Structure, Function and Stability of the BSE Prion

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Brief background

This section is based on the author's personal experience and knowledge of the development of BSE saga from the identification of the first BSE case at the Central Veterinary Laboratory (CVL), Weybridge, UK (where the author was Head of Toxicology at that time) to the first cases of new variant Creutzfeldt-Jakob Disease (nvCJD) in humans several years later. Dr Ray Bradley was Head of Pathology at CVL and was intimately involved in the elucidation of BSE and its cause: for his detailed account of the development of the disease see Bradley (1997).

Origins of BSE

The first cases of bovine spongiform encephalopathy (BSE, Mad Cow Disease) occurred in the UK in 1986. Epidemiological evidence pointed strongly to a point source of 'infection' emanating from a meat works in the south of England. This led to one of the most intense research efforts to date; the, until then, unknown disease was quickly identified as a spongiform encephalopathy akin to sheep scrapie. At this point the discoverer of the prion that caused scrapie in sheep (Professor Stanley Prusiner, awarded the Nobel Prize for Physiology or Medicine in 1997 for his discovery) was brought to the UK to help elucidate the causative agent of BSE. It was concluded that the unknown disease was a prion-associated transmissible spongiform encephalopathy (TSE) – the term BSE was coined.

Then began one of the biggest crises the UK agricultural sector had ever experienced. The impact was driven by concerns that BSE was transmissible between cattle (it was) and that it might be contracted by humans (this was later shown to be the case). The UK beef market collapsed.

For many years beef exports from the UK (and other countries that had BSE cases) were tainted (and to some extent still are) by concerns about the safety of their beef. NZ has never recorded a case of BSE, which puts our beef (and beef product; e.g., bile salts for pharmaceutical use) export market in a superior world position. NZ guards this status avidly and trades on the safety of its beef and beef products.

Prions and prion infectivity

The TSEs are all caused by prions – proteinaceous infectious particles – discovered by Prusiner (1982). Prions are proteins, they are not conventional infectious agents because they cannot reproduce. Their proliferation is via an induced protein conformational change which converts native prions to TSE prions simply by contact between the two proteins. When Prusiner discovered this, it revolutionised infectious disease thinking (hence his Nobel Prize).

Normal prions (designated PrP^{C}) are present in cell membranes, they appear (but this is not fully understood) to play a key role in cell-cell communication, particularly in neurological systems (hence the extreme neurological symptoms associated with the TSEs). PrP^{C} has a predominantly α-helix secondary (2°) protein structure, whereas TSE prions (designated PrP^{Sc} – 'Sc' denotes scrapie, but the term is used for all TSE prions) have a greater proportion of β-pleated sheet 2° protein structure (Table 1). The change from PrP^{C} to PrP^{Sc} results in a very significant change in function of the prion, even though the proteins' primary (1°) structures are the same. The protein conformational change causes significant neurological aberrations due to changes in cell-cell communications.

Table 1 Prion 2° protein structure (Riesner, 2003)

Prion	**α-helix** %	**β-pleated sheet** %
PrP^{C}	43	-
PrP^{Sc}	20	34

If PrP^{Sc} comes into contact with PrP^{C} it pulls the PrP^{C}'s 2° structure from α-helix to β-pleated sheet – this is termed 'induced conformational change' and is the basis of PrP^{Sc} proliferation in the central nervous system (CNS) of exposed animals (including humans) – this will be discussed in more detail later.

Origins of the BSE prion

There is much conjecture about what initiated the BSE epidemic; but at a basic molecular biology level, this must have involved some change in the structure of PrP^{C} that supported (thermodynamically) the spontaneous conformational change to PrP^{Sc}. Various theories for this have been expounded ranging from cattle's exposure to organophosphorus pesticides (Purdey, 1994) to mutations in the prion gene leading to prion misfolding (Hwang & Nicholson, 2018). The latter is the most convincing explanation because it relies on a single amino acid mutation (glutamic acid to lysine) in the gene product which introduces instability into the PrP^{C} α-helix.

