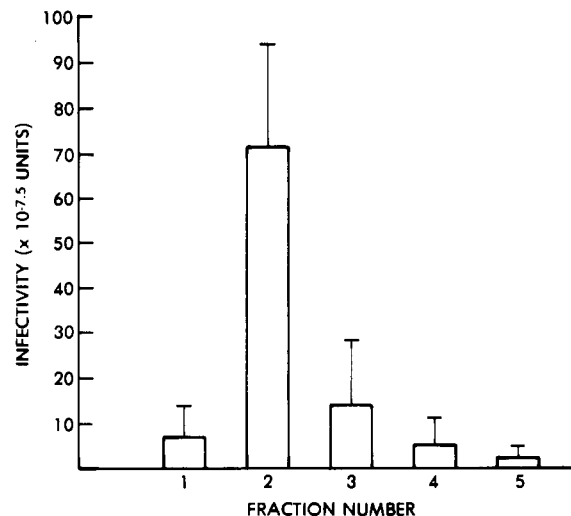


FIGURE 3: Recovery of the scrapie agent from preparative sucrose gradients. Data for 13 gradients similar to that shown in Figure 2 were averaged. The recovery of the agent in fractions 1-5 is shown. Variation is given by standard error bars.

Table II: Purification of the Scrapie Agent from Hamster Brain^a

fraction	specific infectivity (ID ₅₀ units/mg of protein)	purification (x-fold)	recovery (%)
homogenate	3.5×10^7		
S ₂	5.1×10^7	1.5	75
P ₃	5.9×10^7	1.7	12
P ₄	9.9×10^8	28	7.1
F ₂	1.4×10^{10}	410	6.2

^a Data are from two representative preparations (KB 81 and KB 85); average values are given.

contains no detergent. The excellent reproducibility of the procedure is shown in Figure 3 for 13 different gradients. Greater than 70% of the scrapie agent in the five fractions from the bottom of the gradient was found in fraction 2. The recovery of the scrapie agent from all fractions of the sucrose gradients ranged from 65% to 190%.

The overall purification of the scrapie agent in fraction 2 obtained by this procedure (Figure 1) varied from 100- to 1000-fold with respect to cellular protein. The progressive increase in specific infectivity expressed as ID₅₀ units per milligram of protein during two representative purifications is shown in Table II. The recovery of total scrapie agent infectivity after purification varied from 1% to 10%. Fraction 2 generally contained $>10^9$ ID₅₀ units/mL scrapie agent, 20-100 μg/mL protein, and <10 μg/mL each of RNA and DNA.

Both the digestions with micrococcal nuclease and proteinase K were important in the purification scheme. Decreasing the micrococcal nuclease from 12.5 to 5 units/mL resulted in an increase in the concentration of protein to >100 μg/mL in fraction 2. Omitting the digestion with proteinase K increased the recovery of protein even more; measurements on combined fractions from the lower half of the gradient showed that the concentration of protein increased from an average value of 28 μg/mL to 791 μg/mL.

The addition of Triton X-100 and NaDodSO₄ to fraction P₄ prior to rate-zonal sucrose gradient centrifugation was also found to be critical for purification of the scrapie agent. Substitution of 5% octyl glucoside or 10% Triton X-100 for the Triton X-100/NaDodSO₄ combination resulted in vastly different distributions of proteins as well as prions (Figure 4).

