EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C1 - Follow-up and dissemination of scientific opinions

OPINION ON:

HYPOTHESES ON THE ORIGIN AND TRANSMISSION OF BSE

ADOPTED BY THE SCIENTIFIC STEERING COMMITTEE AT ITS MEETING OF 29-30 NOVEMBER 2001

OPINION ON HYPOTHESES ON THE ORIGIN AND TRANSMISSION OF BSE IN CATTLE

When preparing its opinions on BSE-risks, the Scientific Steering Committee has frequently been confronted with the unresolved issues related to the "Origin of BSE" and with "Alternative hypotheses for the transmission" of this disease other than via animal proteins and maternal transmission. It therefore invited the TSE/BSE ad hoc group to prepare two scientific reports on the current the state of affairs on both issues. Having taken note of the two reports adopted by the the TSE/BSE ad hoc group at its meeting of 15 November 2001, the SSC concludes as follows:

With regard to the responsible agent of BSE

The prion protein theory remains central to BSE and other TSEs. However, it is wise at present to maintain an open mind on the nature of the agent(s) responsible of BSE.

A number of other hypotheses have been proposed on the nature of the responsible agent(s) of BSE (e.g. a toxin, a bacteria, alkaloidal glycosidase inhibitors, auto-antibodies and a single stranded DNA), but for none of them is sufficient scientific evidence available and some are clearly implausible or very difficult to investigate.

With regard to the origin of BSE

The origin of the BSE prion is not known. Many hypotheses have been suggested, including for example an origin from mammalian species other than cattle (a mutant form of scrapie agent, a natural TSE in Bovidae or Felidae or other wild animals whose carcasses were rendered into MBM, the existence of a form of sporadic TSEs like CJD of humans, a spontaneous mutation of normal bovine PrP into an infectious and protease resistant TSE prion etc.). For none of these hypotheses is there enough data to either substantiate or to reject it.

With regard to BSE transmission

Epidemiological studies, rendering studies and the effect of feed bans in all countries with BSE very clearly support the hypothesis of infected mammalian protein in the form of MBM being the major vehicle for BSE transmission in cattle. It can enter the feed deliberately, or accidentally by cross-contamination that can occur readily during

feed preparation in feed mills, during transportation or on farm. Other infected materials, such as gelatine and/or fat, incorporated in feed represent another possibility of transmission.

Maternal transmission is theoretically a possible route of transmission, although it has not yet been demonstrated. There is statistical support for some possible maternal transmission of BSE in cattle. However, even if it exists, it cannot account for more than 10% (c.i. 5-15%) of the offspring of all cases with BSE and probably less if transmission to calves occurs only if the dam is in the late stage of BSE incubation. Moreover, no plausible mechanism for the maternal transmission of BSE has been identified so far.

For what concerns routes of transmission other than those mentioned above (so-called third route), no one of the many possibilities considered has been substantiated by scientific evidence so far.

With regard to factors which might increase susceptibility of cattle to BSE

Factors which have been suggested as having a potential for affecting susceptibility of cattle to BSE include some metal ions (e.g. copper and manganese), organo-phosphorous compounds and antioxidants. However, supporting data for all these hypotheses are very limited and do not allow any conclusions to be drawn.

The SSC intends to continue to review the situation as it develops.

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HYPOTHESES ON THE ORIGIN AND TRANSMISSION OF BSE

REPORT ADOPTED BY THE TSE/BSE AD HOC GROUP AT ITS MEETING OF 15 NOVEMBER 2001

EXECUTIVE SUMMARY

When preparing its opinions on BSE-risks, the Scientific Steering Committee (SSC) has frequently been confronted with the unknowns related to the 'Origin of BSE' and with 'Alternative hypotheses for the transmission' of this disease other than *via* animal proteins and maternal transmission. It therefore invited the TSE/BSE *ad hoc* Group to prepare two scientific reports presenting the state of affairs on both issues. At its meeting of 15 November 2001 the TSE/BSE *ad hoc* Group discussed and adopted the attached reports which will be updated according as new firm, data-supported evidence or soundly supported hypotheses become available.

These reports can be summarised as follows:

With regard to the origin of BSE

a) The origin of BSE remains unknown. Given the available data, the prion protein is central to TSE science and that MBM is the main vehicle for BSE transmission with accidental cross-contamination of ruminant rations with MBM being an important feature in perpetuating BSE epidemics after feed bans were in place.

b) The origin of the BSE prion is also not known, and many hypotheses have been suggested, including *for example* an origin from mammalian species other than cattle (a mutant form of scrapie agent from sheep, an unmodified scrapie agent from sheep, a natural TSE in *Bovidae* or *Felidae* or other wild animals whose carcasses were rendered into MBM, the existence of a form of sporadic BSE akin to sporadic CJD of humans, a spontaneous mutation of normal bovine PrP into an infectious and protease resistant TSE prion, etc). For none of these hypotheses is there enough data to either substantiate or to reject it. To differentiate these hypotheses the crucial issue is whether the nature of the epidemic is an extended common source or a point source followed by repeated recycling before being recognised. Regarding the origin of BSE, both hypotheses remain open.

c) Disease in an extended common source epidemic occurs more or less concurrently in multiple, widely dispersed different geographical locations that each have the same, or similar, exposure to the same contaminating infection at approximately

the same time. The hypothesis of an extended common source epidemic would fit with the observations that BSE appeared in most parts of Great Britain within a short space of time, shorter than the mean incubation period of BSE and that regional differences could be explained by the epidemiological findings.

d) A point source epidemic is one originating from a singleton event, or focus, and then spreading from that point. An example would be importation of a bovine animal incubating, or affected with foot and mouth disease, but was undetected and mixed with other cattle which then became infected and dispersed the virus to other susceptible animals and species in the same or distant geographic locations. The discrimination between a point source and common source is thus not easy because a point source, at the end of the initial stages of spread, would take the characteristics of a common source. A point source epidemic is thus feasible but it would imply that in the intervening years (say 10-15 years or 2-3 incubation periods) between initial exposure and the first detected cases coming to light no veterinarian detected a new disease, nor was confident enough to submit a brain to a competent laboratory for microscopic investigation. This is considered uncertain. However, if more evidence for a point source epidemic would come forward in the future, then many currently rejected or partially rejected hypotheses (e.g., the BSE infectious agent could originate from any mammal susceptible to TSE) would become viable.

e) The report addresses the view adopted in the Horn Review dated 5 July 2001 that the unique combination of demography (large sheep population compared to cattle population and large amount of sheep waste generated for rendering), events (rendering changes) and particularly calf feeding practices in the UK is a plausible explanation of why BSE was initiated on such a scale there and not elsewhere. The Horn review also considered that there might be an age susceptibility to BSE infection and that this could be investigated experimentally.

f) It is acknowledged, however, that other alternative hypotheses on the origin of BSE exist. Some are not supported and can be rejected as not being possible to cause BSE under any condition (*e.g.* the autoimmune hypothesis, the bacterial (*Spiroplasma* sp.) hypothesis, the single stranded DNA hypothesis or an origin from *Coenurus cerebralis*) and others are implausible and difficult to investigate

at the present time. Some of the latter hypotheses are related to the nature of the agent and how it causes its effect, such as by a toxic action (*e.g.* fat-associated chemical toxins in tallow or organo-phosphorous compounds) or deficiency such as an inadequate exposure to prostaglandins). If at all, they are likely to only partially and minimally contribute to the BSE epidemic, for example by altering susceptibility of an animal to TSE infection. They do not help particularly in identifying an alternative origin for BSE, but they could be important to consider once the real nature of the agent is defined and accepted. It is therefore perhaps wisest at present to still keep an open mind on the nature of the agent and to consider rather that its structure is unknown or at least uncertain.

On BSE transmission

a) There is very clear and strong support from epidemiological studies, rendering studies and the effect of feed bans in all countries with BSE, for the hypothesis of infected mammalian protein in the form of MBM being the major vehicle for BSE transmission in cattle. It can enter the feed deliberately, or accidentally by cross-contamination. However no-one has reported so far, an experiment to test this hypothesis using compound feed with MBM containing the BSE agent rather then infected cattle tissues only.

b) The actual occurrence of cross-contamination of ruminant diets with infected mammalian protein (especially MBM), even though it is not suspected, is not considered to be a possible "third way" of BSE transmission, but part of the feed route. Cross-contamination can occur readily during feed preparation in feed mills, during transportation or on farm, unless stringent measures are taken to avoid it. Usually, cross-contamination would have been accidental. It is possible that the accidental 'cross-contamination' route of exposure could account for the bulk of, if not all, assumed 'Third Way' cases.

c) The incorporation of infected ruminant- or mammalian-derived materials in feed other than MBM is another possibility of transmission which also is not a "third way". Such materials might have been gelatine, fat or blood (or protein products derived from them) in which the starting materials were contaminated. Effectively enforced SRM bans and improved and authorised ruminant stunning and

processing methods (including for rendering, and for gelatine and fat manufacture) should now eliminate such causes.

d) Maternal transmission is theoretically a possible route of transmission since it would appear to occur in natural scrapie in sheep. There is some statistical support for the possibility of some form of maternal transmission of BSE in cattle, but if existent it cannot account for more than 10% [c.i. 5-15%] of the offspring of all cases with BSE and probably less if transmission to calves occurs only if the dam is in the late stage of BSE incubation. However, there is no evidence so far that this so called 'maternal transmission' occurs in the absence of a feed borne source and no plausible mechanism for the so-called maternal transmission has been identified[1]. Nevertheless, it is not currently possible to eliminate maternal transmission completely as an occasional cause of BSE.

e) Any other cause than from feed or maternal transmission becomes a potential 'Third Way'. Possible genuine 'Third Ways' are listed and discussed in detail in the report. Some, though unproven, may increase susceptibility to the disease. Many are theoretically possible (e.g., environmental contamination after unauthorised burial of carcasses of non-declared BSE cases) but, if existent, unlikely to have significantly contributed to the BSE epidemic. They may, however, initially have been overshadowed by the feed and maternal transmission routes of transmission and eventually become a factor in the current trend of the epidemic impeding the rapid total elimination of the disease.

[1] In sheep a plausible mechanism has been identified, *i.e.*, from the placenta of infected sheep. However, comparable investigations in cattle were not conclusive.

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PART 1

HYPOTHESES ON BSE ORIGIN

(A SUMMARY OF ORIGINAL HYPOTHESES AND THOSE INCLUDED IN THE REPORT OF THE BSE INQUIRY AND THE HORN REVIEW)

AS DISCUSSED BY THE TSE/BSE *AD HOC* GROUP AT ITS MEETING OF 15 NOVEMBER 2001

PART I: THE ORIGIN OF BSE

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I. MANDATE AND SCOPE

When preparing its opinions on BSE-risks, the Scientific Steering Committee has frequently been confronted with the unknowns related to the **'Origin of BSE'** and with **'Alternative hypotheses for the transmission'** of this disease other than *via* animal proteins and maternal transmission.

There are several hypotheses on the **'O*rigin of BSE'*** (because it is still disputed). These have been the subject of separate scientific papers and reports, the most recent one being the Horn *et al* review (2001). The TSE/BSE *ad hoc* Group acknowledges this review. It is not the purpose of its own report to further examine the origin of BSE. However, alternative hypotheses for the origin of BSE have been presented to the European Commission (EC) services by correspondence, in addition to the ones also discussed in Horn *et al.,* (2001). These hypotheses have often been submitted without accompanying published or peer-reviewed descriptions, but nevertheless merit being inventoried and, if appropriate, discussed.

Regarding **'*Alternative hypotheses for the transmission of BSE'*,** a comprehensive overview does not currently exist.

This Report therefore has a dual purpose:

a. To give consideration to unpublished, alternative, hypotheses for the **'Origin of BSE'** that have been presented to services of the European Commission (EC) in addition to the ones also discussed in Horn *et al* (2001). **This forms Part 1 of the Report.**

b. To present an inventory and briefly comment on the currently existing hypotheses regarding possible '**Alternative, or third ways'** in which BSE might be transmitted to cattle, using the historical theories for the transmission of scrapie in sheep as a baseline and supplementing them with current published hypotheses that relate only to cattle. **This forms Part 2 of the Report.**

The reader will notice that there is inevitably a degree of overlap between 'the origin' and possible 'third ways'. In this context it is important to note that historically, before anti-BSE measures were applied for the first time in 1988, there were a number of possible 'third ways' for BSE to be transmitted like *via* tallow, gelatin, blood, fertilisers and a range of other ruminant-derived by-products. Currently the measures introduced to reduce possible risks from these sources have blocked all recognised pathways if they are properly enforced. However, there remain a wide range of more obscure potential 'third ways' for transmitting BSE and it is these that will receive most attention.

The TSE/BSE *ad hoc* Group and the SSC gratefully acknowledge Dr.R.Bradley who prepared the basis version of the current inventory.

Key words: bovine spongiform encephalopathy, transmissible spongiform encephalopathy, TSE, BSE, transmission, hypotheses, third way, third route.

II INTRODUCTION INCLUDING SOME HISTORICAL FEATURES OF TSE ORIGIN AND TRANSMISSION

There is epidemiological (Wilesmith *et al.,* 1988, Wilesmith, Ryan and Atkinson, 1991) and other evidence (Taylor, Woodgate and Atkinson, 1995, Schreuder *et al.,* 1998) that BSE is a disease of cattle in which exposure is attributed to the feeding of concentrate rations containing mammalian proteins that are believed to be the vehicle for the infection. There is also some statistical evidence from a cohort study (Wilesmith, *et al.,* 1997, Donnelly *et al.,* 1997a*)* and analysis of the UK BSE database for dam calf pairs that, on statistical grounds, suggest a risk from maternal transmission of around 10% (Donnelly *et al.,* 1997b). However, there are no data to support the occurrence of maternal transmission in the absence of a feed-borne source (Curnow and Hau, 1996, J.W. Wilesmith, personal communication). Of the six cases of BSE reported in cattle in the UK born after 1 August 1996, dams of some of these cases were still alive years after the birth of the case, which makes maternal transmission unlikely in these cases, though it cannot be completely excluded. By the end of 2000, up to 19 cases of BSE in cattle were predicted by experts, (MAFF, 2001), but there are still only six to mid 2001. Furthermore, despite the fact that BSE appears clinically identical in UK, Irish and continental cattle and the strain types of agent isolated and so far tested are biologically identical, there are no data supporting the occurrence of maternal transmission outside of the UK and including from Switzerland where a specific analysis has been undertaken (Braun *et al* 1994, Fatzer *et al.,* 1998).

No male or female bovine reproductive tissues, mammary gland, milk or placenta have shown infectivity. Placenta, like the other tissues from natural cases of BSE, has been bio-assayed not only in mice but also in cattle by the oro-nasal route (MAFF 2000, Bradley, 1996) and no detectable infectivity has been found. Thus, there is no plausible explanation of how infectivity moves from dam to calf. Even if maternal transmission did occur, it could not sustain the UK epidemic since the necessary contact ratio is not achieved. From the above it can be concluded that in the UK most, if not all, cases of BSE can be attributed to feed exposure and the residue is resultant upon some form of imprecisely determined transmission that may not occur at all in the absence of a feed-borne source. It is not intended or necessary here to review all the evidence for and against maternal transmission but rather to indicate that, like many aspects of BSE, it is not proved beyond doubt that it occurs in the absence of feed-borne infection and if it does, what the mode of transmission is. For a concise account of the pros and cons of maternal transmission see the annex to the Opinion of the SSC of 29-30 November 2001 on the six BARB BSE cases in the UK since 1 August 1996.

In the spring of 2000, BSE was accelerating in France at a rate higher than expected although a feed ban had been imposed in 1990 to protect cattle. Furthermore, as in the UK, it is rarely possible to firmly establish in individual cases when, or how, exposure occurred. Initially some farmers, or indeed veterinarians, reasonably concluded that their animals had never received a diet containing mammalian proteins, or ruminant proteins. However, they usually ignored the importance of TSE-contamination of feed ingredients and/or the accidental cross-contamination of ruminant diets with such materials intended for use in feed for non-ruminant species. In some instances,

the dam of the case was still alive and healthy, no other offspring of the dam had clinical signs and thus maternal transmission was highly unlikely. Such a situation (even if erroneous because cross-contamination of feed had been ignored) led to the hypothesis that there was a 'Third way' of exposure of cattle to infectivity. However, there is no firm evidence to support any 'Third way' of transmission.

Scrapie, the TSE of sheep that has been known in Europe for over 250 years at least, is maintained as an endemic disease in some flocks and countries over long periods (Bradley, 2001a, Woolhouse *et al.,* 2001). Sheep to sheep transmission is responsible, placenta from infected animals being one possible vector as it is known to be infective by the oral route for sheep and goats (Pattison *et al.,* 1972,1974) and carries infectivity (Onodera *et al.,* 1993, Race, Jenny and Sutton, 1998) and PrP (Race, Jenny and Sutton, 1998). As with cattle, maternal transmission alone cannot explain why the disease becomes endemic. Some form of horizontal transmission (that could involve the placenta being eaten by related or unrelated sheep) and/or environmental transmission is necessary to maintain the infection.

Before scrapie was formally identified as an infectious disease by Cuillé and Chelle (1938), alternative causes had been proposed, such as infection with *Sarcocystis* species (M'Gowan, 1914), a ubiquitous protozoon parasite of grazing ruminant species. Interestingly the adult parasite is virtually a commensal organism normally causing very little reaction in the host. In this respect the outer coat of a Miescher's tube, as PrP seems to stimulate no immediate reaction in the tissue in which it resides. The adult stages are found intracellularly in skeletal and cardiac muscle, but part of the lifecycle involves the intestine and sometimes the placenta. Occasionally parasites are found in the brain, usually with no inflammatory reaction to them. All the above-mentioned tissues are involved, or potentially involved, in scrapie-like diseases. M'Gowan's (1914) report was suggesting that *Sarcocystis* species were responsible for scrapie. However, an alternative view could be that the juvenile forms of the parasite might carry infectivity from the gut occasionally to the brain. This would mean that the infectivity would somehow have to escape from the parasite into the brain tissue and there is no evidence to support this notion, so all is conjecture. Nevertheless, *Sarcocystis* species are only one of several parasites that might rarely and theoretically act as carriers of infectivity into the brain.

Amongst other causes for scrapie from the French literature have been sexual excess and exposure to lightning (Roche-Lubin, 1848). Curiously, as recently as 1996, a modern writer is suggesting that inadequate direct sexual contact is responsible for BSE (Gjorgov, 1996, 2001, Gjorgov *et al,* 1999). This is discussed in a later section. However, there was much speculation in the historical literature that scrapie was an inherited disease and spread within families. This was supported strongly by James (H.B.) Parry who studied the disease in Suffolk sheep over many years (Parry, 1983).

A vaccine was implicated in an epidemic of scrapie in Great Britain in the 1930s (due to using sheep tissues unknowingly infected with scrapie in its manufacture Greig, 1950). Since then other hypotheses have been put forward such as nematode worms and mites carrying the infection.

The next section will deal more specifically with the origin of BSE seen through the eyes of the epidemiologists who first had the opportunity of investigating the cause of BSE, the BSE Inquiry and finally the Horn Committee. Included is a short review of the autoimmune hypothesis and alternative origins of BSE not discussed in the Horn report.

III HYPOTHESES AND REVIEWS ON THE ORIGIN OF BSE

III. 1 ORIGINAL (1988 - 1991) HYPOTHESES

Wilesmith *et al,* (1988) made extensive studies of the epidemiological features of the first c.200 cases of BSE that occurred in Great Britain in period up to the end of 1987. From this study, the meat-and-bone-meal (MBM) hypothesis was founded. Subsequently, studies on methods of MBM feeding practices (case control study, Wilesmith, Ryan and Hueston, 1992) and tallow production as a result of processing waste animal products through the rendering industry were undertaken and reported (Wilesmith, Ryan and Atkinson, 1991). Essentially the hypothesis is that MBM is the vehicle that carried the BSE agent. MBM introduced the agent into cattle particularly, but not exclusively to dairy calves, in concentrate rations. The MBM was used to supply essential proteins to these fast growing animals that were unnaturally separated from their dams at, or soon after, birth and fed artificially. The feeding of MBM was the factor that was common to all the first cases that were examined.

In regard to the rendering procedures, Wilesmith *et al,* (1991) showed a relatively steady change from batch to continuous rendering in the 1970s and 1980s that had no close correlation with the calculated first exposures of cattle to BSE in feed, namely in the period 1981-1982. There were also no close correlations with time or temperature parameters used to process waste into MBM. However, the study did reveal that hydrocarbon solvent extraction of tallow was curtailed, particularly in the risk period for the first exposures. This led to the hypothesis that the cessation of hydrocarbon solvent extraction of tallow was a critical factor leading to sufficient infection to be passed in the MBM consumed by a calf, to establish an infective dose and thus disease, once the incubation period was complete. These views on the origin are supported by Brown, 2001 and Brown *et al,* 2001. However, Brown (1998) in a critical review of BSE and vCJD, suggested an alternative hypothesis, that makes the rendering changes irrelevant, namely that a strain of scrapie or spontaneous BSE emerged that had a thermal or other resistance sufficient to withstand the rendering process.

Wilesmith *et al*, (1991) also proposed two possible hypotheses for the origin of BSE. The first was that it was caused by a scrapie-like agent from sheep (also supported by Brown *et al,* 2001). The second was that it was due to a cattle-adapted scrapie-like agent. They rejected the hypothesis that a mutant scrapie agent was responsible because this was inconsistent with the epidemiological findings.

