

## Discussion Paper

# REFLECTIONS ON SCRAPIE AND RELATED DISORDERS, WITH CONSIDERATION OF THE POSSIBILITY OF A VIRAL AETIOLOGY

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### ABSTRACT

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The transmissible spongiform encephalopathies of domesticated animals, scrapie in sheep and bovine spongiform encephalopathy (BSE), and transmissible mink encephalopathy are more than a scientific curiosity; under certain circumstances their impact on commercial activities can be calamitous. Knowledge of their causation and pathogenesis is still rudimentary, but many consider than an unconventional agent, the prion (a brain protein, PrP), that is not associated with nucleic acid is involved in both. Others believe that conventional viruses, which replicate by virtue of their nucleic acid-defined genes, are involved in the causation and progression of the encephalopathies but that technical problems have prevented their identification. Others postulate even more exotic causative agents. While this paper will particularly address the possibility of a viral aetiology for these diseases, it is also emphasized that our knowledge of the state of the immune system in animals with encephalopathy needs broadening. There are remarkable gaps in our knowledge of the histopathology of these diseases, particularly the nature of the characteristic vacuoles. Much further work is needed on the biochemical changes in the brain and the serum, particularly of the latter as it could lead to an additional means of recognizing clinical cases without waiting for the animal to die with subsequent examination of the brain for characteristic lesions and the presence of protease-K-resistant PrP.

*Keywords:* aetiology, Creutzfeldt–Jakob, genes, prion, scrapie, spongiform encephalopathies, transmissible mink encephalopathy, virus

*Abbreviations:* AI, artificial insemination; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; ET, embryo transfer; GSSD, Gerstmann–Sträussler–Scheinker disease; HDV, hepatitis delta virus; MCF, mink cell focus; PK, proteinase K; PrP, prion protein; PrP<sup>Sc</sup>, scrapie prion protein; PrP-C, the proteinase-K sensitive homologue in normal brain; SAF, scrapie-associated fibrils; TME, transmissible mink encephalopathy

*Like the story of the blind men trying to describe an elephant, the view one gets of the scrapie and CJD agents depends on how, and where, one looks. [Bolton and Bendheim, 1991]*

### INTRODUCTION

In the transmissible spongiform encephalopathies (Kimberlin, 1990), a variety of neurological signs follow non-inflammatory, vacuolar, degeneration in the brain and spinal cord. Accumulations of amyloid and other proteinaceous fibrils occur. These fibrillar accumulations can form plaques similar in appearance to those seen in

Alzheimer's disease in man. After a very long quiescent latent period following infection, the spongiform encephalopathies progress very slowly over a period of weeks to months. This group of diseases provides a good example of disastrous events in the field of veterinary medicine that have put into question all our current concepts of the genesis of disease. These events have also had the positive effect of focusing attention on enormous gaps in our knowledge and stimulating a great deal of research. Reference to older literature on scrapie can be made in books edited by Kimberlin (1976a) and Prusiner and Hadlow (1979). The advent of BSE in Britain, starting in the 1980s, resulted in the appearance of numerous articles on the significance of the spongiform encephalopathies. These include a number of more recent reviews (Little and Thorsen, 1989; Chesebro, 1991; Powell, 1993).

Scrapie, a disease of sheep, was the first of these diseases to attract enough attention to stimulate research into its causation and recognition. The disease had been recognized for some time but it did not become the subject of experimental work until the 1930s, when Cuillé and Chelle, cited by Gordon (1946), reported on its transmission. Its accidental transmission during large-scale field trials of vaccination of sheep against louping-ill also gave impetus to research on this disease<sup>1\*</sup>. The vaccine had been prepared from formalinized brain and spleen suspensions from louping-ill-infected yearling sheep (Gordon, 1946). Starting 2½ years after vaccination, scrapie started to appear in animals of all breeds vaccinated with one lot of vaccine. This unfortunate experience prompted transmission studies designed to determine the nature of the infective agent and the pathogenesis of the disease in sheep. Conclusions drawn from the vaccination trials and transmission experiments were that scrapie, given by subcutaneous inoculation, had a latent period of 2 years and longer; that the infective agent was resistant to 0.35% formalin; that the disease appeared more quickly and in a higher percentage of recipients following intracerebral than following subcutaneous injection; and that the causative agent was probably a filtrable virus.

A similar disease to scrapie is bovine spongiform encephalopathy (BSE), of which the first case of a major enzootic was confirmed in November 1986 in England (Bradley and Matthews, 1992). The emergence of this disease was considered to be related to the ingestion of meat and bonemeal derived from ruminant sources following countrywide changes in the rendering process (Wilesmith *et al.*, 1991). The feeding of ruminant-derived meat and bonemeal to cattle in Britain was stopped by law in 1988 (Wilesmith *et al.*, 1992). Since the implementation of this ban, the occurrence of new cases of this disease in cattle has been reduced (Wilesmith and Ryan, 1993).

The BSE outbreak drew attention to spongiform encephalopathies that occurred around the same time in other bovids, kept in zoological and wildlife park collections in Britain (Jeffrey *et al.*, 1992; Cunningham *et al.*, 1993). These cases could have developed through the animals being fed similar rations to the cattle population. Spongiform encephalopathy has also been found in mule deer and captive elk in North America (Williams and Young, 1980, 1982; Wells and McGill, 1990), evidence that agents causing encephalopathy are present in wild ruminants in that continent. Spongiform encephalopathy has recently been demonstrated in domesticated and wild felids (Wells and McGill, 1990; Willoughby *et al.*, 1992).

\*Please see Notes section at end of text.