If only a very few protein molecules in an individual cow were affected, the PrP^{Sc} present would induce the conformational change $PrP^{C} \rightarrow PrP^{Sc}$ in very many others. If this cow was (via meat and bone meal) incorporated into cattle feed many other animals would be infected. This scenario fits well with the epidemiologically determined point source of BSE in a single abattoir in the south of England. If this hypothesis is correct, a single mutation in a single cow sparked the BSE epidemic.

Transmission of TSE prions

The primary route of TSE prion transmission is oral (Palmer & Collinge, 1997) although it has also been suggested that the inhalation (of dust from bovine meat and bone meal-containing animal feed) route might account for some 'rapid onset' cases of nvCJD in farmers (Shaw, 1995) (Fig. 1).

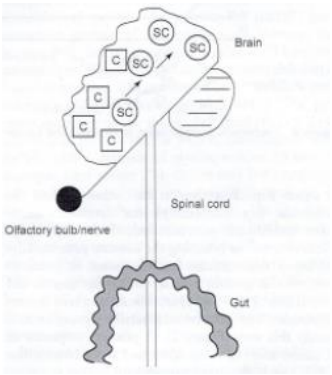

Figure 1 Schematic representation of infection by the BSE prion. Absorption in the gut followed by diffusion up the spinal cord leads to delivery of PrP^{Sc} to the brain, where it induces a conformational change in endogenous PrP^{C}, so generating more PrP^{Sc}. A quick route to the brain might be via the olfactory nerve following inhalation of prion dust [from Shaw, 2004].

In the case of BSE, PrP^{Sc} is consumed by cattle (or other mammals) as a contaminant of CNS-containing meat and bone meal as a component of proprietary cattle feed. The prion progresses down the gastrointestinal tract (GIT) with the food as it goes through the digestion process and is taken up into the lymphatic system probably in the small intestine; interaction with lymphoid cells, particularly B-cells, is thought to be important in this uptake process. The prion progresses with via lymph to peripheral nerves and then on to the CNS (Davies *et al*., 2006) (Fig. 1). This progression is passive and the time taken to travel from the GIT to the CNS is possibly linked to the distance between the two. The size of the animal orally infected with PrP^{Sc} therefore, in part, determines the disease's latent period (akin to incubation period for conventional pathogen) as does the size of the animal's brain,

which is not directly related to the animal's overall body size (unlike other organs; e.g., liver). This suggests that a simple stoichiometric relationship between number of prions and brain size might influence the disease's aetiology (Bae *et al*., 2012) – this points to a purely chemical relationship and links to the mechanism of the induced conformational change $PrP^{C} \rightarrow PrP^{Sc}$.

Molecular biology of the BSE prion

The induced conformational change $PrP^{C} \rightarrow PrP^{Sc}$ is the key to understanding the infectivity of the BSE prion (Clark *et al*., 2000; Weissmann, 2004; Figs 1 and 2). In addition, the chemical nature of both PrP^{C} and PrP^{Sc} is important in understanding the stability and effects of chemical insults (e.g., pH changes) and temperature on the stability and infectivity of BSE prions.

Where the original PrP^{Sc} came from is unknown. However, as discussed above it likely resulted from a mutation that slightly altered the prions 1° sequence which led to misfolding to form a β-pleated sheet structure, which by induced conformational change began the chain reaction conversion of PrP^{C} to PrPSc. Transmission might have resulted from cannibalism - as is the case for the human TSE kuru (Will, 2003). This means that spontaneous mutation might lead to some idiosyncratic BSE cases, rather than transmission via food (this, of course, remains a possibility in NZ). It has also been suggested that a chemical intervention (e.g., exposure to organophosphorus pesticides (OPs) used as veterinary medicines) in cattle led to the initial $PrP^{C} \rightarrow PrP^{Sc}$ transformation (Purdey, 1994); however, exposure of PrP^{C} to the OP Phosmet did not form PrP^{Sc} (Shaw *et al*., 2002).