III.2. BSE INQUIRY REPORT (INQUIRY 2000) ON HYPOTHESES

The BSE Inquiry (Inquiry, 2000), agreed that MBM was the major vector of BSE in cattle, including *via* unintentional cross-contamination of ruminant diets with feed, or MBM, intended only for monogastric species. They supported the view that maternal transmission could account for some cases of BSE but were uncertain of the role environmental contamination and could not absolutely rule out the transmission of BSE *via* hormones and veterinary preparations.

Importantly they stated that scrapie agents were not responsible for BSE. Furthermore, in contrast to the Wilesmith reports (above) they claimed that there had been several cycles of BSE transmission in the 1970s and 1980s particularly in the South West of England. These were mainly pre-clinical cases of BSE with a few unrecognised clinical cases too. To initiate the infection they propose a genetic mutation in the *PrP* gene. However, they concede that this could have had an environmental cause such as from a toxic chemical.

The Inquiry conceded the history of rendering changes but were forthright in their comment that the changes in rendering that Wilesmith, Ryan and Atkinson (1991) put forward were erroneous. No rendering system, even to-day, could inactivate the BSE (or scrapie) agents with certainty.

The Inquiry (2000) supported the view that PrP was an essential component of the BSE story and had a central role in the transmission of disease. They also accepted the role of the *PrP* gene in familial CJD and in the determination of susceptibility of sheep (but not cattle) to TSE agent exposure. They also agreed that there was a single biological strain of agent that caused BSE, feline spongiform encephalopathy (FSE) and encephalopathy in captive wild BOVIDAE and FELIDAE.

In regard to alternative theories on the origin the Inquiry dismissed the autoimmune theory and thought the organophosphorus theory was inconsistent with the epidemiology. However, they conceded that Phosmet might possibly be able to modify the susceptibility of cells to prion attack.

III.3. THE HORN REVIEW AND HYPOTHESES (HORN, 2001)

The Horn Committee concluded that MBM in cattle feed was the vehicle that spread BSE within the cattle population and the evidence was strong. Furthermore the Committee strongly supported the Inquiry's view on the role of accidental cross-contamination of cattle diets with feed containing MBM intended for pigs and poultry.

Whereas the origin of the BSE agent remains obscure, once it had initiated disease in cattle, recycling of infection from infected cattle tissues was the main way in which further cattle became infected. By contrast, other considered possible origins (such as from dam to offspring, contamination of pastures (environmental spread) and/or, through the use of veterinary preparations) if these occurred, were likely to have made only a small contribution. Doubt was cast on the statistically calculated figure of 10% for maternal transmission, as the number of cases in the cohorts approaching five years of age is now low compared with the number expected. It is noted that

Bradley and Wilesmith (1993) reported that in no year between 1988 and 1993 (when the epidemic was at its height) did the actual incidence of BSE in the offspring of confirmed cases exceed the expected incidence of BSE from the feed-borne source alone.

As to the Inquiry's hypothesis of several undetected cycles of infection in the SW of England, the Committee thought although this was plausible but it is also speculative.

They were very firm that it was not tenable to exclude an unmodified scrapie agent in sheep being responsible for BSE.

A proposed origin from an African antelope with a natural TSE could not be substantiated but also could not be completely excluded.

PrP was agreed to play a central and essential role in TSE in general and BSE in particular. However, the Committee noted that the properties of the abnormal prion protein were incompletely understood and thus, how the normal form was converted to the abnormal form and why the properties of the two protein forms were so different, is also unclear. Whilst the Committee could not exclude that environmental factors and/or toxic chemicals could be implicated in the initiation or maintenance of disease, there was no convincing evidence to support this notion. These factors might however influence susceptibility to disease or infection.

The Committee pointed to the continuing puzzles of BSE, such as why it commenced in the UK and did not arise spontaneously and independently in other parts of the world where similar rendering systems, feeding practices and scrapie were evident. They sought to explain this observation as follows:

- Whilst they agree that no rendering system so far evaluated can guarantee to eliminate TSE infectivity completely, a tenfold increase in the amount of infectivity remaining in MBM (compared with that in the pre-BSE era) could be critical and could have contributed significantly to the epidemic.
- In the period 1970 to 1988 feed manufacturers introduced MBM into calf diets in the UK. This type of feeding was less prevalent in continental Europe (and in Ireland) and in the USA. However, it is noted that the Horn Review (Horn, 2001) stated that the incorporation of MBM into concentrate feed of calves in the UK was an essentially new phenomenon between 1970 and 1988. Even so, before 1970, unless completely separated feed manufacturing, transportation and storage facilities were available and used for calf feed, there would still have been an opportunity to cross-contaminate calf feed with MBM, or with feed for adult cattle or non-ruminant species that contained MBM. Cross contamination of ruminant rations is widely regarded as the main reason for cases of BSE born after feed bans were in place.
- Because of the nature of feeding beef calves the incidence of BSE in the offspring of suckler cows was low compared with that in the offspring of dairy cows.
- Most cattle with BSE became infected when calves (as determined by modelling).

In regard to the origin of the BSE agent the Committee noted as follows:

- The UK had the largest sheep and third largest cattle population in the EU and the highest ratio of sheep to cattle.
- If the proportion of sheep waste material was proportional to the respective sizes of the sheep and cattle populations, then because there were estimated to be between 5,000 and 10,000, cases of scrapie *p.a.* in the UK (noting there were no corresponding data for other Member States) a high level of scrapie infectivity could be in MBM (if it was not inactivated).
- This infectivity could have included a BSE strain, a fact that cannot yet be ruled out despite no such strain having been found in individually typed brains of sheep with scrapie. The Committee recognises that TSE agents mutate and strains might be selected when transferred over species boundaries. Alternatively (and speculatively) a new strain could have occurred as a sporadic event (see sporadic BSE below) or from other animal sources like goats or exotic ungulates.

The committee thus summarise that many of the above events, although not individually unique to the UK were collectively unique. They raise the additional point that feeding MBM to calves (apparently much more common in the UK than elsewhere) as distinct from adults, that the youth of the animal could be an important susceptibility factor. They recommended this be investigated experimentally.

III.4. AUTOIMMUNE HYPOTHESIS

This hypothesis has been discussed by the BSE Inquiry and the Horn Committee. Because it is a unique attempt to explain some of the mysteries of BSE and, if true would have significant repercussions, it is briefly summarised here.

Ebringer, Pirt and Wilson (1997, 1998) Ebringer *et al* (1997, 1998), Tiwana et al., (1999), Wilson, Hughes and Ebringer, (2001) claim that BSE is an autoimmune disease. The basis of the theory is that the neurological signs and damage are due to autoimmune damage of nervous tissue by bacterial antibodies that cross-react with nervous tissue. The bacterium that gives rise to the antibodies is *Acinetobacter calcoaceticus,* which is a ubiquitous organism found in soil. The view of most scientists in the field is that the hypothesis does not explain many important facets of BSE and the data so far produced are unconvincing. Importantly, as observed by Lachmann (1997) and Bruce (1998), experimental murine BSE can be transmitted to mice lacking a functional immune system by i/c inoculation which invalidates the theory. Furthermore, PrP knockout mice with a functional immune system do not develop scrapie when challenged lending further weight to the argument. In addition, the hypothesis does not explain why cattle alone have been singled out from other species as alone being at risk from this autoimmune phenomenon. The BSE Inquiry and the Horn Committee as well as other individuals have, on the basis of sound scientific evidence, firmly rejected this theory as a plausible hypothesis for the origin or maintenance of BSE.

IV. ALTERNATIVE ORIGINS NOT DISCUSSED IN THE HORN REPORT

IV.1. CATTLE ORIGINS

IV.1.1. *CATTLE ADAPTED SCRAPIE-LIKE AGENT ORIGIN*

In this regard, the possible origin of BSE from a cattle-adapted scrapie-like agent (Wilesmith *et al.,* 1988, Kimberlin, 1993) is of particular importance to consider as a possible explanation for a 'Third Route'. If this hypothesis is true there must have been some natural way of transmission from one cattle generation to the next. This would be necessary in order to maintain a geographically widespread reservoir of infection without causing more than an occasional clinical case that would have been difficult to detect at such a low incidence. If the agent from which BSE was derived, by mutation followed by selection, was either not neuro-invasive or only poorly so, and/or had low neuro-pathogenicity, the rarity of disease could be explained by a long incubation period that for the most part, exceeded the commercial lifespan of cattle. In this hypothesis, BSE resulted from the selection of a new strain with a relatively shorter incubation period (within the normal commercial lifespan of breeding cattle), neuro-invasive and pathogenic properties and which was recycled by the rendering system as detailed by Wilesmith and others (Wilesmith *et al.,* 1988, Wilesmith, Ryan and Atkinson, 1991). Thus during the BSE epidemic there would be two cattle agents circulating. One the BSE agent as we know it to day, re-circulated *via* infected feed and the other a less virulent form maintained naturally by an unknown mechanism. It could well be that BSE, as we know it, could be eliminated from the cattle population by the measures currently in place in the EU. However, these may be insufficient to eliminate the cattle strain that was the origin of BSE and so this may still be present and be responsible for sporadic cases of 'BSE' now detectable because of much improved quality of surveillance for neurological disease. It is possible that following the BSE epidemic therefore, the situation would return to the historical norm, a cattle-adapted lowly pathogenic agent present in a substantial number of cattle but giving rise only to rare clinical cases.

It is beyond the scope of this paper to justify the hypothesis. However, challenges to it could be made because the 'historical' agent has not been detected following bioassay in mice. This could be explained in several ways. Not all cattle with BSE would need to be infected with both agents. It could be that cattle infected with the historical agent alone might even confer a degree of protection from genuine BSE by occupying receptor sites (Dickinson and Outram, 1979). Therefore, those cattle brains used for bioassay may have been infected only with the 'real' BSE agent. Even if by chance one or more of the brains used for bioassay were infected with the 'historical' and current BSE agents the former may not be transmissible to mice.

IV.1.2. 'SPORADIC' BSE

The Horn Review (Horn, 2001) did briefly mention (page 12 of the Review, response to paragraph *2.4*) the possibility of a sporadic form of BSE (and scrapie) in the reply to suggestions from the BSE Inquiry that BSE had resulted from a gene mutation in the early 1970s. However, there was insufficient elaboration on this comment to enable comparison with the hypothesis described here.

A modification of the 'cattle adapted, scrapie-like agent hypothesis' described in the preceding paragraph, is that BSE could hypothetically exist in a sporadic form, as does sporadic CJD. Sporadic CJD is the commonest type of CJD accounting for around 85% of cases. The disease is geographically widespread and occurs at a similar low incidence of between 1 and 2 cases per million annually in all countries where its true incidence has been investigated. An incidence of a comparable disease in cattle, occurring at a similar low incidence, could have escaped identification until BSE was discovered and surveillance, especially in affected countries, was increased. During the period of the epidemic, it would probably have been impossible to distinguish the hypothetical 'sporadic' BSE, if it occurred, from the epidemic type assuming there is a difference. However, once the epidemic type is eliminated there could still be the rare occurrence of the sporadic case attributed to 'sporadic' BSE. These could occur in any country and theoretically, not just in cattle, but in any species. To date there is no evidence of such a disease but it could explain isolated cases in the future, especially if they occur in native-born cattle in countries with BSE, which have a low geographical BSE risk and where adequate tissues for investigation are available at necropsy. This assumes that the hypothetical 'sporadic' BSE has a different phenotype.

It is as well to note that the cause of sporadic CJD is not known. There are suggestions that it may be due to a somatic mutation in the *PrP* gene, though proof of this is lacking and it will be difficult to prove.

IV.2. SHEEP ORIGIN MASKED BY SCRAPIE AGENT

Studies in transgenic mice expressing bovine PrP (Scott *et al.,* 1997, 1999) showed that they were susceptible to both BSE and scrapie prions (Prusiner, 2000) and that the scrapie prions produced disease more rapidly than did BSE prions. Since it is already known that natural scrapie isolates may yield more than one scrapie agent strain, it is possible that in some sheep with scrapie, there are mixtures of scrapie and BSE agents. If the BSE agent were to be present, it could be masked by the more rapidly growing scrapie strain. Prusiner (2000) went on to suggest that changes in rendering could have inactivated the scrapie agent but allowed the BSE agent to accumulate and spread to the cattle population. The hypothesis is thus that sheep could harbour a low (harmless to humans) level of BSE infectivity. However, it is noted that RIII mice replicate BSE at a faster rate than scrapie agents yet to date the BSE agent has not been identified in more than 150 investigated incidents of scrapie in British sheep using biological strain typing methods.

IV.3. NEW TSE AGENTS AND DUAL INFECTIONS

Cattle fed MBM could have been exposed to both the BSE agent and the scrapie agent and probably were if these were to be present in the raw material. This gives rise to two possibilities.

The first is the occurrence of 'scrapie' in cows. This has only been demonstrated as an experimental disease and seems to provoke no concern so far, but is no different in principle from the other hypothetical disease 'BSE in sheep' over which there has been abundant concern. There is no evidence for a large epidemic of 'scrapie' in sheep in the UK in the period 1980-2000

(Gravenor, 2000). However, if there was significant scrapie infectivity in MBM and this was fed to sheep, it would be more likely to have been recognised in this species (or goats) than in cattle, since there is no species barrier. With no detectable change in the neuropathology of cattle with BSE during the whole epidemic, this also suggests no significant 'scrapie' has occurred in cattle, though infection cannot be ruled out.

The second is that there could be (in the UK, and France for example, where BSE and scrapie exist) exposure to both the BSE and scrapie agents in MBM. Results from dual infection of mice with BSE and scrapie agents has been published (Baron and Bicabe, 2001) but at present it is not known in cattle if dual infections can be established and what are the consequences. One consequence could be the blocking of replication sites (Dickinson and Outram, 1979) by the scrapie agent that would not allow the BSE agent to replicate. It is unwise to believe in advance of proof, that when the last BSE case has been found that infection could not remain, either with scrapie agents or BSE agents or perhaps both. This scenario might be perpetuated silently from generation to generation by some, as yet unidentified mechanism

IV.4. SPORADIC SPONTANEOUS CONVERSION OF PrP^{C} TO PrP^{Sc}

The hypothesis (Sulkowski, 1992) is that the octapeptide repeat regions of the prion protein that contain histidine and tryptophan residues function as ligands (electron donors) for transition metals Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. It is proposed that combination of these metals with the octa-peptide repeats, results in the spontaneous conversion of PrP^{C} to PrP^{Sc}, with subsequent dimerisation, thus explaining the build up of prion protein. The hypothesis could be tested *in vitro.* The conclusion by the author is that if the hypothesis is valid then transition metals such as those listed above could be recognised as the aetiological agent(s) of sporadic and familial CJD.

In humans, mutation of the octapeptide repeat region of PrP is associated with some forms of familial CJD. Also there is undeniably an interaction (binding) between copper and PrP in the octapeptide repeat region *via* histidine and this may have a role in transmission (Brown, 1999) and cellular resistance to oxidative stress (Brown *et al.,* 1999). To suggest that metal ions are the agents responsible for prion diseases, is an exaggeration of the truth. No comments upon the cause of BSE have been proposed in this paper.

Leclerc *et al* (2001) have recently reported that conversion to the infectious form is particularly associated with major structural rearrangement in the central part of the protein. By using antibodies to different parts of PrP they have shown that equivalent major structural rearrangements occur in the same central part when the protein is immobilised on a surface. Other parts of the protein remain unaltered. This phenomenon appears to occur when PrP molecules are not in contact with one another. Clearly these findings, if confirmed and repeated in other laboratories could, as stated by the authors, have important implications for prion biology.

Some allusion to the spontaneous generation of PrP^{Sc} has been made by Safar (1996), who reported that the solid state of aggregated proteins is non-crystalline and amorphous. The transition from monomer in solution to an aggregated solid state induces no, or only minor changes to, the protein or

peptide secondary structure. However, exposure of protein films to high temperature, changes the various types of secondary structure, like turns and α helices and random elements, into uniform β sheets. Once the sheets are formed the structure is very stable and the changes are irreversible and remain after cooling to room temperature. These general observations may lend some weight to the view that there is potential for a spontaneous conversion of protein from an innocuous form, into an aggregated form rich in β sheet, that is life threatening. There is a large energy barrier that needs to be overcome before amyloid formation is effected. This leads to the possibility that such changes could happen *in vitro* under certain conditions during the processing of animal products containing PrP^C. All the above assumes that the agent of TSE is a prion. If it is not and has a conventional genome of its own (virino hypothesis or unconventional virus hypothesis, see Schreuder, (1994)), the spontaneous conversion of PrP^C into PrP^{Sc} may still occur, but could not, on its own generate infectivity. To date it has not been possible to generate infection from non-infectious PrP^C in the laboratory under any conditions. There is still considerable doubt about what is the nature of the agent that causes TSE.

IV.5. OTHER SOURCES, INCLUDING THOSE THAT MIGHT MIMIC BSE, OR BECAUSE OUR CURRENT UNDERSTANDING OF TSE IS LATER SHOWN TO BE WRONG

In regard to mimicry, there are numerous real examples of this in animal and human disease. Mimicry can be at the clinical or pathological level and scientifically can be distinguished at the level of aetiology. Examples are, at the clinical level in pigs, swine vesicular disease that is very difficult to distinguish from foot and mouth disease, yet although clinically similar, the viruses that cause the two diseases are quite different. At the pathological level in cattle, congenital arthrogryposis can be a hereditary disease, or caused by viruses, plant toxins or nutritional deficiencies. Such diseases are phenocopies. If we translate these well-known, but not particularly common, phenomena to BSE, it is quite possible that individual cattle could show clinical signs that mimic those of BSE, yet have a quite different cause. During the height of the BSE epidemic in the UK, some 15% of clinical suspects turned out not to have BSE at all, but rather other diseases (about 40% of the 15%), or to have no lesions (about 60% of the 15%) (Cockcroft, 1999, Jeffrey, 1992, Wells, Sayers and Wilesmith, 1995). BSE suspects are reported in most countries with good surveillance including those that have been classified as being in Geographical BSE Risk Assessment Category 1 (BSE highly unlikely). In fact, as in the UK, either other diseases are responsible or no lesions are found. It is therefore absolutely essential that clinical diagnosis of BSE is confirmed by use of one of the methods recommended in the OIE *Manual* of Standards (OIE, 2000). Failure to do this could result in support being given to invalid causes and permit the current number of different hypotheses of possible "Third Ways" to be perpetuated without reason.

There are some well-recorded cattle diseases that could be confused with BSE and thus form genuine differential diagnoses. These include idiopathic brainstem neuronal chromatolysis and hippocampal sclerosis (Jeffrey and Wilesmith, 1992) cerebral listeriosis, polioencephalomalacia, hypo-magnesaemia, hepatic encephalopathy (including bovine bonkers Morgan and

Edwards, 1986), various encephalomyelitides, crazy cow disease associated with *Solanum dimidiatum* toxicity, (Menzies, Bridges and Bailey, 1979).

In none of the following hypotheses have authors presented data that show the hallmark pathology of BSE or the presence of PrP^{Sc}. On this basis, they can be dismissed as serious contenders for being a cause of BSE.

IV.5.1. *THE AGENT IS A TOXIN*

Shaw (1993) does not argue that a different kind of agent (a poison) is responsible for BSE. Rather he argues that PrP is a toxin and not an infectious agent. Thus this view is one relating to the mechanism of establishing the disease *i.e.,* by a toxic process by which incoming 'toxic' PrP^{Sc} invokes a change in non-toxic' PrP^{C} to convert it into the toxic form. This may lead to cell death by apoptosis. Giese and Kretschmar, (2001) explain that there are two main concepts that are not mutually exclusive: the 'gain of function' hypothesis where the accumulation of PrP^{Sc} may be directly or indirectly neurotoxic. Alternatively the 'loss of function' hypothesis argues that conversion of PrP^{C} to PrP^{Sc} may lead to a reduction of the functional PrP^{C} available.

On the other hand, Stockdale (1997) concludes the agent is a bacterial toxin, possibly from *E.* coli O157 that circulates in the blood and enters the brain as a result of leaky membranes. The hypothesis is chemically complex and side issues are also introduced such as the seasonal winter occurrence of BSE due to low levels of melatonin secretion at this time. The author claims that in the UK after 1970 wheat was used in compounded cattle rations, especially dairy cake and this increased in the period up to 1987 when he erroneously believed the epidemic started (first exposures were in 1981-1982). The absence of BSE in the USA is attributed to not using wheat but rather maize in cattle diets, though both used animal protein. Then importance of wheat in the diet is explained as follows: if large quantities of wheat are fed the intestinal contents become liquefied (this happens seasonally in the spring when cattle are on grass), mucus protection is decreased, epithelial cells become colonised by toxin-producing bacteria and the toxins are absorbed into the blood stream. The toxin is claimed to catalyse the dissociation of nicotinamide adenine dinucleotide (NAD) in the cells of the liver and other organs with the formation of adenosine dinucleotide phosphate-ribose. In an unexplained way, there is a shortage of lipids for membrane maintenance and the regulatory mechanism for cyclic adenosine monophosphate (cAMP) is damaged and cAMP is over-produced. If the toxin leaks into brain tissue over a considerable period specialised macrophages respond by producing beta amyloid (which is not a feature of TSE) causing senile dementia in humans, but small quantities enter neurons and initiate processes that result in BSE or sporadic CJD. The hypothesis ignores the fact that the incidence of BSE declines in cattle following the introduction of mammalian feed bans and does not occur in other species like pigs and poultry that have traditionally been fed on wheat.