Transmissible mink encephalopathy (TME) (Marsh and Hanson, 1969; Marsh, 1976), which is rarely observed in ranch-raised animals, is caused by an agent with the same physicochemical properties as the scrapie agent. In the first outbreaks of TME, it could not be excluded that the occurrence of the disease had followed feeding of meat and offal from sheep, but the feeding of sheep meat was not involved in a more recent and major outbreak of TME on a ranch in the United States (Marsh *et al.*, 1991). Three diseases are associated with spongiform encephalopathy in man: Kuru, Creutzfeldt–Jakob disease (CJD) and Gerstmann–Straüssler–Scheinker disease (GSSD). Kuru, which is very similar to scrapie, is believed to be associated with funeral practices involving ritualistic cannibalism (Gajdusek, 1977). A very full account of this and other human spongiform encephalopathies and follow-up experimental work is given by Brown and Gajdusek (1991). CJD usually occurs sporadically at a very low incidence but Deslys and colleagues (1994) refer to a group of 25 cases of CJD that occurred in France among children treated between January 1984 and June 1985 with human growth hormone (hGH). The hormone had been extracted from large numbers of cadaveric hypophyses. The cases were confined to individuals with the same genetic status as sporadic cases of CJD, who were homozygous at codon 129 of the PrP gene. GSSD shows a clinical overlap between Kuru and CJD but is familial and related to a point mutation on chromosome 20, which encodes an amyloidogenic protein PrP (Brown, 1990). As in scrapie and BSE, the causative agent of CJD can be transmitted to experimental animals (Gibbs *et al.*, 1979; Manuelidis and Manuelidis, 1979; Tateishi *et al.*, 1979).

Thus spongiform encephalopathies have been described in several species, and in most of them the disease has been shown to be transmissible to animals of the same species, where this is ethically possible, and to certain laboratory animals. The purpose of this paper is to give a condensed overview of the aetiology, pathology and familial nature of some of the spongiform encephalopathies as well as aspects of diagnosis and serology; it will particularly address the possibility of a viral aetiology for these diseases.

## DIFFICULTIES IN STUDYING SPONGIFORM ENCEPHALOPATHIES

There are many difficulties in studying either the natural or experimentally induced diseases: the animals involved, the incubation period required for the emergence of the disease, the innate resistance of a proportion of the population seen as an expression of genetic influences, the differing behaviour of strains of agents isolated from a given species, the symptomatology, the pathology, the uncertain nature of the agent and its means of transmission, the perceived 'lack' of an immunological response or changes in the immune system, and the biological hazards involved in conducting experiments.

## THE ANIMALS INVOLVED

None of the infective agents that cause the spongiform encephalopathies can be demonstrated except by animal inoculation. Sheep were the first experimental animals used for research on this group of diseases (scrapie) and the intracerebral route of

inoculation was found to be better than the subcutaneous route for obtaining the maximum incidence of disease. There are ethical, logistical and humanitarian considerations against using large animals for this type of study. The finding that the spongiform encephalopathies were transmissible to certain laboratory animals (mice, hamsters and laboratory primates) was a critically important advance. It then became possible to titrate the infectivity of spleen, brain and other tissues in small laboratory animals, which was essential if any real advance in our understanding of the causative agents was to be obtained. However, a problem with the use of laboratory animals is that they may carry other disease agents in a latent form. The presence of such agents might even determine whether or not scrapie appears following inoculation with infected material and also the progress of the disease. This possibility has attracted attention, and 'caesarian-derived, barrier-maintained colonies have been the rule for most serious work in small laboratory animals. Neoplasia, however, remains a problem in some colonies of laboratory rodents' (R.M. Barlow, personal communication, 1994).

The need to hold and observe animals for long periods of time is a great inconvenience in studies on natural and experimental transmission of the spongiform encephalopathies. In BSE, for instance, some cases are still emerging that may reflect exposure to the agent in the early 1980s. In ovine experiments, the 4½-year period of observation noted in the early Scottish transmission experiments was not excessive, although enough data can usually be collected within a 2-year period in sheep. In mice, scrapie infection progresses more quickly, and if 4–5-week-old animals are inoculated intracerebrally with large amounts of the agent most of them will develop the disease within 10 months (Chandler, 1961); in hamsters the disease may proceed even more rapidly (Kimberlin and Walker, 1977). Contributing to the incubation period is the phenomenon that has been termed the 'zero phase' (Dickinson and Outram, 1979). This is a period after infection when no infectivity can be demonstrated from such organs as the brain, spleen and liver.

## THE DIFFERING BEHAVIOUR OF STRAINS OF AGENTS ISOLATED FROM A GIVEN SPECIES

The severity and type of lesions vary, and a quantitative assessment of these can be used as a characteristic of the biological behaviour of scrapie isolates. Transmission experiments in inbred mice have shown that there are mixtures of agents in a natural case of scrapie in a sheep, and as many as 15 distinct stains have been so identified. At least four murine strains of scrapie have been derived from the 'drowsy goat' source, the result of scrapie transmission experiments in goats (Bruce and Dickinson, 1979). Such a spectrum of behaviour by different isolates of field cases would be expected in studies with the spongiform encephalopathies of other species, especially if they truly represent passages of the scrapie agent.

The agent causing BSE is transmissible to mice (Fraser *et al.*, 1992; Jeffrey *et al.*, 1992) and can be orally transmitted to C57B1 mice by feeding BSE brain (Barlow and Middleton, 1991). However, the nature and distribution of pathological lesions in experimentally infected mice were similar even when the source of the infection was different species of bovidae (Jeffrey *et al.*, 1992), and there was no evidence of strain variation. Although transmissible encephalopathy is not observed naturally in pigs, it

can be produced experimentally in this species (Dawson *et al.*, 1990) by transmission of BSE. Differently located neural lesions and different signs from those seen in TME were observed in mink receiving brain tissue from BSE-infected cattle. Transmission was effected both parenterally and orally (Robinson *et al.*, 1994).

In transmission experiments with CJD and Kuru, chimpanzees and squirrel monkeys are the most sensitive, and are uniformly susceptible, but the disease has also been transmitted to laboratory animals. Guinea-pigs died quite rapidly of hydrocephalus, which did not appear to be due to the presence of any additional conventional virus in the inoculum. Hamsters seemed uniformly susceptible and developed paralysis of the hind limbs and skin hypersensitivity. Mice that were infected with guinea-pig-derived CJD developed signs of skin hypersensitivity and locomotor problems after a long latent period (Manuelidis and Manuelidis, 1979).

Not all transmissible spongiform encephalopathies are due to scrapie-like agents. Mice, homozygous for the grey tremor trait, develop a transmissible encephalopathy that is not accompanied with the coat pigmentation, the seizures, tremors and ataxia characteristic of the clinical disease in the donor animals. Further, brain homogenates from these mice do not show the presence of proteinase-K-resistant PrP<sup>Sc</sup> (Bendheim *et al.*, 1988).