Structure of prions

As outlined above the conversion $PrP^{C} \rightarrow PrP^{Sc}$ is the key to prion infectivity and pathogenicity. Further, when the β-pleated sheet PrP^{Sc} is formed it spontaneously forms a β-oligomer (12-16 molecules associated) which aggregates to form insoluble amyloid (Riesner, 2003). PrP^{Sc}, its oligomers and amyloid are incredibly stable.

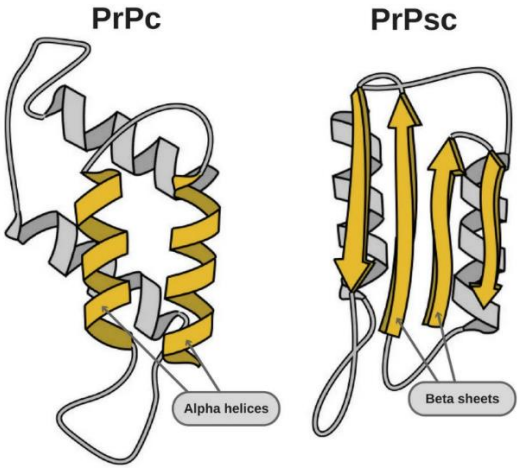

Figure 2 The conformational change $PrP^{C} \rightarrow PrP^{Sc}$, showing the transformation from α-helix to β-pleated sheet (from Prions: a new approach to Alzheimer's disease – Science for everyone (wordpress.com))

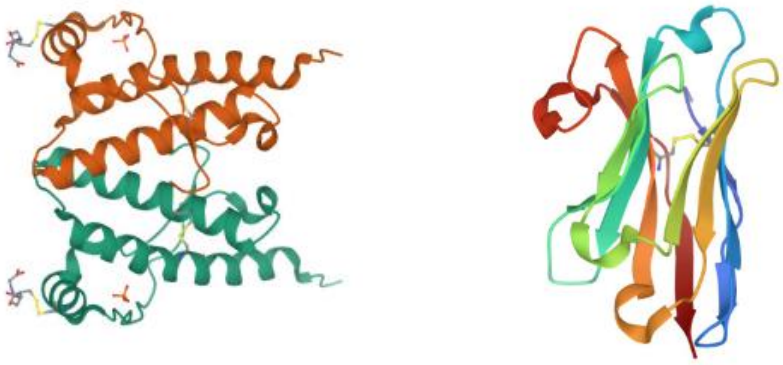

Figure 3 X-ray crystallographic structures of prions. **Left:** globular domain of sheep PrP^{C} showing the α-helices (PDB1UW3: Haire *et al.*, 2004). **Right:** mouse PrP^{Sc} showing its β-pleated sheet structure (PDB6HEQ: Abskharon *et al.*, 2019).

Infectivity of PrP^{Sc} to humans

Following the identification of BSE in the UK in 1986, concerns began to grow about the possibility that the disease could transmit to humans via the food chain. To some extent, the concern about this possibility was ameliorated by the knowledge that sheep scrapie appeared not to jump the sheep/human species barrier. Despite this, concerns grew, particularly in the media and thus consumers. This had a huge impact on beef consumption in the UK and significantly affected beef exports from the UK. The concerns about transmission to humans were, no doubt, augmented by

the fact that there existed several human prion diseases (e.g., kuru, Creutzfeldt-Jacob Disease (CJD): now prions are implicated in many more diseases; e.g., Alzheimer's Disease), which underpinned the potential for the BSE prion to cross the species barrier and infect humans (for a detailed review of human prion diseases see Collinge & Palmer, 1997; and for a simplified overview see Shaw, 2004).