IV.5.2. THE ROLE OF FAT-ASSOCIATED CHEMICAL TOXINS

Parish and Parish (2001), suggested in 1996 that the use of hydrocarbon solvents, such as trichlorethylene, used by some renderers to increase the amount of tallow extracted from the raw material, removed with it certain

toxic substances including dioxins that can accumulate in fatty tissues. In other words these toxic chemicals are proposed as the cause of, or trigger factor, for BSE. They suggest that feeding 'toxic' MBM to cattle could initiate the disease in the unborn offspring (or in offspring after birth through milk) even without disease occurring in the dam (which would depend on the amount and duration of exposure). However, the milk hypothesis does not fit epidemiologically with the higher incidence of disease in dairy cattle than in beef cattle, nor with the occurrence of BSE in countries or regions that never used the solvent extraction system for preparing MBM. Furthermore, dioxin toxicity does not produce spongiform encephalopathy and the hypothesis appears to ignore the fact that the tallow fraction prepared by the solvent extraction system would contain the hypothetical toxic chemical in higher concentration than otherwise and this too could be fed to a range of species without clear evidence of an increased risk of TSE developing. These authors claim that there is no evidence to support the transmission of BSE between cattle horizontally or to other species and that this is supported by a statement on the USDA website. (See Reference list under Parish and Parish, 2001 for appropriate website addresses).

IV.5.3. *THE CAUSES ARE ALKALOIDAL GLYCOSIDASE INHIBITORS (AGI)*

AGI are produced by plants and micro-organisms in the environment. They are consumed by grazing animals and absorbed from the gut, distributed throughout the body and are concentrated within cells. AGI alter the glycan chains of cellular glycoproteins (CGP) during their formation so that CGP produced by different clones of cells and thus with different glycan chains become structurally identical. Prion protein (PrP^{Sc}) is a CGP that is resistant to destruction by cellular processes. Dealler, (1994) proposes that AGI ingested by healthy sheep with a specific *PrP* genotype, stimulates the conversion of PrP^{C} into an aggregated form of PrP^{Sc}. The geographical distribution of plants with the necessary AGI is suggested for the geographical distribution of scrapie in sheep. The author suggests the AGI changes the primary, secondary and tertiary structure of PrP. However, in fact the primary structure of PrP^{C} and PrP^{Sc} are the same. No evidence is produced to show the geographical coincidence of AGI and scrapie. The author does not propose this mechanism for the occurrence of BSE.

IV.5.4. THE AGENT IS A BACTERIUM

This hypothesis proposed by Bastian, (1991, 1993) and Bastian and Foster, (2001) has been developed partly as a result of electron microscopic findings of spiroplasma-like inclusions in the brain from patients with CJD, suggests that the agent causing CJD is a filterable bacterium. The most convincing evidence comes from experiments where *Spiroplasma* organisms are treated with sodium deoxycholate that releases slender fibrils which form a helically twisted ribbon within the *Spiroplasma* cells. The fibrils comprise a protein with a molecular mass of 55 kD (20 kD more than full length PrP), are protease resistant, react with antibody to PrP and are morphologically indistinguishable from scrapie associated fibrils. The author claims that the major surface protein of *Spiroplasma* resembles PrP as it is an acylated protein with a molecular mass of 26-30 kD. In some respects this hypothesis includes

some of the features of the agents known as intestinal fluid dependent organisms (IFDO) isolated from humans (Burdon, 1989). The latter also resemble prions in their resistance to heat and some chemical and physical disinfectants including ionising radiation. However, IFDO can be distinguished from prions because they are readily inactivated by some chemicals that have no effect on prions.

IV.5.5. *THE AGENT IS A SINGLE-STRANDED DNA*

This view, based on some experimental studies including ultrastructural studies of tubulofilamentous particles has been put forward vigorously by Narang (1993) but there are several elements in the work that leaves considerable doubt that there is any validity in the hypothesis. The first is that the tubulofilamentous structures described by Narang have the morphology of ciliary organelles (Chasey, 1994), that are not unique to scrapie or even to TSE-affected brain. They could have arisen from ciliated cells within the brain that are normal structures found in ependymal cells for example. The identification of DNA has also been disputed following attempts to recreate Narang's work independently but using his unconventional techniques (Bountiff, Levantis and Oxford, 1996). Notwithstanding, these findings some scientists support the view that TSE agents are in fact unconventional viruses and point out the virus theory has yet to be disproved.

IV.5.6. *THE AGENT IS NOT AN INFECTIOUS PROTEIN BUT RATHER ITS STRUCTURE IS UNKNOWN*

It is perhaps wiser to keep an open mind on the structure of TSE agents until it is determined independently in several different laboratories. No one has yet generated infectious PrP from non-infectious pre-cursors. The consequences of finding that there is a nucleic acid genome in association with PrP (the virino hypothesis) could be considerable. Not least, it would have consequences for developing a test for the agent and possibly for developing vaccines and for more appropriate methods of destruction than currently exist. According to Farquhar, Somerville and Bruce (1998) "There is more to comprehending the biological diversity of TSEs than 'prion' protein".

V CONCLUSIONS ON THE REPORT OF THE BSE INQUIRY AND THE HORN REVIEW AND DISCUSSION ON ALTERNATIVE ORIGINS NOT DISCUSSED IN THE HORN REVIEW.

Most authorities and individuals seem agreed that MBM is the main vehicle for BSE transmission and that prion protein is central to TSE science and transmission. They also in general agree that accidental cross-contamination of ruminant rations with MBM in feed for monogastric species was an important feature in perpetuating BSE epidemics after feed bans were in place.

There is a dispute about the origin of BSE, which is truthfully unknown. However, an unmodified scrapie agent from sheep cannot be excluded as the cause. The unique combination of factors described above in the Horn Review seem to be a reasonable and plausible explanation of why BSE was initiated on such a scale in the UK and not elsewhere. The introduction of MBM into feed for calves in the UK from 1970 could explain the initiating event that was

finally responsible (after recycling of infection) for the occurrence of BSE from1985-1986. However, the reason why there had been no effective exposure from calf feed before 1970, as a result of cross-contamination from MBM or feeds containing MBM, such as pig or poultry feed, is not fully explained, bearing in mind that accidental cross contamination of feed for calves is widely regarded as being responsible for most cases of BSE born after feed bans were in place. It is possible however, that historically an insufficient oral infective dose could be delivered as a result of any cross-contamination that occurred. In other words the infecting dose was insufficient to produce detectable disease within the commercial lifespan of cattle. Some alternative theories are not supported and others, whilst they cannot be totally excluded, could only partially and minimally contribute to the BSE epidemic, for example, by altering susceptibility.

Whereas the Horn Report and Part 1 of this dual report is essentially a review of the *'Origin'* of BSE, Part 2 is directed at possible methods for the *'Perpetuation'* of BSE when effective measures to control exposure *via* feed are certainly in place. If methods that could establish new BSE infections are verified, they might delay or prevent elimination of the disease from countries or regions. The goal of eradication of BSE from all herds everywhere could then be thwarted. Eradication is the long-term. In the sister report on 'Alternative Hypotheses of BSE Transmission' there is some discussion of possible ways of maintaining the BSE epidemic that might also explain the origin. For example, it is known that a number of BOVIDAE and FELIDAE species are susceptible naturally to 'BSE' though the diseases are collectively rare. The BSE Inquiry could not exclude a BSE origin from mammalian species other than cattle, whose carcases were rendered into MBM. The Horn Review could not substantiate this origin, neither could they find sufficient evidence to reject the hypothesis entirely. However, the point at issue here is perhaps a more general one not dealt with in any detail in the Horn Review, namely whether the epidemic of BSE in the UK was a point source or extended common source epidemic.

Wilesmith *et al,* 1988 and Wilesmith, Ryan and Atkinson, 1991, were firmly of the view that BSE was an extended common source epidemic. This was because BSE appeared in most parts of Great Britain within a short space of time, shorter than the mean incubation period of BSE. There were regional differences of course, but these were readily explained by the epidemiological findings.

An origin from a rare inclusion of an TSE-infected single carcass in the raw material would produce a point source epidemic as also would a mutant form of scrapie agent from sheep, the main reason Wilesmith, Ryan and Atkinson, 1991 rejected this latter hypothesis. There was no epidemiological evidence to support a point source epidemic but there was strong evidence to support an extended common source epidemic. If however more than anecdotal evidence (such as that presented to the Inquiry and the Horn Committee) for a point source epidemic comes forward in the future, then many currently rejected or partially rejected hypotheses (*e.g.,* from any mammal susceptible to TSE), become viable.

To accommodate such hypotheses the crucial issue is therefore the nature of the epidemic: extended common source (as originally reported) or point source followed by repeated recycling before being recognised, as suggested by some people. It is understood that modelling shows that a point source epidemic is feasible but it would imply that in the intervening years (say 10-15) between initial exposure and the first detected cases coming to light no detected a new disease, nor was confident enough to submit a brain to a competent laboratory for microscopic investigation. This is unlikely but not completely impossible.

In regard to alternative origins not discussed in the Horn Review, some can be rejected as not being possible to cause BSE under any condition (*e.g.* the autoimmune hypothesis). At the other end of the spectrum some other hypotheses are plausible but difficult, if not impossible, to investigate or prove at the present time. One such is the possibility that there is a form of sporadic BSE akin to sporadic CJD of humans.

Between these extremes, other hypotheses are related to the nature of the agent and how it causes its effect, such as by a toxic action. Although important to appreciate, they do not help particularly in identifying an alternative origin for BSE, though they could be immensely important to consider once the real nature of the agent is defined and accepted. It is perhaps wisest at present to still keep an open mind on the nature of the agent and to consider rather that its structure is unknown or at least uncertain.

One intriguing theoretical possibility is that under certain specific conditions, PrP can spontaneously convert into the so-called infectious form. This has, potentially at least, important consequences if this conversion can initiate the chain reaction of conversion that is known to occur in the presence of the disease-specific form of the protein. It is also important to know if the generated new form of protein is infectious, and whether it could establish infectivity by renewed conversion of further PrP. Wherever animal parts are, so is the normal form of the protein, especially in central nervous tissue. Even if spontaneous conversion is very rare, it is scary to think that infectivity might be generated from what has been considered to be innocuous material. If this is true, it is important to pursue the issue through targeted research as it could possibly inform on the origin of BSE and of ways of preventing such occurrences in the future.

VI REFERENCES

BARON, T.G.M., & BICABE, A-G, 2001. Molecular analysis of the abnormal prion protein during co-infection of mice by BSE and the scrapie agent. Journal of Virology, **75,** 107-114.

BASTIAN, F.O., 1991. Creutzfeldt-Jakob disease and other transmissible spongiform encephalopathies. Mosby Year Book, Inc.Chicago.

BASTIAN, F.O., 1993. Bovine spongiform encephalopathy: relationship to human disease and nature of the agent. Am. Soc. Microbiol., **59,** 235-240.

BASTIAN, F.O. & FOSTER, J.W., 2001. Spiroplasma sp. 16S rDNA in Creutzfeldt-Jakob disease and scrapie as shown by PCR and DNA sequence analysis. J. Neuropath. and Experimental. Neurology, **60,** 613-620.

BIRD, S., 2001. BSE transmission routes: vertical or other parenteral or oral (excluding MBM). Unpublished article and correspondence submitted to the Commission September 2001.

BOUNTIFF, L., LEVANTIS, P. & OXFORD, J., 1996. Electrophoretic analysis of nucleic acids isolated from scrapie-infected hamster brain. Journal of General Virology, **77,** 2371-2378.

BRADLEY, R., 1996. Experimental transmission of bovine spongiform encephalopathy. In : Transmissible subacute spongiform encephalopathies, L.Court, B Dodet, Eds., Elsevier, Paris. Pp51-56.

BRADLEY, R. 2001a. Will scrapie in sheep in Britain disappear? Trends in Microbiology, **9,** 260-261.

BRADLEY, R. 2001b. Comments from R. Bradley on the letter from Professor Knudsen in regard to the third way. E-mail to the Commission 10 Sep 2001.

BRADLEY, R., & WILESMITH, J.W., 1993. Epidemiology and control of bovine spongiform encephalopathy (BSE). British Medical Bulletin, **49**, 932-959.

BRAUN, U., AMREIN, E., ESTERMANN, U., EGLI, J., SCHWEIZER, T., LUTZ, H., EHRENSPERGER, F., VANDEVELDE, M. & KIHM, U., 1998. Untersuchungen an 182 Nachkommen von an boviner spongiformer Enzephalopathie (BSE) erkrankten Kﬁhen in der Schweiz. Teil 1: Klinische Befunde. Schweiz. Arch. Tierheilk., **140,** 240-249.

BROWN, D.R., 1999. Prion protein expression aids cellular uptake and veratridine induced release of copper. J. Neurosci. Res., **58,** 717-725.

BROWN, D.R., WONG, B.S., HAFIZ, F., CLIVE, C., JONES, I.M. HASWELL,S.J. & JONES, M., 1999. Normal prion protein has an activity like that of superoxide dismutase. Biochemical Journal, **344,** 1-5.

BROWN, P., 1998. On the origins of BSE. Lancet, **352,** 252-253.

BROWN, P., 2001. Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease. British Medical Journal, **322,** 841-844.

BROWN, P., WILL, R.G., BRADLEY, R., ASHER D.M. & DETWILER, L. 2001. Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease: background, evolution and current concerns. Emerging Infectious Disease, **7,** 6-16.

BRUCE, M.E., 1998. Autoimmunity or infection in the TSEs. A comment on the 'Friendly fire' theory. SGM Quarterly, November 1998, p137.

BURDON, W., 1989. A novel replicating agent isolated from human intestinal tract having characteristics shared with Creutzfeldt-Jakob and related agents. Journal of Medical Microbiology, **29,** 145-157.

CHASEY, D., 1994. Comment on the paper of H.K. Narang: Evidence that scrapie-associated tubulofilamentous particles contain a single-stranded DNA. Intervirol, **37,** 306.

COCKCROFT, P.D., 1999. Pattern-matching models for the differential diagnosis of BSE. Veterinary Record, **144,** 607-610.

CURNOW, R.N. & HAU, C.M., 1996. The incidence of bovine spongiform encephalopathy in the progeny of affected sires and dams. Vet. Rec., **138,** 407-408.

CUILLÉ, J. & CHELLE, P.L., 1938. La maladie dite tremblante du mouton est-elle inoculable? C R Acad Sci., **203,** 1552-1554.

DEALLER, S., 1994. Alkaloidal glycosidase inhibitors (AGIs) as the cause of sporadic scrapie, and the potential treatment of both transmissible spongiform encephalopathies (TSEs) and human immunodeficiency virus (HIV) infection. Medical Hypotheses, 42, 69-75.

DONNELLY C.A., FERGUSON N.M., GHANI A.C., WILESMITH J.W., ANDERSON R.M. 1997a. Analysis of dam-calf pairs of BSE cases: confirmation of a maternal risk enhancement. Proc. R. Soc. Lond. B., **264**, 1647-1656.

DICKINSON, A.G. & OUTRAM, G.W., 1979. The scrapie replication site hypothesis and its implications for pathogenesis. In: Slow Transmissible Diseases of the Nervous System. S. B. Prusiner and W. J. Hadlow, Eds., Vol. 2, pp. 33-54. Academic Press, New York. Pp 13-31.

DONNELLY C.A., GHANI A.C., FERGUSON N.M., WILESMITH J.W., ANDERSON R.M. 1997b Analysis of the bovine spongiform encephalopathy maternal cohort study: evidence for direct maternal transmission. Appl. Statist., **46**, 321-344.

EBRINGER, A., PIRT, J. & WILSON, C., 1997. Is BSE an autoimmune disease? Immunology news, **4,** backchat.

EBRINGER, A., PIRT, J. & WILSON, C., 1998. Friendly fire; molecular mimicry and BSE. SGM Quarterly, November 1998, p 136-137.

EBRINGER, A., PIRT, J., WILSON, C., et al 1997. Bovine spongiform encephalopathy: Is it an autoimmune disease due to bacteria showing molecular mimicry with brain antigens. Environmental health Perspectives, **105,** 1172-1174.

EBRINGER, A., PIRT, J., WILSON, C., THORPE, C., TIWANA, H., CUNNINGHAM, P. & ETTELAIE, C., 1998. Bovine spongiform encephalopathy: Comparison between the 'Prion' hypothesis and the autoimmune theory. J Nutritional and Environmental Medicine 1998, **8,** 265-276.

E.C. (European Commission), 2001. Opinion of the Scientific Steering Committee on the six BARB BSE cases in the UK since 1 August 1996. Adopted on 29-30 November 2001

FARQUHAR, C., SOMERVILLE, R.A. & BRUCE, M.E. 1998. Straining the prion hypothesis. Nature, **391,** 345-346.

FATZER, R., EHRENSPERGER, F., HEIM, D., SCHMIDT, J., SCHMIDT, A., BRAUN, U. & VANDEVELDE, M., 1998. Untersuchungen an 182 Nachkommen von an boviner spongiformer Enzephalopathie (BSE) erkrankten K hen in der Schweiz. Teil 2: Epidemiologische und pathologische Befunde. Schweiz. Arch. Tierheilk., **140,** 250-254.

GIESE, A., & KRETSCHMAR, H.A., 2001. Prion-induced neuronal damage – the mechanisms of neuronal destruction in the sub-acute spongiform encephalopathies. Current Topics in Microbiology and Immunology, **253,** 203-217.

GJORGOV, A.N., 1996. Prevention and eradication of the bovine spongiform encephalopathy (BSE): a solicited response and proposal. Macedonian Veterinary Review, 25, 97-101.

GJORGOV, A.N., 2001. Letter to European Commission 14 February 2001.

GJORGOV, A.N., JUNAID, T.A., BURNS, G.R. & TEMMIM, L., 1999. Efficacy of preventive prostaglandin treatment of malignant mammary lesions in rats. Journal of the Balkan Union of Oncology, **4,** 295-306.

GRAVENOR, M.B., COX, D.R., HOINVILLE, L.J., HOEK, A. & McLEAN, A.R., 2000. Scrapie in Britain during the BSE years. Nature, 406, 584-585.

GREIG, J.R., 1950. Scrapie in sheep. Journal of Comparative pathology, 60, 263-266.

HORN, G., 2001. Review of the origin of BSE. DEFRA, London. Pp. 66.

INQUIRY, 2000. Report, evidence and supporting papers of the Inqquiry into the emergence and identification of BSE and variant CJD and the action taken in response to it up to the 20 March 1996. Stationery Office Ltd., Norwich.

JEFFREY, M., 1992. A neuropathological survey of brains submitted under the bovine BSE Orders in Scotland**.** Veterinary Record**, 131:** 332-337**.**

JEFFREY, M. & WILESMITH, J.W., 1992. Idiopathic brainstem neuronal chromatolysis and hippocampal sclerosis: a novel encephalopathy in clinically suspect cases of bovine spongiform encephalopathy. Veterinary Record, **131,** 359-362.

KIMBERLIN, R.H., 1993. Bovine spongiform encephalopathy: an appraisal of the current epidemic in the United Kingdom. Intervirol, **35,** 208-218.

KNUDSEN, I, 2001a. Letter to Commission posing various questions stimulated by the paragraph in the 'Third Way' report concerning the possibility of 'Sporadic BSE'. September 2001.

KNUDSEN, I, 2001b. Letter to Commission concerning the response to answers to the queries posed in Knudsen 200a. September 2001

LACHMANN, P.J., 1997. 'Best' scientific evidence on BSE. The Times, 14 July 1997 p. 21.

LECLERC, E., PERETZ, D., BALL, H., SAKURAI, H., LEGNAME, G., SERBAN, A., PRUSINER, S.B., BURTON, D.R. & WILLIAMSON, R.A., 2001. Immobilised prion protein undergoes spontaneous rearrangement to a conformation having features in common with the infectious form. EMBO Journal, 20, 1547-1554.

MAFF, 2000. Bovine Spongiform Encephalopathy: a progress report. June 2000. MAFF, London.

MAFF, 2001. Report of the Chief Veterinary Officer: Animal Health 2000. MAFF, London.

M'GOWAN, J.P., 1914. Investigation into the disease of sheep called "scrapie" (Traberkrankheit; la tremblante) with especial reference to its association with sarcosporidiosis. Edinburgh and East of Scotland College of Agriculture. William Blackwood and Sons. Edinburgh.

MENZIES, J.F., BRIDGES, C.H. & BAILEY, E.M., 1979. A neurological disease of cattle associated with *Solanum dimidiacum.* Southwestern Veterinarian, **32,** 45-49.

OIE, 2000. Manual of standards for diagnostic tests and vaccines. OIE, Paris.

ONODERA, T., IKEDA, T., MURAMATSU, Y. & SHINAGAWA, M., 1993. Isolation of scrapie agent from the placenta of sheep with natural scrapie in Japan. Microbiol. Immunol., **37,** 311-316.

PARISH, A.R. & PARISH B., 2001. To determine the 1985/6 BSE epidemic trigger factor. http://www.bse.org.uk/report/volume2/chapter2.htm see also: http://www.onshop.co.uk/bse/bse.htm http://www.aphis.usda.gov/oa/pubs/fsbse.html; http://www.onshop.co.uk/bse/us_bse.htm

PARRY, H.B., 1983. Scrapie disease in sheep. D.R. Oppenheimer, Ed. Ac. Press, London. Pp. 192.

PATTISON, I. H., HOARE M. N., JEBBETT J. N., WATSON W. A. 1974. Further observations on the production of scrapie on sheep by oral dosing with foetal membranes from scrapie affected sheep, Br. Vet. J. **130**, lxv-lxvii.

