Novel Proteinaceous Infectious Particles Cause Scrapie

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Summary. After infection and a prolonged incubation period, the scrapie agent causes a degenerative disease of the central nervous system in sheep and goats. Six lines of evidence including sensitivity to proteases demonstrate that this agent contains a protein that is required for infectivity. Although the scrapie agent is irreversibly inactivated by alkali, five procedures with more specificity for modifying nucleic acids failed to cause inactivation. The agent shows heterogeneity with respect to size, apparently a result of its hydrophobicity; the smallest form may have a molecular weight of 50,000 or less. Because the novel properties of the scrapie agent distinguish it from viruses, plasmids, and viroids, a new term "prion" is proposed to denote a small *pro*teinaceous *in*fectious particle which is resistant to inactivation by most procedures that modify nucleic acids. Knowledge of the scrapie agent structure may have significance for understanding the causes of several degenerative diseases.

A major, unanswered question in molecular biology concerns the chemical structure of the scrapie agent. Until recently, mysteries surrounding the scrapie agent were so commonplace that investigators had come to accept rather than question its enigmatic properties. The scrapie agent causes a degenerative disorder of the central nervous system (CNS) in sheep and goats (*1*).

The extraordinary resistance of the scrapie agent to Formalin was responsible for the inadvertent inoculation of sheep in Scotland. Eighteen thousand animals were vaccinated against louping ill virus with a formalin-treated suspension of ovine brain and spleen that, as was shown subsequently, had been contaminated with the scrapie agent (*2*). Two years later, 1500 sheep developed scrapie. Subsequently, studies on CNS diseases (including scrapie) of sheep provided the foundation for Sigurdsson's concept of slow infections (*3*). In 1959, Hadlow suggested that kuru, a CNS degenerative disease of New Guinea highlanders, might be similar to scrapie because the pathologies of these disorders share many features (*4*). The transmission of kuru to chimpanzees in 1965 by Gajdusek, Gibbs, and Alpers forced a major reconsideration of the etiology of all degenerative disorders and made scrapie a subject of intense medical interest (*5*). Subsequently, Creutzfeldt-Jakob disease (CJD), a progressive, presenile dementia, was shown by Gibbs, Gajdusek, and co-workers to be caused by a transmissible agent (*6, 7*).

A recent study suggests that there may be similarities between the agents causing scrapie and CJD (*8*). Goats inoculated with brain tissue from demented patients dying of CJD developed a neurological disorder 3 to 4 years after inoculation (Fig. 1). Five out of ten CJD inocula have produced disease in goats (*9*). Experimental CJD in goats is indistinguishable both clinically and neuropathologically from natural scrapie. Monkeys have been used as a common experimental host for scrapie and CJD; curiously, chimpanzees are susceptible to CJD but not scrapie (*10*). Numerous attempts to link scrapie epidemiologically to CJD have been unsuccessful (*11*). At present, there is no direct evidence that the scrapie agent causes disease in humans.

In contrast to CJD which occurs worldwide, kuru is found only in a small mountainous region of Papua New Guinea. Epidemiological studies of kuru provide evidence for incubation periods of 20 to 30 years (*12, 13*). Although considerable evidence implicates cannibalism in the spread of kuru, no direct observations of cannibalistic acts in the "endemic" region have been recorded. Attempts to transmit kuru by feeding infected brain tissue to chimpanzees have been unsuccessful although one monkey developed a kuru-like illness 36 months after oral ingestion of the kuru agent (*14*). In contrast, goats fed scrapie-infected tissue frequently develop disease (*15*). Recently, we have taken advantage of the natural cannibalistic activities of hamsters to develop an experimental model of scrapie transmitted by cannibalism (*16*). Oral transmission of the scrapie agent appears to be extremely inefficient. Cannibalism requires a dose of agent 10^9 times greater than that needed to produce scrapie by intracerebral injection. These results provide compelling evidence for oral transmission of the scrapie agent and may offer new insights into the spread of kuru by cannibalism among the Fore people and their neighboring tribes.

Bioassay of the Scrapie Agent

Studies on the scrapie, kuru, and CJD agents have been greatly limited by the slow, tedious, and costly bioassays used to detect these agents. Since tissue culture systems are not available for the replication and assay of these agents and they appear to be nonantigenic in their native forms, animal bioassays must be used. For many years all assays for the scrapie agent were performed in sheep and goats (*17*). In 1961, transmission of the scrapie agent to mice transformed research (*18*), but the murine end-point titration assay was still heroic. Quantifying a single sample required eight to ten serial tenfold dilutions and injection of each dilution into six mice (*19*). Then 50 to 60 mice were held for 1 year and examined weekly for signs of scrapie. The number of animals developing scrapie at the highest dilution was used to calculate an end point. The time required for titration of a sample was reduced to 200 days when a more rapid form of the disease in hamsters was discovered (*20, 21*).

Several investigators have estimated scrapie titers by measuring the time interval from inoculation to onset of illness (incubation period) in mice (*22, 23*). Reluctance to refine such measurements has prevented its wide use in mice.

With hamsters, studies on the scrapie agent have been accelerated by development of a bioassay based on measurements of incubation time (*24, 25*). It is now possible to assay samples with the use of four animals in 60 to 70 days if the titers of the scrapie agent are high. As is shown in Fig. 2, the interval from inocu-

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lation to onset of illness (y) was inversely proportional to the dose injected intracerebrally into random bred weanling Syrian hamsters. The logarithm of the mean interval ($\bar{y}$) in days minus a time factor of 40 is a linear function of the logarithm of the dose over a wide range; the time factor was determined by maximizing the linear relation between the time interval and dose. With a factor of 40, the regression coefficient of the line is 0.87. A similar analysis was performed for the time interval from inoculation to death (z). With a time factor of 61, the regression coefficient of the line is 0.86. Linear relationships were also obtained when the reciprocals of the time intervals were plotted as a function of the logarithm of the dose.

