Papers and Articles

Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents

B. E. C. Schreuder, R. E. Geertsma, L. J. M. van Keulen, J. A. A. M. van Asten, P. Enthoven, R. C. Oberthür, A. A. de Koeijer, A. D. M. E. Osterhaus

The efficacy of the procedures in use at the two rendering plants in the Netherlands was assessed on a laboratory-scale using procedures that simulated the pressure cooking part of the rendering process. A pool of bovine spongiform encephalopathy (BSE)-infected brainstem from the United Kingdom and a pool of scrapie-infected brainstem from Dutch sheep were used to spike the rendering materials. The mixtures were subjected to various time-temperature combinations of hyperbaric heat treatment related to the conditions used in Dutch rendering plants in the early 1990s, and to the combination of 20 minutes at 133°C required by the EU Directive on rendering of 1996. The efficacy of the procedures in inactivating BSE or scrapie infectivity was measured by titrating the materials before and after heat treatment in inbred mice, by combined intracerebral and intraperitoneal inoculations at limiting dilutions. Two independent series of experiments were carried out. The design of the study allowed for minimum inactivations of up to 2·2 log (2·0 in the second series) to be measured in the diluted infective material and 3·1 log in the undiluted material. After 20 minutes at 133°C there was a reduction of BSE infectivity of about 2·2 log in the first series (with some residual infectivity detected), and in the second series more than 2·0 log (with no residual infectivity detected). With undiluted brain material there was an inactivation of about 3·0 log (with some residual infectivity detected). With the same procedure, scrapie infectivity was reduced by more than 1·7 log in the first series and by more than 2·2 log in the second series. With undiluted brain material there was an inactivation of more than 3·1 log. In each case no residual scrapie infectivity was detected. The BSE agent consistently appeared to be more resistant to heat inactivation procedures than the scrapie agent, particularly at lower temperatures and shorter times.

BOVINE spongiform encephalopathy (BSE) has become a major veterinary problem in cattle over the last decade. Not only animal health is at stake, there are also implications for public health. The recognition of the BSE agent as a possible cause of a new variant of Creutzfeldt-Jakob disease (vCJD) in human beings (Will and others 1996) had placed BSE in a spotlight which was accentuated by confirmatory data indicating that there is a link between this form of CJD and BSE (Bruce and others 1997, Hill and others 1997). The resulting public concern about the zoonotic potential of the disease reaches far beyond the borders of the UK and has determined the need for the assurance of a 'safe food chain'.

Early in the BSE epizootic, it was concluded on the basis of epidemiological data that cows had contracted the disease through eating meat and bone meal infected with a transmissible spongiform encephalopathy agent, possibly originating from scrapie-infected sheep (Wilesmith and others 1988, 1991). In the UK in the late 1970s and early 1980s rendering conditions had been changed to conditions which apparently inactivated the BSE agent insufficiently. It is now generally accepted that the recycling of BSE-infected material in cattle was the key factor in driving the epizootic. The risks associated with the import of products possibly contaminated with BSE or of BSE-infected animals, and the possibility that BSE might emerge as a result of the recycling of indigenous scrapie infectivity, prompted the evaluation of rendering procedures in the Netherlands.

The difficulty of inactivating the agents causing transmissible spongiform encephalopathies (TSEs) or prion diseases, had been established before BSE emerged by thermal inactivation studies with the agents causing scrapie or CJD (Kimberlin and others 1983, Taylor 1991). For the inactivation of most strains of scrapie moist heating at hyperbaric pressures of 20 psi (≈2·4 bar) at 126°C seemed to be effective. For the most thermoresistant scrapie strain, 22A, four minutes exposure at 30 psi (≈3·1 bar) at 136°C proved to be effective, when porous load autoclaving procedures were used. However, the results of later studies proved to be less reassuring (Taylor and others 1994).