PATTISON, I.H., HOARE, M.N., JEBBETT, J.N., WATSON, W.A., 1972. Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie affected sheep. Vet. Rec., **90**, 465-468.

PRUSINER, S.B., 2000. Letter to Dr P Vossen 2 August 2000.

RACE, R., JENNY, A. & SUTTON, D., 1998. Scrapie infectivity and proteinase K- resistant prion protein in sheep placenta, brain, spleen, and lymph node: implications for transmission and antemortem diagnosis. J. Infect. Dis., **178,** 949-953.

ROCHE-LUBIN, M., 1848. Mémoire pratique sur la maladie des bêtes à laine connue sous le nom de prurigo-lombaire, convulsive, trembleuse, tremblante, etc. Rec Méd. Vet., **25,** 698-714.

SAFAR, J., 1996. Structure and biologic characteristics of prion protein (scrapie amyloid): implications for the safety of naturally derived biologics. In Bovine spongiform encephalopathy: the BSE dilemma. C.J. Gibbs, Ed. Springer-Verlag, New York. Pp179-191.

SCHREUDER, B.E.C., 1994. BSE agent hypotheses. Livestock Prod. Sci..38, 23-33.

SCHREUDER, B.E., GEERTSMA, R.E., van KEULEN, L.J.M., van ASTEN, J.A.A.M., ENTHOVEN, P., OBERTHÜR, R.C., de KOEIJER, A.A. & OSTERHAUS, A.D.M.E., 1998. Studies on the efficacy of hyperbolic rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents. Vet. Rec., **142,** 474-480.

SCOTT, M.R., *et al,* 1997. Identification of a prion protein epitope modulating transmission of BSE prions to transgenic mice. Proceedings of the National Academy of Sciences, **94,** 14279- 14284.

SCOTT, M.R., *et al,* 1999. Compelling transgenetic evidence for transmission of BSE prions to humans. Proceedings of the National Academy of Sciences, **96,** 15137-15142.

SHAW , I., 1993. Mad cows and a protein poison. New Scientist, **140,** 50-51.

STOCKDALE, T., 1997. Are bacterial toxins involved in the aetiology of transmissible spongiform encephalopathies? Nutrition and Health, **11,** 301-307.

SULKOWSKI, E., 1992. Spontaneous conversion of PrP^{C} to PrP^{Sc}. Fed. European Biochem. Soc., **307,** 129-130.

TAYLOR, D.M., WOODGATE, S.L. & ATKINSON, M.J., 1995. Inactivation of the bovine spongiform encephalopathy agent by rendering procedures. Veterinary Record, **137,** 605-610.

TIWANA, H., WILSON, C., PIRT, J., CARTMELL, W. & EBRINGER, A., 1999. Autoantibodies to brain components and antibodies to *Acinetobacter calcoaceticus* are present in bovine spongiform encephalopathy. Infection and Immunity, **67,** 6591-6595.

WELLS, G.A.H., SAYERS, A.R. & WILESMITH, J.W., 1995. Clinical and epidemiological correlates of the neurohistology of cases of histologically unconfirmed, clinically suspect BSE. Veterinary Record, 136, 211-216.

WILESMITH, J.W., RYAN, J.B.M. & ATKINSON, M.J., 1991. Bovine spongiform encephalopathy: epidemiological studies of the origin. Veterinary Record, **128,** 199-203.

WILESMITH, J.W., RYAN, J.B.M. & HUESTON, W.D., 1992. Bovine spongiform encephalopathy: case control studies of calf feeding practices and meat-and-bone-meal inclusion in propritary concentrates. Research in Veterinary Science, **52,** 325-331.

WILESMITH, J.W., WELLS, G.A.H., CRANWELL, M.P. & RYAN, J.B.M., 1988. Bovine spongiform encephalopathy: epidemiological studies. Veterinary Record, 123, 638-644.

WILESMITH J.W., WELLS G.A.H., RYAN J.B.M., GAVIER-WIDEN D., SIMMONS M., 1997. A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. Veterinary Record, **141**, 239-243.-

WILSON, C., HUGHES, L. & EBRINGER, A., 2001. Antibodies to *Acinetobacter radioresistens, Pseudomonas aeruginosa,* myelin basic protein and neurofilaments in Bovine Spongiform Encephalopathy. In Press.

WOOLHOUSE, M.E.J., COEN, P., MATTHEWS, L., FOSTER, J.D., ELSEN, J-M., LEWIS, R.M., HAYDON, D.T. & HUNTER, N., 2001. A centuries-long epidemic of scrapie in British sheep? Trends in Microbiology, **9,** 67-70.

PART 2

HYPOTHESES ON BSE TRANSMISSION

(A SUMMARY REVIEW WITH COMMENTS ON HYPOTHESES ON THE TRANSMISSION OF BSE IN DOMESTIC CATTLE).

DISCUSSED BY THE TSE/BSE *AD HOC* GROUP AT ITS MEETING OF 15 NOVEMBER 2001

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I. MANDATE AND SCOPE

When preparing its opinions on BSE-risks, the Scientific Steering Committee has frequently been confronted with the unknowns related to the 'Origin of BSE' and with 'alternative hypotheses for the transmission' of this disease other than *via* animal proteins and maternal transmission.

There are several hypotheses on the 'O*rigin of BSE'* (because it is still disputed). These have been the subject of separate scientific papers and reports, the most recent one being the Horn *et al* review (2001). The TSE/BSE *ad hoc* Group acknowledges this review. It is not the purpose of its own report to further examine the origin of BSE. However, alternative hypotheses for the origin of BSE have been presented to the European Commission (EC) services by correspondence, in addition to the ones also *discussed* in Horn *et al.,* (2001). These hypotheses have often been submitted without accompanying published or peer-reviewed descriptions, but nevertheless merit being inventoried and, if appropriate, discussed.

Regarding '*Hypotheses for the transmission of BSE'*, a comprehensive overview does not currently exist.

This Report therefore has a dual purpose:

c. To give consideration to unpublished, alternative, hypotheses for the 'Origin of BSE' that have been presented to services of the European Commission (EC) in addition to the ones also discussed in Horn *et al* (2001). This forms Part 1 of the Report.

d. To present an inventory and briefly comment on the currently existing hypotheses regarding possible 'Alternative, or third ways' in which BSE might be transmitted to cattle, using the historical theories for the transmission of scrapie in sheep as a baseline and supplementing them with current published hypotheses that relate only to cattle. This forms Part 2 of the Report.

The reader will notice that there is inevitably a degree of overlap between 'the origin' and possible 'third ways'. In this context it is important to note that historically, before anti-BSE measures were applied for the first time in 1988, there were a number of possible 'third ways' for BSE to be transmitted like *via* tallow, gelatin, blood, fertilisers and a range of other ruminant-derived by-products. Currently the measures introduced to reduce risks from these sources have blocked all recognised pathways if they are properly enforced. However, there remain a wide range of more obscure potential 'third ways' for transmitting BSE and it is these that will receive most attention.

The TSE/BSE *ad hoc* Group and the SSC gratefully acknowledge Dr.R.Bradley who prepared the basis version of the current inventory.

Key words: bovine spongiform encephalopathy, transmissible spongiform encephalopathy, TSE, BSE, transmission, hypotheses, third way, third route.

II. SUMMARY OF MOST COMMONLY ACCEPTED POSSIBLE SOURCES OF BSE TRANSMISSION IN CATTLE

II.1. FEED AND THE ORAL ROUTE

II.1.1 MAMMALIAN PROTEIN AND MBM FROM DOMESTIC RUMINANTS

The most widely accepted hypothesis is that feed is the major and perhaps only method that definitively transmits BSE to cattle. Only feed that contains infected mammalian protein is considered a TSE risk. The possible feed ingredients that might deliver the mammalian protein are considered below. The route of infection is oral, perhaps entirely so, but conjunctival or respiratory points of entry cannot be excluded, particularly the former, as this is known in experimental studies in mice to be as efficient as the oral route (Scott, Foster and Fraser, 1993). No reports have been found where experimental challenge has been by the respiratory route.

It has generally been assumed, and supported by epidemiological studies, that the main vehicle of infection is mammalian protein in the form of meat-and-bone-meal (MBM) derived from domestic ruminants (Wilesmith *et al,* 1998, Wilesmith Ryan and Atkinson, 1991). This is discussed in the next paragraph.

II.1.2. FEEDING OF MAMMALIAN PROTEIN

Deliberate inclusion of mammalian protein, mostly in the form of MBM, in cattle diets was the norm for some types of cattle (including dairy calves in some countries, including Australia – a country without BSE and scrapie) for decades before BSE was discovered in 1986 and before feed bans were introduced (Cooke, 1998).

In the UK in the 1950s (Statutory Instrument 1950) a recommended formulation for a national, high protein concentrate food for cattle included 2.5% animal protein-rich substances, with a maximum dried blood content of 1/5. A national grain balancer food for cattle was similarly constituted. The animal protein-rich substances contained at least 40% protein. Whilst the latter were not specifically described as MBM, it is likely that the food animal species were the source of the protein and up to 4/5 of it might have been MBM.

However, it is noted that the Horn Review (Horn, 2001) stated that the incorporation of MBM into concentrate feed of calves in the UK was an essentially new phenomenon between 1970 and 1988 (See Part 1, Opinion on BSE origin). Even so, before 1970, unless completely separated feed manufacturing, transportation and storage facilities were available and used, there would still have been an opportunity to cross-contaminate calf feed with MBM, or with feed for adult cattle or non-ruminant species that contained MBM. Cross contamination of ruminant rations (see below) is widely regarded as the main reason for cases of BSE born after feed bans were in place, so called, 'born after the ban', or BAB cases. However, the reason why feed cross-contamination events do not appear to have been evident historically, but have been prominent at least in the 1990s, is not fully explained. It is possible that historically an insufficient oral infective dose could be delivered through cross-contamination.

II.1.3. SPECIFIED RISK MATERIALS (SRM)

The highest risk for cattle would be from BSE-infected tissues. There is a limited range of infected tissues in cattle with BSE (CNS (including eyes and ganglia) and intestine, especially the distal ileum). A wider range of tissues from TSE-infected small ruminants can carry TSE-infectivity, namely the lymph nodes, tonsil, thymus and spleen. Some other tissues may occasionally be infected at low titre (Hadlow *et al,* 1979, 1980, Hadlow, Kennedy and Race, 1982). The World Health Organisation (WHO) has classified ruminant tissues into four categories including a category of 'no detectable infectivity' to which category belongs blood, muscle and milk amongst a range of products that form part of the human diet (WHO, 1997). SRM is the name given to the tissues that must be removed by law at slaughter or death from ruminant animals because they may harbour BSE infectivity.

The UK experience reveals that, despite the original specified bovine offals (SBO) ban of 1989/1990 being scientifically sound, it was not completely complied with, or well enforced, particularly in regard to protecting animal health. Thus, the starting material used to prepare MBM for feed for non-ruminant species contained significant quantities of SRM, some of which would be infected with the BSE agent. This provided the opportunity for any cross-contamination event to introduce BSE infectivity into cattle diets (see below). In the light of experience, extensions to the SBO ban were introduced to secure better protection (elimination of brains was followed by elimination of skulls and then heads for example (Collee and Bradley, 1997a,b).

Blood from healthy cattle is devoid of detectable TSE infectivity (as determined by current methods) but could become infected at slaughter if various severe (now prohibited) stunning methods are used and if appropriate hygiene is not practised at slaughter. In addition, the risk from embolism following pithing created a potential risk in blood, heart and lungs from animals in the late stages of incubation from BSE, thus effectively converting them from TSE safe tissues, into SRM (see SSC 2001e for a full discussion on the subject). There could have been potential secondary risks for cattle because unprocessed blood was spread on land and processed blood was used in feed. Furthermore, all three tissues may have entered the rendering system and returned to animal feed and to ruminants by accidental cross-contamination of cattle diets.

Human food waste (swill or plate waste), that traditionally has been fed to pigs in some countries (where it has been incriminated, from time to time, in the initiation and spread of conventional diseases) might be processed into feed pellets for feeding to ruminants. Clearly, if countries unknowingly were incubating BSE in their cattle populations and had no effective SRM ban, there could be a risk for cattle fed this type of material if it was infected. Mechanically recovered meat (MRM) derived from cattle in such waste might be of particular concern, as it has been suggested to be a vehicle for the transmission of BSE infectivity to the human diet and thereby could cause vCJD. MRM from ruminant species is now prohibited.

II.1.4. CROSS CONTAMINATION OF RUMINANT DIETS

Accidental inclusion of mammalian protein in the form of MBM in cattle diets (by cross-contamination events) was commonplace in the UK both before and since the ruminant feed ban was introduced in 1988. A similar situation occurred in other countries with BSE, but at later dates. This origin of infection accounted for all, or most, of the cases of BSE that occurred after the feed bans were put in place. In Great Britain over 42,000 of the c.180,000 confirmed cases of BSE were in cattle born after the 1988 feed ban (BAB cases, MAFF, 2000b). Most BSE cases in cattle in other countries are now occurring in BAB cattle. However, it should be noted that the dates of introduction of feed bans in each country differ. It is noted that the number of cases of BSE reported in all other affected countries is very low in comparison to the UK (total to June 2001 c.3,200 compared with c.180,000, respectively).

Mammalian protein, presumably in the form of MBM, has been found in such feed ingredients as fishmeal. Thus, accidental or deliberate/fraudulent adulteration of an otherwise TSE-safe commodity could theoretically arise and be responsible for some cases of BSE.

II.1.5. CRIMINAL ACTIVITIES

Criminal introduction of TSE-risk materials into cattle diets is a rare possibility. But other than it being used as a scare tactic, or to dispose of a small quantity of such material this is unlikely to be fertile ground for the criminal if the intention is to cause disease, because there is never certainty about infection being present and even if it was there would, on average, be no result for five years when the incubation period was complete. In any case, it is highly unlikely that a sufficiently large quantity of such risk material would be readily available for criminal use.

II.1.6 MBM DERIVED FROM CAPTIVE WILD RUMINANTS AND OTHER SPECIES WITH TSE

This hypothesis has been put forward by Professor Roger Morris of Massey University, New Zealand. His modelling studies show this to be the most compelling of several hypotheses for the origin of BSE (R. Morris, Personal communication). The idea is that wild BOVIDAE species have a naturally occurring TSE, at least one animal incubating such a disease was imported and was rendered some time in the past, by an inefficient rendering system and the epidemic was started by the MBM produced being fed to cattle that subsequently amplified the epidemic. However, no such TSE disease has ever been found. It would be a major coincidence for such a rare disease (if it occurred naturally) to be imported in such a small consignment, or alternatively if it was not rare, why was the UK the only country to import such cases? There is no dispute that captive wild BOVIDAE became infected with the BSE agent after importation so the importance in regard to 'Third Ways' is to be sure that there is no way of recycling any infectivity in captive wild animals back to food animal species.

Clinical signs of a scrapie-like disease have been reported in Reindeer in Iceland but there is no convincing evidence that this species harbours

infection. TSE with a presumed origin from cattle has been reported in domestic cats and a number of captive wild FELIDAE species. Clearly if any carcases from TSE-infected individuals entered an ineffective rendering facility and the resultant MBM was incorporated into feed for farmed, food animal species a TSE risk would result. Such practices are now prohibited.

II.1.7. MAMMALIAN PROTEIN OTHER THAN MBM

Several bovine-derived materials other than MBM are, or have been, included within concentrate ruminant diets. These include gelatine, hydrolysed proteins, amino acids, tallow and tallow derivatives, blood and blood products and dicalcium phosphate. Scientific opinions on all these items have been prepared by expert groups and Opinions have been expressed by the Scientific Steering Committee (SSC, 2001a-e).

II.1.7.1 Gelatin

In regard to gelatin, this can be prepared from pig skin, pig bones and cattle skin that are not considered to be a BSE risk because inherent infectivity is undetectable and risk management procedures can eliminate significant cross contamination.

Gelatin can also be prepared from bovine bone. Certain bovine bones used as starting materials potentially create significant BSE risk because skulls and vertebrae (excluding tail vertebrae) may contain, or have attached, residual central nervous tissues that have been shown to harbour BSE infectivity. The skull could contain brain, retina and trigeminal ganglia and vertebrae could contain spinal cord and dorsal root ganglia.

Whereas the process of gelatin manufacture is a severe one, there is now more knowledge about the effectiveness of the various chemical treatments in reducing TSE infectivity and measures have been introduced accordingly (See SSC 2001a,b for a more complete analysis of effective gelatin processing).

II.1.7.2 Dicalcium phosphate from bovine bones

Dicalcium phosphate prepared from bovine bones could have BSE risks for the same reason that bones used for gelatin manufacture may create a risk. Furthermore, in the case of fallen stock that have died of BSE, or in any cattle in which, BSE infectivity is present in the CNS, there is possible infectivity in bone marrow (Wells *et al,* 1999*).* The risks would be higher in cattle over 30 months of age than in cattle under this age. Furthermore, historically a variety of processing methods were used and none was completely effective at inactivating TSE agents, though titre reductions of significance were obtained. It is still not clear that the commonly used processes for preparing calcium phosphate from bones are completely inactivating and therefore the importance of using safe sources is paramount (SSC, 1998).

II.1.7.3. Constituents of cattle diets that might contain gelatin

Waste human foods like, broken biscuits and confectionery of similar kinds have been used in cattle diets. These might contain significant quantities of animal derived ingredients, like gelatin. Gelatin is also used in small quantities

to protect vitamin supplements that are an essential component of some cattle diets.

II.1.7.4. Amino acids and *Polygeline* manufactured from bovine bone gelatin.

Amino acids could be used in cattle diets. If prepared from safe starting materials or generated from fermentation processes that excluded animal tissues, there would be no risk. But, if prepared from bovine bone gelatin, there is a theoretical risk that small amounts of BSE infectivity could be present in such gelatin if the bones from which gelatin was derived, contained infected CNS material and an ineffective inactivation procedure was not used during manufacture.

A process whereby amino acids are prepared from gelatin by using heated hydrochloric acid has been examined and reported by Appel *et al,* (1999). The experimental method (1N HCl for 1 hour at >65˚C) almost completely inactivated high titre (8.6 logs of hamster, i/c ID_{50}/g) spike-induced infectivity. The actual commercial method uses HCl to provide a pH of 0.8 at 120˚C for four hours so is even more harsh in terms of temperature, time and pH (more acidic). Any residual risk that survived the gelatin production process would be negligible if the above process or an equivalent one was used.

Polygeline is a plasma substitute agent manufactured from bovine bone gelatin and designed for use in humans, though it is feasible that it could rarely be used in cattle. As in the case of amino acids derived from bovine bone gelatin the starting material might not be guaranteed to be free of BSE infectivity. However, Peano *et al,* (2000) has experimentally shown a marked (additive) titre reduction of between 9 and 14 logs in experimental studies using high titre spiked starting material. Measures introduced to ensure bovine bone gelatin was safe to use for certain processes would also ensure the safety of derived materials.

II.1.8 FAT (TALLOW)

Historically, tallow has been produced from raw materials from mixed mammalian species including from fallen stock and condemned tissues (any of which might have contained TSE infectivity) as well as from TSE agent-free tissues passed fit for human consumption but chosen not to be used, for human food. Furthermore, in experimental BSE in cattle, bovine bone marrow may occasionally be infected with the BSE agent (Wells et al., 1999). This wide range of material, of often uncertain quality and possible TSE risk, may in the past, not have been processed by methods that ensure its safety. Even so, no detectable infectivity has been found in either unfiltered or filtered tallow even from inadequate processing (Taylor, Woodgate and Atkinson 1995, Taylor, Woodgate, Fleetwood and Cawthorne, 1997, Schreuder *et al,* 1998). These studies used mice that may have underestimated any residual infectivity. Recent studies by Appel et al, (2001) indicate that PrP could still be detected after autoclaving at temperatures now authorised for rendering if there were very high contents of fat. No incidents of BSE have been epidemiologically attributed to the feeding of tallow following rendering but this possibility cannot be excluded.

The source materials from which tallow is extracted could be contaminated with SRM. New Opinions on the safety of adipose tissue associated with the digestive tract of ruminants and on the safety of tallow from ruminant slaughter by-products have recently been adopted by the SSC (SSC, 2001c,d). This leads to the possibility that some tissues, historically believed to be 'safe' could in fact present a risk.

One such tissue could be fat derived from bone degreasing (where the bones in question include the skull and/or vertebral bone, excluding the tail). Bone degreasing is a prelude to gelatin manufacture from bovine bones. They are treated only at a low temperature (c 85ºC) and could be cross-contaminated with BSE-infected CNS tissue. Another source could be fat derived from mesenteric fat attached to infected lymphoreticular tissue in the gut, particularly the distal ileum.

Bovine fat is included in the formulation of milk-replacers for calves and if it included such fat as described above, it could introduce BSE infectivity into the calf diet, particularly of dairy calves, or orphaned calves fed artificially. Measures have been, or are, being introduced to improve the TSE safety of fat derived products in food and feed.

A detailed study dealing with the changes in the tallow production process and its use in milk replacers in dairy cattle in UK in the seventies and eighties, the chance of these replacers being contaminated at that time by proteins and the sensibility of young monogastric calves (before making their forestomacs) to BSE infection compared to older calves , has not been carried out so far. Its outcome may also shed light on the question whether this route of transmission possibly was / is [currently] important in certain low incidence countries.