Replication of the Scrapie Agent

The kinetics of scrapie agent replication in hamsters and mice are well documented (*21, 26*). After intracerebral inoculation of hamsters with 10^7 ID_{50} (median infectious dose) units, about 10^2 ID_{50} units can be recovered in the brain 24 hours later. During the next 50 days the amount of agent in the brain increases to 10^9 ID_{50} units. At this time the agent is widely distributed throughout the brain and no regional differences are apparent (*27*). The neuropathology is minimal and the animals exhibit no neurological dysfunction. During the next 10 to 15 days the animals develop ataxia, difficulty righting themselves from a supine position, generalized tremor, and head bobbing. By 60 to 70 days, vacuolation of neurons and astrogliosis are found throughout the brain, even though the titer of the agent remained constant. Thus, the "pathological hallmarks" of this spongiform encephalopathy do not correlate with the extent of agent replication. In addition, the spongiform pathology that characterizes kuru and CJD is inconspicuous in natural scrapie (*28*).

Hypothetical Structures for the Scrapie Agent

Investigators have been aware of the unusual properties of the scrapie agent for more than three decades. Hypotheses on the chemical structure of the scrapie agent have included: sarcosporidia parasite (*29*), "filterable" virus (*30*), small DNA virus (*31*), replicating protein (*32*), replicating abnormal polysaccharide within membranes (*33*), DNA subvirus controlled by a transmissible linkage substance (*34*), provirus consisting

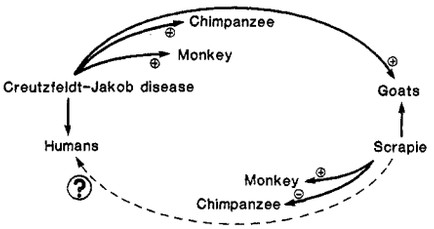

Fig. 1. Experimental relation between Creutzfeldt-Jakob disease of humans and scrapie of goats and sheep.

of recessive genes generating RNA particles (*35*), naked nucleic acid similar to plant viroids (*36*), unconventional virus (*12, 37*), aggregated conventional virus with unusual properties (*38*), replicating polysaccharide (*39*), nucleoprotein complex (*40*), nucleic acid surrounded by a polysaccharide coat (*41*), spiroplasma-like organism (*42*), multicomponent system with one component quite small (*43*), and membrane-bound DNA (*43, 44*).

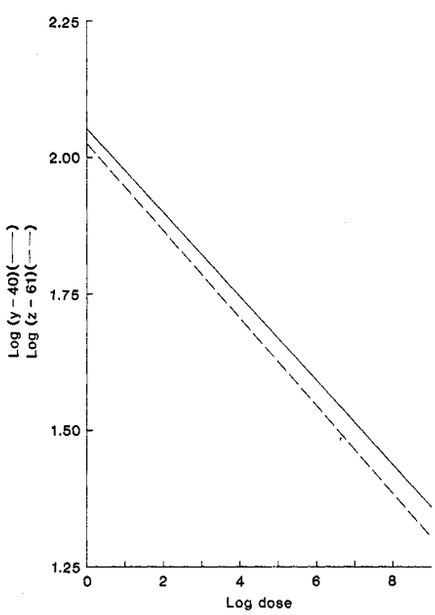

Fig. 2. Assay of the scrapie agent by measurements of the incubation time interval. The intervals from inoculation to onset of illness (y) and to death (z) are plotted as a function of the injected dose. Equations were written to describe these linear functions which relate the titer of the inoculum to the time intervals both from inoculation to onset of illness (Eq. 1) and from inoculation to death (Eq. 2):

$$\text{Log } T_y = 26.66 - (12.99) \log (\bar{y} - 40) - \log D \quad (1)$$

$$\text{Log } T_z = 25.33 - (12.47) \log (\bar{z} - 61) - \log D \quad (2)$$

where T is the titer expressed in ID_{50} units per milliliter, D is the dilution defined as the fractional concentration of the diluted sample, $\bar{y}$ is the mean interval from inoculation to onset of clinical illness in days, and $\bar{z}$ is the mean interval from inoculation to death in days. The most precise estimate of titer is obtained by calculating a weighted average for T_y and T_z.

Purification and Hydrophobicity of the Scrapie Agent

Several investigators have mounted major efforts to purify and characterize the scrapie agent over the past two decades (*45–48*). Early studies suggested that the scrapie agent was distributed throughout virtually all subcellular fractions (*45, 46*). The interpretation of those observations was complicated by the imprecision of the end-point titrations of the agent. Nevertheless, the scrapie agent was reported to be intimately associated with cellular membranes, and from this association the "membrane hypothesis" evolved (*33*). When various extraction procedures failed to release the agent from membrane fractions, it was concluded that the agent is a replicating membrane fragment that cannot be separated from cellular membranes.

Several different purification procedures have been reported. One involved copurification of the scrapie agent and microsomes (*49*). Another involved isolation of a "membrane-free" fraction after prolonged ultracentrifugation (*50*). This fraction contained 1 to 10 percent of the scrapie agent and was precipitated with ammonium sulfate. Sodium dodecyl sulfate (SDS) gel electrophoresis was used to obtain a further purification (*51*). Although the results of these studies seemed encouraging initially, subsequent work has been disappointing (*52*).

Using equilibrium sucrose and sodium chloride density gradients, Siakotos, Gajdusek, Gibbs, and co-workers have attempted to purify the scrapie agent from murine brain (*53*). They suggested that there was a peak of infectivity at a sucrose density of 1.19 g/cm^3. However, multiple peaks of infectivity were found throughout the gradients, an indication of considerable heterogeneity with respect to density and showing that the technique when applied to crude suspensions of membranous material from brain is probably not useful in isolating the scrapie agent. Other studies from the laboratory of Gajdusek have shown considerable heterogeneity of the agent in metrizamide and cesium chloride density gradients (*54*).