## THE SYMPTOMATOLOGY

'Most neurological diseases follow the same general sequence of events: excitement → hyperaesthesia → dementia → dullness → coma → death; incoordination → ataxia → paresis → paralysis. It is the duration of each phase which varies with the cause and the neural centres critically damaged' (R.M. Barlow, personal communication, 1994). The signs seen in scrapie, which are not specific, reflect damage to the neural centres, following the two alternative progressions noted above. For instance, with reference to the louping-ill vaccination trials in the 1930s, louping-ill itself is also characterized by muscle tremor and incoordination of the hind limbs. There may be hypersensitivity to noise and touch. Unlike scrapie, the disease progresses more rapidly. Death follows within 7–10 days of signs of developing. Some of the signs seen in scrapie show points of resemblance. In scrapie, there are also signs of increased skin reactions to pressure and other stimuli. There is a characteristic 'nibbling' reaction and a pruritus manifested by the sheep rubbing, leading to loss of wool over the rump, thighs and tail base. There is impairment of locomotion, starting with problems in the hind limbs (Blood *et al.*, 1983). Although scrapie is a far more chronic disease than louping-ill, it is curious that variations of hyperaesthesia are observed in both these diseases, with very different aetiologies.

In bovine spongiform encephalopathy (BSE), hypersensitivity to touch and sound are also common signs, but there are many other subtle changes in behaviour. However, cases of disease with these very signs, but which are not BSE in terms of their histopathology, can occur in cattle (Jeffrey and Wilesmith, 1992).

It has also been suggested (Gibbs *et al.*, 1990) that BSE may have occurred in the United States under the clinical guise of the 'downer cow' syndrome, even though cattle disease due to BSE has not been reported in that country. These workers attempted experimental transmission of the scrapie agent in brain homogenate from sheep or goats to 10 cattle. Three cattle developed neurological signs 27–48 months

after inoculation, consisting of progressive difficulty in rising, stiff-legged stilted gait, incoordination, disorientation and terminal recumbency. From onset to terminal signs, the disease lasted 1–2.5 months. There were resemblances to the ‘downer cow’ syndrome. While autopsy revealed insufficient changes to confirm a clinical diagnosis of BSE, the protease-resistant protein PrP<sup>27-30</sup> was detected in each of the 3 animals with neurological signs; none was detected in the other 7 animals.

If the results of this experiment can be replicated, the consequences are serious. The ‘downer cow’ is a familiar one to veterinarians and, while the usual aetiology is a Ca/P imbalance, successful repetition of this experiment would mean that, where that imbalance cannot be demonstrated, the presence or absence of BSE will have to be verified.

The signs and symptoms that follow the onset of the human spongiform encephalopathies show some differences between types: in Kuru there is a fine tremor of the head, trunk and limbs, together with the onset of a progressive ataxia; in CJD the symptoms are of dementia and, instead of the fine tremor, there is a myoclonic jerking; in GSSD there is an overlap with the signs of Kuru and CJD and there is also a marked dementia. The type of hypersensitivity associated with scrapie and BSE is absent.

Returning to the pruritus seen in scrapie and BSE, there seems to have been no investigation of the cause of the irritation. One must question whether the hyperaesthesia is evidence solely of nerve irritation or whether it is a pointer to a development of the hypersensitivity or skin lesions seen in some virus infections (Fenner *et al.*, 1974) or even related to the local release of histamine or a related compound.

## THE PATHOLOGY

There are no immunological tests to aid recognition of the diseased living animal. Clinical signs are not sufficiently specific to diagnose disease and diagnosis depends on the examination, by experienced pathologists, of stained sections of brain tissue for characteristic lesions (Wells and McGill, 1990), electron microscopy to detect the characteristic SAF fibrils (Scott *et al.*, 1990) and tests to detect PrP<sup>Sc</sup>, the prion protein characteristically found in the spongiform encephalopathies (Cho, 1986; Rubenstein *et al.*, 1986). This latter protein is characteristically resistant to proteinase-K. PrP<sup>Sc</sup> contributes to the formation of the amyloid aggregates or plaques seen in the brain of animals with scrapie. PrP<sup>Sc</sup> is encoded by the PrP gene, which expresses a similar protein PrP<sup>C</sup> (sensitive to the action of proteinase-K) in uninfected tissues. In the past, one histological characteristic of the brain lesions seen in spongiform encephalopathies drew much interest, this being the occurrence of large vacuoles outside the outer wall of neurons. No good explanation has yet been found for these vacuoles, which invariably appeared empty by both light and electron microscopy – the larger vacuoles appearing to have formed by a coalescence of smaller vacuoles. Although interesting, these vacuoles are not pathognomonic, as similar vacuolation has been seen in the cerebrum of mice infected with a parainfluenza virus (Baringer *et al.*, 1979) and also in lymphoma-prone wild mice (Gardner *et al.*, 1979). Nevertheless, such vacuoles are a good diagnostic aid. As ‘empty’ inclusions, they presumably represent unstainable material, fluid, gas or volatile hydrocarbon<sup>2</sup>.

The possibility of these vacuoles representing unstainable material was addressed many years ago by Darcel and colleagues (1961). These authors were aware of two familial diseases in man: Castaigne–Lhermite’s and Wilson’s diseases. In both diseases, the patient’s behaviour shows some resemblance to certain signs seen with the spongiform encephalopathies, namely a motor disability in the first disease, a progressive rigidity, a coarse tremor of the limbs, and some degree of dementia in the second. In Castaigne–Lhermite’s disease, histological examination of the nervous system reveals the accumulation of glycogen within the nerve cells, varying from small droplets to large masses. In Wilson’s disease, where the onset is most common in the second decade of life, copper accumulates in large amounts in the corpus striatum and in other tissues. While there are differences in the clinical signs and the histopathology shown by both diseases from those seen in scrapie, it was thought worthwhile to determine the glycogen and copper levels in the brains of sheep with experimentally induced scrapie. The levels of neither copper nor glycogen were elevated compared to the values found in the brain of normal sheep<sup>3</sup>.