II.I.9. TALLOW DERIVATIVES

Tallow derivatives are most unlikely to have been a source of BSE infectivity for cattle. This is partly because they are not used to any real extent in cattle diets (though they may be used during the manufacture of vaccines) and more importantly because they are derived from already processed material (tallow) in which infectivity has not been found. Furthermore, the hydrolytic processes of high pressure (50 bar), high temperature (250ºC) and long time (3 hours) are generally regarded as secure methods for inactivating TSE agents although specific testing has not been reported.

II.1.10 EFFICIENCY OF THE ORAL ROUTE

In regard to the oral or intra-gastric route of infection, these are generally regarded as the least efficient of all routes. The relative efficiency between the i/c and oral routes in rodents has varied in different publications from between 10^5 in mice, (Kimberlin and Walker, 1989) and 10^9 in hamsters (Prusiner *et al,* 1985), the latter figure being derived from incubation period data rather than the more reliable end point titration method. Discrepancies between the two methods can be explained by the degree of aggregation of PrP^{Sc}. (For a discussion on the relative merits of the two methods see Masel and Jansen, 2001). However, in various risk analyses estimates of the efficiency have been reduced in case the guidance derived from experimental studies in

rodents do not apply in practice to ruminants and cattle in particular. It is therefore interesting and important to note that Taylor *et al.,* (2001) reported the comparative efficiency of the i/c over the oral route was a mere 700 times compared with about the 200,000 times previously reported using C57 black mice (Kimberlin, 1994), though the latter studies did not use the same inoculum. This means that if the BSE agent somehow shows a higher efficiency for oral transmission in ruminants than do other strains of TSE agent under natural conditions, a much lower dose might be able to establish infection than was hitherto thought.

II.2. MATERNAL TRANSMISSION

II.2.1. GENERAL

It is not intended or necessary here to review all the evidence for and against maternal transmission but rather to indicate, like many aspects of BSE, it is not proved beyond doubt that it occurs in the absence of feed-borne infection and if it does, what the mode of transmission is. For a concise account of the pros and cons of maternal transmission see the annex to the SSC opinion of 29-30 November 2001 on The six BARB BSE cases in the UK since 1 August 2001 (prepared by S.Bird).

Maternal transmission is theoretically a possible route of transmission, and has been investigated (Wilesmith, *et al.,* 1997, Donnelly *et al.,* 1997 a, b, Curnow and Hau, 1996) if only for the reason that it would appear to occur in natural scrapie. Furthermore, in sheep a plausible mechanism has been identified. That is to say from the placenta of infected sheep. However, comparable investigations in cattle have led to different results (no experimental transmission) and thus different conclusions *i.e.* that if maternal transmission occurs, either the placenta (and other reproductive tissues and milk) are not involved or, that the event is infrequent (see below).

Supporting evidence for the natural transmission of scrapie via the placenta

"There is convincing evidence that the placenta of scrapie infected and affected sheep carries scrapie infectivity and can transmit this infectivity to other sheep and to goats by the oral and i/c routes, and to mice by the i/c route. The results of the experimental studies reported below sit comfortably with the epidemiological studies of natural scrapie and the presumed exposure of the majority of sheep at lambing time. Pattison et al (1972, 1974) showed that placenta obtained from late pregnancy from six Swaledale ewes each transmitted scrapie to six goats and to four sheep either by the oral (3 goats and 2 sheep) or by the i/c route. In total, 10 of 12 animals inoculated i/c and 9 of 11 dosed orally succumbed to scrapie. Interestingly the incubation period ranges in the sheep were no different between routes of challenge (21-57 months orally, and 25-55 months by i/c inoculation) but the infecting doses were not reported.

In Japan Onodera et al., (1993) reported the isolation of scrapie infectivity, but not PrP^{Sc}, from the placenta of a three months pregnant, clinically normal Corriedale sheep with microscopically confirmed scrapie. Infectivity and PrP^{Sc} were found in the parental brain.

More recently Race, Jenny and Sutton, (1998) identified PrP^{Sc} and scrapie infectivity in the brain and spleen in all of ten sheep with microscopically confirmed disease. They also studied the infectivity and PrP status in some placentas recovered from sequential pregnancies and at necropsy. They reported scrapie infectivity in the placenta of eight of these sheep, four shed

naturally 126-470 days before the onset of clinical scrapie and four at necropsy, but not in two others shed 252 and 109 days before necropsy. There was a complete correlation between the finding of infectivity and the finding of PrP^{Sc}. There was however an inconsistency in the occurrence of infectivity in placentas in successive pregnancies such that in two sheep placentas positive at the previous pregnancy, were negative at the next. This might be explained by variation in the genotype of the fetal placentas that was not reported. There is clearly a value in checking placentas for PrP^{Sc} when scrapie is suspected in a flock because it is a non-invasive method and could be utilised as part of a control or surveillance programme." (Abstracted from the original papers and quoted from 'Infectious Diseases of Livestock, with special reference to Southern Africa, Scrapie chapter", in Press). (See also Dickinson, Stamp and Renwick, (1974) and Tuo et al, (2001) who showed that the sheep embryo/fetus was not exposed to scrapie in utero because, although PrP^{C} is widely distributed in the fetus, fetal fluids and placenta, PrP^{Sc} is confined to the caruncular endometrium and cotyledonary chorioallantois of pregnant scrapie-infected ewes. The fetus is separated from these structures by the PrP negative amnion.

Two pieces of information lend some support to the occurrence of maternal transmission in cattle namely the results of the cohort study (Wilesmith *et al.,* 1997, Donnelly *et al*, 1997b) and analysis of dam calf pairs in the large UK BSE database (Donnelly *et al.*, 1997a). Each suggests a transmission rate of around 9-10% by this means though part of it (about 50%) may have an unidentified genetic basis. However, unlike in sheep, there is no plausible evidence for a mechanism from transmission studies. There have been limited epidemiological studies on this aspect in other countries (Braun *et al* 1994, Fatzer *et al.,* 1998) and there is no evidence for transmission in this way in any country in the absence of a feed-borne source. In any case in Member States of the EU, offspring of BSE cases are traced and compulsorily slaughtered.

II.2.2. INFECTIVITY STUDIES ON CATTLE PLACENTA

The number of bioassays of bovine placenta from cows with BSE in the UK (Bradley, 1996) have been limited. Since maternal transmission, if it occurs at all, does not exceed 10% [c.i.: 5-15%], a substantial number of placentas from BSE-affected animals would need to be bio-assayed before it could be concluded that infectivity in this tissue was unlikely.

If placenta was even rarely infected in cattle with BSE, this would provide the potential for a 'Third way' by establishing a means of horizontal transmission since other cattle could consume the placenta or become infected by indirect means from a contaminated environment, as may occur in scrapie in sheep.

II.2.3. INFECTIVITY IN COLOSTRUM AND MILK

Colostrum from cattle with BSE has not been bio-assayed, but milk has, and there is no positive evidence that milk is infectious for susceptible mice challenged by the oral (Middleton and Barlow, 1993, Taylor *et al*, 1995) or by the i/c route (Taylor *et al*, 1995). Furthermore, it is normal farming practice to ensure that all cattle, whether dairy or beef, obtain colostrum usually, but not exclusively, from the dam, since this provides essential immunoglobulins that protect the young animal from lethal or disease-inducing pathogens in the environment. Epidemiological studies in relatively small numbers of beef cattle indicate that milk does not transmit BSE (Donnelly, 1998, Wilesmith, 1996, Wilesmith and Ryan 1997).

III THIRD WAYS OF TRANSMISSION

III.I GENERAL CONCEPTS ABOUT 'THIRD WAYS'

These could include:

- Different (parenteral = non-oral) routes of delivery
- Different infected materials as sources of infectivity
- Genetic factors
- Temporal changes
- Magnitude changes

These concepts are now considered in turn.

III.1.1. DIFFERENT (PARENTERAL) ROUTES OF DELIVERY

Until now, it has generally been accepted that the main route of transmission is by the oral route. If maternal transmission does rarely occur, the oral route could even be involved in *in utero* and in post-natal maternal transmission. If vaccines or medicinal products are a source of rare infection then, although the oral route is not excluded, other routes such as intra-muscular and sub-cutaneous, conjunctival, intra-peritoneal, intra-nasal, and intravenous routes should be considered. Other than the conjunctival route (equal efficiency to the oral route, Scott *et al*, 1993), these are likely to be more efficient than the oral route, so that a lower dose could theoretically produce infection and disease.

Other routes that theoretically might be implicated are the dental route and by skin scarification. The former route has been identified as an efficient route to the brain *via* the trigeminal nerve, at least in Syrian hamsters (Ingrosso *et al*, 1999). It is assumed that cattle are mostly infected by the oral route when young, so a dental route during the period when teeth are erupting is a possible route of infection (Grant, 2001). It is difficult to see how this could occur other than when infection is present and presented orally. Smaller doses than those required to induce infection by the oral route might be effective by this route.

Skin scarification in mice has an efficiency as high as the intravenous and intraperitoneal routes (Taylor *et al*, 1996). This is again without significance unless there is a source of infection that is delivered to broken skin. If it was, it would suggest that smaller doses of infection than by the oral route might result in disease. Epidemiological investigations could reveal if these routes have any significance in isolated cases of BSE occurring long after effective feed bans were in place.

Since abdominal operations are relatively frequent in cattle practice (*e.g.* for correction of displacement of the abomasum, rumenotomy and Caesarean section) the intra-peritoneal route of infection is possible if infected instruments or materials are used. However, it could be viewed that abdominal surgery is comparable to exposure by the intravenous route and would therefore be more efficient than the intra-peritoneal route. Since neuro-surgery, especially in adult cattle, is a rare procedure, the chance for instruments to become contaminated from other cattle is low. Should the same

instruments be used on both sheep and cattle there are possibilities for instruments to become contaminated from sheep incubating TSE. The theoretical risk from adult sheep that not infrequently have Caesarian operations is greater than from cows because infectivity is more widely distributed in sheep than cows including in the placenta during the incubation period (Race, Jenny and Sutton, 1998). Cleaning and effective decontamination is necessary to reduce this risk. For obvious reasons, the intra-cerebral route is not commonly directly available, other than in experimental situations. Epidemiological study of incidents should reveal or eliminate the possibility of most parenteral exposures in cattle. If they occur at all, they are, on the basis of current epidemiological evidence, extremely rare.

III.1.2. DIFFERENT INFECTED MATERIALS AS SOURCES OF INFECTIVITY

The highest risk for cattle would be from infected cattle tissues, but risks from exposure to infected tissues from other species, notably small ruminants, cannot be excluded. There is a limited range of infected tissues in cattle (CNS and intestine, especially the distal ileum). Blood, heart and lungs can be cross-contaminated with brain tissue if certain stunning methods or pithing is practised. Some processed products like gelatin and tallow theoretically could be cross-contaminated with these SRM. A wider discussion on these tissues is in Section I.

III.1.3. GENETIC FACTORS

These do not involve infection events but rather genetic mutations of a sporadic or familial type as has been reported in Creutzfeldt-Jakob disease (CJD). There is only a hypothetical risk for the occurrence of either form, which at present seems unique to man. The occurrence would not, unlike environmental exposures, be restricted by geography. The risk should be considered to be similar in all geographical locations, at least until genuine familial and genetic cases have been identified. Apart from the absence of epidemiological data on genetic disease there is no evidence from the molecular genetic studies so far reported that there is any connection between polymorphisms in the *PrP* gene of cattle and the occurrence of BSE (Goldmann *et al.,* 1991, McKenzie *et al.*, 1992, Grobet *et* al., 1994, Hunter *et al.,* 1994). Modelling studies reported by Hau and Curnow, (1996) concluded there was still no evidence, molecular or statistical, for genetic variations in susceptibility.

III.1.4. TEMPORAL CHANGES

It is as well to recognise that during the course of the BSE epidemic in Europe there has been an increasing global awareness of the disease and especially since 1996 when its importance as a zoonosis was firmly established. Thus, if there were originally a variety of origins for BSE they may well have been concealed by the enormity of the exposures from infected feed and especially MBM. Since 1996, it could be that these subsidiary causes have been eliminated, primarily as a result of the imposition of measures and also because of awareness of risks and their independent management.

Contrariwise, if there really is a 'Third Way' and the risk from it has not been managed adequately, there is an improved opportunity to identify it, specifically by epidemiological means. Six cases of BSE have occurred in cattle in the UK that were born after 1 August 1996, the date that a ban was believed to have been effectively enforced. However, this is fewer than anticipated by modelling and there is little supporting evidence for maternal transmission in the majority of the incidents. It will take from 5 to 7 years from the date of the last born case (currently this is May 1997) to be sure that the disease is extinct. In the UK, that time is fast approaching and provided the current controls continue to be properly enforced, it should be possible to determine if there is a significant 'Third Way' or not. In other countries with BSE, the effectiveness of the bans will take longer to determine because the dates for effective enforcement are later. Explaining solitary incidents of BSE whilst there is still a risk of feed exposure is unlikely to ever be possible (other than possibly for genetic causes involving the *PrP* gene) since exposures would have been distant in the past. Nevertheless, if incidents of BSE do occur in the absence of feed-borne exposure the possibility of detecting the source of infection by epidemiological means becomes greater unless they are equivalent to sporadic CJD, the cause/origin of which has avoided detection for over 70 years.

III.1.5. MAGNITUDE CHANGES

Because of the introduced measures to eliminate BSE from EU herds it is clear that infectivity available to cattle, in man-controlled situations (feed, medicinal products and vaccines for example), is likely to be reduced so much that an infective dose sufficient to cause disease would be unlikely to be delivered. However, if there is a third way it is still theoretically possible to maintain an infected cattle population, and therefore all possible routes and sources should be followed up to ensure there is a minimal risk of this happening.

III.2. HYPOTHESES FOR OTHER 'THIRD WAYS'

In theory, these might include environmental, iatrogenic and genetic transmission.

III.2.1 ENVIRONMENTAL TRANSMISSION

III.2.1.1 General

The evidence for the transmission of natural scrapie from an infected environment is circumstantial (Hoinville, 1996), but two points are worthy of note when considering this issue. Firstly, strains of high titre hamster scrapie agent retain infectivity after burial for three years, though over 99% of the infectivity was lost (Brown and Gajdusek, 1991). Secondly, scrapie eradication programmes in several countries have failed to eliminate the disease. In Iceland, where the greatest effort has been made, success is close to achievement. This has followed close attention to the removal of the hazard of possible environmental contamination. The measures included extremely thorough cleaning and disinfection of farm buildings (flaming, burning, disinfection, creosoting, oil-based painting), leaving farms devoid of sheep for

up to three years and removal of the topsoil from around farm buildings and other contaminated areas (Sigurdarson, 1991, 2000). Restocking was from scrapie-free flocks in fenced areas of Iceland.

Environmental transmission embraces all possible non-genetic methods of transmission. These methods include:

Direct horizontal transmission from cattle sources other than by placenta, milk or colostrum:

- *Direct contact – Experimental – Mice*
- *Direct contact – Experimental - Sheep and goats*
- *Direct contact – Natural disease*

Indirect transmission from cattle or other animal sources to the alimentary tract of cattle:

- *Risks from soil*
- *Experimental studies*
- *Experiences in Iceland*
- *Risks from tissues and excretions*
- *Faeces*
- *Saliva (and faeces)*
- *Urine*
- *Risks from plants*
- *Risks from fertilisers and sewage sludge*
- *Risks from burial*
- *Contaminated water*
- *Risks from other mammalian species susceptible to TSE or carrying infection – General*
- *Risks of transmission from MBM derived from captive wild ruminants with TSE*
- *Tallow and gelatin*
- *Composted manure and stomach and intestinal contents*
- *Enteric nematodes (and other organisms) carrying infection*

  Historical data

  - Blow flies and oribatid mites carrying infection

  More recent studies

  - Hay mites carrying infection

Indirect transmission to the CNS:

- *Protozoon and other parasites*
- *Coenurus cerebralis*

Collateral factors **(factors that might increase susceptibility):**

- *The role of copper and manganese*
- *Exposure to organo-phosphorus compounds*
- *Green cluster nutrients antioxidants and BSE*
- *Inadequate exposure to prostaglandins*

Other hypotheses unsupported by published articles or singleton presentations in 'one off' articles

Each of these will be discussed in turn.

III.2.1.2. Direct horizontal transmission from cattle sources other than by placenta, milk or colostrum

a) Direct contact – Experimental - Mice

Dickinson, Mackay and Zlotnik, (1964) established that scrapie could be transmitted by contact between mice of different genetical constitution. Three uninoculated mice caged with five i/c inoculated mice developed scrapie after prolonged incubation periods. The strain of agent in two of these cases was typed and found to be the same as that inoculated into the cage mates. One mouse had been attacked and bitten by cage mates after inoculation but other possible routes of infection were not regarded as plausible. Pattison, (1964), reported contact transmission (possibly as a result of fighting) in 15 of 49 caged mice. These reports contrast with subsequent unpublished experiences in numerous mouse experiments using BSE and scrapie agents, that transmission of infection by close association, if not contact, is at the worst a rare event.

b) Direct contact – Experimental - Sheep and goats

Under experimental conditions Pattison (1964), was unable to demonstrate contact transmission in 17 sheep housed with scrapie-inoculated sheep or 192 goats maintained in a scrapie contained environment or in 33 goats, the progeny of goats inoculated at mating with scrapie that came down with disease subsequently. This was despite being housed together and being able to suck milk from their dams. However, Brotherston *et al.,* 1968, conducted contact experiments where 17 goats were kept for long periods in the same small pen as sheep with natural scrapie. Ten goats developed scrapie. Control goats remained normal. Contact infection did not occur within the same time scale when sheep with experimental scrapie were used. Three cases of scrapie occurred in Scottish Blackface sheep (a breed that at the time had not been reported to be affected with scrapie), kept in continuous contact in a small building from birth with naturally affected sheep of various breeds for periods of 3 years, 9 months and 4 years , 4 months.

c) Direct contact – Natural disease

Amongst naturally occurring TSE, only in scrapie is horizontal transmission a proven event, though it cannot be excluded in chronic wasting disease of deer and elk. Where clusters of TSE cases have occurred (for example kuru in Papua New Guinea, CJD in Orava, Slovakia, BSE in Western Europe, transmissible mink encephalopathy (TME) of farmed mink in North America) they can be attributed to common source exposure and to consumption of infected body parts (kuru) to feed (BSE and TME), or familial occurrence due to gene mutation (CJD in Orava). There is no evidence that human TSE has ever transmitted to animals, notwithstanding the single report from Italy of the concurrent occurrence of sporadic CJD in a single human patient and a spongiform encephalopathy in a cat (Zanusso *et al.,* 1998). This is because the

precise nature of the disease in the cat is not confirmed and the causal agents have not been isolated and shown to have the same biological strain properties.

In regard to BSE, even though an origin from a scrapie-like agent from sheep has been proposed (Wilesmith *et al.,* 1988) this is not believed to be the result of horizontal transmission, since 20% of farms with BSE cases had not held sheep in living memory. Once BSE had been introduced into the cattle population in the UK, horizontal transmission might then have been possible. Possible sources might theoretically be urine or faeces that are massive in quantity and deposited naturally or spread as fertiliser on the farmland following composting. These are dealt with in the next section. However, it is noted in passing that rarely individual young cattle may develop a habit of drinking/sucking urine, particularly from steers.

A study of possible horizontal transmission of BSE by Hoinville *et al* (1995) revealed that, although there may have been an increased risk of BSE occurring in animals that were born on the same day, or between one and three days after an affected animal had calved, there was no plausible mechanism for this. In any case, a direct route of transmission could not have been responsible for the majority of cases of BSE that had occurred after the introduction of the 1988 feed ban in the UK. Alternative sources of infection were suggested to be the use of cross-contaminated feed (supported by epidemiological evidence) or indirect routes that could only be investigated by comparing exposures in affected and unaffected herds that was not investigated.