The resistance of TSE agents to dry heat was even more notorious, especially after reports that scrapie agent even resisted ashing, at least in part (Brown and others 1990). So far, no experiment had mimicked actual rendering conditions because almost all experiments used brain macerates heated in autoclaves. The first rendering experiments with the BSE agent were reported by Taylor and others (1995), and they were followed by similar rendering studies with scrapie-infected materials (Taylor and others 1997). In these

B. E. C. Schreuder, DVM, L. J. M. van Keulen, DVM, A. A. de Koeijer, MSc, DLO-Institute for Animal Science and Health (ID-DLO), PO Box 65, 8200 AB, Lelystad, The Netherlands
R. E. Geertsma, MSc, J. A. A. M. van Asten, Ing, BSc, A. D. M. E. Osterhaus, DVM, PhD, National Institute of Public Health and Environment (RIVM), Bilthoven, The Netherlands
P. Enthoven, MSc, Cooperative Central Laboratory, Veghel, The Netherlands
R. C. Oberthür, PhD, Fleischmehlfabrik Brögbern, Lingen-Brögbern, Germany
J. A. A. M. van Asten's present address is PO Box 2644, 3000 GD Amersfoort, The Netherlands
A. D. M. E. Osterhaus' present address is Erasmus University Rotterdam, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

experiments, a range of rendering processes in use in the European Union, including hyperbaric procedures, were evaluated in pilot-scale facsimiles.

The present studies have been confined to variations within the hyperbaric process, and were designed to assess the efficacy of the Dutch rendering processes in inactivating the causative agents of BSE and scrapie. Because heat treatment at high temperature under hyperbaric conditions ('pressure cooking') is considered the critical part of the inactivation process, only this part of the rendering process was evaluated. The possible effects of other components of the rendering process were not considered.

In the early 1990s, this pressure cooking was performed at one of the two Dutch rendering plants (plant A) as a batch process, and at the other plant (plant B) as a continuous process. The Dutch Rendering Directive, in force at the time of inception of the study (1990/91), specified minimum holding times of 10 and 15 minutes, for the continuous and batch processing plants, respectively, at temperatures above 130°C under hyperbaric conditions. These minimal requirements were in addition to specified longer holding times at lower temperatures. In validation studies, using chromium and cobalt as markers for the solid and liquid phase, respectively, the actual conditions in the continuous process were evaluated (results not reported here). Since 1996 both plants have used a batch processing system with a minimum holding time of 20 minutes at 133°C, thus complying with current EU directives.

This paper describes laboratory studies with rendering materials spiked with BSE or scrapie agent in different well defined, hyperbaric inactivation cycles, which were based on the procedures in use at the plants when the study began and also included the present EU-recommended procedure. The aims of the study were first to assess the efficacy of an essential part of the Dutch rendering process in eliminating BSE and scrapie agents from the animal food chain, and secondly, to determine the kinetics of the inactivation processes.

Materials and methods

Collection and preparation of the material used for spiking

One of the limitations of previous rendering experiments (Taylor and others 1995) was the low initial titre in the spiking material. Because brainstem material, especially in the case of BSE, would contain higher titres of infectivity than a mixture of whole brain (Hadlow and others 1979, Scott and others 1990, R. Bradley, personal communication), only brainstem material was used. The following materials were collected and used for spiking:

a) Brainstems from six histologically confirmed BSE-affected cattle, originating from five different farms (made available by kind permission of Dr Keith Meldrum, CVO, UK).

b) Brainstems from 60 histologically confirmed scrapie-affected sheep, collected from 38 different farms throughout the Netherlands. No more than five brainstems per farm were included. Materials from at least eight different breeds of sheep (including foreign breeds) or their crosses were involved.

Approximately half of each paramedically cut brainstem was available for these experiments. Both the BSE and scrapie brain materials were homogenised in a Sorvall Omni-mixer. The BSE material was homogenised without adding other material, the scrapie material was diluted to a 66·6 per cent suspension in saline, to facilitate mixing. The scrapie material was first mixed in batches of five samples each, after which a final pool was prepared from these initial batches. The brain materials were divided into appropriate aliquots and stored at -20°C until used.

Material tested

The infectious spiking materials were mixed with raw material for dilution. The raw material, mainly abattoir waste and predominantly of porcine origin, was obtained from one of the rendering plants as crushed and heat-treated (<95°C) material containing 70 per cent water, 14 per cent protein, 10 per cent fat and 6 per cent

ash; it was considered to have a typical composition for this stage in the process. This crushed material was further homogenised in a Sorvall Omni-mixer and finally mixed with the spike material to obtain a 10 per cent w/w mixture of spike material in partially rendered material. Thirty millilitre glass vials, 'universal bottles', with a screw-cap were then two-thirds filled with the spiked rendering material for exposure to various hyperbaric heat treatment cycles.