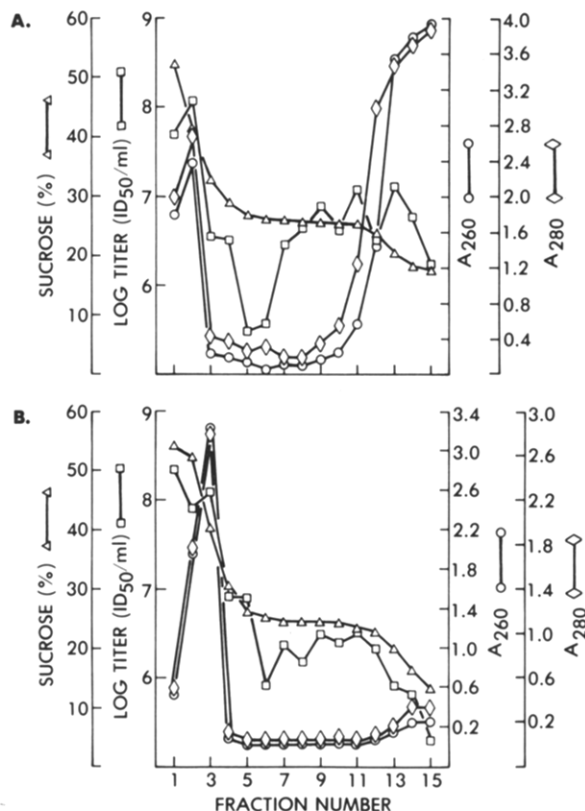


FIGURE 4: Purification of the scrapie agent by discontinuous sucrose gradient centrifugation depends on the detergent used. (A) Triton X-100 [10% (v/v)] or (B) octyl glucoside [5% (w/v)] was substituted for the mixture of Triton X-100/NaDodSO₄ used in Figure 2. Symbols are the same as those in Figure 2 except A₂₈₀ (◇).

The gradient with 10% Triton X-100 exhibited a biphasic distribution of protein. Although the majority of the protein was found at the top of the gradient, nearly 30% sedimented to the bottom with most of the scrapie agent. When 5% octyl glucoside was used, nearly all the protein was found at the bottom of the gradient associated with the majority of the scrapie agent. In other studies, no purification was achieved with linear metrizamide gradients (15–55%) containing 2% sarkosyl or sarkosyl plus one of the following detergents: 4% Triton X-100, 5% lyssolecithin, or 3% dodecyltrimethylamine oxide (Ammonyx LO). The metrizamide gradients were centrifuged at 50,000 rpm for 15 h in a fixed-angle 60Ti rotor at 4 °C. Attempts to isolate the scrapie agent in cesium chloride or cesium sulfate density gradients after isopycnic centrifugation were also unsuccessful. Substantial losses of infectivity as well as our inability to obtain scrapie agent of uniform density precluded the use of cesium salt gradients.

Electron microscopy of selected fractions from the Triton X-100/NaDodSO₄ discontinuous sucrose gradient (Figure 2) was performed to search for macromolecular structures uniquely present in scrapie preparations. Rotary shadowing with tungsten was employed because negative staining frequently gave artifactual results due to residual detergents in the preparations. Grids were coated with poly(lysine) after ionization by glow discharge to maximize the attachment of proteinaceous particles. As shown in Figure 5A, aggregates composed of amorphous material and flattened rodlike structures were found in our most purified samples (fraction 2 of a Triton X-100/NaDodSO₄ discontinuous sucrose gradient). The rods measured 25 nm in diameter by 100–200 nm in length. While these rods are frequently found in scrapie preparations, they do not appear to be unique since a few rods of similar size and shape have been found in control fractions.

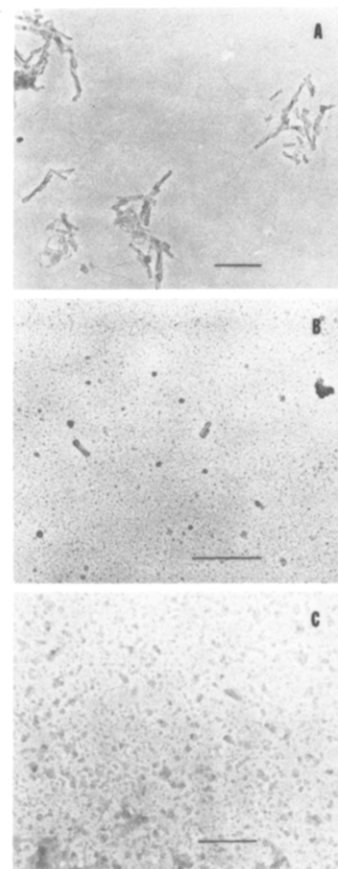


FIGURE 5: Rotary-shadowed electron micrographs of fractions from a Triton X-100/NaDodSO₄ discontinuous sucrose gradient. Samples were applied to grids coated with poly(lysine) and then shadowed with tungsten. (A) Fraction 2, (B) fraction 8, and (C) fraction 14. The solid bars are 400 nm.

Examination of fraction 8 from the middle of the gradient revealed a few rods but no aggregates (Figure 5B); a few spherical particles were also observed. At the top of the gradient, numerous particles without any distinct shape or size were observed in fraction 14 (Figure 5C). The significance of the numerous rodlike structures found at the bottom and middle of the sucrose gradients containing scrapie samples is unclear. Since the titers of fractions 2 and 14 are similar, these results diminish the likelihood that the rods are related to prions. However, further experiments are needed to assess the possibility that the rods represent an aggregated form of the infectious agent. It is of interest that identical rods have been observed in purified fractions of scrapie prions prepared by sarkosyl-agarose gel electrophoresis (Prusiner et al., 1981b).