Since the initial purification of many biological macromolecules involves a series of differential centrifugations (*55*), we began our studies on the scrapie agent by defining its sedimentation properties in fixed-angle rotors in order to develop a preparative protocol (*56*). These studies showed that the agent from both murine spleen and brain sedimented over a range of particle sizes from 60*S* to 1000*S* (*19*).

Table 1. Molecular properties of the scrapie agent: evidence that it contains a protein. Abbreviations: PMSF, phenylmethylsulfonylfluoride; DEP, diethyl pyrocarbonate; TX-100, Triton X-100; OGS, octylglucoside; SB 3-14, sulfobetaine 3-14; ET-12H, 1-dodecyl propanediol-3-phosphorylcholine; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; Gdn, guanidinium; NP40, Nonidet P-40; Sarkosyl, sodium dodecyl sarcosinate; TCA, trichloroacetic acid; SCN, thiocyanate.

Treatment	Stable	Labile
Protease digestion		Proteinase K, trypsin
Chemical modification		DEP, butanedione, PMSF
Detergents	TX-100, NP40, OGS, SB 3-14, ET-12H, cholate, Sarkosyl	SDS, LDS
Ions	Na^+, K^+, Cl^-, SO_4^{-2}, $EDTA^{-4}$, PO_4^{-3}	Gdn^+, SCN^-, TCA^-
Denaturants		Urea
Organic solvents	Methanol, ethanol	Phenol

On the basis of the information derived from these sedimentation profiles, a partial purification scheme for the murine scrapie agent from spleen was derived (*57*). The preparation was devoid of cellular membranes, and enriched for the scrapie agent 20- to 30-fold with respect to protein and DNA. Studies on the agent by rate-zonal sucrose gradient centrifugation gave sedimentation coefficients for the agent ranging from 40*S* to > 500*S*. Sucrose density gradient centrifugation revealed a particle density ranging from 1.08 to more than 1.30 g/cm^3, an indication that some forms of the agent might be associated with lipids. Further sedimentation studies showed that the agent aggregated with cellular elements on heating the agent in a partially purified fraction (*58*). The agent was stable in nonionic and nondenaturing anionic detergents, but was inactivated by SDS. Free-flow electrophoresis showed that most of the agent has a net negative charge, but significant charge heterogeneity was found.

Heterogeneity of the scrapie agent with respect to size, density, and charge suggested that hydrophobic domains on its surface might be responsible for these phenomena. Such domains are usually formed by the juxtaposition of nonpolar side chains of amino acids within a protein (*57, 59*).

These initial studies on the murine agent from spleen revealed the complexities of scrapie agent purification. We then developed an improved assay based on measurements of the incubation time. With this new bioassay, we created a purification scheme for the agent from hamster brain, where the titers are highest (*21*). The initial steps of the purification were similar to those for the murine agent (*57*). Deoxycholate extracts (P_4) were digested sequentially with micrococcal nuclease and proteinase K. The digestions were performed at 4°C to prevent aggregation of the agent, which is observed at elevated temperatures (*59*). The digested preparations were then subjected to cholate-Sarkosyl extraction followed by ammonium sulfate precipitation (P_5). Most of the remaining digested proteins and nucleic acids were separated from the scrapie agent by Sarkosyl agarose gel electrophoresis at 4°C (*60*). Such preparations of the eluted scrapie agent (E_6) were 100- to 1000-fold purified with respect to cellular protein (*60*). With these enriched preparations we demonstrated that a protein within the agent is required for infectivity (*60*). Fraction E_6 contains (per milliliter) $10^{6.5}$ to $10^{8.5}$ ID_{50} units of agent, 20 to 50 μg of protein, < 1 μg of DNA, and < 10 μg of RNA.

Since the ratio of particle number to infectivity unit for the scrapie agent is unknown, the extent of purification required to obtain homogeneous preparations is unknown. Each hamster brain contains 10^9 ID_{50} units of the scrapie agent and 100 mg of protein. Assuming an average molecular weight of 50,000 for all proteins and a ratio of particle number to infectivity unit of 1, a 10^7-fold purification will be required to prepare a pure preparation of the agent. However, if the particle to infectivity ratio is 1000, then a 10^4-fold purification will be required. Experience with assaying conventional viruses in animals suggests that the particle to infectivity ratio for the scrapie agent may be considerably greater than 1. These calculations indicate the need for a source of the scrapie agent with considerably higher titers. Attempts to propagate the scrapie agent in cell culture have been disappointing.

The hydrophobicity of the scrapie agent has complicated purification as described above. Further evidence for the hydrophobicity of the scrapie agent comes from its binding to phenyl-Sepharose (*60*). The agent could not be eluted in 8.5*M* ethylene glycol; however, inclusion of 4 percent Nonidet P-40 and 2 percent Sarkosyl in the ethylene glycol eluate resulted in the almost quantitative recovery of the agent from phenyl-Sepharose. In addition, the hydrophobicity was reflected by diminished titers when detergent was removed from fraction E_6 (*60*). Presumably this decrease in infectivity was due to aggregation.

Not only is the hydrophobic nature of the scrapie agent important with respect to purification, it may also explain some of its enigmatic properties. That hydrophobic interactions increase with elevated temperature may be reflected in the extreme heat stability of the agent (*47*). Numerous unsuccessful attempts to detect antibodies against the native scrapie agent in fraction E_6 might be explained by its hydrophobicity (*61*). Hydrophobic proteins in their native state are sometimes poor antigens (*62*). An alternative (and attractive) explanation for the apparent lack of immunogenicity of the scrapie agent evolves from the possibility that the agent may be closely related to a normal cellular protein to which the host does not produce antibodies.

