The chemistry of scrapie infection: implications of the 'ice 9' metaphor

The transmissible spongiform encephalopathies pose an increasing problem for animal, and perhaps human, health. The infectious agent seems to lack a nucleic acid component, posing the question of how it can reproduce. A model of reproduction by nucleated polymerization suggests a number of novel approaches to the problem.

The transmissible neurodegenerative disease scrapie has fascinated neurobiologists and virologists alike since the discovery, over thirty years ago, that the infectious agent is much more resistant to inactivation than a typical virus. Subsequent work suggested that the agent consists of a single, host-encoded protein, designated the prion protein. The proposal that the scrapie infectious agent is an ordered aggregate of a normal cellular protein has broad implications for experimental approaches to the mechanism of infection and pathogenesis. Here we briefly review the aggregate hypothesis and its implications for research into scrapie and related diseases such as Creutzfeldt–Jacob disease (CJD), Kuru, bovine spongiform encephalopathy (BSE), and perhaps also Alzheimer's disease.

Ice nine revisited?

In Kurt Vonnegut's novel *Cat's Cradle* [1], the physicist Felix Hoenikker creates ice 9, the most terrible weapon the world has ever seen. Hoenikker's ice 9 is more stable than normal ice (it melts at 114.4° Fahrenheit!), but is kinetically inaccessible under standard conditions, presumably because nucleation is extremely slow. Felix's son Newt demonstrates that ice 9 crystals seed their own replication, and, in the process, wipes out the world's water supply. This concept was revived by the popular press in the mid 1960's, in the wake of Russian reports of a new form of water, 'polywater' [2]. Polywater later proved to be an experimental artifact [2]. But the notion that a kinetically inaccessible, but thermodynamically stable, form of a natural material could be very dangerous may be relevant to a fascinating class of neurodegenerative diseases, the transmissible spongiform encephalopathies (TSEs).

The TSEs, like Alzheimer's disease, are characterized by the deposition of insoluble protein in the brain [3–5]. Despite their rare occurrence in man, these diseases have received a huge amount of attention due to their prevalence in the livestock population (scrapie in sheep and BSE in cattle) and the discovery that they can be transmitted by a virus-like agent [3,4]. Recent cases of TSE among other mammalian species in Great Britain, apparently due to BSE-infected feed [6], have caused

concern that this disease could cross species barriers and spread to the human population [7,8]. There is some precedent for oral transmission of this disease in humans: kuru, a prevalent TSE among the primitive tribes of New Guinea, was spread by the traditional funeral ritual, which included consumption of the brains of ones ancestors [3]. The agent which causes all of the TSEs has been called a slow virus [3], a prion [4], and, aptly, an unconventional virus. Like a computer virus, it may bear little physical resemblance to a typical virus.

The infectious agent is virus-like, but no agent-specific nucleic acid has been detected

In the early 1960's, scrapie was transmitted to rodents and the study of the infective agent began. The agent proved to be resistant to harsh chemical and enzymatic treatments which inactivate typical viruses [9,10]. Analysis of the infective agent suggested that it comprises primarily one protein [11,12], with no specific nucleic acid component, despite the fact that it exhibits three biological characteristics that are traditionally associated with viruses. First, the agent replicates *in vivo*. Second, infectivity is subject to species barriers. Last, strains of the scrapie agent have been isolated. Despite being derived from the same host, these infective isolates produce distinct symptoms and pathologies, showing differences both in the time course of pathogenesis and onset of symptoms and in the location of the abnormal pathology in the brain [13]. The strains have been propagated by serial passage in experimental animals. In 1993, one of us presented a testable mechanistic explanation of scrapie infection [14,15], based on experimental models [14,16] and existing general proposals [3,17,18], which reconciles the apparently contradictory biological and chemical properties of the infectious agent [14–16]. That mechanism is elaborated below.

How could a protein self-replicate?

The insoluble proteinaceous deposits that characterize scrapie pathology are infectious [3,4,12]. These deposits appear to consist predominantly of a single protein [11,12], designated the prion protein (PrP) [4]. The form of the prion protein associated with infection (PrP-scrapie, PrP^{Sc}) is extremely insoluble and protease-

resistant, in contrast to the normal, cellular form of the protein (PrP-cellular, PrP^C). No covalent chemical difference between PrP^C and PrP^{Sc} has been demonstrated, however. Host PrP^C is the precursor of PrP^{Sc} [19] and is required for scrapie infection [20].