To further explore the composition of fraction 2, we radioiodinated aliquots with Bolton–Hunter reagent. The ¹²⁵I-labeled samples were disaggregated by boiling in the presence of NaDodSO₄ and β-mercaptoethanol and analyzed by polyacrylamide gel electrophoresis. In all preparations of fraction 2 from scrapie-infected hamster brains, a unique protein was found (identified by the arrow in Figure 6). The protein, which migrated as a diffuse band in electrophoretic gels, was not seen in preparations of fraction 2 from uninoculated animals as well as animals inoculated with normal brain extracts. The unique protein identified by radioiodination studies was also detected following radiolabeling with [¹⁴C]-DEP.

Although the unique protein could be readily degraded upon digestion with proteases following denaturation, it was resistant to enzyme-catalyzed proteolysis prior to denaturation (Bolton



FIGURE 6: ^{125}I -Labeled sucrose gradient fractions 2 and 3 analyzed by NaDodSO₄-polyacrylamide gel electrophoresis. Aliquots were radiolabeled with ^{125}I -labeled Bolton-Hunter reagent. The samples were boiled for 2 min in 1.25% NaDodSO₄ and 1.25% β -mercaptoethanol prior to electrophoresis in 5–20% linear gradient polyacrylamide gels. From the left: lanes 1 and 2, scrapie fraction 2; lane 3, scrapie fraction 3; lane 4, control fraction 2; lane 5, control fraction 3. Autoradiographic exposure was for 3 h. See Materials and Methods for further details. The position of the unique protein is denoted by an arrow.

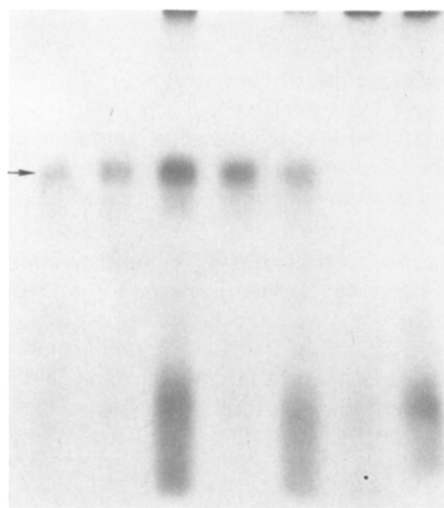


FIGURE 7: [^{14}C]DEP-labeled sucrose gradient fraction 2 digested with proteinase K prior to denaturation. After the proteolytic digestion was completed, the samples were boiled for 2 min in 1.25% NaDodSO₄ and 1.25% β -mercaptoethanol prior to electrophoresis in 15% polyacrylamide gels. From the left: lanes 1–5, scrapie fraction 2; lanes 6 and 7, control fraction 2. Autoradiographic exposure was for 720 h. The position of the unique protein is denoted by an arrow.

et al., 1982). Since most of the other proteins in the purified fraction 2 were degraded by protease digestion under non-denaturing conditions, the unique protein could be readily separated from cellular contaminants. Five different preparations of the scrapie agent and two control preparations were labeled with [^{14}C]DEP before digestion with proteinase K. After proteolysis, the radiolabeled proteins were then denatured prior to separation by NaDodSO₄-polyacrylamide gel electrophoresis (Figure 7). It is important to note that chemical modification of purified preparations of the scrapie agent by DEP resulted in reversible inactivation of the infectious prion (McKinley et al., 1981).

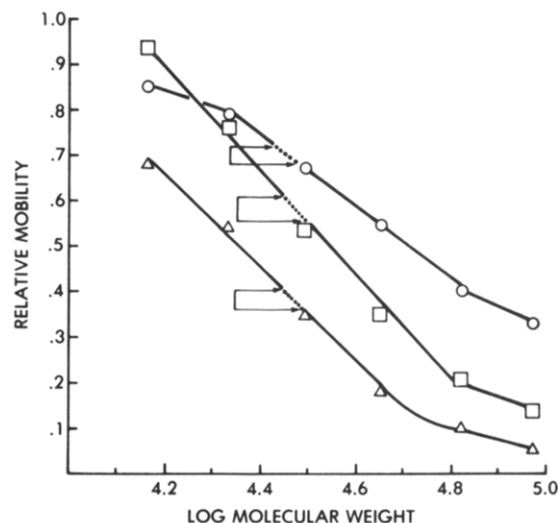


FIGURE 8: Molecular weight of a unique protein in purified preparations of the scrapie agent. The migration of the protein in three different polyacrylamide gels is shown: 15% (w/v) polyacrylamide concentration (Δ), 13% polyacrylamide concentration (\square), and 5–20% linear gradient polyacrylamide concentration (\circ).