Scrapie Agent Contains Protein

Six separate and distinct lines of evidence show that the scrapie agent contains a protein that is required for infectivity: (i) inactivation as a result of digestion with proteinase K, (ii) inactivation by chemical modification with diethyl pyrocarbonate, (iii) inactivation by SDS, (iv) inactivation by chaotropic salts such as guanidinium thiocyanate, (v) inactivation by phenol, and (vi) inactivation by urea (*60*). The cumulative evidence for a protein within the scrapie agent appears to be compelling (Table 1).

Digestion with crystalline proteinase K inactivated the scrapie agent from hamster brain (*60*); the decrease in titer was a function of enzyme concentration, temperature, and time of digestion. Prior treatment of proteinase K with the protease inhibitor phenylmethylsulfonylfluoride (PMSF) completely abolished the protease-catalyzed degradation of the agent. Digestion with trypsin also destroyed the scrapie agent. The protease sensitivity of the scrapie agent was revealed only after considerable purification (fraction E_6). Other investigators have occasionally observed decreases in scrapie titers after addition of proteases (*48, 63*).

Carbethoxylation by diethyl pyrocarbonate also inactivated the purified scrapie agent (*60, 64*), but activity was restored by treatment with hydroxylamine. This reversibility of the inactive, chemically modified agent provides a further

Table 2. Resistance of the scrapie agent to procedures that attack nucleic acids. Abbreviations: AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen; HEP, 1-α-4′ hydroxyethylpsoralen; HMT, 4′hydroxymethyl 4,5′,8-trimethylpsoralen; MMT, 4′-methoxymethyl-4,5′,8-trimethylpsoralen; TMP, 4,5′,8-trimethylpsoralen.

Procedure	Resistant	Labile	Possible explanations
*p*H	H^+	OH^-	Hydrolysis of RNA genome, denaturation of dsDNA genome, or protein denaturation
Nucleases	Ribonucleases, deoxyribonucleases		Enzymes unable to penetrate protein shell
UV irradiation	254 nm		Shielded by protein shell or no critical nucleotide dimers formed
Divalent cation hydrolysis	Zn^{2+}		Ions unable to penetrate protein shell
Psoralen photoreaction	AMT, HEP, HMT, MMT, TMP		Monoadducts of single-stranded genome do not inactivate or psoralens unable to penetrate protein shell
Chemical modification	Hydroxylamine		Nucleophiles react only with surface protein and are unable to penetrate the shell or react minimally with double-stranded genome

argument for a protein target. More recent data on chemical modification indicate that the scrapie agent is also inactivated by 10 m*M* butanedione and 2 m*M* PMSF. Butanedione modifies arginine (*65*), lysine, and histidine residues; modifications of one or more of these amino acid residues may explain its effect on the scrapie agent.

Three reagents used to denature proteins and isolate biologically active nucleic acids (*66*) also inactivate the agent. First, SDS diminished the scrapie agent titer when the ratio of SDS (grams) to protein (grams) exceeded 1.8 (*24*). In contrast, the agent was stable in various nondenaturing ionic and nonionic detergents (Table 1). Simultaneous addition of a nonionic detergent and SDS to a preparation containing the scrapie agent prevented the inactivation observed with SDS alone. Second, studies with chaotropic ions have shown that in low concentrations they inactivate the agent (*24, 67*). Irreversible inactivation of the agent was found upon exposure to 1*M* guanidinium thiocyanate at 4°C for 3 hours. Higher concentrations of less potent chaotropic salts were required to achieve irreversible inactivation. Third, phenol, useful in the isolation of nucleic acids, inactivates the scrapie agent (*68*). In contrast, the agent is stable in methanol and ethanol, but is readily precipitated. Extraction with phenol, a potent denaturant of protein, under various salt and *p*H conditions destroyed infectivity (*24*). In the above studies partially purified preparations were first digested with proteinase K to prevent the formation of an interface in which the agent might be trapped. We have attempted to restore scrapie agent infectivity from phenol extracted preparations by incorporation into liposomes and by transfection into cultured cells. Using reverse-phase liposome formation (*69*), no infectivity was recovered from the aqueous phase, dialyzed phenol phase, or combination of these two phases (*70*). Preparations of phenol extracted DNA and RNA from scrapie-infected murine spleen failed to produce infectious scrapie agent upon transfection of L cells (*71*). Similar transfection experiments with murine fetal brain cells and embryonic fibroblasts also failed to produce infectious agent (*72*). From all of these studies with chemical reagents that denature proteins but permit isolation of biologically active nucleic acids, we conclude that denaturation of a protein within the scrapie agent leads to inactivation of the infectious particle. Moreover, CJD agents adapted to guinea pigs and mice are also inactivated when extracted with phenol (*73*).

Hunter and co-workers showed that exposure of the scrapie agent to 6.0*M* urea decreased the titer by a factor of 100 (*74*). This high concentration of urea could have denatured protein or nucleic acid. We have found that exposure of the scrapie agent in partially purified fractions to 3*M* urea at 4°C decreases the titer by a factor of 50 (*70*). Removal of the urea after 2 hours was not accompanied by a return of infectivity. This observation contrasts with other findings where removal of the KSCN was accompanied by an apparent return of infectivity (*67*). Whether urea or cyanate ions are responsible for the loss of scrapie infectivity in these experiments (*75*) is not known. From our data the most likely target within the scrapie agent for denaturation by urea is a protein.

The functions of a protein or proteins within the scrapie agent are unknown. The hydrophobicity of the protein should allow it to penetrate membranes, but whether or not there are specific receptors on cell surfaces to which the scrapie protein might bind is unknown. Studies on transmission of scrapie by cannibalism in hamsters suggest that the scrapie agent is transported across epithelial cells and then presumably enters the bloodstream (*16*). Manuelidis and co-workers have found the CJD agent in white blood cells (*76*). Studies by Kimberlin and Walker suggest that the agent may be transported within axons much like rabies virus (*77*). One possibility is that the protein is a polymerase that is necessary for replication of a putative nucleic acid within the agent. This would explain the protein requirement for infectivity and would be similar to negative strand viruses. We also must consider the possibility that the scrapie protein acts as an inducer or as a template for its own synthesis.