In 1967, J. S. Griffith [17] suggested that the infectious scrapie protein is a conformational isomer of a normal host protein. Subsequent isolation of the prion protein allowed Fourier-transform infrared spectroscopy studies of PrP^C and PrP^{Sc}, which suggested a structural difference, supporting this notion [21,22]. The in vivo conversion of PrP^C into PrP^{Sc} must be very slow, since endogenous levels of PrP^C do not normally result in disease (sporadic CJD is extremely rare). Scrapie infection may result from acceleration of this conversion by exogenous PrP^{Sc}.

Griffith [17] suggested several possible ways in which a 'protein-only' infectious agent could self-replicate in vivo. One suggested mechanism, which has been modified and elaborated [4,22–24], holds that PrP^{Sc} and PrP^C are stable, noninterconverting, monomeric conformational isomers of PrP and that the barrier to sporadic disease is the slow conformational change from PrP^C to PrP^{Sc}. No protein is known to exist in several noninterconverting monomeric conformations [25], however, let alone the fifteen to twenty required to explain the isolated strains of PrP^{Sc} [26]. Furthermore, the mechanism proposed to explain infection, catalysis of the slow PrP^C-to-PrP^{Sc} conformational change via PrP^C–PrP^{Sc} heterodimer formation, is unprecedented [14,15].

An ordered aggregate can self-replicate by nucleated polymerization

We believe that the difference between the cellular and infectious forms of the prion protein is primarily one of quaternary structure, and that the differences in protein conformation seen are a consequence of this. This type of phenomenon has been observed in other systems. The components of many insoluble ordered protein aggregates (for example, bacterial flagella) are conformationally distinct from their soluble, monomeric protein subunit [27]. The aggregating species is often not the most stable monomeric conformer, but an easily accessible minor conformer which is trapped and stabilized by intermolecular interactions. Indeed, the allosteric transition model of Monod and Changeux [28] is based on the stabilization, by intermolecular interactions, of an accessible, albeit rare, soluble conformer. One striking example of the stabilizing effect of intermolecular interactions is a lysozyme mutant that crystallizes in two forms containing five distinct conformers [29]. By analogy, the conformation of PrP monomer found in PrP^{Sc} may be sparsely populated at equilibrium but easily accessible under physiological conditions. Exchange between the two PrP forms would pose no problems unless the PrP concentration exceeded the critical concentration, at which point nucleation could occur, leading to formation of PrP^{Sc}. Nucleus formation is slow relative to subsequent rapid growth (Fig. 1) and can be extremely sensitive to protein concentration [15].

Nucleation could be the slow step in spontaneous disease

We believe that nucleation of polymerization, rather than a unimolecular conformational change, is the rate-determining step in PrP^{Sc} formation [14,15]. At a physiologically realistic PrP concentration, slightly in excess of its thermodynamic solubility, nucleation would be extremely slow, explaining the rarity of sporadic disease. Slight increases in the concentration of PrP in vivo would have dramatic effects on the rate of nucleation, however. Mutations in the PrP gene that either increase the concentration of PrP or favor the protein–protein associations which precede nucleation even by a small amount would have a large overall effect on the rate of nucleus formation, explaining the 'all or none' genetics of CJD [14–16]. This would also explain the observed correlation between PrP genotype and the occurrence of sporadic CJD [16], and the variation in the severity of different PrP point mutations. Similarly, a point mutation on the hemoglobin surface facilitates the
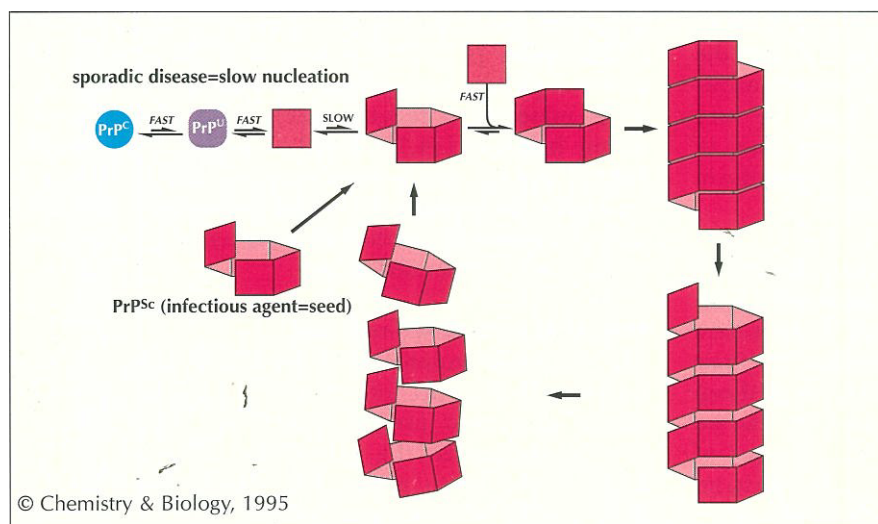