The unique protein exhibits microheterogeneity during electrophoresis in polyacrylamide gels. The radioiodinated protein has an apparent molecular weight between 27 000 and 30 000 as judged by its electrophoretic migration in polyacrylamide gels of three different concentrations (Figure 8).

Additional radiolabeling studies were performed on fraction 2 to search for a unique nucleic acid associated with prions. After phenol extraction of fraction 2, the aqueous phase was treated with alkaline phosphatase and subjected to 5'-end labeling with [γ - ^{32}P]ATP. The labeled nucleic acids were then separated on 10% polyacrylamide gels. Autoradiograms failed to show any distinct nucleic acid species. No significant differences between scrapie and control preparations were detectable. These polyacrylamide gels were capable of separating nucleic acids of molecular weights ranging from 1.3×10^4 to 1.9×10^6 .

Discussion

A major problem confronting investigators attempting to purify prions over the past two decades has been the extraordinary heterogeneity of the scrapie agent with respect to size, density, and charge (Prusiner, 1982; Prusiner et al., 1978, 1979, 1980a). Early studies clearly indicated the apparent heterogeneity of the agent was so extreme that it was probably not due to intrinsic changes within the agent; rather, this seemed to be a consequence of the hydrophobicity of the agent (Prusiner, 1982; Prusiner et al., 1978, 1979, 1981a,b).

We have taken advantage of the hydrophobicity of scrapie prions in our purification scheme. A partially purified fraction, P₄, was mixed with Triton X-100/NaDodSO₄ prior to rate-zonal sedimentation through a sucrose step gradient containing no detergent. Presumably, large sedimenting forms of the agent were present either as aggregates that were not dissociated in Triton X-100/NaDodSO₄ or as aggregates that formed as the agent entered the sucrose gradient. The behavior of the scrapie agent under these conditions may be analogous to that observed for the Ca²⁺-ATPase (Warren et al., 1974a,b). Studies with Ca²⁺-ATPase have shown that the enzyme forms aggregates as it enters a sucrose gradient that does not contain detergent. At several stages of the purification protocol (Figure 1), a combination of nonionic/anionic detergents were used. This contrasts with an earlier protocol, which employed only anionic detergents.

Purification of both proteins and viruses using PEG precipitation or phase separations has been widely employed (Polson et al., 1964; Yamamoto & Alberts, 1970). More recently, PEG has been employed to precipitate membrane proteins such as Ca^{2+} -ATPase from detergent mixtures (Dean & Tanford, 1977). As shown in Table I, prions were also efficiently precipitated from suspensions containing a variety of detergents. Since precipitation of proteins by PEG has been reported to be most selective at 10–20 °C with the pH between 3 and 5 (Polson et al., 1964), these same conditions have been investigated for the scrapie agent. We have found that neither acidic conditions nor elevated temperatures substantially influence the precipitation of the scrapie agent by PEG.

Determining the extent of purification of the scrapie agent is complicated by the imprecision of the bioassay. Since differences of 1 log ID_{50} units/mL are not considered significant, changes in the level of purification by a factor of 10 or less are difficult to verify (Prusiner et al., 1982). In general, the purification protocol reported here resulted in a 100–1000-fold enrichment for the scrapie agent. Data given in Table II are representative of that obtained by this protocol.

Progress in preparing highly enriched fractions of scrapie prions has made important contributions to our knowledge of the properties of this unusual agent. Six lines of evidence have accumulated, showing that the scrapie agent contains a protein that is required for infectivity (Prusiner, 1982; Prusiner et al., 1981b). Two of these lines of evidence, sensitivity to proteases and reversible chemical modification by DEP, were only seen after substantial purification of the agent was achieved (Prusiner et al., 1981b; McKinley et al., 1981). Attempts to destroy infectivity in crude homogenates by protease digestion of the agent or chemical modification of the agent by DEP failed.