III.2.1.3 Indirect transmission from cattle or other animal sources to the alimentary tract of cattle:

a) Risks from soil

The German, Federal Ministry for the Environment, Nature Conservation and Reactor Safety have produced a report of an International Expert Discussion on the Occurrence and Behaviour of BSE/TSE prions in soil, held on 8 December 2000 in Bonn (Report 2001). This report indicated the lack of knowledge about the contamination and degradation following pollution of soil and water and made useful recommendations for the correction of this deficiency but no conclusive final statement that had the support of all participants could be made. Nevertheless, they considered various routes for the pollution of soil and water including from organic fertilisers containing for example mammalian protein, industrial fertilisers from farms (faecal and urine sources), from composting of infected material after biogas production, sheep placentas, sewage sludge and dog and cat faeces. Deficits in particular knowledge were indicated in the role of soil nematodes but there was a consensus that prions might be bound in the superficial layers of soil and that degradation would be a slow process. This report noted that cattle could consume up to 1kg of soil per day suggesting a risk might be present should an effective oral dose of the BSE agent be present. Cattle would be less likely to consume leaves contaminated with dust from any distributed source of infection. In addition, the plants themselves would be devoid of risk because the roots cannot absorb protein molecules the size of PrP molecules. The role

of dog and cat faeces was considered a negligible risk. It is also known that the scrapie agent is highly resistant to inactivation (Taylor 1996).

b) Experimental studies

Greig (1940), found it was very difficult from the analysis of field incidents of scrapie to determine whether or not scrapie could be transmitted from pasture upon which scrapie animals had grazed. He therefore undertook an experimental study in which 3 or 4 scrapie sheep and 20 sheep from flocks and regions with no history of scrapie. These animals were kept on separate fields without direct contact. About twice each week, the sheep in each pasture were exchanged, again without direct contact with each other. The ewes were mated and lambed. After three years, no cases of scrapie were seen in the contact sheep. They were moved to another farm that had never reported scrapie but between a further three months and a further two years and three months a total of 9 cases of scrapie developed and it was concluded that the origin of infection was the pasture that had been grazed by scrapie-affected sheep.

c) Experiences in Iceland

Indirect transmission of scrapie from a contaminated environment has been reported, notably from Iceland (Pálsson, 1979; Sigurdarson, 1991). This is plausible because there has been a high incidence of scrapie (rida) in some Icelandic flocks in fenced-off scrapie-affected regions, especially as there is close confinement of housed breeding sheep over the long winter period. In one recent occurrence in Iceland, scrapie returned to a flock following strict depopulation cleaning and disinfection after a period of seven years (S. Sigurdarson, personal communication). This suggests that the site might previously have been highly infected and was responsible for the new occurrence (Wilson, Anderson and Smith, 1950) or that an alternative source such as hay mites (see below) might be responsible. Scrapie infectivity could be transferred to the environment from infected placenta. Sheep placenta is a known source of scrapie infection and, if not consumed by the dam or unrelated sheep, could be taken by foxes or other carnivorous species across farm boundaries. Ravens in Iceland (Sigurdarson, 1991) and Black-backed gulls (Moon, 1978) have been suggested as vectors. However, ravens do not succumb to challenge with scrapie (S. Sigurdarson, Personal communication). Furthermore laboratory strains of high titre hamster scrapie can survive for up to three years in the soil, though at very much reduced titre, but still sufficient to produce disease by the i/c route in hamsters (Brown and Gajdusek, 1991). Transmission by the oral route following three years in soil has not been demonstrated.

d) Risks from tissues and excretions

Other sources of infectivity could be postulated from study of the infectivity of various tissues, secretions and excretions from sheep and goats in the clinical phase of disease and in sheep during the incubation period (Hadlow, Kennedy and Race, 1982). Results from natural scrapie are perhaps more pertinent to the practical situation than results from experimental studies. However, few experiments have been done using the natural host to detect infectivity so there is a loss of sensitivity due to the so-called species barrier, that may only be

partially recovered by using parenteral routes (including the i/c route) of challenge. Of the positive tissues derived from natural scrapie cases, the intestine, amniotic fluid and nasal mucosa are pertinent. Several vaccines used in cattle to protect them from respiratory disease are administered by the intra-nasal route (though there is no evidence at all for transmission in this way or indeed from any commercially produced vaccines by any route). However, faeces show no detectable infectivity and amniotic fluid positivity is inseparable under practical conditions from the positive infectivity in the placenta mentioned above. Hadlow (1991) comments on potential risks from nasal mucosa.

Faeces

In cattle with natural or experimental BSE, the only plausible source of infection is from faeces (urine is discussed separately below), as other infected organs have no direct connection with the environment in the living animal. In this context there are theoretically three ways in which faeces might become infected and then theoretically transmit disease indirectly after composting (that is unlikely to completely inactivate TSE agents).

The first is by direct passage of unaltered, infected material through the gut. In mice, it is recognised that when an experimental oral infection is administered only a small proportion is absorbed, altered or destroyed and the greatest proportion is passed out in the faeces. The same is likely in the natural situation where cattle are infected following consumption of infected feed. Nevertheless, due to the effects of rumination, dilution with imbibed water (up to 160 litres per day for an adult, lactating cow), saliva (up to 40 litres per day produced daily by an adult cow), feed, and the long average passage time through the gut of ruminants, it would seem likely that most infectivity would be more widely dispersed than it was in the original feed. An exception might be in experimental high oral dosage of cattle. Thus, in the natural situation it would be less likely that feed or forage contaminated with faeces would deliver an infectious oral dose, especially as cattle are not intentionally coprophagic.

The second is by shedding of infected intestinal epithelium into the lumen of the gut. Although substantial numbers of cells are removed in this way, the epithelial layer of the bovine intestine, although the possible point of entry, is not known to replicate BSE or to harbour detectable levels of PrP.

The third is by direct excretion into the lumen from sites of replication (Peyer's patches) possibly in lymphocytes and macrophages which are found in the superficial layers of the intestine and are also presumably shed into the lumen. Again, the immense dilution is an important factor in making this an apparently low risk route for transmission. Such a conclusion is supported by the relatively low within-herd incidence of BSE in herds affected by the disease (below 3% in any six months period in the UK epidemic (Bradley and Wilesmith 1993)).

In regard to faeces, from cattle with BSE, these have not been bio-assayed but they have been in sheep (Hadlow, Kennedy and Race, 1982) and goats (Hadlow *et al.,* 1980) with scrapie with no detectable infectivity being found. Evidence that is more convincing comes from the epidemiological findings in

BSE including the low within herd incidence (Bradley and Wilesmith, 1993). Pattison and Millson (1961) failed to detect infectivity in the faeces of clinically affected goats challenged orally with experimental, goat-passaged scrapie. This, although an experimental study, has some relevance as the route of challenge with faeces was i/c and the species was caprine so there was no species barrier. However, infection from faeces cannot be entirely ruled out.

Saliva (and faeces)

Pattison (1964) reported an experiment where either 142 oral doses of saliva (10 ml on each occasion) or faeces (2.5 g on each occasion) derived from scrapie inoculated goats did not produce disease within a period of 40 months. The risk of transmission in this way, at least in goats, seems to be low.

Urine

At its meeting of 6-7 September 2991, the SSC discussed the Shaked *et al* (2001) paper announcing the presence of a protease resistant PrP isoform in the urine of animals and humans affected with prion diseases[2]. The SSC considered that the announced results are interesting and the authors may have identified an important phenomenon. It considered, however, that the work needed to be further pursued, independently verified in other qualified laboratories, replicated and extended before the results could be considered proved and final conclusions drawn. The identified phenomenon has possibilities for exploitation for pre-clinical diagnosis should the results in experimental hamsters be confirmed during the incubation period in humans and animals. The SSC further considered it premature to revise the SSC opinions with respect to the safety of animal and human tissues or products. It was, however, important to obtain as soon as possible, the results of urine infectivity studies related to the Shaked *et al* (2001) research.

The (Shaked *et al,* 2001) report has demonstrated that cattle with BSE excrete a protease-resistant form of PrP (UPrP) in the urine. This also occurs in experimental scrapie in hamsters and in humans with familial CJD. The hamster form is also excreted in the incubating phase and therefore might enable a pre-clinical test to be developed for use at least in this species. However, the hamster form of UPrP is not yet proven to be infectious for hamsters as none have succumbed to a clinical disease to date (270 days post-challenge), though one out of three sacrificed at 120 days showed PrP^{Sc} in the brain. If this same feature results in cattle then there should be no risk of disease occurring from the exposure of cattle to urine. Pattison and Millson (1961) failed to transmit scrapie from experimentally scrapie infected goats following challenge of goats by the i/c routes. Furthermore, though urine from cattle with BSE has not been bio-assayed, the kidney has, and shows no detectable infectivity (MAFF, 2000a).

e) Risks from plants

Higher plants including forage plants or conserved forage like hay and silage are not expected to transmit BSE. This is because plants cannot take up large

[2] **Gideon M. Shaked, Yuval Shaked, Zehavit Kariv-Inbal, Michele Halimi, Inbal Avraham and Ruth Gabizon, 2001.** A Protease Resistant PrP Isoform Is Present In Urine of Animals and Humans Affected with Prion Diseases. Journal of Biological Chemistry, July 2001.

molecules like proteins with high molecular weights (like PrP) *via* the roots. However, it is noted that yeasts and fungi contain some proteins that can behave as prions that can be propagated from generation to generation (Caughey, 2000). There is little information about the possible existence of PrP-like prions in plants likely to eaten by ruminant species.

Mechanical contamination of plant leaves by prions is theoretically possible following the spreading of organic fertilisers, manure or stomach/intestinal contents from animals fed MBM or infected with TSE, blood, incinerator ash, sewage sludge, rendering condensate and the like. No evidence has been found that BSE has been transmitted in this way and many risk analyses have shown that any risks from at least some of these items is likely to be very small.

f) Risks from fertilisers and sewage sludge

Gale and Stanfield (2001) have made a quantitative risk assessment for BSE in sewage sludge based on the source, pathway receptor approach. The main sources of uncertainty in the risk assessment are the degree to which sewage sludge treatment inactivates the BSE agent, whether there is a threshold dose and the amount of CNS material that enters the sewage system from abattoirs. They conclude that the dose consumed by grazing cattle is insufficient to sustain the epidemic of BSE in the UK. The important issue is to restrict the amount of bovine CNS entering the sewage system.

Waste animal by-products converted to organic fertilisers used on land grazed by cattle historically, could have been a BSE risk because fallen stock that might include TSE-affected animals and SRM might have been starting materials. Once these are excluded and the standard rendering processing parameters are achieved, the risks are regarded as low. Sewage sludge is prohibited from use as a fertiliser because of lack of traceability and risks from cross-contamination.

Risks from sewage sludge of human origin or from other species, from housed pigs and chickens are theoretical. From humans (if human TSE agents are excreted in urine or faeces) there is a theoretical risk of exposure to human TSE agents including vCJD. However, for all human prion diseases other than possibly vCJD, there is no known voiding of TSE agents and to date no risk has been attributed to humans from this source. In regard to pigs and poultry, these species are not affected by TSE agents but both species in the past could have received MBM in the diet and this could have been infected with either scrapie or BSE agents. The infection could have been voided in faeces. However, to date no episode of BSE has been attributed to this kind of exposure.

g) Risks from burial

The risks from the burial of BSE infected carcases or materials in licensed landfill sites are only likely to cause a potential risk of contamination of leachate. A risk analysis published by Det Norske Veritas (DNV, 1997) for the UK Environment Agency, reveals that estimates for the contamination of the water supply by leachate from licensed landfill are below any level that would be considered to be of significance.

Note: However, on-farm burial of fallen cattle was not uncommon in the UK in the 1980s and early 1990s. Rapid BSE testing of fallen stock was not then available. An unknown proportion will have been late in their incubation of BSE, numerically many more than considered by the UK's Spongiform Encephalopathy Advisory Committee's (SEAC) risk analysis in respect of on-farm burial of foot-and-mouth culled bovines in 2001.

h) Contaminated water

Water might theoretically become contaminated if burial of BSE-infected carcases in unlicensed sites or sites where a risk assessment for possible contamination has not been done, yet there is a hazard.

There are several possible circumstances when high-risk waste (*i.e.* BSE-infected material) might be buried. The first is when fallen cattle stock is buried on-farm whether or not BSE is suspected, but the animal is nevertheless infected. The second is illegal burial when BSE is suspected and the owner wishes to conceal the event. The third is during an epidemic of a highly contagious notifiable disease such as foot and mouth disease when some cattle destroyed as part of the control measures may also be actually infected with BSE. Risks only arise when an aquifer or water-course becomes polluted and is a source of water for cattle, other animals or humans. The risk depends on a series of factors such as the amount of infectivity present in the buried animal(s), the proximity of the burial site to water-courses and the geological nature of the burial site and its drainage. The greatest risk would be for cattle as there would be no species barrier. In practice, it is likely that any risk there may be from such events will long be past by the time the discovery is made since significant pollution from decay would follow soon after burial. Whether or not an effective oral exposure could arise from such an event is not known but cannot be entirely ruled out. However, in general the risks are believed to be small. A risk analysis from burial of carcases has been published as a SEAC report (SEAC, 2001).

i) Risks from other mammalian species susceptible to TSE or carrying infection – General

In countries with BSE, the only farmed animals that are known to be affected by TSE (scrapie) are sheep and more rarely goats and moufflon. CWD has not been reported in deer species in Europe. The only other animal species affected by TSE are domestic and captive wild cats with feline spongiform encephalopathy (FSE), captive wild ruminants with spongiform encephalopathy and farmed mink with transmissible mink encephalopathy (TME). TME has not been reported in wild mink. Only sheep, and to a lesser extent goats and moufflon, are likely to co-graze with cattle. Farm cats could be closely associated with cattle whilst in buildings. Indirect contact with feline faeces is also possible. However, any risk from domestic cats (other than theoretically from protozoon parasite carriers such as *Toxoplasma* and *Sarcocystis* species) is likely to be negligible (see below).

j) Composted manure and stomach and intestinal contents

Theoretical risks from spreading composted dung from captive wild ruminant species on farmland exist but should easily be detectable by epidemiological

investigation. A greater risk might theoretically have occurred in the past (before the introduction of feed bans for non-ruminant animals) if stomach/intestinal contents or dung/droppings from abattoirs dealing with pigs and poultry fed mammalian protein was spread on to farmland grazed by cattle. The same could apply to composted dung or droppings from these species fed mammalian protein. This is a more realistic risk because it would be expected that the inclusion rate of such protein could be quite high (c. 15%) and infectivity if present in the raw material would mostly pass through the gut to enter the faeces. Whether or not an infectious oral dose of BSE for cattle could be consumed is more difficult to ascertain but it is not beyond the bounds of possibility if land was grazed quickly after distribution of these products.

k) Enteric nematodes (and other organisms) carrying infection

Historical data

There are conflicting data on the possible role of parasitic nematodes (and presumed scrapie infectivity in them) such as *Haemonchus contortus* and the occurrence of scrapie. Hourrigan *et al.,* (1979), reported that one cage of mice succumbed to scrapie after challenge with *H. contortus,* presumably derived from sheep or goats with natural scrapie (the experimental details are not given). However, Fitzsimmons and Pattison, (1968) failed to transmit scrapie with *H. contortus* either following i/c inoculation of sheep or goats with ground up parasites or by feeding third stage larvae derived from scrapie affected animals. Laplanche *et al.,* (1996), reported a sudden and severe outbreak of scrapie in a Romanov flock following oral exposure to third stage nematode larvae (*Teladorsagia circumcincta*) and further occurrence in un-dosed sheep in a second (parent) flock from which the challenged animals were derived and kept in a separate enclosure. A total of 236 cases of scrapie out of 1000 occurred in the two flocks. The hypothesis proposed that there was a low level of scrapie infection in the field but that the gastro-intestinal parasites caused inflammatory changes in the bowel that aided the penetration of the scrapie agent and thus induced infection.

Blow flies and oribatid mites carrying infection

Post *et al,* (1999) reported on the results of experimental feeding scrapie infected and non-infected hamster brain to fly larvae and pupae from *Sarcrophaga carnaria* and to oribatid mites, followed by feeding the exposed larvae, pupae or mites back to hamsters. The mites did not transmit disease but larvae and pupae did, though the attack rate was not 100%. The negative mite study might have been due to feeding back too few mites. Testing larvae for PrP two days after feeding gave a strongly positive result but not after several days from feeding (unless the larvae were dead). It was concluded that it was possible for fly larvae and pupae to carry scrapie infectivity but it could not be concluded that they replicate infectivity. Thus, a theoretical 'Third Way' is established. In practice it would seem unlikely that many cattle would have access to, or consume sufficient infected fly larvae or pupae to establish infection. However, the route cannot be completely discounted, say from an undiscovered, rotting and infected carcases on moorland. Such incidents

would be expected to be very rare and speculative. It is improbable they could ever be proved, even if an effective oral dose could be consumed.

More recent studies

A collaborative project between Institutes in France, Iceland and Spain funded by the EC under the FAIR programme is designed to examine the comparative epidemiology and ecology on scrapie-affected and scrapie-free sheep farms. Gruner (2001) reports that studies include the assessment of the role of sheep nostril fly (*Oestrus ovis),* enteric nematodes including *Oesophagostomum venulosum,* mites, field voles (*Microtus arvalis) and wood voles (Apodemus sylvaticus)* in the occurrence of scrapie. Experimental studies in mice infected with nematodes (*Heligmosoides polygyrus)* and sheep infected with *Teladorsagia circumcinta* are in progress. Contrarily the incubation period of scrapie in mice is lengthened in parasitised mice compared with un-parasitised mice. Studies in sheep currently remain negative (Gruner, 2001).

Hay mites carrying infection

Rubenstein *et al* (1998) studied hay mites that had been concentrated from hay from farms in Iceland that had kept scrapie-infected sheep. Mites from three of five farms tested caused scrapie when parenterally inoculated into mice. PrP^{Sc} was demonstrated in the brains of these mice and in mite concentrates from one of the farms. It is too early to say whether these observations can explain the re-occurrence of scrapie on a few farms in Iceland that have had recurrence of disease after complete depopulation and thorough cleaning and disinfection of the buildings and equipment. In Iceland the numbers of incidents in the last three years have been 1999 – 2, 2000 – 2 and 2001 – 1 to July 2001. In at least one incident, the last previous case of scrapie was 7 years earlier and the only possible route for transmission was a previous existence of infection on the farm (S Sigurdarson personal communication). Hay mites are still regarded as possible sources of infection.

III.2.1.4. Indirect transmission to the CNS

Protozoon and other parasites

As mentioned in the introduction, there are several protozoon parasites that frequently (*e.g. Toxoplasma* species), or rarely (*e.g. Sarcocystis* species) enter the brain. Acute sarcocystosis has been observed experimentally in sheep due to the effect of meronts on capillary epithelia and clinically are manifest as anaemia, muscle weakness, fever and mild nervous disease. Diagnosis of naturally occurring disease in sheep is usually associated with the presence of meronts in the brain and spinal cord though the mere presence of meronts does not mean they caused the disease (Sargison *et al.,* 2000) since nearly all grazing ruminants are exposed to infection and have sarcocysts in their muscles. Should these parasites be infected with, or carry the infective agent, this could be a means of seeding the brain with TSE infection. No evidence has been found to explore this hypothesis. Sargison *et al.,* (2000) made one very unexpected observation and that was that in the particular outbreak described, uncooked venison was fed to dogs kept on the property suggesting that there may have been a deer/dog/sheep cycle of *Sarcocystis* infection that introduces the idea that perhaps other infections of deer might rarely have a

route of transmission to sheep. No similar circumstances have been described in regard to cattle and it is perhaps stretching the hypothesis too far to suggest that BSE could in any way be transmitted from another species *via* Sarcocystis infection. Rare incidents of abortion in cattle have been associated with *Neosporum caninum* infection (Barr *et al,* 1991) that can also cause encephalitis. There are no data available to indicate any association with the occurrence of BSE but should outbreaks occur concurrently with protozoal infections this species should not be ignored as a potential, even if unlikely, carrier of infection.

Coenurus cerebralis tapeworm cysts from *Multiceps multiceps* from sheep have been proposed by a German scientist as a source of BSE infectivity in cattle. The scientist hypothesised that the tapeworm was the cause of BSE but there is no evidence to support this theory. These cysts are relatively resistant to inactivation (though not as resistant as prions) and it is proposed that rendered material from sheep containing these cysts could still be viable in MBM that is fed to cattle. The possibility that such cysts from brains of sheep with scrapie could become contaminated with scrapie agent, and might act as a mechanical vector of infectivity, whilst unlikely, cannot be completely rejected as a possible rare mechanism for transmission. However, it is very clear that the majority of cattle could not possibly have been infected in this way. Furthermore, current legislation and proposed legislation controlling the use of animal by-products and processed animal protein, should ensure that such a route of transmission is impossible in the future.

III.3. IATROGENIC TRANSMISSION

Iatrogenic transmission of BSE has not been reported, or even suspected, in cattle but there are some definite occurrences of scrapie in sheep that have been reliably attributed to the use of non-commercial vaccines containing ovine starting materials. For this reason, the issue is discussed below. Other forms of iatrogenic transmission of TSE have been restricted to humans and human tissues. For the sake of completeness and convenience, these subjects are briefly discussed below.

III.3.1 VACCINES

Reference has already been made to the occurrence of at least several hundred cases of scrapie in British sheep as a direct result of the use of a vaccine against the tick transmitted, viral disease, louping-ill (Gordon, Brownlee and Wilson, 1939, Gordon, 1946 and Greig, 1950). This occurrence resulted from the accidental use of scrapie-infected source material and processing methods that did not inactivate the scrapie agent that was unknowingly present.

A more recent possible occurrence of possible iatrogenic scrapie has recently been reported in Etna Silver crossbred goats in Italy by Cappucchio *et al.,* (1998). The goats were kept at grass and concentrate rations were not fed, thus eliminating a source of infection from feed *via* mammalian proteins. Animals over two months old were annually vaccinated against contagious agalactia caused by *Mycoplasma agalactiae*. The vaccine included central nervous system from 'pathogen-free' sheep. The mortality rate in the goats reached 28% in 1 herd, 60% in the second and 5.5% in a third herd. About half the

goats were between 2.5 - 3 years old. Only 1.15% of sheep that were kept with the goats developed scrapie. Scrapie was confirmed by microscopic examination of the brain and by detection of PrP^{Sc} including by immunocytochemistry. PrP^{Sc} was widespread in the brain and beyond sites of vacuolar change. The high mortality, severe loss of weight and simultaneous appearance in the three herds were distinctly unusual features in this outbreak. The source of infection remains uncertain and unproven but iatrogenic transmission must be considered.

A larger epidemic involving 20 outbreaks of scrapie in sheep and goats, also in Italy, has been even more recently reported by Agrimi *et al.,* (1999). The annual incidence ranged from 1% to 90% with a mean incidence for goats of 26% and for sheep of 10%. The total number of cases in sheep and goats together was 1040. The clinical disease was confirmed by microscopic examination of the brain and PrP immunocytochemistry or Western blotting. The high incidence in goats, the high within-flock/herd incidence, the temporal clustering, absence of commercial concentrate feeding in eight flocks and association with the use of a sub-cutaneously administered *M. agalactiae* vaccine, prepared locally using brain and mammary tissue from clinically healthy sheep, strongly suggests an iatrogenic origin. Scrapie appeared between 23 and 35 months after the vaccine was administered.