Search for Nucleic Acid in Scrapie Agent

In our search for a nucleic acid genome within the scrapie agent, we subjected the agent to changes in *p*H (Table 2). Although other investigators had indicated that the agent was stable over a *p*H range from 2 to 10.5 (*78*), our observations do not agree with some of these earlier studies. We have found that the titer of the scrapie agent is irreversibly reduced by alkali (*67*). The titer was reduced by a factor of 1000 on exposure to *p*H 10 for 1 hour at 4°C or by a factor of 100 on exposure to *p*H 9 for 1 hour at 37°C (*70*). Neutralization with acid did not restore infectivity. In contrast, no loss of infectivity at *p*H 3 was observed over a 16-hour period at 37°C. One interpretation of these studies is that alkali hydrolyzed a few phosphodiester bonds within a scrapie nucleic acid rendering the agent inactive. The covalent backbone in RNA is labile to alkali while that in DNA is generally stable; however, base modifications such as methylation of purines render DNA labile in alkali (*79*). Denaturation of double-stranded DNA (dsDNA) in alkali is also well doc-

umented (*80*). Alternatively, inactivation by alkali under rather mild conditions could be due to protein denaturation. Unfortunately, the lack of specificity in these *p*H stability studies does not allow us to make a definitive statement concerning the presence or absence of a nucleic acid within the scrapie agent.

Over the past 15 years, two techniques with high degrees of specificity have suggested that the scrapie agent might not contain a nucleic acid. The scrapie agent in crude preparations has been found to be resistant to nuclease digestion (*46, 48, 59*) and to ultraviolet (UV) irradiation at 254 nm (*81, 82*). The objection to these studies was that a protective coat prevented nucleases from penetrating the agent, as well as shielding it from radiation.

At several different stages of purification we have searched for susceptibility of the agent to nuclease digestion. No decrease in scrapie infectivity has been observed with micrococcal nuclease, nuclease P, deoxyribonucleases I and II, ribonucleases A and T_1, and phosphodiesterases I and II at 10, 100, and 500 μg/ml for 3 to 30 hours at 37°C. Ribonucleases III and H at 1 and 10 unit/ml also showed no effect. Although nuclease sensitivity has been described for the scrapie agent (*44*), we have been unable to confirm this observation (*52*).

The complete lack of scrapie agent sensitivity to nucleases in view of inactivation by proteases is of interest. Numerous viruses are resistant to nucleases; presumably, these enzymes do not penetrate the viral protein coats (*83*). In contrast, addition of ribonuclease A at 0.1 μg/ml to a crude nucleic acid extract containing potato spindle tuber viroid (PSTV) decreased the PSTV titer by a factor of $>10^6$ in 1 hour at 25°C (*84*). Hydrolysis of a single phosphodiester bond within a viroid probably inactivates it (*85, 86*). There are many examples of proteins that retain their biological activities after limited proteolysis (*87*). We do not know in the case of the scrapie agent how many peptide bonds must be cleaved to cause inactivation.

Studies with the optically clear fraction E_6 have confirmed the resistance of the scrapie agent to UV-inactivation (*81, 82*). Fractions S_2, P_5, and E_6 were irradiated at 254 nm with increasing doses. Although no inactivation of the agent in fraction S_2 was observed, a minimal but probably significant decrease was found in fractions P_5 and E_6 as a function of dose (*88*). The kinetics of inactivation by irradiation at 254 nm suggest a single-hit process. The survival of 37 percent of the scrapie agent in fractions P_5 and E_6 was observed after a UV dose (D_{37}) of 42,000 J/m^2. The resistance of the scrapie agent to irradiation at 254 nm is compared to that observed for viruses and viroids in Table 3. Clearly, the inactivation of the scrapie agent at these extreme energy levels indicates a photochemistry of a far different nature from that observed for virus inactivation through the formation of thymine or uracil dimers. Proteins are relatively resistant to irradiation at 254 nm (*89*) and are probably the target within the scrapie agent in these irradiation studies.

Table 3. Inactivation of small infectious agents by UV irradiation at 254 nm.

Example	D_{37} (J/m^2)*
Bacteriophage T2	4
Bacteriophage S13	20
Bacteriophage φX174	20
Rous sarcoma virus	150
Polyoma virus	240
Friend leukemia virus	500
Murine leukemia virus	1,400
Potato spindle tuber viroid	5,000
Scrapie agent	42,000

*Data from (*82, 85, 88*). D_{37} is the dose of irradiation that permits 37 percent survival.

Observations on the resistance of the scrapie agent to procedures attacking nucleic acids have been extended by means of three other techniques (Table 2). The agent has been incubated at *p*H 7 in the presence of 2 m*M* $Zn(NO_3)_2$ at 65°C for periods as long as 24 hours without loss of infectivity (*70*). Under these conditions polymers of RNA are completely reduced to mononucleotides, and polymers of DNA undergo considerable hydrolysis (*90*). Photochemical inactivation of the scrapie agent with psoralens was attempted with samples at several levels of purification, both from murine spleen and hamster brain. Five different psoralens of varying degrees of hydrophobicity were used (*91*). It was expected that the most hydrophobic psoralens readily partitioned into the scrapie agent. No inactivation of the scrapie agent was observed with any of these psoralens over a wide range of dosages (*92*). Psoralens may form diadducts upon photoactivation within base-paired regions of nucleic acids and monoadducts within single-stranded regions (*93*). Psoralens have several advantages in searching for a nucleic acid genome: (i) low reactivity with proteins, (ii) penetration of viral protein and lipid coats, and (iii) formation of stable covalent linkages on photoactivation. Psoralens have been found to inactivate numerous viruses, but not, for example, picornaviruses (*94*). Psoralens, like acridine orange and neutral red dyes (*95*), do not penetrate the protein coat of poliovirus. Photoadducts with viral RNA were formed when psoralens or the above tricyclic dyes were added to cultured cells replicating the poliovirus.