Electron microscopy studies have consistently failed to identify virus-like particles in thin sections from hamster brain where the titer of the scrapie agent is $>10^{9.5}$ ID_{50} units/g of tissue (Baringer et al., 1979, 1981). Examination of fractions from preparative Triton X-100/NaDodSO₄ discontinuous sucrose gradients has revealed no macromolecular structures clearly unique to preparations from scrapie-infected brain. However, scrapie preparations did contain many more flattened rods than control preparations. The significance of these rods remains to be established. If the agent possessed a distinctive structure similar to that of a conventional virus, we would expect to observe scrapie particles in both fractions from the bottom and top of the gradient since the titers of the scrapie agent in fractions 2 and 14 were $>10^{9.5}$ ID_{50} units/mL. Furthermore, if the scrapie agent behaved like a conventional virus, then its particle to infectivity ratio would be expected to be considerably greater than unity when the titer was determined in animals (Wildy & Watson, 1962; Schwerdt & Fogh, 1957). That being the case, the number of scrapie particles would be expected to be $>10^{11}$ /mL, and this would be sufficient for detection by electron microscopy (Schwerdt & Fogh, 1957; Luria et al., 1951; Monroe & Brandt, 1970). Our inability to identify a unique scrapie particle in fractions 2 and 14 suggests that the scrapie agent may be of a size smaller than we can readily detect by electron microscopy of rotary-shadowed samples. Preliminary studies have shown that, after mixing fraction 2 and 5% sulfobetaine 3-14, all of the agent passes through nucleopore filters with 15-nm pores (S. B. Prusiner and D. F. Groth, unpublished experiments). These data are consistent with other studies, suggesting that the minimum size of the scrapie agent is considerably less than that of the smallest viruses (Prusiner, 1982).

While our conclusions about the significance of the rodlike structures observed by electron microscopy must remain tentative, protein radiolabeling studies have clearly revealed a unique protein in fractions highly enriched for the agent. The protein has been found in twelve different scrapie agent preparations and not observed in eight control preparations (Bolton et al., 1982). Four lines of evidence suggest that this unique protein may be a structural component of the scrapie agent: (1) the protein is only present in purified fractions prepared from scrapie-infected brains and not from uninoculated controls; (2) the properties of the protein are sufficiently similar to those of the agent that they allow for copurification; (3) one or more [¹⁴C]ethoxyformyl groups are probably incorporated into a structural prion protein since DEP reversibly inactivates the infectious agent (McKinley et al., 1981); (4) preliminary results indicate that the amount of the protein correlates with the titer of the agent and that both the protein and the infectious agent are resistant to limited proteolysis, which degrades all other proteins in fraction 2. Further studies are required to determine whether or not the unique protein isolated under nondenaturing conditions possesses infectivity.

The microheterogeneity of the unique protein is evident in electrophoretic gels where it migrated as a diffuse band. This may be due to glycosylation, phosphorylation, fatty acid acylation, or another chemical modification process (Wold, 1981). Alternatively, the microheterogeneity of the protein might result from the proteinase K digestion used in the purification protocol (Figure 1). The apparent molecular weight of the protein is between 27 000 and 30 000; thus, it is sufficiently small to be contained within the infectious particle. On the basis of titer measurements of fraction 2 and the amount of unique protein in this fraction, we estimate that approximately 1 μg of this protein is found per $10^{8.5}$ ID_{50} units of scrapie agent. From the molecular weight of the protein we calculate that there are approximately 10^4 molecules of the protein per ID_{50} unit in fraction 2.

Our findings that the unique protein is resistant to degradation by proteases have allowed us to distinguish it from contaminating proteins found in normal brain. In our experiments, fraction 2 was incubated with 100 μg /mL proteinase K for 30 min at 25 °C. Preliminary observations indicate that under these conditions no change in the titer of the agent occurs. Earlier studies with fractions purified by sarkosyl gel electrophoresis showed that a significant reduction of infectivity required incubation of the agent with 100 μg /mL proteinase K for 3 h or more at 37 °C (Prusiner et al., 1981b). Studies are in progress to determine whether or not under these nondenaturing conditions the unique protein is susceptible to protease-catalyzed hydrolysis. Changes in conformation of the agent may modulate its susceptibility to proteolysis since both denaturation and surface hydrophobicity have been shown to be important factors in the hydrolytic degradation of proteins (Rupley, 1967; Holzer & Heinrich, 1980). The hydrophobicity of the agent is well documented (Prusiner et al., 1978, 1979, 1980a, 1981b).