A third outbreak in southern Italy attributed also to the same vaccine has been described by Caramelli *et al,* (2001) in a mixed flock of Comisana sheep and half-bred goats in an upland area of southern Italy. High crude mortality and scrapie incidence occurred in both species and a large proportion of aged animals were affected. The neuropathology was similar to that in other sheep in Italy with iatrogenic disease but different from conventional natural scrapie. Affected sheep were all of the most susceptible genotype (Codon 171 QQ). It is stressed that the vaccines incriminated in the transmission of scrapie in all these incidents are not commercially produced. They have been prepared and distributed locally within the country.

Dr Subash Arya has repeatedly drawn attention to the possible risk of transmitting CJD to humans vaccinated with sheep-brain derived vaccines in India, *e.g*. Arya, (1994). However, neither Dr Arya nor any of his colleagues has yet found any such case. The episodes of scrapie resulting from the use of vaccines prepared from infected sheep tissues emphasises the need for caution and mandatory selection of safe sources for starting materials used in the manufacture of vaccines. Such vaccines could theoretically at least, be used in cattle thus creating a potential risk, though it is most unlikely that they would be licensed for this purpose in Europe.

Vaccines have not been incriminated in the transmission of BSE (Wilesmith *et al.,* 1988, J.W.Wilesmith, personal communication). Furthermore, large numbers of doses of commercially produced vaccines that have used bovine starting materials, have been inoculated by parenteral and oral routes into cattle throughout the world and a substantial proportion have been produced in Europe, but no incident of BSE has been attributed to their use. This is important because, since there is no species barrier, any chink in the armour protecting vaccines from contamination would have been revealed, but none has.

III.3.2. OTHER MEDICINAL PRODUCTS DERIVED FROM TSE-SUSCEPTIBLE SPECIES

Animal sources of material used in medicinal products vary, but mostly are derived from cattle. There is thus at least a possibility that unless strict precautions are taken, disease could be transmitted in this way. It cannot be ruled out that no case ever arose by this means, but it is clear that the majority did not, even at the very beginning of the BSE epidemic before publication of information on BSE, and before any legislation was in place (Wilesmith *et al.,* 1988). The highest risk tissue is bovine brain from a clinically affected animal or one in the immediate pre-clinical phase. Posterior pituitary extract (now prepared biosynthetically), was available and used in veterinary practice mainly in adult female cattle at the time of parturition, to assist treatment of retained placenta or to assist in milk let down. However, no association was found between its use and the occurrence of BSE (Wilesmith *et al.,* 1988).

III.3.3. SURGERY (INCLUDING USE OF CATGUT AND TRANSMISSION BY INSTRUMENTS)

The transmission of CJD in humans by surgical instruments has only very rarely been reported and then only following cross contamination from central nervous tissue. There is currently some concern in human surgery about transmission from surgical instruments as a result of the emergence of vCJD (Frosch *et al*, 2001) that has a much wider distribution of infectivity in peripheral organs than occurs in sporadic CJD. Steps are in hand to reduce any risks from this source in the UK such as introduction of single use instruments for tonsillectomies.

In regard to surgery in cattle, the risks are different. The distribution of BSE infectivity in cattle is very restricted. Neuro-surgery is infrequently carried out. Thus, any risks for instrument contamination with the BSE agent would be very small. Abdominal surgery is however carried out frequently and if the same instruments were used also in sheep that might be infected with the scrapie agent without adequate cleaning and disinfection, there could be a theoretical risk for transmission form this source. Again, epidemiological studies would be likely to reveal the source of infection.

An additional risk could arise from the use of catgut that traditionally has been manufactured from cleaned and polished cattle small intestine. This may present a risk if the intestine came from a BSE infected animal and the processing was not effective. As a result of the fore-mentioned action of the CPMP, CVMP and VPC and the SC MPMD alternative sourcing of cattle gut away from countries at risk from BSE should ensure that risks are historical. This presumes that old stocks of catgut that might theoretically have been at risk are destroyed. Should any such iatrogenic cases arise, epidemiological studies would be likely to reveal the source of infection.

Blood transfusion

Blood transfusion in cattle is rarely undertaken. Usually, it is performed by taking several litres of blood from a herdmate donor. Indications are severe haemorrhage and following acute babesiosis (redwater fever). However blood and components of blood from cattle with BSE show no evidence of infection following bioassay either in mice (MAFF, 2000a) or with buffy coat alone from cattle experimentally challenged with BSE and collected at 32 months post-challenge followed by bioassay by the i/c route in cattle > 4 years after

inoculation (G.A.H. Wells and S.A.C.Hawkins, personal communication). Any risks from blood transfusion are therefore most improbable but if they did rarely occur, epidemiological investigation should reveal them.

Transmission of prion disease by transfusion of 400ml of blood to one sheep of 19 from a sheep experimentally infected orally with BSE, has been reported by Houston *et al,* (2000). However there has been significant criticism (*e.g.* Brown, 2000) over the reporting of this preliminary result because at the time of writing no other sheep had succumbed and it was not proven that BSE had been transmitted or what the origin of the infection was (SSC 2000). The findings cannot be directly translated to risks of transmitting BSE to cattle *via* blood.

III.4. GENETIC TRANSMISSION

Genetic mutation (familial or sporadic)

This has been discussed above in Section III.3. There is no evidence for either in cattle, but neither can these methods of occurrence be completely eliminated. They could theoretically occur anywhere at any time and are likely overall to be of minor importance provided there is no way of recycling infectivity to cattle or other species. Germ-line mutation, if it occurred, could be transmitted paternally and maternally. However, the data that we have does not incriminate bulls used for artificial insemination (AI) in the transmission of BSE (Bradley and Wilesmith, 1993). If later it were discovered that rare instances of familial disease occurred it would be potentially possible to control such events by rigorous testing of bulls used for service (particularly bulls used for AI, since they produce a disproportionately high number of offspring). This implies of course that the gene responsible is identified and its mode of inheritance is known. No protection can be provided in advance of the first occurrence for either sporadic or familial cases. Sporadic BSE akin to sporadic CJD in man, if it occurred, is likely to be a purely chance phenomenon and attributable perhaps to a somatic mutation that would be difficult to prove.

III.5. COLLATERAL FACTORS (FACTORS THAT MIGHT INCREASE SUSCEPTIBILITY)

a) The role of copper and manganese

Purdey (2000), has reported that analyses of food chains supporting isolated clusters of sporadic TSE (CWD in Colorado, scrapie in Iceland, CJD in Slovakia) demonstrate a consistent 2.5 times increase of the pro-oxidant divalent cation manganese in relation to normal levels recorded in adjoining TSE-free localities. Deficiencies of the anti-oxidant co-factors Cu, Se, Zn, Fe, Mg, P and Na were also consistently recorded in TSE food chains. Purdey goes on to suggest that sporadic TSE results from early life dependence of TSE-susceptible genotypes on ecosystems characterised by a specific pattern of mineral imbalance. Although there is a relationship between Cu^{2+} and PrP because PrP is a copper binding protein (Brown, 1999, Brown *et al.,* 1997) and Mn^{2+} competes with copper (because it binds to PrP but with a lower affinity than for copper and may change its conformation and create partial protease resistance, Brown *et al.,* 2000) some of the complex biochemical

processes that Purdey describes have a degree of validity. Lack of copper such as that that might be induced by competition with dietary excess of manganese could result in lack of protection of the CNS from oxidative damage, such as might be provided by the copper-containing enzyme copper/zinc superoxide dismutase. Brown *et al.,* (1999) claim that normal PrP (PrP^C) has an activity like superoxide dismutase. Furthermore Wadsworth *et al.,* (1999) have demonstrated that two distinct sub-types of PrP from humans with CJD can be inter-converted *in vitro* by altering their metal ion (Cu^{2+} / Zn^{2+}) occupancy thus providing a basis for the generation of different molecular strains of agent. The role of copper and competing ions in the diet like manganese in TSE is still being investigated and developed. However, it is incorrect to say there are clusters of sporadic CJD in Slovakia. This only applies to familial CJD related to the codon 200 mutation. There are also no localised clusters of CWD in Colorado or scrapie in Iceland that cannot be explained by the occurrence of conventional infectious TSE agents. It cannot be totally excluded yet that manganese may be involved in competitive depletion of copper in certain important metabolic pathways including in the brain and may thus increase the susceptibility of individuals to prion disease.

By contrast Waggoner *et al.,* (2000) dispute some of the current views on the role of copper in prion diseases. Using transgenic mice expressing different levels of PrP^C they studied the levels of brain copper and the properties of two brain cupro-enzymes and found that the brain copper levels and enzymic activities of Cu-Zn superoxide dismutase and cytochrome C were no different in the different mice strains. Notwithstanding these seemingly contradictory results from various sources, it seems possible that variations in the mineral content of the diet may influence the susceptibility of animals to prion disease.

b) Exposure to organo-phosphorus (OP) compounds

The use of OP compounds, particularly those used to treat warble fly has been hypothesised by Purdey (1991, 1994) to be a cause of BSE, or to make cows susceptible to the disease, or cause 'mutation' of PrP^C in *in utero* calf brains if the warble treatment is done at a certain critical stage of early gestation. The hypothesis is incompatible with BSE epidemiology. It does not explain why BSE in Japan has not been found until 2001 and then in only so far in one native-born animal. OP compounds have historically been widely used in Japan and humans have had wide exposure to them. The hypothesis also does not explain the absence of scrapie in sheep treated by OP compounds for fly strike and sheep scab in many countries and the occurrence of BSE in Guernsey where no warble-fly existed and no OP treatments were given. In microbiology, the basic law is to ensure that prospective 'agents' are tested to see they follow Koch's hypotheses. Anyone seeking a microbial cause of a disease ignores this at his peril (McManus, 1996). The pathology of OP toxicity is quite unlike that of TSE. The possibility of occasional cases being mistaken for BSE through mimicry, particularly at the clinical level, cannot however, be entirely excluded.

c) Green cluster nutrients, antioxidants and BSE

This hypothesis suggests that a lack of green cluster nutrients and in particular of α-linolenic acid (the omega 3 precursor for docosahexaenoic acid needed for brain growth) and linoleic acid could create increased susceptibility to BSE (Crawford *et al.,* 1991). The study is based on comparative biochemical studies in wild ruminants and the same species held captive in zoos. It is also based upon the changed feeding practices in farmed ruminant species namely the feeding of animal rather than vegetable protein. However, there is no evidence to show that cattle with BSE are deficient in these acids. There is abundant evidence from numerous sources however, to show that free radicals damage cell membranes leading to disturbances in calcium metabolism and mitochondrial, calcium overload, a final common pathway leading to cell death (Wrogemann and Pena, 1976). Though originally applied mostly to muscle diseases and in the context of acute cell necrosis (rather than death by apoptosis as is believed to occur in TSE), there is a growing literature on the role of oxidative stress in prion diseases.

For example Guentchev *et al.,* (2000) found widespread immunolabelling of neurones with nitrotyrosine, a marker of oxidative stress, in scrapie-affected mouse brains. Brown *et al.,* (1999), showed that acquisition of copper by PrP^C during re-folding endowed superoxide dismutase activity on the protein and interpreted their experimental results to suggest that PrP^C had an enzymic function to protect cells from oxidative stress. Wong *et al.,* (2000) go further and propose that imbalances of metal-catalysed reactions result in an alteration in antioxidant function. These result in an increased level of oxidative stress and trigger the neurodegenerative cascade. Collectively all these studies suggest that there is a role for free radicals in the causation of TSE and that antioxidants present in green-cluster nutrients (such as vitamin E), trace minerals like selenium (as a constituent of glutathione peroxidase), copper and copper antagonists contribute to protect from or accelerate the disease process. Interpreted simply, deficiency of the various protective mechanisms may render individuals or groups of animals more susceptible to prion disease.

d) Inadequate exposure to prostaglandins

Gjorgov *et al.,* (1999), published a paper showing the efficacy of prostaglandin treatment, or permitting natural sexual contact and insemination of tubal-ligated female rats, in preventing the development of malignant mammary tumours. The science behind this approach was that prostaglandins, administered by injection or naturally introduced with semen, reduced the risk of tumours developing. Applying the same principle to cattle and humans in respect of CJD and BSE respectively, Gjorgov (1996), proposed that the enforced lack of mating due to health disorders and heavy manipulation with steroid hormones (and possibly the reduced access to prostaglandins in semen), increased the risk of disease. However, this fails to explain the approximately equal occurrence of CJD in humans by sex and a similar proportional occurrence of BSE in bulls and cows. Clearly in Western countries, there are far fewer bulls than cows due to the use of artificial insemination (AI). Furthermore, there are no data to support an increased occurrence of BSE in barren cows. Efficient farming depends upon a cow producing approximately one calf a year whether AI or natural mating is used.

Matings by AI have been the norm for the majority of dairy cattle in Western Europe and many other countries for many years before BSE was recognised. Also, even under natural conditions, assuming fertile animals, only about one to three natural matings per season might be expected, as mating in cattle depends on the occurrence of oestrus outside the period of gestation (283 days).

III.6. OTHER HYPOTHESES UNSUPPORTED BY PUBLISHED ARTICLES OR 'ONE OFF' ARTICLES

A number of other hypotheses for a possible 'Third Way' of transmission of BSE have been described in letters, singleton publications in the journal 'Hypotheses' or other forms of communication. It is not proposed to discuss these in detail because they are either obscure, vague, insufficiently detailed or can be dismissed as serious contenders for consideration. They include for example, excessive use of high nitrogen fertilisers, the use of aluminium in equipment used for feeding, application of paper sludge on land grazed by cattle, the effects of either too little of the right kind of protein in the diet and the consequences of that on a particular part of the nervous system, or an insufficiency of protein plus an insufficiency of minerals specifically of calcium, sulphur, water, and potassium and/or chromium, in this order, and the results of this on specific tissues of the body.

IV. CONCLUSIONS

There are a large number of hypotheses for 'Third Ways'. However, there is very clear and strong support from epidemiological studies, rendering studies and the effect of feed bans in all countries with BSE, for the infected mammalian protein (meat-and-bone-meal) hypothesis to prevail above all others. Mammalian protein in the form of MBM is the major vehicle for BSE transmission in cattle. It can enter the feed deliberately (before the practice was made illegal) or accidentally by cross-contamination. The latter route could be the major 'Third way'.

Maternal transmission is theoretically a possible route of transmission since it would appear to occur in natural scrapie in sheep. In sheep a plausible mechanism has been identified. That is to say from the placenta of infected sheep. However, comparable investigations in cattle have led to different results (no experimental transmission from relevant tissues) and thus different conclusions *i.e.* that if maternal transmission occurs, either the placenta (and other reproductive tissues and milk) are not involved, or if they are, infectivity is infrequent.

Based on the results of a cohort study, there is some support for the possibility of some form of maternal transmission. Even so, maternal transmission cannot account for more than 10% of all cases of BSE and about 50% of them may be attributable to a genetic cause, the molecular basis for which has not been identified. However, there is no evidence so far that this so called 'maternal transmission' occurs in the absence of a feed borne source. No plausible mechanism for the so-called maternal transmission has been identified. If in spite of these conclusions there is some rare form of maternal transmission by a biological mechanism (infected, placenta, milk or colostrum for example),

this opens the door to a potential for horizontal transmission either directly or indirectly. Such a means of spread is unproven and appears not to be of any great significance if it occurs at all. Maternal transmission could not sustain the BSE epidemic in the UK. In all Member States of the EU the offspring of BSE cases are now traced, compulsorily slaughtered and destroyed completely. This is a two-edged sword (une arme à deux trenchants) as, although residual risks from hypothetical maternal transmission will be virtually eliminated, the availability of field evidence for the occurrence of maternal transmission will be reduced. Nevertheless, it is not currently possible to eliminate maternal transmission completely as an occasional cause of BSE. Any other cause than from feed or maternal transmission becomes a potential 'Third Way'. There could be concealed 'Third Ways' and/or real 'Third Ways'.

In regard to concealed 'Third Ways' there are basically two kinds. The first is the actual occurrence of cross-contamination of ruminant diets with infected mammalian protein (especially MBM) even though it is not suspected. Cross-contaminations can occur readily during feed preparation in feed mills, during transportation or on farm unless stringent measures are taken to avoid it. Usually these would have been accidental. It is possible that the accidental 'cross-contamination' route of exposure could account for the bulk of, if not all, 'Third Way' cases.

The second way is by the incorporation of infected ruminant or mammalian derived materials in feed other than MBM. Such materials might have been gelatin, fat or blood (or protein products derived from them) in which the starting materials were contaminated. To be infected they would have had to be derived from or contaminated with infected SRM such as CNS (that could be attached to certain bones) and intestine that might have contaminated fat. Effectively enforced SRM bans and improved and authorised ruminant stunning and processing methods (including for rendering, gelatin and fat manufacture), now should eliminate such causes. Use of rapid PrP tests and selection only of young animals for slaughter for human consumption have contributed to consumer confidence. Thus if all routes of transmission from BSE infected tissues are cut off the only remaining sources of infection could be genuine 'Third Ways'.

Possible 'Third Ways' have been discussed above. Many are theoretically possible but none can explain the majority of BSE cases. Some, (*e.g.,* mineral and vitamin imbalances) though unproven, may increase susceptibility to the disease.

It is well to remember that even the meat and bone meal hypothesis is a hypothesis and no-one has reported an experiment to test it using the BSE agent. However, in contrast to the other hypotheses there is so much epidemiological and other information to support it, that it standing is supreme. None of the other hypotheses explain the epidemiological features of the disease. They are rather theories or hypotheses that are in most cases not backed up by data or experimentation.

It cannot be stressed enough that safe sources of starting materials must be used for any purpose connected with the raising of cattle. Based on current

knowledge, current measures in place or proposed, if enforced, provide assurance that the recycling of BSE in cattle is, in future, an unlikely event.

V. REFERENCES

AGRIMI, U., RU, G., CARDONE, F., POCCHIARI, M. & CARAMELLI, M., 1999. Epidemic of transmissible encephalopathy in sheep and goats in Italy. Lancet, 353, 560-561.

APPEL, T.R., WOLFF, M., von RHEINBABEN, F., HEINZEL, M. & RIESNER, D., 2001. Heat stability of prion rods and recombinant prion protein in water, lipid and lipid-water mixtures. Journal of General Virology, **82,** 465-473.

APPEL, T.R., GROSHCUP, M.H. & RIESNER, D., 1999. Amino acids from gelatin. Poster at Tűbingen, TSE meeting.

ARYA, S., 1994. Transmissible spongiform encephalopathies and sheep-brain rabies vaccines. Biologicals, 22, 73.

BARR, B.C., ANDERSON, M.L., DUBEY, J.P. &CONRAD, P.A., 1991, *Neospora*-like protozoal infections associated with bovine abortions. Veterinary Pathology, **28,** 110-116.

BIRD, S., 2001. BSE transmission routes: vertical or other parenteral or oral (excluding MBM). Unpublished article and correspondence submitted to the Commission September 2001.

BRADLEY, R., 1996. Experimental transmission of bovine spongiform encephalopathy. In: Transmissible sub-acute spongiform encephalopathies, L.Court, B Dodet, Eds., Elsevier, Paris. Pp 51-56.

BRADLEY, R., & WILESMITH, J.W., 1993. Epidemiology and control of bovine spongiform encephalopathy (BSE). British Medical Bulletin, **49**, 932-959.

BRAUN, U., AMREIN, E., ESTERMANN, U., EGLI, J., SCHWEIZER, T., LUTZ, H., EHRENSPERGER, F., VANDEVELDE, M. & KIHM, U., 1998. Untersuchungen an 182 Nachkommen von an boviner spongiformer Enzephalopathie (BSE) erkrankten K[illegible]hen in der Schweiz. Teil 1: Klinische Befunde. Schweiz. Arch. Tierheilk., **140,** 240-249.

BROTHERSTON, J.G., RENWICK C.C., STAMP J. T., ZLOTNIK I., PATTISON I. H. 1968. Spread of scrapie by contact to goats and sheep, Journal of Comparative Pathology, **78**, 9-17.

BROWN, D.R., 1999. Prion protein expression aids cellular uptake and veratridine induced release of copper. J. Neurosci. Res., **58,** 717-725.

BROWN, D.R., QIN, K., HERMS, J.W., MADLUNG, A., MANSON, J., STROME, R., FRASER, P.E., KRUCK,T., von BOHLENS, A., SCHULZ-SCHAEFFER, W., GIESE, A., WESTAWAY, D. & KRETZSCHMAR, H., 1997. The cellular prion protein blinds copper *in vivo*. Nature, **390,** 684-687.

BROWN, D.R., HAFIZ, S., GLASSMITH, L.L., WONG, B.S., JONES, I.M. CLIVE, C. & HASWELL, S.J., 2000. Consequences of manganese replacement of copper for protein function and proteinase resistance. EMBO Journal, **19,** 1180-1186.

BROWN, D.R., WONG, B.S., HAFIZ, F., CLIVE, C., JONES, I.M. HASWELL,S.J. & JONES, M., 1999. Normal prion protein has an activity like that of superoxide dismutase. Biochemical Journal, **344,** 1-5.

BROWN, P., 2000. BSE and transmission through blood. Lancet, **356,** 955-956.