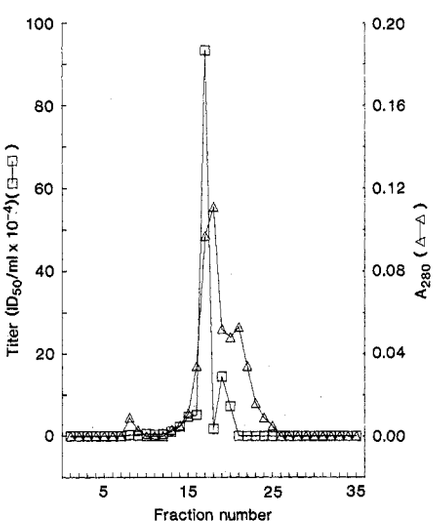

Fig. 3. Molecular sieve chromatography of the scrapie agent in sulfobetaine 3-14. Fraction P_5 was exposed to 10 percent (weight to volume) sulfobetaine 3-14 for 16 hours at 4°C and then chromatographed in 60 m*M* tris-acetate, *p*H 7.2 containing 1 m*M* EDTA and 0.024 percent sulfobetaine 3-14 over a molecular sieve (Toya Soda TSK 4000) column (0.75 cm, inside diameter, by 50 cm, length). The column was developed at 4°C (Varian 5000 HPLC) at 1 ml/min. Fractions (1 ml) were collected and assayed in hamsters by measurements of incubation time intervals. Blue dextran, bovine serum albumin, and tryptophan under identical conditions eluted in fractions 7, 15, and 22, respectively.

In contrast to psoralens, hydroxylamine readily inactivates poliovirus at neutral *p*H (*96*). Hydroxylamine does not generally react with proteins at neutral *p*H, but it does decarbethoxylate modified proteins and it does modify cytosine bases (*97*). At concentrations up to 0.5*M* at neutral *p*H hydroxylamine failed to alter scrapie agent infectivity (*64*). Under these conditions, most animal and plant viruses as well as bacteriophage are inactivated by hydroxylamine (*98*), except for the paramyxoviruses, which are resistant. In contrast, inactivation of the scrapie agent by carbethoxylation upon treatment with diethyl pyrocarbonate was found to be reversible with NH_2OH (*64*).

The extreme resistance of the scrapie agent to inactivation suggests that its structure is different from that of viruses. While there are examples of viruses that are resistant to inactivation by two or even three of the six procedures in Table 2, we are unaware of any viruses which, like the scrapie agent, are resistant by all

of these procedures. However, the possibility must be considered that the putative genome of the scrapie agent is buried within a tightly packed protein shell which excludes nucleases, UV irradiation, Zn^{2+}, psoralens, and NH_2OH. Also, we cannot exclude an unusual nucleic acid with a different base structure or polymer packing that might exhibit the resistant characteristics described for the scrapie agent.

Of interest are studies showing a large oxygen effect upon exposure of the scrapie agent to ionizing radiation (*99*). Viruses and nucleic acids characteristically show a small oxygen effect. Biological membranes and probably lipoproteins show large oxygen effects. The increased sensitivity of the scrapie agent to ionizing radiation in the presence of oxygen presumably reflects the hydrophobic protein with bound lipids that is required for infectivity (*60*). These data do not eliminate the possibility that the agent also contains a nucleic acid.

Molecular Size of the Scrapie Agent

The extreme resistance of the scrapie agent to inactivation by ionizing radiation raised the possibility that the agent is quite small (*100*). Target calculations have given minimum molecular weights ranging from 64,000 to 150,000 (*82, 100*). However, two important factors could not be taken into account in these calculations. The first is the possibility that multiple copies of the agent might exist within a single infectious particle as would occur with aggregation. We have good evidence that the agent readily associates with cellular elements and probably aggregates with itself in purified preparations (*47, 57, 58*). The second is the efficiency of the cellular repair processes. For example, polyoma virus dsDNA (3×10^6 daltons) has been found to be almost as resistant to ionizing radiation as either viroids or the scrapie agent (*101*). The extreme efficiency of the cellular repair processes for the polyoma virus dsDNA genome accounts for its apparent resistance to damage by ionizing radiation (*82*).

Studies on the scrapie agent in murine spleen have shown a continuum of sizes ranging from 40*S* or less to more than 500*S* by rate-zonal sucrose gradients (*47, 57*). Parvoviruses are among the smallest viruses identified and they have sedimentation coefficients of 100*S* to 110*S* (*83*). The scrapie agent in preparations extracted with sodium deoxycholate associated with cellular elements when heated to form large infectious particles of >10,000*S* (*47, 58*). Such particles are the size of mitochondria. Sedimentation studies of CJD agents adapted to both guinea pigs and mice suggest that the sizes of these agents are similar to that observed for the scrapie agent (*73*).

Table 4. Properties of the scrapie agent.

Stable at 90°C for 30 minutes
Low molecular weight infectious particles (minimum estimate, 50,000 daltons or less)
Hydrophobic protein (or proteins) is required for infectivity
Resistant to ribonucleases and deoxyribonucleases
Resistant to UV irradiation at 254 nm
Resistant to psoralen photoadduct formation
Resistant to Zn^{2+} catalyzed hydrolysis
Resistant to NH_2OH chemical modification

Gel electrophoresis has also shown that the scrapie agent exists as a succession of particles of varying size (*52, 59*). Sarkosyl agarose gel electrophoresis of partially purified fractions showed that some forms migrated more slowly than DNA restriction endonuclease fragments of 15×10^6 daltons. Some smaller forms of the agent migrated ahead of 3×10^5 dalton DNA fragments. Digestion of crude preparations with nucleases and proteases facilitated the entry of the agent into these gels. One report showed that most of the scrapie agent migrated with 5*S* RNA molecules in the presence of SDS (*51*). We were unable to confirm these findings since SDS inactivated the agent (*24, 52*).