Equipment

The heat treatment cycles were carried out in an autoclave with internal monitoring of conditions. For this purpose a 25 litre steam steriliser was adapted to allow the temperature to be set adequately and the exposure time to be interrupted immediately after the required test period had elapsed. To control and monitor the exposure conditions, a pressure-temperature measuring system was used, because after all air has been removed, pressure is the most accurate way to control the temperature. This system complies with the requirements of EN 285 (EN 285 1997) in that it measures one pressure with an accuracy of 1 kPa and at least five temperatures with an accuracy better than 0·5°C. Before each test series the entire measuring system was calibrated against in-house standards.

Dummy vials were used to monitor the temperature; they were identical to the sample vials but contained clean, unspiked material, that is, rendering material to which 10 per cent negative brain material had been added. One of the temperature sensors was placed inside a dummy sample vial in the centre, at one third of the material level (from the bottom). This position had been demonstrated to be the most critical position within the material to be treated (J. van Asten, unpublished observations). During the heat treatment cycles, the dummy vial was placed adjacent to the sample vials. Owing to the relatively small volume of the vials and the saturated steam heat-up cycle, the temperature differences within each vial and between the vials were considered negligible.

Both the sample vials and the dummy vials containing the temperature sensors were hermetically sealed, and no additional steam was added or injected into the vials. With 70 per cent water, the moisture in the sample materials is considered sufficient to create an internal liquid- or steam-saturated micro-environment within the vials which mimics the large-scale rendering processes in the Netherlands, where heating takes place in closed, steam-jacketed vessels, without steam being injected into the vessels.

The temperature in the dummy vial was monitored throughout the cycle. The exposure time started when the recorded temperature reached the planned test temperature, and this temperature was maintained for the required period after which the steriliser was vented and the contents allowed to cool down. The sample vials were stored at -20°C pending bioassay.

Experimental procedures

All the experiments, including the preparation of the samples, were carried out in duplicate, in two independent series of experiments (series 1 and 2) and with both BSE and scrapie brainstems as spike material. The following temperature-time cycles were tested in separate test runs:

A) three minutes at 105°C (survival of infectivity expected);

B) three minutes at 121°C (survival of infectivity expected);

C) fifteen minutes at 125°C (theoretical worst case scenario in plant A in 1993*);

D) three minutes at 134°C (theoretical worst case scenario in plant B in 1993*);

E) twenty minutes at 133°C (EC directive 90/667, for high risk material);

P) twenty minutes at 133°C (EC directive 90/667), in series 2 with undiluted brainstem material only.

*These conditions were considered the theoretical worst case scenarios for any material at the start of the experiments (around 1993) for both plants in the Netherlands. Both plants now operate as batch processing plants and comply with current EU directives (Commission Decision 1996).

In order to maximise the initial titre, one set of tests was run with undiluted spike material at the temperature/time combination having what was presumed to be the highest efficiency (20 minutes at 133°C) (process P in series 2).

Bioassay in mice

For the bioassay, parent stock of three mouse strains RIII/FaDk-ro, C57BL and VM mice, was obtained from the Neuropathogenesis Unit (NPU), Edinburgh, of the Institute of Animal Health, by kind permission of Dr C. Bostock. The material used for spiking was titrated in 10-fold dilutions (six or seven steps) in groups of six to eight mice for each dilution: BSE brainstem material in RIII/FaDk-ro mice, and scrapie brainstem material in C57BL and VM mice. The heat-treated spiked material was also assayed undiluted and in 10- and 100-fold dilutions, in groups of 15 or 16 mice of the same strains. In addition, in the lowest time-temperature combination (three minutes at 105°C) one extra dilution step was included. Not all the tests with scrapie material were done in C57BL and in VM mice because of breeding difficulties with the VM strain and because of hypersensitivity to the antibiotic solution with the C57BL strain. It was therefore necessary to resort temporarily to RIII mice for the assay of scrapie material. Aliquots of the pooled brainstem material used for spiking were diluted in antibiotic solution containing 5000 iu penicillin and 5000 μg streptomycin/ml. Aliquots of the heat-treated material were also diluted, initially in the same solution, but after problems with the C57BL mice it was replaced by sterile saline. No bacteriological screening was done and no problems related to possible bacteriological contamination were encountered.