Fig. 1. Nucleus formation is the kinetic barrier to sporadic TSE, but is bypassed by infection. Nucleus formation is very slow at concentrations slightly exceeding the critical concentration. Small increases in PrP concentration would greatly increase the rate of nucleation [15]. Mutations in PrP that cause CJD may affect the unfolding equilibrium (PrP^U is an unfolded form of PrP) or may affect the association equilibria [14]. Infection results from seeding of PrP polymerization, by preformed PrP^{Sc} nuclei. All of the oligomers shown would be effective seeds, however, only the high molecular weight oligomers would be easily observable by electron microscopy.

Fig. 2. Barriers to interspecies infection result from packing defects. Slight structural differences between the species PrPs may affect the efficiency of seeding or may prevent seeding altogether. In the figure, three species variants of PrP are represented by three shapes. The intraspecies seeding is clearly most efficient. Interspecies heterologous seeding can occur if the structural differences are small (for example, the species barrier between mouse and hamster is permeable). However, if the structural differences are large, interspecies seeding may not be effective at all (we hope, for example, that the barrier between sheep or cow and human is impermeable). The best known example of heterologous nucleation is cloud seeding, which was the field of Kurt Vonnegut's brother.

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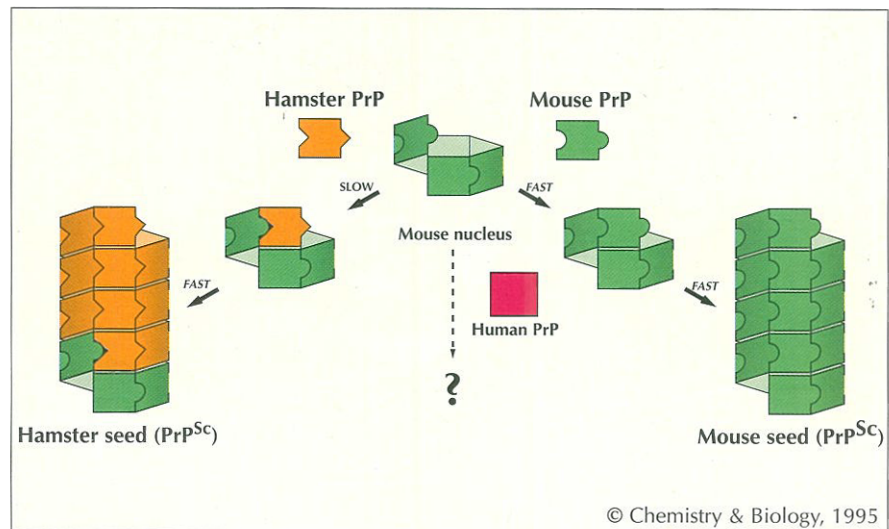
nucleation-dependent formation of the ordered sickle-cell hemoglobin fibril [15].

Infection may involve transfer of a preformed seed
We have proposed that the scrapie infectious agent contains an oligomeric form of PrP (PrP^{Sc}), which acts as a nucleus, or seed, for the polymerization of the cellular protein (PrP^{C}) [14,15]. The nucleus, shown in Fig. 1 as a six-mer, is defined as the smallest oligomer which can act as a seed. At a PrP concentration slightly exceeding its thermodynamic solubility, nucleation would be very slow (sporadic disease, Fig. 1), but seeded growth (infection, Fig. 1) would readily occur [15]. Achieving such PrP concentrations *in vivo* may require sequestration of the protein in a cellular compartment such as an endosome or a lysosome. In fact, there is evidence that PrP^{Sc} is formed along the endocytic pathway from the plasma membrane. [19, 30,31].