Until recently, gel filtration studies with anionic detergents and chaotropic ions have given results similar to those described for rate-zonal sucrose gradients and gel electrophoresis. Typically most of the agent eluted in the void volume followed by a continuum of particles apparently of decreasing size (*59, 67*). In contrast, incubation of the scrapie agent overnight with 10 percent (weight to volume) sulfobetaine 3-14, a zwitterionic detergent, appears to have dissociated the agent (Fig. 3) (*70*). Under these conditions the scrapie agent eluted as a peak behind bovine serum albumin (BSA), but slightly ahead of ovalbumin. If the agent has a globular shape in sulfobetaine 3-14, then it may have a molecular size of 50,000 daltons or less. How much detergent is bound to the agent and how the detergent influences the apparent molecular weight of the agent remains to be determined (*102*). Similar observations have been recorded with another detergent, 1-dodecyl propanediol-3-phosphorylcholine, which is a synthetic derivative of lysolecithin. Confirmation of these findings by rate-zonal sucrose gradient centrifugation is awaited since anomalous behavior of proteins during gel filtration is well known (*103*). Thus, the monomeric form of the scrapie agent may indeed be considerably smaller than that of a viroid, which until now has been the smallest infectious agent known.

If the scrapie agent does have a molecular weight of 50,000 or less, then a nucleic acid within such a globular structure will be too small to code for a protein. A spherical scrapie agent of molecular weight 50,000 would have a diameter of 4 to 6 nm (*104*). Let us assume that the agent has a protective protein which is 1 nm (10 Å) thick. The volume of the core will be 14.1 nm^3. From measurements of DNA packing in crystals and bacteriophage (*105*), there is space for a 12-nucleotide polymer consisting of six base pairs. Dehydration of the polymer would permit 32 nucleotides to be encapsidated. Indeed, if such oligonucleotides exist within the agent, they must have a function other than that of a template directing the synthesis of scrapie coat proteins.

Novel Properties of the Scrapie Agent

The foregoing summary of experimental data indicates that the molecular properties of the scrapie agent differ from those of viruses, viroids, and plasmids (Table 4). Its resistance to procedures that attack nucleic acids, its resistance to inactivation by heat, and its apparent small size all suggest that the scrapie agent is a novel infectious entity. Because the dominant characteristics of the scrapie agent resemble those of a protein, an acronym is introduced to emphasize this feature. In place of such terms as "unconventional virus" or "unusual slow virus-like agent," the term "prion" (pronounced *pree-on*) is suggested. Prions are small *pro*teinaceous *i*nfectious particles which are resistant to inactivation by most procedures that modify nucleic acids. The term "prion" underscores the requirement of a protein for infection; current knowledge does not allow exclusion of a small nucleic acid within the interior of the particle.

Our data and that of other investigators suggest two possible models for the scrapie agent: (i) a small nucleic acid surrounded by a tightly packed protein coat or (ii) a protein devoid of nucleic acid, that is, an infectious protein. While the first model might seem the most plausible, there is no evidence for a nucleic acid within the agent. The sec-

ond model is consistent with the experimental data but is clearly heretical. Skepticism of the second model is certainly justified. Only purification of the scrapie agent to homogeneity and determination of its chemical structure will allow a rigorous conclusion as to which of these two models is correct.

There seems to be little advantage in championing one model over another; however, several previously postulated structures for the scrapie agent can now be discarded. The requirement of a protein for infectivity eliminates the possibilities that the scrapie agent is composed entirely of polysaccharide or nucleic acid. Thus, the replicating polysaccharide and naked nucleic acid–viroid hypotheses are no longer viable. The hypothetical nucleic acid surrounded by a polysaccharide coat can also be eliminated. Studies demonstrating the small size of the scrapie agent clearly distinguish it from conventional viruses, spiroplasma-like organisms, and parasites such as sarcosporidia.

Rigid categorization of the scrapie agent at this time would be premature. Determination of its molecular structure will be required prior to deciding whether prions represent a distinct subgroup of extraordinarily small viruses or a completely different type of pathogen which lacks a nucleic acid genome.

How Do Prions Replicate?

One of the fascinating questions about prions concerns their mode of replication. If prions do not contain a nucleic acid genome, then studies on the replication of prions may reveal unprecedented mechanisms of reproduction.

The first possibility is that prions contain a protected nucleic acid and that, like a viral genome, it codes for the protein shell (Table 5). The hypothetical prion genome could derive its protection from the protein-lipid coat or from an unusual chemical structure. Such an unusual genomic structure might confer upon prions the characteristics of proteinaceous particles that are resistant to most procedures that attack nucleic acids.

Alternatively, prions may contain an oligonucleotide that acts as a regulatory element instead of a coding template. This oligonucleotide might act as an inducer to promote the synthesis of prions. Small nuclear RNA's are thought to be regulatory elements controlling the splicing of genes (*106*). If the postulated nucleic acid within the scrapie agent does not code for the protein (or proteins) in its coat, then this would be a major feature distinguishing prions from viruses.

Table 5. Possible mechanisms of prion replication.

Prions contain undetected nucleic acids
Code for prion protein (or proteins)
Activate transcription of host genes coding for prion protein
Prions are devoid of nucleic acids
Activate transcription of host genes coding for prion protein
Code for their own replication by
Reverse translation
Protein-directed protein synthesis

The second possibility is that prions are, in fact, devoid of nucleic acid. If this is the case, then alternative modes of replication for these infectious proteins must exist (Table 5). The macromolecular information required for the synthesis of prions must be contained either in the host cell or in the prion itself.