With respect to the vacuoles representing fluid accumulations, this is judged unlikely because body fluids, even cerebrospinal fluid, contain protein which is stainable by routine histological procedures. We are left with the possibility that these vacuoles result from the accumulation of gas in the form of bubbles or the presence of inclusions of volatile hydrocarbons.

The most likely gases to be released would be oxygen or carbon dioxide. The latter would be taken up by the buffering action of the tissues, while oxygen free radicals are released by enzymes such as the O<sub>2</sub>- and H<sub>2</sub>O-forming oxidases<sup>4</sup>. In the blood, molecular O<sub>2</sub> would be quickly bound by haemoglobin in the red cells, but most locations in the brain are beyond the ‘blood–brain barrier’ and, there, oxygen would be poorly soluble and quite toxic.

With regard to the possibility of the vacuoles being due to volatile hydrocarbons, both ethane and pentane are released from cells when there is peroxidation of polyunsaturated lipids (Müller and Sies, 1984). Efforts to detect evidence of such peroxidation in brain tissue from cases of transmissible encephalopathy have yet to be made.

Although Hunter (1979) lists a number of changes in the brain with the progress of scrapie, neither this nor a survey of the literature shows any indication that the possibility that these vacuoles are gas inclusions has been vigorously researched. Resolution of this problem could have led to a more directed approach to the biochemical changes that occur in the encephalopathies. Biochemical changes that have been noted include changes in amino acids in the blood plasma and brain tissue (Kasting and Darcel, 1963) but, except for great increases in the activity of hydrolytic enzymes, evidence of other biochemical alterations in the brain, plasma and urine is scanty (Kimberlin, 1976b). Liver function is normal (Darcel *et al.*, 1963). However, there has been the recent observation in one case of BSE of a very high plasma glucose concentration (Scott *et al.*, 1988). More data are needed as consistently high blood glucose could point to other biochemical investigations that should be made.

Millson and Bountiff (1973) found a group of five glycosidases that showed greatly increased activity in the brain of mice with clinical signs following inoculation of the agent, four of these showing an increase before clinical signs appeared. One wonders whether such increased activity would spill over into the plasma and be usable for an early diagnostic test for the development of a spongiform encephalopathy.

## THE UNCERTAIN NATURE OF THE AGENT

Scrapie is transmissible, but the causative agent has not been shown to be a virus. One of the difficulties in accepting a conventional virus as the agent has been its resistance to damage with a variety of agencies. In spite of this, a viral aetiology has not yet been excluded. The possibility has recently been advanced that mollicutes (spiroplasmas) are responsible (see below), but most current thinking envisages a different kind of transmissible agent. Two such agents have been postulated – ‘prions’ and ‘virinos’. The ‘prion’ was postulated to be an infectious protein that directs its own replication (Prusiner, 1982). The ‘virino’ hypothesis suggests the existence of a very small scrapie-specific nucleic acid (Dickinson and Outram, 1988). Evidence for the prion and virino and other possible hypotheses for the causation of the encephalopathies will now be considered.

### *Prions*

The variety in the infective agents that can cause disease is awe-inspiring, particularly with regard to the way they are propagated, retain their characters, and cause problems for the host. With bacteria and viruses, the infective agent carries its own genetic material in the form of either RNA or DNA, but a decade ago it was suggested that an altered protein can itself transmit the encephalopathies. This is the prion PrP<sup>Sc</sup> (Prusiner, 1982), or scrapie-associated fibril protein (SAF), which is present as fibrils or rods, 33–35 kDa in size, that can be seen by electron microscopy intracellularly and extracellularly in the brain of animals affected by scrapie. PrP<sup>Sc</sup> is heterogenous on electrophoresis, although this may be due to different degrees of glycosylation of this sialo-glycoprotein (Somerville and Ritchie, 1990). Species differences have been found in bovine, murine and ovine PrP<sup>Sc</sup> (Groschup and Pfaff, 1993). The homozygous PrP genotype has been found to predispose to sporadic CJD (Palmer *et al.*, 1991) and a prion protein missense variant is linked to the occurrence of GSSD (Hsiao *et al.*, 1989).

The possibility that protein can be infectious in the absence of an association with nucleic acid is intriguing. Prion protein and scrapie-associated fibrils are especially characteristic of brain lesions in scrapie (Merz *et al.*, 1981; Bolton *et al.*, 1982). However, although hamster and mouse spleen contain only low levels of PrP mRNA (Caughy *et al.*, 1988), they can contain large amounts of the transmissible agent. This is hardly good evidence that the prion is the infective agent. Kitamoto and colleagues (1989) were able to demonstrate a correlation between titres of the CJD agent in the brain and spleens of infected mice and their content of PrP, while Czub and colleagues (1986) were not able to do so for scrapie. Unfortunately, it is difficult to resolve this question, since titration of the scrapie agent is attended with wide errors (Marsh *et al.*, 1984) and the specificity of serological methods for determining SAF/PrP<sup>Sc</sup> is open to question, since PrP<sup>Sc</sup> is produced by the same gene as its homologous normal protein, differing only in its sensitivity to proteinase-K.

Another fact suggesting that the ‘prion’ is not the infective agent for scrapie is that PrP<sup>Sc</sup> expressed *in vitro* by a cloned PrP gene is ineffective in inducing the disease. When the hamster gene for expressing this protein is introduced into mice, this species now has the susceptibility to scrapie characteristic of hamsters rather than the



susceptibility of mice; the transgenic mice still have to be infected with the scrapie agent (Scott *et al.*, 1989). Other evidence indicating that the 'prion' or SAF is not the causative agent of scrapie is given by Braig and Diringler (1985). Finally, Bueler and colleagues (1993) showed that mice bred for lack of expression of the PrP gene are resistant to the development of scrapie. After the introduction of Syrian hamster PrP transgenes, these mice became highly susceptible to hamster but not mouse prions. Again, it has been found (Xi *et al.*, 1992; Demaimay *et al.*, 1994) that, following treatment of hamsters with amphotericin B or a derivative of this antibiotic, the accumulation of PrP was not correlated with replication of the scrapie agent.