BROWN P. & GAJDUSEK, D.C.1991. Survival of scrapie virus after 3 years' interment. Lancet, **337,** 269-170.

CAUGHEY, B., 2000. Transmissible spongiform encephalopathies, amyloidoses and yeast prions: common threads? Nature Medicine, **6,** 751-754.

CAPUCCHIO, M.T., GUARDA, F., ISAIA, M.C., CARACAPPÀ, S. & Di MARCO, V., 1998. Natural occurrence of scrapie in goats in Italy. Veterinary Record, **143,** 452-453.

CARAMELLI, M., RU, G., CASALONE, E., BOZZETTA,E., ACUTIS, P.L., CALELLA, A & FORLONI, G., 2001. Evidence for the transmission of scrapie to sheep and goats from a vaccine against *Mycoplasma agalactiae.* Veterinary Record, **148,** 531-536.

COLLEE, J.G. & BRADLEY, R., 1997a. BSE a decade on – part 1. Lancet, **349,** 636-641.

COLLEE, J.G. & BRADLEY, R., 1997b. BSE a decade on – part 2. Lancet, **349,** 715-721.

COOKE, B., 1998. A brief history o n the use of meat products, especially meat and bone meal in the feeds for ruminant animals. The BSE Inquiry, Statement No 27. London.

CRAWFORD, M.A., BUDOWSKI, P., DRURY, P., GHERBREMESKEL, K., HARBIGE, L., LEIGHFIELD, M. PHYLACTOS, A. &WILLIAMS, G., 1991. The nutritional contribution to bovine spongiform encephalopathy, Nutritional Health, **7,** 61-68.

CURNOW, R.N. & HAU, C.M., 1996. The incidence of bovine spongiform encephalopathy in the progeny of affected sires and dams. Vet. Rec., **138,** 407-408.

DICKINSON, A.G., STAMP, J.T. & RENWICK, C.C., 1974. Maternal and lateral transmission of scrapie in sheep. J. Comp. Pathol, **84**, 19-25.

DICKINSON, A.G., MACKAY, J.M.K. & ZLOTNIK, I., 1964. Transmission by contact of scrapie in mice. J. Comp. Path., **74,** 250-254.

DNV, 1997. Risks from BSE via environmental pathways for the Environment Agency. 4. Assessment of risk from BSE carcases from landfill. DNV Technica, London.

DONNELLY C.A. 1988. Maternal transmission of BSE: interpretation of the data on the offspring of BSE-affected pedigree suckler cows. Veterinary Record, **142**, 579-580.

DONNELLY C.A., FERGUSON N.M., GHANI A.C., WILESMITH J.W., ANDERSON R.M. 1997a. Analysis of dam-calf pairs of BSE cases: confirmation of a maternal risk enhancement. Proc. R. Soc.Lond. B., **264**, 1647-1656.

DONNELLY C.A., GHANI A.C., FERGUSON N.M., WILESMITH J.W., ANDERSON R.M. 1997b Analysis of the bovine spongiform encephalopathy maternal cohort study: evidence for direct maternal transmission. Appl. Statist., **46**, 321-344.

FATZER, R., EHRENSPERGER, F., HEIM, D., SCHMIDT, J., SCHMIDT, A., BRAUN, U. & VANDEVELDE, M., 1998. Untersuchungen an 182 Nachkommen von an boviner spongiformer Enzephalopathie (BSE) erkrankten K□hen in der Schweiz. Teil 2: Epidemiologische und pathologische Befunde. Schweiz. Arch. Tierheilk., **140,** 250-254.

FITZSIMMONS, W.M. & PATTISON, I.H., 1968. Unsuccessful attempts to transmit scrapie by nematode parasites. Res. Vet. Sci., 9, 281-283.

FROSCH, A., JOYCE, R.,&JOHNSON, A., 2001. Iatrogenic vCJD from surgical instruments. British Medical Journal, **322,** 1558-1589.

GALE, P. & STANFIELD, G., 2001. Toward a quantitative risk assessment for BSE in sewage sludge. Journal of Applied Microbiology, **91,** 563-569.

GJORGOV, A.N., 1996. Prevention and eradication of the bovine spongiform encephalopathy (BSE): a solicited response and proposal. Macedonian Veterinary Review, **25,** 97-101.

GJORGOV, A.N., JUNAID, T.A., BURNS, G.R. & TEMMIM, L., 1999. Efficacy of preventive prostaglandin treatment of malignant mammary lesions in rats. Journal of the Balkan Union of Oncology, **4,** 295-306.

GOLDMANN, W., HUNTER, N., MARTIN, T., DAWSON, M. & HOPE, J., 1991. Different forms of the bovine PrP gene have five or six copies of a short , G-C-rich element within the protein-coding exon. Journal of General Virology, **72,** 201-204.

GORDON, W.S., 1946. Louping ill, tick-borne fever, and scrapie. Vet Rec., **58,** 516-525.

GORDON, W.S., BROWNLEE, A. & WILSON, D.R., 1939. Studies in louping-ill, tick-borned fever and scrapie. third International Congress for Microbiology. Section III, Various Diseases of animals, p 362-363.

GRANT, H., 2001. Humans can live with BSE as long as they do not eat brains. British Medical Journal, **322,** 1545.

GREIG, J.R., 1940. Scrapie. Observations on the transmission of the disease by mediate contact. Veterinary Journal, **96,** 203-206.

GREIG, J.R., 1950. Scrapie in sheep. Journal of Comparative pathology, **60,** 263-266.

GROBET, L., VANDEVENNE, S., CHARLIER, C., PASTORET, P.P. & HANSET, R., 1994. Polymorphisme du gêne de la protéine prion chez des bovines belges. Ann. M□d. V□t., **138,** 581-586.

GRUNER, L., 2001. Environmental and host factors in scrapie transmission. Abstract in TSE Co-ordinator's Meeting, Brussels, 31 May – 1 June 2001. EC. Brussels.

GUENTCHEV, M., VOIGTLÄNDER, T., HARBELER, C., GROSCHUP, M.H. & BUDKA, H., 2000. Evidence for oxidative stress in experimental prion disease. Neurobiology of Diseases, **7,** 270-273.

HAU, C.M. & CURNOW, R.M., 1996. Separating the environmental and genetic factors that may be the causes of BSE. Philosophical Transactions of the Royal Society, London B., **351,** 913-920.

HADLOW W.J., 1991. To a better understanding of natural scrapie. In: Current topics in Vet. Med. and Anim. Sci., Sub-acute spongiform encephalopathies, Bradley R., Savey M., Marchant B., eds. Proceedings of an EC seminar, Brussels**,** 12-14 November 1990. Kluwer, Dordrecht. **Pp.** 117-130.

HADLOW W.J., KENNEDY R.C., RACE R.E., Natural infection of Suffolk sheep with scrapie virus. Journal of Infectious Diseases, 1982, **146**, 657-664.

HADLOW W.J., KENNEDY R.C., RACE R.E., EKLUND C.M., 1980. Virological and neurohistological findings in dairy goats affected with natural scrapie. Veterinary Pathology, **17**, 187-199.

HADLOW W.J., RACE R.E., KENNEDY R.C., EKLUND C.M., 1979. Natural infection of the sheep with scrapie virus. In : Slow Transmissible Diseases of the Nervous System. (S.B. Prusiner and W.J. Hadlow, Eds), **Vol.2,** pp 3-12, Academic Press, New York.

HOINVILLE L.J., 1996. A review of the epidemiology of scrapie in sheep. Rev sci tech Off int Epiz **15,** 827-852.

HOINVILLE, L.J., WILESMITH, J.W. & RICHARDS, M.S., 1995. An investigation of risk factors for cases of bovine spongiform encephalopathy born after the introduction of the 'feed ban'. Vet. Rec., **136,** 312-318.

HORN, G., 2001. Review of the origin of BSE. DEFRA, London. Pp. 66.

HOURRIGAN J., KLINGSPORN A., CLARK W.W. & de CAMP M. Epidemiology of scrapie in the United States. In: Slow Transmissible Diseases of the Nervous System **(Vol. I).** Editors: Prusiner, S.B. and Hadlow, W.J., Academic Press, New York, 1979, 331-356.

HOUSTON, F., FOSTER, J.D., CHONG, A., HUNTER, N. & BOSTOCK, C.J., 2000. Transmission of BSE by blood transfusion in sheep. Lancet, **356,** 999-1000.

HUNTER, N., GOLDMANN, W., SMITH, G. & HOPE, J., 1994. Frequencies of PrP gene variants in healthy cattle with BSE in Scotland. Vet. Rec., **135,** 400-403.

INGROSSO, L., PESANI, F. & POCCHIARI, M., 1999. Transmission of the 263K scrapie strain by the dental route. J. Gen. Virol, **80,** 3043-3047.

KIMBERLIN, R.H., 1994. A scientific evaluation of research into bovine spongiform encephalopathies. In: Transmissible Spongiform encephalopathies: A consultation with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels from 14-15 September 1993. R. Bradley and B. Marchant, Eds. EC, Brussels. Pp. 455-477.

KIMBERLIN, R.H. & WALKER, C.A., 1989. Pathogenesis of scrapie in mice after intragastric infection. Virus Research, **12,** 213-220.

LAPLANCHE, J.-L., ELSEN, J.-M., EYCHENNE, F., SCHELCHER, F., RICHARD, S., AMIGUES, Y. & LAUNAY, J.-M., 1996. Scrapie outbreak in sheep after oral exposure to infective gastrointestinal nematode larvae. L.Court, B Dodet, Eds., Elsevier, Paris. Pp**.** 41-46.

MAFF. 2000a. Bovine Spongiform Encephalopathy: a progress report. June 2000 MAFF, London.

MAFF. 2000b. Bovine Spongiform Encephalopathy: a progress report. December 2000 MAFF, London.

MASEL, J., **& JANSEN, V.A.A., 2001.** The measured level of prion infectivity varies in a predictable way according to the aggregation state of the infectious agent. Biochimica et biophysica Acta, **1535,** 164-173.

McKENZIE, D.I., COWAN, C.M., MARSH, D.F. & AIKEN, J.M., 1992. PrP gene variability in the US cattle population. Animal Biotechnol., **3,** 309-315.

McMANUS, K., 1996. The prion hypotheses. New Zealand Veterinary Journal, **44,** 119.

MIDDLETON D.J & BARLOW R.M. 1972, Failure to transmit bovine spongiform encephalopathy to mice by feeding them with extraneural tissues of affected cattle. Veterinary Record, 1993, **132,** 545-547.

MOON, G.J.H., 1978. Scrapie quarantine. New Zealand Veterinary Journal, 24 July 1978, 235.

ONODERA, T., IKEDA, T., MURAMATSU, Y. & SHINAGAWA, M., 1993. Isolation of scrapie agent from the placenta of sheep with natural scrapie in Japan. Microbiol. Immunol., **37,** 311-316.

PÁLSSON, P.A., 1979. Rida (scrapie) in Iceland and its epidemiology. In : Slow Transmissible Diseases of the Nervous System. (S.B. Prusiner and W.J. Hadlow, Eds), **Vol.1,** Academic Press, New York.Pp. 357-366.

PATTISON, I.H., 1964. The spread of scrapie by contact between affected and **healthy sheep**, goats or mice. Veterinary Record, **12**, 333-336.

PATTISON, I. H., HOARE M. N., JEBBETT J. N., WATSON W. A. 1974. Further observations on the production of scrapie on sheep by oral dosing with foetal membranes from scrapie affected sheep, British Veterinary journal, **130**, lxv-lxvii.

PATTISON, I.H., HOARE, M.N., JEBETT, J.N., WATSON, W.A., 1972, Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie affected sheep. Veterinary Record. **90**, 465-468.

PATTISON, I.H. & MILLSON, G.C., 1961. Experimental transmission of scrapie to goats and sheep by the oral route. Journal of Comparative Pathology, **71,** 171-176.

PEANO, S., REINER, G., CARBONATTO, M., BODENBENDER, L., BOLAND, P. & ABEL, K. -J., 2000. Determination of the clearance factor for TSE agents during the manufacturing process of Polygeline. Intensive Care Med, **26,** 608-612.

POST, K., RIESNER, D., WALLDORF, V. & MEHLHORN, H., 1999. Fly larvae and pupae as vectors for scrapie. Lancet, **354,** 1969-1970.

PRUSINER, S.B., COCHRAN, S.P. & ALPERS, M.P., 1985. Transmission of scrapie in hamsters. Journal of Infectious Diseases, **152,** 971-978.

PURDEY, M., 1991. Mad cows and warble flies: a link between BSE and organophosphates. The Ecologist, 22, 52-57.

PURDEY, M., 1994. Are organophosphate pesticides involved in the causation of bovine spongiform encephalopathy (BSE)? Hypothesis based on a literature review and limited trials on BSE cattle. Journal of Nutritional Medicine, 4, 43-82.

PURDEY, M., 2000. Ecosystems supporting clusters of sporadic TSEs demonstrate excesses of the radical-generating divalent cation manganese and deficiencies of antioxidant co factors Cu, Se, Fe, Zn. Medical Hypotheses, **54,** 278-306.

RACE, R., JENNY, A. & SUTTON, D., 1998. Scrapie infectivity and proteinase K- resistant prion protein in sheep placenta, brain, spleen, and lymph node: implications for transmission and antemortem diagnosis. J. Infect. Dis., **178,** 949-953.

REPORT, 2001. Occurrence and behaviour of BSE/TSE prions in soil. Fraunhofer-Institute for Environmental Chemistry and Ecotoxicology, Scmallenburg. Pp. 25.

RUBINSTEIN, R., KASCSAK, R.J., CARP, R.I., PAPINI, M., La FAUCI, G., SIGURARSON, S & WISNIEWSKI, H.M., 1998. Potential role of mites as a vector and/or reservoir for scrapie transmission. Alzheimer's Disease Review, **3,** 52-56.

SARGISON, N.D., SCHOCK, A., MACLEAN, I.A., RAE, A., HECKEROTH, A.R. & CLARK, A.M., 2000. Unusual outbreak of sporozan encephalomyelitis in Bluefaced Leicester ram lambs. Vet. Rec., **146,** 225-226.

SCHREUDER, B.E., GEERTSMA, R.E., van KEULEN, L.J.M., van ASTEN, J.A.A.M., ENTHOVEN, P., OBERTHÜR, R.C., de KOEIJER, A.A. & OSTERHAUS, A.D.M.E., 1998. Studies on the efficacy of hyperbolic rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents. Vet. Rec., **142,** 474-480.

SCOTT, J.R., FOSTER, J.D. & FRASER, H., 1993. Conjunctival instillation of scrapie in mice can produce disease. Vet. Microbiol., **34,** 305-309.

SEAC 2001. Note of SEAC working group on disposal of carcasses of cattle culled for foot and mouth disease (FMD) control, 24/5/01. DEFRA/DoH London.

SHAKED, G.M., SHAKED, Y., KARIV Z., HALIMI, a, M., AVRAHAM, I. & GABIZON, R., 2001. A protease resistant PrP isoform is present in urine of animals and humans affected with prion diseases. Journal of Biochemistry, Journal of Biological Chemistry, July 2001.

SIGURDARSON S., 1991. Epidemiology of scrapie in Iceland and experience with control measures. In: Current topics in Vet. Med. and Anim. Sci., Sub-acute spongiform encephalopathies, Bradley R., Savey M., Marchant B., eds. Proceedings of an EC seminar, Brussels**,** 12-14 November 1990. Kluwer, Dordrecht. **55,** 233-242.

SIGURDARSON S., 2000. Scrapie eller rida I Island. Norsk Veterinaertidsskrift, 112, 408-413.

SSC, 1998. The safety of dicalcium phosphate precipitated from ruminant bones and used as a feed additive. Opinion of the Scientific Steering Committee, adopted 25-26 June 1998. EC Brussels.

SSC, 2000. Opinion on the implications of the Houston *et al,* paper in the Lancet of 16 September 2000 on the transmission of BSE by blood transfusion in sheep. EC, Brussels.

SSC, 2001a. http://europa.eu.int/comm/food/fs/sc/ssc/ en.htm

SSC, 2001b.. The safety of gelatin. Opinion of the Scientific Steering Committee, adopted 28-29 June 2001. EC Brussels.

SSC, 2001c. Adipose tissue associated with the digestive tract of cattle, sheep and goats : an appreciationof possible TSE risks. Opinion of the Scientific Steering Committee, adopted 28-29 June 2001. EC Brussels.

SSC, 2001d. The safety of tallow obtained from ruminant slaughter by-products. Opinion of the Scientificv Steering Committee, adopted 28-29 June 2001. EC Brussels.

SSC, 2001e. Preliminary Scientific Opinion and Report on Stunning Methods and BSE risks. adopted 6-7 September 2001. EC Brussels.

STATUTORY INSTRUMENT, 1950, No 1988. Supplies and services, Food (Feeding Stuffs) GB. The Feeding Stuffs (Manufacture) Order, 11 December 1950. HMSO, London.

TAYLOR, D.M., 1996. Exposure to, inactivation of, the unconventional agents that cause transmissible degenerative encephalopathies. In: Methods in molecular medicine: Prion diseases. Eds., H Baker and R.M. Ridley. Humana Press Inc. Totowa, New Jersey. Pp 105-118.

TAYLOR, D.M., FERNIE, K., STEELE, P.J. & SOMERVILLE, R.A., 2001. Relative efficiency of transmitting transmissible spongiform encephalopathy to RIII mice by the oral route. Veterinary Record, **148,** 345-346.

TAYLOR, D.M., FERGUSON, C.E., BOSTOCK, C.J., & DAWSON M., 1995. Absence disease in mice receiving milk from cows with bovine spongiform encephalopathy. Veterinary Record, **136**, 592.

TAYLOR, D.M., McCONNELL, I. & FRASER, H., 1996. Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. J. Gen. Virol., **77,** 1595-1599.

TAYLOR, D.M., WOODGATE, S.L. & ATKINSON, M.J., 1995. Inactivation of the bovine spongiform encephalopathy agent by rendering procedures. Veterinary Record, **137,** 605-610.

TAYLOR, D.M., WOODGATE, S.L., FLEETWOOD, A.J. & CAWTHORNE, R.J.G., 1997. Effect of rendering procedures on the scrapie agent. Veterinary Record, **141,** 643-649.

TUO, W., ZHUANG, D., KNOWLES, D.P., CHEEVERS, W.P., SY, M-S, & O'ROURKE, K.I., 2001. PrP^{C} and PrP^{Sc} at the fetal-maternal interface. Journal of Biological Chemistry, **276,** 18229-18234.

WADSWORTH, J.D.F., HILL, A.F., JOINER, S., JACKSON, G.S., CLARKE, A.R. & COLLINGE, J., 1999. Strain-specific prion-protein confirmation determined by metal ions. Nature Cell Biol., **1,** 55-59.

WAGGONER, D.J., DRISALDI,B., BARTNIKAS, T.B., CASARENO, R.L.B., PROHASKA,J.R., GITLIN, J.D. & HARRIS, D.A., 2000. Brain copper content and cuproenzyme activity do not vary with prion expression level. Journal of Biological Chemistry, **275,** 7455-7458.

WELLS, G.A.H., HAWKINS, S.A.C., GREEN, R, B, SPENCER, Y.I., DEXTER, I & DAWSON, M., 1999. Limited detection of sternal bone marrow infectivity in the clinical phase of experimental BSE. Veterinary Record, **144,** 292-294.

WHO, 1997. Report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies with the participation of the Office International des Epizooties (OIE). Geneva, Switzerland, 24-26 March 1997. WHO, Geneva. Pp. 17.

WILESMITH, J.W., 1996. Confidential Report CH25/IX: BSE, Follow up investigation of female offspring of homebred cases of BSE in pedigree beef suckler herds. VI/7521/96, European Commission, Brussels.

WILESMITH, J.W. & RYAN, J.B.M., 1997. Absence of BSE in the offspring of pedigree suckler cows affected by BSE in Great Britain. Vet. Rec., **141,** 250-251.

WILESMITH, J.W., RYAN, J.B.M. & ATKINSON, M.J., 1991. Bovine spongiform encephalopathy: epidemiological studies of the origin. Veterinary Record, **128,** 199-203.

WILESMITH, J.W., WELLS, G.A.H., CRANWELL, M.P. & RYAN, J.B.M., 1988. Bovine spongiform encephalopathy: epidemiological studies. Veterinary Record, **123,** 638-644.

WILESMITH J.W., WELLS G.A.H., RYAN J.B.M., GAVIER-WIDEN D., & SIMMONS M., 1997. A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. Veterinary Record, 141, 239-243.

WILSON, D.R., ANDERSON, R.D. & SMITH, W., 1950. Studies in scrapie. Journal of Comparative, Pathology, **60,** 267-282.

WONG, B-S., PAN, T., LIE, T., LI, R., PETERSEN, P.B., JONES, I.M., GAMBETTI, P., BROWN, D.R. & SY, M-S, 2000. Prion disease: a loss of antioxidant function? Biochemical and biophysical Research Communications, **275,** 249-252.

WROGEMANN, K., & PENA, S.D.J., 1976. Mitochondrial calcium overload: a general mechanism for cell-necrosis in muscle diseases. Lancet **I,** 672.

ZANUSSO, G., NARDELLI, E., ROSATI, A., FABRIZI, G., FERRARI, S., CARTERI, A., de SIMONE, F., RIZZUTO, N. & MONACO, S., 1998. Simultaneous occurrence of spongiform encephalopathy in a man and his cat in Italy. Lancet, **352,** 1116-1117.