Although unlikely, the unique protein identified in fractions highly enriched for the scrapie agent may be a pathological product of the infection. During scrapie infection glial cells proliferate and extensive vacuolation of neurons occurs. An increase in GFA protein accompanies astrocytic gliosis; however, GFA protein has a molecular weight of 46 000 or more while the unique scrapie protein is considerably smaller (Eng, 1980). We cannot eliminate the possibility that another glial protein or some neuronal protein possibly generated by specific proteolytic cleavage during vacuole formation might

be the unique scrapie protein. Clearly, further studies are required to establish both the specificity and significance of our observations.

Attempts to demonstrate a scrapie-specific nucleic acid by 5'-end labeling in the same purified fractions where a unique protein was found have been unsuccessful. These results might be explained by one or more of the following postulates. First, the 5' termini may be blocked by unusual nucleotides or by covalent attachment of proteins. Modified nucleotides at the 5' termini of mRNAs as well as protein covalently linked to the 5' termini of viral genomes are well documented (Gerlich & Robinson, 1980; Revie et al., 1979; Stanley et al., 1978; Ambros & Baltimore, 1978; Rekosh et al., 1977; Ghosh et al., 1979). Second, the nucleic acid could be circular as is the case for many viral genomes, thus providing no sites for end labeling. Third, the number of nucleic acid molecules per ID₅₀ unit may approach unity. If this were the case, then our procedures could not detect these polymers. Fourth, and most intriguing, the scrapie agent might be devoid of nucleic acid; our inability to identify a scrapie-specific nucleic acid is consistent with the resistance of the prion to inactivation by most procedures that hydrolyze or modify nucleic acids (Prusiner, 1982; Diener et al., 1982).