Race and colleagues (1988) described experiments with cloned neuroblastoma cultures with a high titre of the scrapie agent. While these workers acknowledged that a proteinase-K-resistant protein developed with scrapie infection in these experiments, they found no evidence that the PK-resistant form of PrP was necessary for infectivity. They concluded that the PK-resistant PrP of scrapie arose *in vivo* as a result of alteration of PK-sensitive PrP by changes in the physiological conditions of cells as they degenerate, and that the evidence for the inseparability of the scrapie agent and PK-resistant PrP was still circumstantial, a view not shared by Bolton and colleagues (1991). Caughey and colleagues (1991) speculated, from further work on these neuroblastoma cultures, that the increased tendency of PrP to aggregate is the origin of this resistance to PK.

Lewin (1982) was ready to accept that the prion is involved in the aetiology of scrapie but only as a carrier of a small protein or peptide that he called a protovirin. He suggested two alternative mechanisms by which such an entity could act either as an RNA template or as an extraneous DNA operon.

It appears that it is very difficult to resolve the problems of whether PrP<sup>Sc</sup> is the causal agent of scrapie or a carrier agent, and even whether the causal agent is a protein, by the approaches that have been so far made. Whatever the outcome, it is certain that PrP plays an important part in the pathogenesis of the transmissible spongiform encephalopathies (Weissmann, 1991a,b). The discussion on the role of prions is taken up again in other sections of this paper.

### *Virinos*

The virino hypothesis suggests that the infectious agent of scrapie is a very small piece of nucleic acid with a behaviour intermediate between that of viruses and that of viroids (Dickinson and Outram, 1988). It has been further suggested that the virino is not translated and is therefore dependent on host-coded protein to form an infectious unit (Kimberlin, 1990). With the nucleic acid probe techniques now available, it would be thought that virinos would be detectable if they exist, but no report of such work has yet appeared.

### *Conventional viruses*

Characteristic features of spongiform encephalopathies include their long latent period for development of overt disease and the need for the presence of 'susceptibility' genes and the PrP gene in the prospective host. This does not rule out

the involvement of a conventional virus in causation of these diseases. Although no such virus has yet been demonstrated in infective extracts, many believe that such viruses may be involved. Chesebro (1992), reviewing the role of PrP in the aetiology of scrapie, raised the possibility that a mutation of this gene increases the susceptibility to an unknown but ubiquitous conventional virus.

Rohwer (1991) summarized in a masterful way why conventional viruses may still be the most likely causes of the spongiform encephalopathies. For example, the long latent period needed for the spongiform encephalopathies to develop is similar to the length of time often required by lentiviruses such as the maedi-visna virus of sheep. However, no connection has been made between such viruses and scrapie. A long latent period for disease to develop is seen with other viruses, for example oncornaviruses. Examples are the lymphoid leukosis viruses of chickens; these are noted for their ability to activate the formation of oncogenes. Oncogenes are involved in tumour formation because their presence leads to perturbation of the normal cell physiology. Scrapie is not characterized by the development of tumours but oncogenes could still be involved, tied as they are to normal cell physiology and to gene changes. Studies similar to those now being made in oncology might be most fruitful. References should be made to oncogenes in the human genome, where as much as 0.1–0.6% may be derived from endogenous sequences. One such sequence is the ERV-3 virus, first recognized by low-stringency hybridization to probes derived from the *pol* region of the chimpanzee CH2 endogenous provirus (Boyd *et al.*, 1993). This work is mentioned as being indicative of a type of approach that should be undertaken on a major scale with bovine and ovine tissues.

Particular attention should be paid to the possibility of retroviruses akin to the mink-cell focus (MCF) inducing viruses being involved in the pathogenesis of the spongiform encephalopathies<sup>5</sup>. The possible involvement of other retroviruses, particularly the lentiviruses, maedi-visna in sheep and visna-like virus in cattle (Evermann, 1990) should not be overlooked as possible contributors to the development of scrapie and BSE, in their respective hosts. Attention is also drawn to the mouse hepatitis viruses, coronaviruses that have the ability to enter into recombination with other viruses (Luytes *et al.*, 1988) and are widely present in mouse stocks; there are also neurotropic strains (Kyuwa and Stohlman, 1990).

Louping-ill virus is a tick-transmitted member of the virus family Flaviviridae (Fenner *et al.*, 1987; Gao *et al.*, 1993) that is closely related to other European tick-borne encephalitis viruses (Jiang *et al.*, 1993). It was the evaluation of a vaccine designed to prevent disease due to louping-ill that led indirectly to the initial major studies on scrapie (see above). Could vaccination against infection with this virus have increased the susceptibility to scrapie?

Another virus that has been mentioned in the context of scrapie causation is the hepadnavirus. Some time ago, Carl Eklund stated that 'the scrapie agent is not a member of a unique group of pathogens but is a medium-sized virus whose resistance to heat is analogous to that of serum hepatitis virus' (Hotchin, 1979). This suggestion seems to have attracted little attention, but there is a strong possibility that hepatitis B viruses (Tiollais and Buendia, 1991) are more widespread than previously believed. Viruses similar to human hepatitis B virus have been found in woodchucks and ground squirrels (Minuk *et al.*, 1986), while antibody to this virus can be demonstrated in many other animals, including pigs, goats, cattle and especially sheep (Hoofnagle *et al.*, 1983). Of particular interest, with relation to the virino hypothesis of scrapie, is the

fact that another agent is associated with hepatitis B virus. This agent is the hepatitis delta virus (HDV). HDV is a small defective viroid-like RNA virus (Negro *et al.*, 1989) that requires another virus, hepatitis B virus, as a helper. Thus, even if hepatitis B viruses are found in farm animals, the search should not stop there but continue with a search for HDV-like viruses.

### *Spiroplasmas*

Humphrey-Smith and colleagues (1992) noted that the presence of spiroplasma-like inclusions has repeatedly been noted in cases of CJD, that spiroplasmas show a number of similarities with the scrapie-associated fibrillar protein (SAF), and that lesions mimicking spongiform encephalopathy are produced by spiroplasma-infected rodents. Spiroplasmas belong to the Class Mollicutes, Order Mycoplasmatales. Like mycoplasmas, spiroplasmas, some of which are pathogenic for insects and plants, are a family within this order. Humphrey-Smith and colleagues (1992) suggested that spiroplasmas should not be ignored as a possible cause of spongiform encephalopathies. There are indeed immunological cross-reactions between the proteinase-resistant proteins of *Spiroplasma mirum* and PrP<sup>Sc</sup> (Bastian *et al.*, 1987) and there are proteinase-K-resistant proteins in other members of the Class Mollicutes (Butler *et al.*, 1991).