If cellular genes coding for the scrapie prion do exist, then they are highly regulated, not readily activated, and present in various mammalian cells ranging from mice to monkeys. It is pertinent that hundreds of mice and hamsters inoculated with homogenates from the brains of control animals have never developed a neurological disorder (*9*, *107*). These animals have been observed for up to 1 year, a period of time sufficient to detect one infectious unit in the inoculum. An occasional activation of such cellular genes might explain the sporadic occurrence of CJD with an incidence of $1/10^6$ (*11*). A few clusters of CJD with higher rates of incidence have been identified, and 10 percent of CJD cases are familial. The molecular mechanism by which prions might activate cellular genes which code for their biosynthesis is unknown. The emerging story of oncogenes within retroviruses and their cellular counterparts provides an interesting analogy (*108*).

In addition, we must account for the evolutionary pressure that preserves such hypothetical cellular genes that code for prions. Perhaps these hypothetical genes code for some necessary, related protein or proteins when they are under normal regulation. It may be that tolerance to a normal, cross-reacting gene product might allow the scrapie prion to replicate unnoticed by the immune system. Another explanation for tolerance toward the scrapie agent involves selective suppression of small populations of potentially reactive lymphocytes (*109*).

Alternatively, prions could code for their own biosynthesis. This hypothesis contradicts the "central dogma" of molecular biology (*110*). Unorthodox mechanisms such as reverse translation or protein-directed protein synthesis would allow prions to replicate (*111*). We have no precedents for either of these synthetic processes in biology. The possibility that prions are devoid of nucleic acid should be compared to early studies on crystalline tobacco mosaic virus where no RNA was found, and Stanley suggested that the protein of tobacco mosaic virus was autocatalytic (*112*).

Relevant to the mechanism by which the scrapie agent replicates are two observations. First, the various strains of the scrapie and CJD agents have been identified by repeated passage at limiting dilution and by their host range in experimental animals (*45*, *113*, *114*). Second, adaptation of the agent has been observed upon repeated passage in the same host species as evidenced by a reduction in the length of the incubation period (*21*, *113*, *115*). Hadlow observed that the scrapie agent when passaged in mink retains its ability to infect goats, but loses its ability to infect mice (*116*). The agent causing mink encephalopathy has a similar host range (*20*). While adaptation is most readily explained by modification of a nucleic acid genome within the agent, multiple host genes coding for several agents could also explain these observations. The presence of multiple genes coding for different proteins with the same biological activities is emphasized by the occurrence of differing interferons (*117*).

The genetics of the host clearly influences the length of the incubation period for scrapie. Dickinson *et al.* have identified in mice two genetic loci that influence the length of the incubation period (*23*, *118*). In a survey of immunodeficient mice, we found that NZB and NZB × W F_1 mice inoculated intracerebrally have an incubation period of similar magnitude to that found in BALB/c and C57/B1 mice (*61*). In contrast, NZW mice have a significantly shorter incubation period. Further studies with F_2 backcrosses are required to determine if a single gene is responsible for these differences. From these studies and those on the murine CJD agent, we conclude that longer incubation time alleles are autosomal dominant. Murine CJD studies have shown that the D subregion of the H-2 complex plays a central role in controlling the length of the incubation period (*119*). The q allele in this subregion resulted in shorter incubation times while the d allele resulted in longer ones.

Conclusion

The consequences of understanding the structure, function, and replication of prions are significant. If prions do not contain a nucleic acid genome which codes for its protein (or proteins), alternative mechanisms of replication and information transfer must then be entertained.

A knowledge of the molecular structure of prions may help identify the etiologies of some chronic degenerative diseases of humans. Development of sensitive probes for detecting prions in such diseases is needed. Diseases where prions might play an etiological role include Alzheimer's senile dementia, multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, diabetes mellitus, rheumatoid arthritis, and lupus erythematosus, as well as a variety of neoplastic disorders (*12*).

The importance of prion research in the potential elucidation of a wide variety of medical illnesses underscores the need for purification of the scrapie agent to homogeneity and the subsequent identification of its macromolecular components. Only then can we determine with certainty whether or not prions are devoid of nucleic acids. Indeed, recent progress in scrapie research has transformed an intriguing yet forbidding problem into an exciting and productive area of investigation.

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Dominance in Fishes: The Relation Between Environment and Abundance

Bernard Einar Skud

Marine fishery studies are replete with comparisons of environmental factors and population abundance or recruitment, and, recently, correlation matrices have been used to compare responses (coefficients) of different species to the same factor (*1, 2*). Although the studies have included dominant and subordinate species that interact (*3, 4*), the responses of these species have not been related specifically to their positions in the dominance hierarchy. The purposes of this articles are to compare the relation of temperature to the catch of Atlantic herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*), species that have alternated as dominant and subordinate in the pelagic biomass off New England and the Canadian Maritime Provinces, and to relate their response to dominance. The relation also is examined for other species, including the California sardine (*Sardinops sagax caerulea*) and anchovy (*Engraulis mordax*).

To paraphrase Daan (*5*), the dominant species is defined as the more abundant of two species that have a functional relation (interact) and whose densities are maintained at distinctly different levels. He specified that replacement, or a change in dominance, required at least a 50 percent reduction in abundance of one stock and a comparable increase in the other and that the change be persistent for a number of years.

Interaction Between Herring and Mackerel

Landings (catch) and other estimates of abundance and biomass of herring and mackerel in the Gulf of St. Lawrence since 1960 indicated that the species interact and have alternated as the dominant species in the pelagic biomass (*4, 6*). The evidence was based on 15 years of data and on the results obtained from simulation models. The estimates of abundance were from cohort analyses and recruitment surveys. The conclusions of the investigators (*4, 6*) were tempered by the constraints of their models and by the need for a longer series of empirical data. Supporting evidence of this interaction is apparent in data from Georges Bank (*7*) and from the North Sea (*8*).

In order to satisfy the need for a long-term empirical series I compared the landings of mackerel and herring from the Gulf of Maine to the Gulf of St. Lawrence from the late 1800's to 1960 (Fig. 1) (*9, 10*). I assumed that long-term

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