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References

- Alper, T., Cramp, W. A., Haig, D. A., & Clarke, M. C. (1967) *Nature (London)* 214, 764-766.
- Alper, T., Haig, D. A., & Clarke, M. C. (1978) *J. Gen. Virol.* 41, 503-516.
- Ambros, V., & Baltimore, D. (1978) *J. Biol. Chem.* 253, 5263-5266.
- Baringer, J. R., Wong, J., Klassen, T., & Prusiner, S. B. (1979) in *Slow Transmissible Diseases of the Nervous System* (Prusiner, S. B., & Hadlow, W. J., Eds.) Vol. 2, pp 111-121, Academic Press, New York.
- Baringer, J. R., Bowman, K., & Prusiner, S. B. (1981) *J. Neuropathol. Exp. Neurol.* 40, 329.
- Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J.* 133, 529-539.
- Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1982) *Science (Washington, D.C.)* (in press).
- Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem.* 46, 83-88.
- Dean, W. L., & Tanford, C. (1977) *J. Biol. Chem.* 252, 3551-3553.
- Diener, T. O., McKinley, M. P., & Prusiner, S. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 5220-5224.
- Eng, L. F. (1980) in *Proteins of the Nervous System* (Bradshaw, R. A., & Schneider, D. M., Eds.) 2nd ed., pp 85-117, Raven Press, New York.
- Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry* 10, 2606-2617.
- Gajdusek, D. C. (1977) *Science (Washington, D.C.)* 197, 943-960.
- Gerlich, W. H., & Robinson, W. S. (1980) *Cell (Cambridge, Mass.)* 21, 801-809.
- Ghosh, A., Dasgupta, R., Salerno-Rife, T., Rutgers, T., & Kaesberg, P. (1979) *Nucleic Acids Res.* 7, 2137-2146.
- Griffith, J., Huberman, J. A., & Kornberg, A. (1971) *J. Mol. Biol.* 55, 209-214.
- Hadlow, W. J., Prusiner, S. B., Kennedy, R. C., & Race, R. E. (1980) *Ann. Neurol.* 8, 628-631.
- Holzer, H., & Heinrich, P. C. (1980) *Annu. Rev. Biochem.* 49, 63-91.
- Kissane, J. M., & Robins, E. (1958) *J. Biol. Chem.* 233, 184-188.
- Laemmli, U. K. (1970) *Nature (London)* 227, 680-685.
- Lillehaug, J. R., & Kleppe, K. (1975) *Biochemistry* 14, 1221-1225.
- Luria, S. E., Williams, R. C., & Backus, R. C. (1951) *J. Bacteriol.* 61, 179-188.
- Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 560-564.
- McKinley, M. P., Masiarz, F. R., & Prusiner, S. B. (1981) *Science (Washington, D.C.)* 214, 1259-1261.
- Monroe, J. H., & Brandt, P. M. (1970) *Appl. Microbiol.* 20, 259-262.
- Palmiter, R. D. (1974) *Biochemistry* 13, 3606-3615.
- Peterson, G. L. (1977) *Anal. Biochem.* 83, 346-356.
- Polson, A., Potgieter, G. M., Largier, J. F., Mears, G. E. F., & Joubert, F. J. (1964) *Biochim. Biophys. Acta* 82, 463-475.
- Prusiner, S. B. (1982) *Science (Washington, D.C.)* 216, 136-144.
- Prusiner, S. B., Hadlow, W. J., Garfin, D. E., Cochran, S. P., Baringer, J. R., Race, R. E., & Eklund, C. M. (1978) *Biochemistry* 17, 4993-4999.
- Prusiner, S. B., Garfin, D. E., Baringer, J. R., Cochran, S. P., Hadlow, W. J., Race, R. E., & Eklund, C. M. (1979) in *Slow Transmissible Diseases of the Nervous System* (Prusiner, S. B., & Hadlow, W. J., Eds.) Vol. 2, pp 425-464, Academic Press, New York.
- Prusiner, S. B., Garfin, D. E., Cochran, S. P., McKinley, M. P., Groth, D. F., Hadlow, W. J., Race, R. E., & Eklund, C. M. (1980a) *J. Neurochem.* 35, 574-582.
- Prusiner, S. B., Groth, D. F., Cochran, S. P., Masiarz, F. R., McKinley, M. P., & Martinez, H. M. (1980b) *Biochemistry* 19, 4883-4891.
- Prusiner, S. B., Groth, D. F., McKinley, M. P., Cochran, S. P., Bowman, K. A., & Kasper, K. C. (1981a) *Proc. Natl. Acad. Sci. U.S.A.* 78, 4606-4610.
- Prusiner, S. B., McKinley, M. P., Groth, D. F., Bowman, K. A., Mock, N. I., Cochran, S. P., & Masiarz, F. R. (1981b) *Proc. Natl. Acad. Sci. U.S.A.* 78, 6675-6679.
- Prusiner, S. B., Cochran, S. P., Groth, D. F., Downey, D. E., Bowman, K. A., & Martinez, H. M. (1982) *Ann. Neurol.* 11, 353-358.
- Rekosh, D. M. K., Russell, W. C., Bellet, A. J. D., & Robinson, A. J. (1977) *Cell (Cambridge, Mass.)* 11, 283-295.
- Revie, D., Tseng, B. Y., Grafstrom, R. H., & Goulian, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 5539-5543.
- Rupley, J. A. (1967) *Methods Enzymol.* 11, 905-917.
- Schneider, W. C. (1957) *Methods Enzymol.* 3, 680-684.
- Schwerdt, C. E., & Fogh, J. (1957) *Virology* 4, 41-52.
- Stanley, J., Rottier, P., Davies, J. W., Zabel, P., & Van Kammen, A. (1978) *Nucleic Acids Res.* 5, 4505-4522.
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974a) *Biochemistry* 13, 5501-5507.
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974b) *Proc. Natl. Acad. Sci. U.S.A.* 71, 622-626.

Wildy, P., & Watson, D. H. (1962) *Cold Spring Harbor Symp. Quant. Biol.* 27, 25-47.
 Williams, R. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 2311-2315.

Wimmer, E. (1982) *Cell (Cambridge, Mass.)* 28, 199-201.
 Wold, F. (1981) *Annu. Rev. Biochem.* 50, 783-814.
 Yamamoto, K. R., & Alberts, B. M. (1970) *Virology* 40, 734-744.

X-ray Crystallographic and Nuclear Magnetic Resonance Spectral Studies of the Products from the Yeast Inorganic Pyrophosphatase-Co(NH₃)₄PP Reaction. Investigation of the Pyrophosphatase Reaction Mechanism[†]