Collinge & Rossor (1996) recognised a new clinicopathological form of CJD which occurred in young people (e.g., a case occurred in a man of 42 years old) rather than the 7th decade for 'conventional' CJD. They termed this early onset disease 'variant' CJD and later 'new variant' (nv)CJD. They speculated that nvCJD was linked to consumption of BSE prion-contaminated meat, and that consumption had occurred in the early 1980s prior to the UK's offals ban (human consumption of offals (including brain) was banned when it was shown that these tissues harboured high prion concentrations and thus represented the highest risk to human consumers). This meant that the latent period following consumption was approx. 12-17 years. Prusiner (1997) supported Collinge & Rossor (1996)'s proposal that nvCJD originated from human consumption of PrP^{Sc}-contaminated beef. Hill *et al*. (1997) demonstrated that nvCJD was caused by the same prion 'strain' as BSE, which was the proof necessary to link PrP^{Sc} to nvCJD.

Stability of PrP^{Sc}

Temperature stability

PrP^{Sc} is partially resistant to proteinase K (EC 3.4.21.64), a powerful serine protease that is able to cleave (often with complete digestion) even the most resilient proteins (e.g., keratin) (Langeveld *et al*., 2021) – this illustrates the prion's unusual resilience. *B. lichemiformis* PWD1 keratinase at 50°C, followed by autoclaving at 115°C for 40 min in the presence of sodium lauroyl sarcosinate (sarkosyl: a non-ionic detergent) reduced the infectivity of PrP^{Sc} by more than 99.9% (Langeveld *et al*., 2002). These are extreme conditions to effect elimination of a protein's bioactivity, again illustrating the resilience of prion protein (for a very detailed review of the molecular biology underpinning prion stability see Cox *et al*., 2007).

Studies on the heat stability of prions have shown that 98°C for 2 h reduces the BSE prion infectivity (using the mouse bioassay) by only a factor of 10. This would have very little impact on the infectivity of prion-contaminated material. Other PrP^{Sc} strains (RML and 22L – both mouse scrapie isolates) showed very much greater diminution of infectivity – RML showed 10^6 and 22L 10^5 infectivity loss. This shows that thermostability is prion strain dependent, with the BSE prion being the most thermostable (Marin-Moreno *et al*., 2019).

The thermostability of prion rods (these are infectious insoluble aggregates of PrP 27-30; i.e., the N-terminally-truncated prion) has also been studied. Autoclaving at 170°C for 20 min resulted in a reduced infectivity factor of 10^4; however, the presence of lipids increased the resistance of the prion rods to heat degradation (Appel *et al*., 2001). Safar *et al*. (1993) investigated prion thermal stability in relation to its infectivity. They found that heating for 30 min at 60°C, 100°C and 132°C did not alter the β-pleated sheet 2° structure of hamster PrP^{Sc}, but that there was a slight decrease in infectivity (hamster model) at 132°C.

A key issue relating to prion destruction by heat is that the inactivation kinetics are exponential (Casolari 1998), which means that very long heat treatment processes would be necessary to reduce prion infectivity to zero (if, indeed, this is possible). This is illustrated by Taylor *et al*. (1994) who autoclaved macerates of BSE-infected bovine brain and found that 138°C for 60 min did not completely inactivate (in a rodent assay) the BSE prion.

In conclusion, BSE PrP^{Sc} infectivity remains even after prolonged high temperature treatment.

Chemical stability

Exposure of BSE-infected bovine brain to sodium hypochlorite (NaClO: 16,500 ppm available chlorine) for 120 min resulted in no residual prion infectivity (hamster assay); however, treatment with sodium dichloroisocyanurate (also 100 ppm available chlorine) did not result in complete inactivation (Taylor *et al*., 1994) – this suggests that the mechanism of inactivation is not solely dependent on available chlorine, but, perhaps, relates to the kinetics of chlorine release leading to different chlorine exposure concentration profiles.

Exposure of infected bovine brain to 1M or 2M sodium hydroxide (NaOH: approx. pH 14) did not result in complete inactivation of PrP^{Sc} (hamster assay) (Taylor *et al*., 1994); therefore, not even the most extreme pH completely denatures prions.