Replication
The infective scrapie agent replicates in the host animal, making it possible, by serial passage of the agent, to infect large numbers of animals starting with a small amount of PrP^{Sc}. In our model, this could be explained by the idea that the PrP^{Sc} aggregate can be fragmented to produce multiple seeds, each of which can be a template for further polymerization of host PrP. Fragmentation of amyloid fibrils by sonication increases seeding efficiency [14,15]. Similarly, dispersion of prion fibrils increases infectivity, presumably by producing many oligomeric seeds; these may be too small to be easily observable by electron microscopy, however [4].

Species barriers
The induction period for interspecies infection is much longer than for intraspecies infection. The species barrier is presumably due to PrP sequence differences among the species (Fig. 2). If the interaction between seed PrP^{Sc} of one species and the host PrP of another species is not optimal, the growth of PrP^{Sc} may be slower, or entirely prevented [32]. Once the initial barrier is overcome,

however, the resulting PrP^{Sc} would be essentially indistinguishable from host agent in subsequent passages [4], since the seed PrP^{Sc} was present only in trace amounts. This is in fact what is observed in interspecies infection; the incubation time for transfer of infection from mouse to hamster is long, but shortens in subsequent hamster to hamster transfers.

Strains
Perhaps the most puzzling phenomenon in scrapie is the existence of scrapie strains which, despite the fact that the PrP^{Sc} isolates are identical with respect to sequence, behave differently with respect to the induction period and the resultant disease pathology [13]. The nucleation-dependent mechanism of PrP^{Sc} formation provides a simple explanation; strains of PrP^{Sc} may represent alternately packed ordered PrP aggregates (Fig. 3), analogous to the various forms of ice that inspired Vonnegut's ice 9 [1]. Alternative PrP packing modes (PrP^{Sc} strains) may arise from nuclei of different sizes (for example, 5,6, or 7 monomers per nucleus, Fig. 3) and/or configurations. (for example, rectangles can pack along the long or the short side, Fig. 3). The PrP monomer conformation in each strain may be identical (as is the case for ices 1–9) or slightly different. Examples of alternately packed protein crystals include aldolase [33] and a lysozyme mutant [29] which have been shown to produce different crystal forms, depending on the crystallization conditions. Identical flagellin monomers can form straight or curly flagella [27]. The preferred PrP aggregation pathway would be very sensitive to PrP concentration (for example, lower PrP concentration would favor smaller nuclei) and various endogenous factors (for example, pH and/or salt concentration may affect intermolecular interactions). If the energetic difference between the nucleation pathways is small, our model would predict that several strains could arise in one animal. The strains could be isolated during the isolation/dilution/injection protocol of experimental serial passage. Propagation of the scrapie agent strains resembles the seeding of a crystallization. In the presence
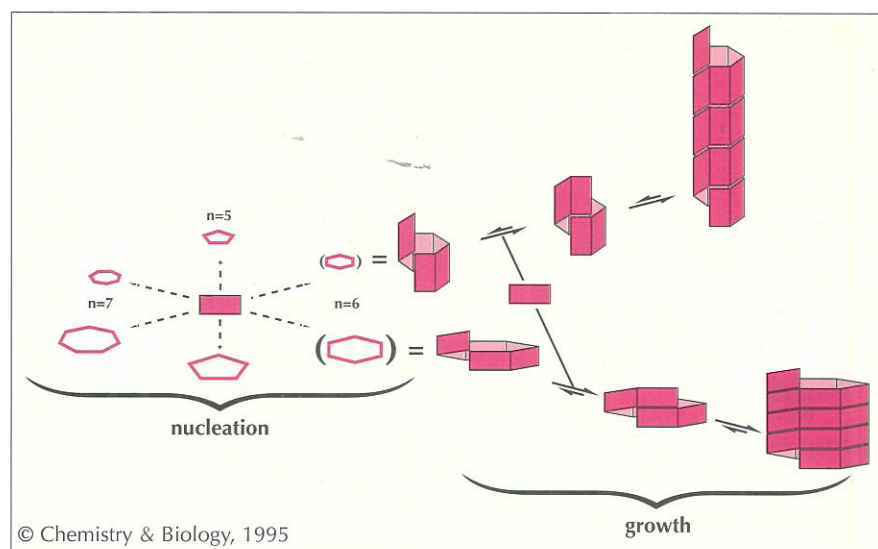
Fig. 3. Strains might originate from alternately packed nuclei and be propagated by seeding. The various scrapie strains may simply be alternately packed PrP polymers, for example having different sizes of nucleus or different configurations of the monomer within the nucleus. Nuclei of five, six and seven monomers, with two different monomer configurations, are shown. Several strains could arise in a single animal if the differences between activation energies for formation of the various nuclei were small. It would then be possible to propagate the strains by seeding.