The mice were inoculated with 0·02 ml intracerebrally and with 0·5 ml intraperitoneally, essentially as described by Taylor and others (1995). Each inoculum was prepared with an Ultra-Turrax T25 homogeniser, and the homogeniser probes were decontaminated between usages by two cycles of porous-load autoclaving for 30 minutes at 136°C, and washed with detergent in between. The homogenates were centrifuged for five minutes at 500 g and the supernatants used for the bioassay.

The mice were observed and clinically monitored daily for a period of up to 900 days. The brains of all mice surviving the first ten days were examined histologically; they were immersion-fixed in 10 per cent formol-saline and histological sections were prepared from five different coronal sections; they were stained with haematoxylin and eosin and examined microscopically for the presence of spongiform lesions. The brains were also examined by immunohistochemistry, consisting of PrP^{Sc} staining with a peptide-based antibody, R505, directed against murine PrP^{Sc}. Positive results were based on both these techniques, and the presence of PrP^{Sc} was considered decisive.

Calculation of infectivity titre and reduction of titre

The results of the animal experiments can be expressed as the infectivity of the samples per ml (or gram) of inoculum, in this case the number of ID50 doses. This was calculated by the method of Kärber (1931) and by an alternative method which uses a generalised linear model (GLM). For the latter it was assumed that an inoculum contains a number of equal BSE or scrapie particles which all have an equal probability of inducing infection. The following dose-response model was therefore used:

$$\text{response} = 1 - e^{-\text{dose} \times \text{titre}}$$

Having thus estimated the average infective dose it was converted, to allow comparison with the Kärber method, to an ID50 titre.

For the titre calculations the number of positive mice was divided by the number of mice which survived until the first mouse in the dilution group was confirmed positive. If there were no positive mice in any group, the data from the next lower dilution were used. If the mortality in the undiluted group did not reach 100 per cent, but the mortality in the next lower dilution did reach 100 per cent, the result from the undiluted group was considered an artefact (probably the best result of difficulties with inoculating undiluted material), and a 100 per cent score was assumed for the undiluted group.

To calculate the reduction in titre caused by a particular process, the titre was calculated back to the initial 100 per cent concentration of the spike. Values calculated by the Kärber method and those calculated by the GLM used their own reference values. The extended incubation periods associated with heat-treated material and the subsequent level of intercurrent mortality resulted in small test groups, and limited the practical applicability of the Kärber method. The GLM method does not suffer from this drawback. However, in the conclusions and discussion, the results calculated by the Kärber method have been used so that they can be related to other research data.

The reduction of the titre of infectivity caused by an inactivation process is an indication of the efficacy of that process, which is determined by the temperature and the holding time. The inactivation mechanism in the rendering process is, just like that in steam sterilisation processes, the effect of moist heat on microorganisms and/or transmissible agents. For steam sterilisation in general the inactivation kinetics of microorganisms can be described as a first order reaction. For this an equation is applicable in which the decimation time D (the time needed for 1 log reduction at a certain temperature) and the resistance coefficient z (the effect of a temperature change on the decimation time) are unknown variables. If the same equation were valid for the inactivation kinetics of prions (which is unlikely given the information from other inactivation studies with TSE agents [Taylor and Fernie 1996]), it should be possible to determine these unknown variables from the results of a series of experiments in which several 'sub-lethal' inactivation processes have been applied.

Results

All the experiments were carried out in duplicate (series 1 and 2). The titres of infectivity in the pooled brainstem materials used for spiking are shown in Table 1.