It would have been thought that articles would have appeared by now listing attempts to demonstrate antibody to *S. mirum* and other spiroplasmas in the sera of sheep with scrapie and cattle with BSE and attempts to correlate the titres against such agents with the onset of signs, but there do not appear to have been any such papers.

## PHYSICAL PROPERTIES OF THE INFECTIVE AGENT

Scrapie is a particularly frightening disease. The transmissible agent is very resistant to heat (Mould and Dawson, 1970) and to other agents normally destructive to biological materials and it gives the appearance of an ability to cross species boundaries. The first clue to the resistance of the agent came from the louping-ill vaccination trials in the 1930s (see introduction), where scrapie developed following the inoculation of formalinized tissue. While later shown not to be completely resistant to the action of formalin, the agent's resistance to a number of other disinfectants is unexpectedly high for the viruses and similar agents with which we are familiar. Further, studies on the critical size of the agent by X-irradiation and by exclusion chromatography give very different estimates of the limiting size of the agent. X-ray studies suggest a size of  $1.5 \times 10^2$  kDa, while the much larger figure of  $5 \times 10^4$  kDa is suggested by chromatographic studies. This difference suggested to Hunter (1979), a source for much data on the physical properties of the scrapie agent, that a two-component system was necessary for scrapie infectivity, a small informational component that provides the radiation target, and a larger component that provides a protective or carrier role. Hunter (1979) also indicated that only proteases have much effect on scrapie activity and that, even for this effect to occur, the substantial amounts of lipid associated with brain tissue must be removed. Part of

the evidence that led to the prion concept was the finding that the proteinase Pronase destroyed the activity of the agent. This pointed to a need for a protein component for its infectivity (Cho, 1980, 1983). However, certain tissues contain proteinase inhibitors that can block the effectiveness of proteinases and, in studies on the kind of activity proteinases have on brain extracts, Darcel (1990) found that Pronase, highly active in control tests on sheep haemoglobin, had no proteolytic effect on the Hirt supernatant of normal mouse brain. Reasons for this lack of activity with brain extracts should be explored and extended to proteinase-K, to which PrP<sup>Sc</sup> is more resistant than its homologue in normal mouse brain<sup>6</sup>. The sensitivity to this enzyme was found to vary between three stains of the mouse-passaged scrapie agent (Kascak *et al.*, 1985)<sup>7</sup>. Without data on what the proteinase-K was really accomplishing, this information cannot be convincing.

## THE UNCERTAINTY CONCERNING THE MEANS OF TRANSMISSION

There will not wittingly be repeats, on any worthwhile scale, of what I have termed the accidental 'experiments'. Thus it is useful to look back at them and see whether any information was missed in the earlier analysis.

The louping-ill vaccination trial provided much of the information on scrapie that we have today: the long latent period before disease appears, the resistance of the agent and the resistance of much of the population to the development of the disease. Rather than implicating the acquisition of infection by ingestion of 'contaminated' feed, that event directly implicated parenteral 'infection'.

No possibility should be overlooked in studies of a disease as strange as scrapie. Even though louping-ill virus affects the same areas of the nervous system as evidenced by clinical signs, inactivated louping-ill virus seems to have been excluded as a contributing factor to the development of scrapie by the design of the vaccination trials. However, the possibility that the injection of a killed louping-ill virus can potentiate infection of the scrapie agent deserves further study<sup>8</sup>. Other controls in attempting to understand the results of the vaccination trials of the 1930s could be to examine brains of sheep with louping-ill for the presence of protease resistant PrP<sup>Sc</sup> and also to test the sera of sheep used in scrapie-transmission experiments for antibodies to a wide spectrum of louping-ill virus isolates.

While the above concerns spread of scrapie by parenteral injection, there has been a suggestion that natural transmission may occur from sheep eating dropped placentae (Dickinson *et al.*, 1974), but this is contraindicated by the fact that ewes usually confine themselves to eating their own placenta.

The interpretation that a change in the rendering of sheep offal led to the outbreak of BSE is based on statistical evidence and on the conclusion that, in the circumstances at the time, no other explanation was possible. It was also an explanation that could be adopted for political reasons and gave some justification for the control procedures that were adopted for controlling BSE. However, it has been pointed out that the human risk from eating mutton and lamb must be negligible since, in a country where scrapie occurs as a disease and where this meat has been eaten for a very long time, the incidence of encephalopathies, such as CJD, that might be expected to follow, is actually very low (0.49 cases per million) (Mathews, 1990). Finally, despite earlier concerns, a connection between the feeding of sheep offal and the incidence of TME has not been proven (see above).

## THE 'LACK' OF IMMUNOLOGICAL RESPONSE OR CHANGES IN THE IMMUNE SYSTEM

Avery and colleagues (1960) considered that, if scrapie were a chronic disease caused by an infective agent, the level of gamma-globulin, the serum protein fraction containing antibody, might well be raised. Unfortunately, the electrophoretic patterns were similar for the sera of both normal and scrapie-affected animals. Nevertheless, despite the 'rather overwhelming evidence for "immunological silence" in scrapie, Stites and colleagues (1979) advanced some evidence for the development of an immunological response. They stressed that the lack of detection of an immune response does not convincingly disprove its existence. Whatever the causal agent, if its tropism were entirely neural, then insufficient agent might reach the blood stream to lead to an immunological response. This explanation appears weak, but another possible explanation is that SAF is too close to its equivalent produced by the PrP gene of normal animals to give a response, although it would be expected that even a small difference would lead to the production of recognizable antibody.

A more direct immunological approach would be to look for antibody to other disease entities; for example, with what frequency are antibodies to BLV encountered in cattle in cases of BSE? with what frequency are maedi-visna antibodies encountered in sheep with scrapie? These viruses are quite prevalent in dairy cattle and in sheep, respectively, and are slow viruses in their own right. Both viruses have a DNA phase and could well have an association with genes for both susceptibility to the encephalopathies and amyloid fibrin formation. Antibodies to hepadnaviruses, to MCF viruses and to spiroplasma should also be sought. Unless there were an associated immunodeficiency, the absence of detectable antibody to these agents would largely remove them from consideration as having primary or secondary roles in the causation of the spongiform encephalopathies.