of seed PrP^{Sc}, the PrP of the host will take on the strain properties of the infecting PrP^{Sc} because the nucleus imposes its own periodicity on the polymer whose formation it seeds.

Implications of the nucleated polymerization hypothesis

Driven by the hypothesis that nucleation of PrP polymerization is the rate-determining step of PrP^{Sc} formation, we set out to develop a cell-free system to study the mechanism of the PrP^{C} to PrP^{Sc} conversion [34]. Previous attempts to develop cell-free conversion systems were based on the premise that monomeric PrP^{Sc} directly catalyzes the conversion of a monomer of PrP^{C} to the PrP^{Sc} conformation, and were unsuccessful. This prompted speculation that a cofactor, possibly ATP or a chaperonin, is required for the PrP conformational change [22,35,36]. We were able to show, however, that by unfolding PrP^{C} and increasing the concentration of PrP^{Sc} relative to PrP^{C} it is possible to catalyze the conversion of PrP^{C} to the PrP^{Sc} form. Presumably this is because our system provides a sufficient number of template nuclei to capture and stabilize the PrP monomers that adopt the PrP^{Sc} conformation.

The success of our system suggests that a separate cofactor is not essential *in vitro*. We cannot rule out the possibility that a cofactor that copurifies with PrP^{Sc} is required, however. The ultimate proof of the 'protein-only' hypothesis will be the *in vitro* creation of PrP^{Sc} from purified PrP^{C} and the proof that this material is infectious. This would not preclude, of course, the possibility that other proteins might be involved in infection or in the generation of spontaneous disease *in vivo*. The recent report that apoE genotype is a major susceptibility factor for CJD [37] suggests that the apoE protein may be involved in the process of spontaneous PrP^{Sc} 'seed' formation, either as an inhibitor or as a promoter. The apoE proteins have been reported, by different research groups, to exert both of these effects on *in vitro* amyloid formation by the β amyloid protein of Alzheimer's disease [38,39].

Consequences for experimental design

Using the cell-free system mentioned above, it should be possible to use existing methods to detect interactions between the PrP protein and apoE or other auxiliary proteins. In designing these experiments, it is crucial to recognize the possibility that the auxiliary protein may interact not with the PrP monomer, but with a PrP oligomer or seed [38]. In addition, the binding of PrP^{Sc} and PrP^{C} to sulfated glycosaminoglycans [40,41] and the potent inhibition of PrP^{Sc} formation and scrapie agent replication by sulfated glycosaminoglycan analogs and Congo red [42–44] suggest that sulfated proteoglycans may have a critical role in *in vivo* PrP^{Sc} formation.

Several aspects of the proposal outlined above now call for detailed testing using cell-free systems. For example, the notion that different strains of PrP^{Sc} represent alternatively packed forms of monomers is now testable. If this hypothesis turns out to be correct, other questions arise. What are the features of the protein structure that make the different forms of aggregate possible? How does a PrP aggregate interact with the PrP monomer of another species? And what bearing, if any, does this have on the function of PrP^{C} in the normal brain?

Finally there is the critical question of how PrP^{Sc} kills neurons. It is possible that the damage simply results from the abnormal protein precipitation caused by aggregation of PrP. Alternatively, PrP^{Sc} may interact with a specific cellular protein. Again, the search for a cellular target of PrP^{Sc} may require methodology specifically designed to discover monomer–aggregate interactions. We look forward to experimental tests of the idea that aggregation is important in the formation of PrP^{Sc}, and, should it be proved correct, to exploring the further implications of the notion as outlined above.

Wisconsin) for comments on the manuscript. The ice 9 metaphor has also been used by Carlton Gadjusek of NIH to describe scrapie infection.

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