Interestingly, health guidelines in the USA, UK and Australia covering manipulation and destruction of CJD clinical samples require incineration where possible or treatment with sodium hypochlorite if incineration is not an option (e.g., Health Australia, 2013). This underlines the point that prions are difficult to fully inactivate except by extreme procedures. It also illustrates that sodium hypochlorite treatment is the only effective (and acceptable by health authorities) chemical method.

Prion stability in the environment

Due to the 'incredible' stability of PrP^{Sc} and its horizontal transmission, it is likely to persist in the environment when shed from infected animals and be a source of infection to other animals grazing the same area (e.g., in mule deer: Miller *et al*., 2003). Therefore, 'the environment likely serves as a stable reservoir for infections prions facilitating a sustained incidence of CWD [chronic wasting disease] in free ranging cervid [deer] populations' (Saunders *et al*., 2008). This conclusion to Saunders *et al*. (2008)'s work makes the important point that depositing PrP^{Sc} onto pastures increases the risk of BSE horizontal transmission to other grazing animals.

The environmental persistence of prions in a particular environment is, at least in part, facilitate by prion-soil particle interactions (Saunders *et al*., 2008). Prion-bound to soil (scrapie hamster model) remains infectious for 26 months (Seidel *et al*., 2007; Wigins, 2009); and, interestingly, soil binding appears to facilitate the stability of PrP^{Sc} or even pull PrP^{C} into the infectious PrP^{Sc} conformation, which might explain the unexpectedly high environmental transmission of TSEs (e.g., in deer) (Johnson *et al*., 2007).

It is possible that environmental degradation of prions might occur; however, the bacterial enzymes capable of degrading prions work best at high pH (10-12) and high temperature (50-60°C), so even though the bacteria occur in the environment it is unlikely that the conditions necessary to facilitate prion degradation will be met (Saunders *et al*., 2008. Interestingly cattle rumen and colon bacteria can degrade (*in vitro*) scrapie PrP^{Sc} (from hamster) to undetectable levels (using immunochemical assays) within 20 h at 37°C under anaerobic conditions; however, despite this significant degradation of the PrP^{Sc} protein, its infectivity (hamster assay) remained (Scherbel *et al*., 2007) – presumably this is because sufficient β-pleated sheet remained intact to effect the $PrP^{C} \rightarrow PrP^{Sc}$ conformational change in the host animal (in this case, the hamster).

The BSE prion is very stable in aquatic environments, including in wastewater. Studies on prion (isolated from BSE-infected cattle brain) infectivity in wastewater and phosphate buffered saline (PBS) showed no significant reduction in infectivity following 9 months incubation in wastewater, whereas incubation in PBS for 8 y resulted in only a 100-fold reduction in infectivity (mouse assay) (Marín-Moreno *et al*., 2016). In terms of environmental persistence of infectivity, this means that the prion is extremely persistent with a half-life probably measured in decades (based on the PBS data).

Prion stability in biogas production

Interestingly Mittelbach *et al*. (2007) assessed the risk of prion infectivity via exposure to biodiesel derived from BSE infected bovine lipids (tallow). The process used for biodiesel production (from tallow) involved:

1. Lipid esterification with concentrated sulfuric acid (H_2SO_4; refluxed with methanol at 65°C for 2 h).
2. Potassium hydroxide (KOH) in methanol-catalysed transesterification (40°C for 20 min).
3. Vacuum distillation (150°C for 30 min) to separate biodiesel.

Prion (hamster scrapie rods) was added to the tallow prior to acidification, prior to base-catalysed esterification, and to the crude biodiesel before vacuum distillation. Prion was determined post process by western blotting (i.e., immunologically following electrophoretic separation). The western blotting technique determines the presence of 'intact' prion – it is important to note that prion fragments can be 'infectious' (Scherbel *et al*., 2007). The degradation of the prion backbone intactness at the three prion spiking stages is shown in Table 2.