TABLE 1: Titres of infectivity in the pooled brainstem material (spike)

Experiment	Brainstem material	Strain of mice	Titre (log ID50/g) Kärber method*	GLM (se)†
Series 1	Scrapie	VM	3·1	2·8 (0·3)
	Scrapie	C57BL	3·5	3·5 (0·3)
	BSE	RIII	3·8	3·7 (0·4)
Series 2	Scrapie	VM	2·4	2·4 (0·2)
	Scrapie	C57BL	3·4	3·4 (0·4)
	BSE	RIII	3·6	3·4 (0·2)

* Calculated by the method of Kärber (1931)
† Calculated using a generalised linear model (GLM) (se = standard error)
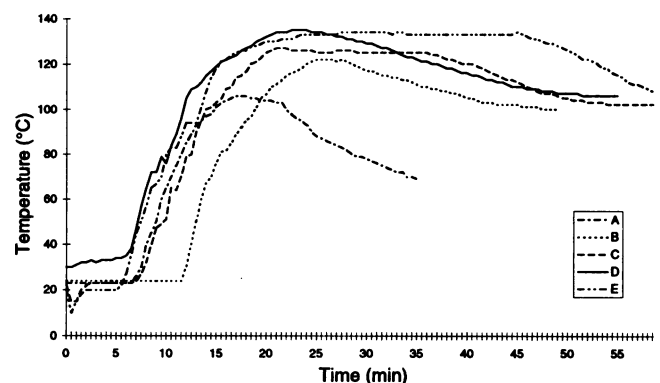For both methods, corrected for the initial dilution step (66·6%) in the case of scrapie

FIG 1: Temperature-time profiles of processes A, B, C, D and E

TABLE 2: Reduction of titres of infectivity in the experiments of series 1

Spiking agent	Code	Process (time/temp)	Strain of mice	Titre after heat treatment (log ID50/g)		Reduction factor* (log 10)	
				Kärber	GLM (se)	Kärber	GLM
Scrapie	A	3 min/105°C	RIII	3·9	3·9 (0·2)	–	–
	B	3 min/121°C	RIII	2·9	2·2 (0·2)	1·0	1·7
	C	15 min/125°C	RIII	2·0	1·2 (0·4)	1·9	2·7
			C57BL	<1·8	<1·1	>1·7*	>2·4*
	D	3 min/134°C	RIII	<1·8	<1·1	>2·1	>2·8
			C57BL	<1·8	<1·1	>1·7*	>2·4*
	E	20 min/133°C	VM	<1·8	<1·1	>1·3*	>1·7*
			C57BL	<1·8	<1·1	>1·7*	>2·4*
BSE	A	3 min/105°C	RIII	4·0	4·0 (0·2)	–	–
	B	3 min/121°C	RIII	3·8	3·8 (0·2)	0·2	0·2
	C	15 min/125°C	RIII	2·4	2·7 (0·3)	1·6	1·3
	D	3 min/134°C	RIII	NC	1·9 (0·5)	NC	2·1
	E	20 min/133°C	RIII	≈1·8	1·7 (0·2)	≈2·2	2·3

All titres were calculated by the method of Kärber and using a generalised linear model (GLM) (se = standard error) and calculated back to 100 per cent brain material
All titres indicated with 'smaller than' (<) had no detectable residual infectivity even in the highest concentration used in the bioassay (in this series applicable to scrapie processes C, D and E only)
* The titre in process A (lowest heat exposure) was used as the reference titre. Only when a different mouse-line was used was the original spike titre used as reference titre (indicated with *)
NC Not calculated owing to limited number of mice

The temperature profiles of the five different time-temperature exposures A, B, C, D and E, as measured at the internal monitoring points in the dummy vials, are given in Fig 1. There was a penetration interval of about five minutes after which the heating up period started, in general requiring between 10 and 15 minutes until the planned temperatures were reached (Fig 1). Only one profile for each cycle is shown, but the temperature profiles of the two series of experiments were virtually congruent.

The impact of the various heat exposure cycles on the titres of infectivity, as assayed in mice, are expressed in log ID50/g of undiluted spike material and shown in Tables 2 and 3 and Fig 2.

There was no reduction in titre as a result of process A, that is three minutes at 105°C, whereas process B (three minutes at 121°C) caused about 1·0 log reduction in titre for scrapie, but not for BSE infectivity. Process C, 15 minutes at 125°C, gave a reduction in titre of almost 2·0 log for scrapie, whereas for BSE the reduction was on average just over 1·0 log. Process D was considerably more effective than process C, especially for BSE infectivity: three minutes at 134°C was thus more efficient than 15 minutes at 125°C. Process E (20 minutes at 133°C) reduced the infectivity of scrapie by more than 1·7 log in series 1, and by more than 2·2 log in series 2. With undiluted brain material (code P in series 2) the

infectivity of scrapie was reduced by more than 3·1 log (all calculated by the Kärber method).