Evidence of an immunological deficiency should also be sought using current technology similar to that used by Rao and colleagues (1990) to study the immune deficiency produced by the avian retrovirus, avian erythroblastosis virus. Stites and colleagues (1979) affirmed that current information could accommodate the possibility that negative regulation or active immunosuppression play an important role in the pathogenesis of scrapie. They noted that mice show a distinct splenomegaly after intracerebral inoculation with the agent, that the agent at first appears in higher concentrations in the spleen than in the brain, and that splenectomy prolongs the incubation period. They also recorded altered responses of spleen cells to mitogens. As in other studies with laboratory animals, they could not entirely rule out the possibility that a passenger virus or microorganism was responsible for these changes.

This possibility of the presence of passenger viruses and microorganisms is a constant obstacle to understanding the results of agent transmission experiments in laboratory animals. Is *Spiroplasma mirum*, proposed as a possible cause of scrapie, merely a passenger responding to a suppressed immune state in the same way as *Pneumocystis carini* in patients with AIDS (Redfield and Burke, 1988)? Any passenger agents involved in the spongiform encephalopathies might well vary according to the host species.

## THE BIOLOGICAL HAZARD

Because of the widespread consumption of products obtained from cattle by most of the human population, the recent outbreak of BSE has caused great alarm and has been the subject of a considerable correspondence in the British medical press. It is not known whether cattle are the final hosts, nor whether the infection can spread to humans by the consumption of beef, milk or products, such as bovine albumin used in various forms of therapy<sup>9</sup>. With the ability for agents causing the spongiform encephalopathies to cross specimen boundaries, the fear that people might acquire human forms of these diseases from cattle has to be very real. It might be assumed, for instance, that, as a result of the epidemic of BSE through which British cattle have passed, slaughterhouse workers will have inadvertently come into contact with preclinical cases (Howard and Castle, 1990; Dealler and Lacey, 1991; Coyle and Harvey, 1992). Scrimgeour and Brown (1991) pointed out that the most efficient means for transmission of scrapie, other than the parenteral routes used experimentally, are via the conjunctival and nasal mucosae and cutaneous abrasions. They suggested that slaughterhouse workers would be prone to this type of contact with microbial zoonoses. The wearing of protective clothing by these individuals when they are extracting the cranial contents from cattle was also advised.

However, these fears of the human population acquiring BSE should be tempered by realism (Collee, 1991). CJD often occurs in clusters with a history of familial relationships, and these seem to have more relevance to the incidence of the disease than do eating habits (Taylor, 1989). If BSE presents a hazard to the human population, especially to veterinarians or to slaughterhouse personnel, a statistical study of the occurrence of spongiform encephalopathy over the next several years should show whether the incidence of CJD has increased as a result of the intimate exposure to cattle. The potential risks to which the British meat-eating population has been exposed have led to funding of the surveillance of cases of CJD that arise in the United Kingdom, with particular reference to the dietary and occupational history and other possible risks (Will *et al.*, 1992). However, Fear and Devakumar (1990) have questioned the accuracy of such an ongoing survey, given the variety of symptoms exhibited by patients with CJD.

A major problem with experimental work on the encephalopathies is the resistance of the agent. In performing experiments on scrapie, etc., great care has to be taken to avoid using contaminated laboratory equipment. Illustrations of the care that has to be taken are the technical details given in papers on the oral transmission of BSE to mice (Barlow and Middleton, 1991) and in the transmission studies with Alzheimer's disease (Manuelidis and Manuelidis, 1991).

## GENETICS AND SPONGIFORM ENCEPHALOPATHIES

The innate resistance of a proportion of the population to experimental transmission of the spongiform encephalopathies is seen as the result of genetic influences. Some animals within a species will prove resistant to experimental infection following infection. However, the degree of resistance will vary according to the route of infection. In most species, the natural route of infection appears to be the oral route and innate resistance is then likely to be more important than when animals are

infected intracerebrally. Offspring from infected ewes are prone to develop scrapie. This may be evidence of a familial trait and the practice has been to remove affected family lines in attempts to control the disease<sup>10</sup>. However, it is uncertain whether this predisposition of sheep to scrapie is due to increased exposure at time of parturition or to gene-dependent susceptibility.

There are specific genes for susceptibility to scrapie: *Sip*, which controls the incubation period of the disease in sheep, and the *Sinc* gene in mice (Bradley and Matthews, 1992; Kimberlin, 1990). *Sinc* has also been found genetically linked to the *Pm* protein gene (Hunter *et al.*, 1987). The finding by Deslys and colleagues (1994) that all the cases of CJD acquired by injections of human growth hormone in 25 French children occurred in individuals who were homozygous at codon 129 of PrP is seen as support for the importance of the PrP gene in the development of encephalopathies.

A complication in the British outbreak of BSE is that the national gene pool among cattle will have changed over the same time-frame as the epidemic owing to the widespread adoption of artificial insemination (AI) and embryo transfer (ET). Perhaps a new study of the data that have been accumulated on this outbreak might point to certain bulls or certain cows that have had an unexpectedly high incidence of BSE in their progeny. If such an effect could be demonstrated, it would mean either that these cases have followed the introduction of genes for susceptibility into previously unaffected herds or that actual infection occurred through the use of these breeding techniques. Wrathall and Brown (1991) stated that there is no evidence to suggest that vertical transmission of BSE occurs and discussed ET and AI studies with scrapie-infected sheep. It has been thought that ET and AI might provide a means for breaking the disease transmission cycle in scrapie and BSE, but such studies will be complicated by the existence of the genes for susceptibility.

## CONCLUSIONS

The research done on the prion theory has greatly expanded our knowledge of these diseases, but the most active promoters of the prion ask us to believe that it, and its gene, can cause the encephalopathies, that it is responsible for the predisposition to and the pathogenesis of these diseases, and that it can ensure its own replication. Much more controlled work on the prion is necessary before conclusions of this kind can be justified. For instance, can the material of which prions are a component (SAF) act as a viral promoter of other neurotropic viruses? An investigation of such a possibility would seem to be essential in understanding the role that the prion plays. Studies with known neurotropic viruses, with the viruses given in the presence and absence of SAF, should show whether this material can have a promoter effect.