Table 2 Degradation of prion backbone intactness in hamster prion-spiked pre-esterification, transesterification and vacuum distillation in the biodiesel production process from bovine tallow (Mittelbach *et al*., 2007).

Process step	**Process temperature °C**	**Reduction factor**
Pre-esterification (conc. H_2SO_4)	65	$>10^2$
Transesterification (KOH in methanol)	40	$>2.5 \times 10^2$
Vacuum distillation	150	$>10^3$

In my opinion, the intact prion reduction factors have little meaning unless considered in the context of the initial contamination (of tallow in this case) and that they are augmented with infectivity studies because the degraded prion might remain infectious. Mittelbach *et al*. (2007) make it clear that tallow (or tissue generally) prion infectivity titre varies enormously (e.g., tissues from a BSE cow have a very high titre, while tissue from a non-BSE cow will have ostensibly zero titre). A 10^2 titre reduction for an initially very high titre arguably has little impact on the infectivity (i.e., it will remain highly infectious). Despite this, Mittelbach *et al*. (2007) carried out a generalised risk assessment for biodiesel based on infectivity levels in

tissue-derived materials (tallow) and the risk of infection in a particular country (i.e., the prevalence of BSE in that country – the lower the prevalence the lower the risk of prion-contaminated tallow).

Mittelbach *et al*. (2007) based their risk assessment on a worst-case scenario (i.e., highly infectious starting tissue) with the UK's then BSE infection rate in the national herd of 44 per million head of cattle and they factored in the expected biodiesel dilution prior to use. From this they calculated that the risk of a human contracting nvCJD following exposure to biodiesel was 10^{-9} that of the background CJD rate (10^{-6}; i.e., one case per million population) worldwide and so was considered negligible.

Mittelbach *et al*. (2007)'s risk assessment refers to biodiesel and not the remaining bovine material that might be used for other (including agricultural) purposes. Interestingly, the European Food Safety Authority (EFSA) approved biodiesel production from bovine materials several years before Mittelbach *et al*. (2007)'s detailed study (EFSA, 2004).

Mittelbach *et al*. (2007)'s study is important because it shows the effects of key steps in the biodiesel process on integrity of PrP^{Sc}, showing approx. 100-1,000-fold reduction in prion protein integrity. The study's significant down point is that no attempt was made to show how this reduction relates to infectivity. It is clear that strong acid (pH ~1) and strong base (pH ~14) reduce prion protein structure integrity, but whether this affects infectivity remains unknown.

Conclusions

- PrP^{Sc} is incredibly stable. It is even partially resistant to powerful proteinase K.
- *B. lichenoformis* PWD1 keratinase at 50°C followed by autoclaving at 115°C for 40 min in the presence of lauroyl sarcosinate reduces PrP^{Sc} infectivity by 99.9%
- Heating BSE PrP^{Sc} to 98°C for 2 h only reduces infectivity by a factor of 10.
- Heating PrP^{Sc} to 170°C for 20 min reduces infectivity by a factor of 10^4 (this is the basis of neurology surgical instrument sterilisation).
- Treatment of BSE-infected brain tissue with sodium hypochlorite (16,500 ppm available chlorine) for 120 min resulted in complete loss of infectivity.
- Exposure to 2M NaOH (pH >14) did not result in complete loss of infectivity.
- PrP^{Sc} persists in the terrestrial environment, partly due to its stabilisation in association with soil particles.
- PrP^{Sc} is very stable in aqueous systems (incubation in PBS for 8 y resulted in only 100-fold reduction in infectivity). Studies in aquatic environments have shown no change in infectivity after 9 months.
- Studies on biodiesel production from tallow show that PrP^{Sc} protein integrity is reduced by the process.

- PrP^{Sc} protein integrity reduction is not necessarily associated with reduced infectivity (i.e., partly degraded PrP^{Sc} might still be infectious).

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