For BSE process E reduced the titre of infectivity by about 2·2 log (calculated by both mathematical methods) in series 1, and by more than 2 log in series 2. With undiluted brain material (code P) the inactivation was about 3 log by the Kärber method.

Discussion

These experiments can be considered to be complementary to the pilot-scale rendering experiments described by Taylor and others (1995, 1997). They used a broad range of time-temperature protocols and simulations of different rendering processes, whereas the present experiments focused on the efficacy of hyperbaric procedures for inactivating BSE and scrapie agents.

Although the study was initially planned to evaluate Dutch rendering conditions (which are largely comparable with the German and the Belgian processes), its results may have a wider bearing since the implementation of the EU Directive on rendering in 1996 (Commission Decision 1996), stipulating that mammalian meat and bone meal may only be fed to mammals if it has been pro-

TABLE 3: Reduction of titres of infectivity in the experiments of series 2

Spiking agent	Code	Process (time/temp)	Strain of mice	Titre after heat treatment (log ID50/g)		Reduction factor* (log 10)	
				Kärber	GLM (se)	Kärber	GLM
Scrapie	A	3 min/105°C	C57BL	4·0	4·0 (0·2)	–	–
	B	3 min/121°C	C57BL	2·8	2·3 (0·3)	1·2	1·7
	C	15 min/125°C	C57BL	2·2	1·8 (0·2)	1·8	2·2
	D	3 min/134°C	C57BL	NC	1·4 (0·5)	NC	2·6
	E	20 min/133°C	C57BL	<1·8	<1·3 (0·3)	>2·2	>2·7
	P‡	20 min/133°C	RIII	<0·8	<0·3 (0·2)	>3·1†	>3·7
BSE	A	3 min/105°C	RIII	3·8	3·8 (0·2)	–	–
	B	3 min/121°C	RIII	3·7	2·8 (0·1)	0·1	1·0
	C	15 min/125°C	RIII	2·8	2·7 (0·2)	1·0	1·1
	D	3 min/134°C	RIII	≈1·6	1·3 (0·3)	≈2·2	2·5
	E	20 min/133°C	RIII	<1·8	<1·3 (0·3)	>2·0	>2·5
	P‡	20 min/133°C	RIII	≤0·8	<0·3 (0·3)	≥3·0	>3·5