The quotation at the beginning of this article describes workers in the field of the encephalopathies very well, each interested in their own particular area. In the author's case, the bias is towards viruses being involved in the causation of these diseases. In considering the experimental work that has been done, the differences in the biological activities of the isolates of the transmissible encephalopathies are impressive. If BSE were really a manifestation of transmitted scrapie, one would expect to find that its isolates behaved with the variability shown by scrapie isolates. This does not occur.

Not knowing the cause of the transmissible encephalopathies makes the approach to controlling these diseases very difficult. The outbreak of BSE in Britain has disrupted the export of pedigree cattle, and there were other ramifications. There is the concern, not substantiated, that once transmitted to a new species, such as bovine, the agent might persist in the new species. There are also fears that the disease might become established in other countries and cross the species boundary to man. The expenditure of money for research on the encephalopathies is therefore justified from the agricultural standpoint alone, and the present paper has tried to suggest areas that are suitable for further exploration.

One interpretation of the cause of the outbreaks of scrapie, TME and BSE noted in this paper is that material in the feed or in the inoculum triggered the growth of a quiescent but unrecognized virus. Is such triggering of a quiescent virus possible? We know that contact with certain compounds can lead to the multiplication of latent oncornaviruses (Robinson *et al.*, 1976) and that corticosteroids can provoke the emergence of viral activity with latent herpesvirus infections (Darcel and Dorward, 1975; Fenner *et al.*, 1987). Other mechanisms may also exist for the stimulation of virus growth.

It is peculiar how refractory the transmissible spongiform encephalopathies are to investigation, and a real understanding of their causation is still elusive. Yet, with research, there is a chance of discovering new types of disease agents and learning much about the nature of important neurological diseases.

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## NOTES

1. Louping-ill is a tick-borne virus-caused disease of lambs, characterized by incoordination of the hind limbs and by hypersensitivity to noise and touch (Blood *et al.*, 1983), seen principally in Great Britain and Russia.
2. Autophagic vacuoles in mice, with sequestered cytoplasmic areas containing ribosomes and occasionally mitochondria and small secondary vacuoles, have been described in neuronal perikarya and neuronal processes of mice with experimental scrapie and CJD (Liberski *et al.*, 1992). There seems to be a renewed interest in these neuropil and neuronal vacuolations in circumscribed areas of the brain, with both scrapie and BSE (Shinagawa *et al.*, 1985; Sasaki *et al.*, 1986; Wells *et al.*, 1987; Wells and Wilesmith, 1989; Liberski, 1990; Scott *et al.*, 1990).
3. Further references to Lhermite's and Wilson's diseases are given in the paper by Darcel *et al.* (1961)
4. To study the possibility that these characteristic vacuoles are due to oxygen would mean the application of a variety of biochemical and histochemical techniques, including a qualitative and quantitative study of the enzymes just mentioned (Brunori and Rotilio, 1984). R.M. Barlow (personal communication, 1994) has suggested that marginal copper deficiency, which is very common in sheep, would affect the superoxide dismutases and facilitate accumulations of oxygen free radicals and toxic peroxides, but the result from studies on copper levels in the brains of sheep with scrapie and those of normal sheep (Darcel *et al.*, 1961) do not support this idea.



5. MCF murine viruses are formed *de novo* by recombination between exogenous, infectious ecotropic virus and endogenous sequences related to xenotropic virus. Members of this group of viruses have been isolated from spontaneous mouse lymphomas and can infect both murine and xenogenic cells (cells of other species). Special attention is drawn to work of Gardner and colleagues (1979) on a murine retrovirus motor disease. MCF-like viruses have also been recovered from feline (Neil *et al.*, 1991) and avian oncogene–murine chimeric systems (Feuer *et al.*, 1993). Other references that provide a useful entry to this field include Adachi *et al.* (1984), Chattopadhyay *et al.* (1991), Di Fronzo and Holland (1993), Khan *et al.* (1987), Koch *et al.* (1984), Levy *et al.* (1985), Makino *et al.* (1990), Oliff *et al.* (1984), Quint *et al.* (1984) and Shields *et al.* (1991).
6. If scrapie-infected brain had been used instead of normal brain and loss of activity of the scrapie agent had been observed, then the effect would probably have been due to non-proteolytic factors in the Pronase. The suggestion was offered (Darcel, 1990) that the brain extracts contained a proteinase inhibitor. This should be looked for, and, if found, removed from the system before applying the proteinase treatment.
7. These authors' protocols show that brain homogenates were homogenized with a solution containing proteinase-K, and the supernatant resulting from centrifugation was incubated for 1 hour. The action of the enzyme was then blocked and the solution was then treated with micrococcal nuclease. Many other steps followed before titres were determined in mice and hamsters and compared with those in the original homogenates.
8. The name 'Berry–Dedrick phenomenon' was coined to describe the results that followed the appearance of myxomatosis after inoculation of rabbits with a heat-inactivated preparation of myxoma virus inoculated into rabbits together with live fibroma virus. This was later explained as resulting from the presence of live, residual myxomatosis virus (Fenner *et al.*, 1974). However, cross-reactivation is a known phenomenon by which infectious virions containing active genes from both viruses can result from genetic recombination between an infectious virus and a related inactivated virus (Fenner *et al.*, 1987)
9. My late colleague Robert Kasting became ill some years after we concluded our studies of amino acids in scrapie and normal brain. He felt that the signs of neurological disease that he exhibited meant he had acquired scrapie from aerosols produced by grinding the brains. His brain was sent to the Rocky Mountain Laboratory after his death for detailed histology and for transmission experiments. The results did not confirm his suspicions.
10. Six of the 26 lambs born to recipients of embryos transplanted from donor ewes that had been experimentally infected with scrapie developed this disease (Foster *et al.*, 1992). No correlation was found between susceptibility and the phenotypes for haemoglobin, blood potassium levels, lactoglobulins, albumin, pre-albumin, esterase, haemoglobin, transferrin, reduced glutathione or  $\alpha$ -mannosidase (Darcel and Avery, 1960; Darcel, 1961; Hunter, 1991).

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