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ABSTRACT: Yeast inorganic pyrophosphatase catalyzes the hydrolysis of P¹,P²-bidentate Co(NH₃)₄ pyrophosphate [Co(NH₃)₄PP] to the cis, bis(phosphate) complex Co(NH₃)₄(P_i)₂, which is not stable at neutral pH and over a period of 24 h converts to HPO₄²⁻ and a mixture of bidentate Co(NH₃)₄(P-O₄) and monodentate Co(NH₃)₄(H₂O)(HPO₄). Concurrent with this process is the reduction and subsequent release of Co(H₂O)₆²⁺ from the cobalt tetraammine bis(phosphate) complex and/or the cobalt tetraammine monophosphate complex. Bidentate tetraammine phosphatocobalt(III), hexaaquocobalt(II), orthophosphate, and two free water molecules cocrystallize [Co(NH₃)₄PO₄·Co(H₂O)₆²⁺·HPO₄²⁻·2H₂O] from

the reaction mixture in the triclinic space group *P* $\bar{1}$ (*Z* = 2) with cell dimensions *a* = 6.849 (1) Å, *b* = 11.693 (2) Å, *c* = 12.630 (2) Å, α = 65.60 (1)°, β = 88.98 (1)°, and γ = 73.04 (1)°. The structure was solved by the heavy atom technique and refined to an *R* index of 0.040 by using 3077 intensities measured up to a 2θ limit of 155°. ³¹P NMR studies of the equilibrium mixture reveal that the equilibrium constant is a sensitive function of solution pH and temperature. Unlike the Co(NH₃)₄PP complex, there is evidence indicating that the Mg(H₂O)₄PP complex is degraded to monodentate Mg(H₂O)₅PO₄ in the enzyme active site.

Yeast inorganic pyrophosphatase catalyzes the hydrolysis of inorganic pyrophosphate. The enzyme requires a minimum of two divalent cations per subunit for activity (Knight et al., 1981; Springs et al., 1981; Cooperman et al., 1981; Rapoport et al., 1973). Several models for metal-pyrophosphate or metal-phosphate interactions during catalysis have been presented (Knight et al., 1981; Konsowitz & Cooperman, 1976; Avaeva et al., 1977; Rapoport et al., 1973; Springs et al., 1981; Sperow & Butler, 1976). The results from studies in our laboratory are most consistent with a mechanism in which water coordinated to a second enzyme-bound metal ion adds to the phosphorus atom of the P¹,P²-bidentate metal-pyrophosphate complex resulting in the cleavage of the phosphoanhydride linkage and formation of *cis*-metal(H₂O)₄(P_i)₂. This complex may then be released directly from the enzyme or undergo further hydrolysis (of the M-O-P bond) prior to release. Magnesium, which serves as the metal cofactor *in vivo*, forms kinetically unstable complexes with polyphosphates, making isolation and structural characterization of the pyrophosphatase substrate, intermediate, and product metal complexes impractical. However, if Co(III)-complexed pyrophosphate is used as the substrate, the product complex should

be of sufficient stability to permit structural studies to be carried out. This study was undertaken to determine the structures and stability of the reaction products resulting from the pyrophosphatase-catalyzed hydrolysis of Co(NH₃)₄PP.

Materials and Methods

Preparation and Structure Analysis of Tetraammine-(phosphato)cobalt(III) Hexaaquocobalt(II) Hydrogen Phosphate (2-) Dihydrate Crystals. A 3-mL solution 10 mM in Co(NH₃)₄PP (Cornelius et al., 1977), 50 mM in Pipes [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (K⁺ salt, pH 7.5), 1 mM in Mg²⁺, and 300 units/mL in pyrophosphatase was allowed to proceed to equilibrium at 25 °C. After 24 h, most of the reaction product had crystallized. X-ray diffractometer intensity data were collected on these crystals (observed density = 2.02 g/cm³, calculated density = 2.010 g/cm³) using Ni-filtered Cu K α radiation (λ = 1.5418 Å). Out of a total of 3703 unique reflections measured up to a 2θ limit of 155°, 3077 reflections with *I*/ σ (*I*) greater than 1.5 were used for the structure analysis. The data were corrected for crystal decay (approximately 4%), Lorentz, and polarization effects. The data were also corrected for X-ray absorption (μ = 15.0 mm⁻¹) by using a two-parameter empirical absorption correction based on both ϕ and θ with a maximum ϕ correction of 33% at θ = 13° and 14% at θ = 64°.

A Patterson synthesis showed a very strong vector at *x*, *y*, *z* = (1/2, 0, 1/2), consistent with an intensity distribution: strong for *h* + *l* even and weak for *h* + *l* odd. This indicates the presence of a cobalt atom at (0, 0, 0) and (1/2, 0, 1/2) or alternatively at (1/4, 0, 1/4) and (3/4, 0, 3/4). The former possibility would be inconsistent with the *cis*, *bis* complex Co(NH₃)₄(P_i)₂, which was believed to be the crystalline py-

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