All titres were calculated by the method of Kärber and using a generalised linear model (GLM) (se = standard error) and calculated back to 100 per cent brain material
All titres indicated with 'smaller than' (<) had no detectable residual infectivity even in the highest concentration used in the bioassay (in this series applicable to processes E and P [scrapie] only)
* The titre in process A (lowest heat exposure) was used as the reference titre. The reduction factor was calculated by the same mathematical method each time
†The titre in process A of series I was used as the reference value, because this was the only one available for scrapie in RIII mice
‡ Code P represents undiluted brain material
NC Not calculated owing to limited number of mice
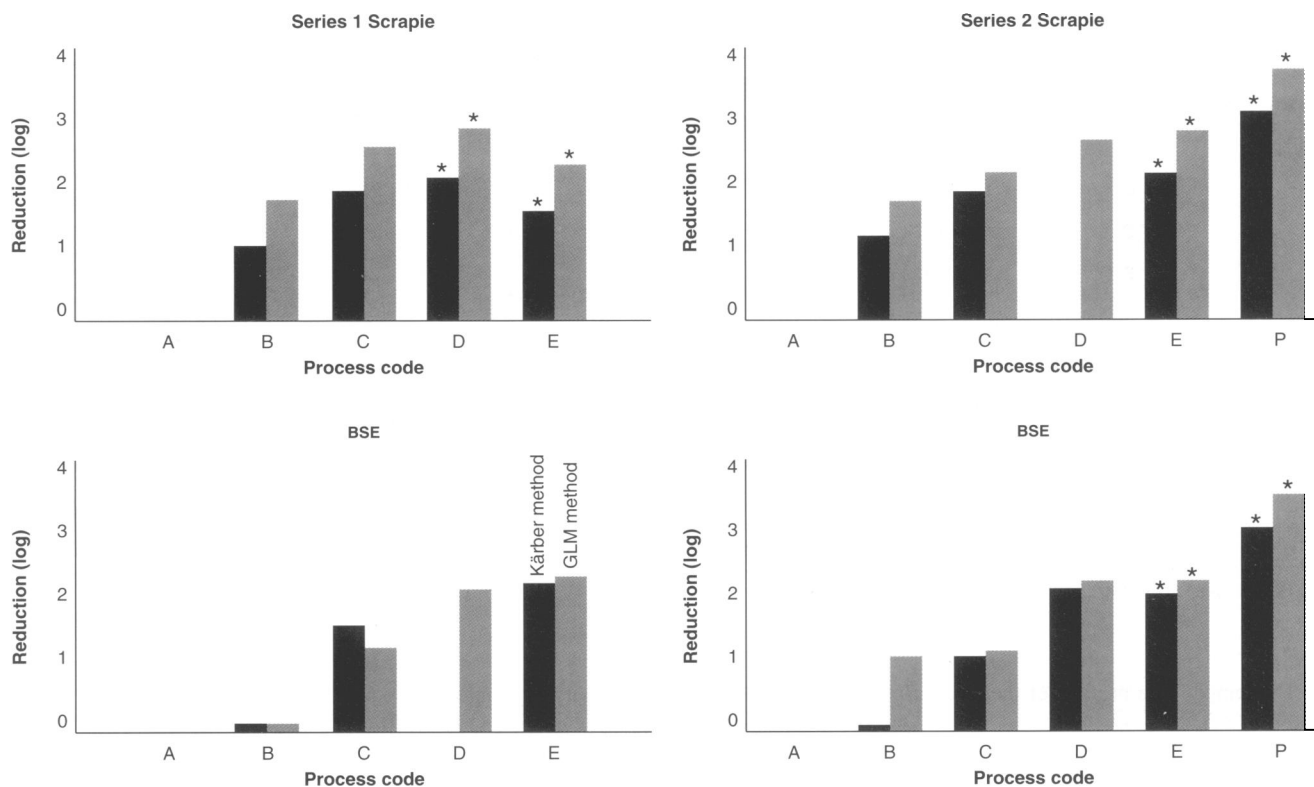
FIG 2: Reductions in the titres of infectivity of scrapie and BSE in the experiments of series 1 and 2. * Reduction exceeded measuring window of study

duced by a hyperbaric process for at least 20 minutes at 133°C at 3 bar.

The experiments covered only part of the total rendering process, but they nevertheless provide an indication of what to expect from the rendering process stipulated by EU directives. The laboratory scale of the experiments also requires to be taken into account when they are compared with commercial scale processes in which additional variables such as the dilution of infective material, particle size, heat penetration time, stirred vessels, and so on need to be considered. To address these issues, studies of the entire rendering process need to be carried out. The pilot-scale experiments carried out in the UK by Taylor and others (1995, 1997) provide part of the answer, but for hyperbaric processes comparable to the 3 bar for 20 minutes process, they only indicate that inactivation will be at least 80-fold in the case of BSE. The present experiments also provide essential inputs for modelling studies to calculate the risks involved by the inclusion of meat and bone meal in feed for ruminants, whether purposely as in the past, or accidentally as the result of cross-contamination within the system.

One of the aims of the study was to determine quantitatively the inactivation kinetics of the agents during hyperbaric heat-treatment processes, so that, for any desired reduction of infectivity, the required treatment could be calculated without the need for animal experiments. Unfortunately, this proved impossible with the data obtained. The main reason was that for a reliable calculation of the inactivation kinetics, a dose-response curve derived from a range of experimental results within the measuring range of the assay is needed. The results which gave either a zero reduction or were below the detection level of $10^{1.8}$ ID50/g (for which it could not be established how far below the detection level they were) could not be used for the calculation, but only as controls of the outcome. The measuring range was only about 2 log (3 log in the undiluted series) and there were unfortunately too few results within this range to make a reliable calculation. Whether the inactivation of prions follows first order inactivation kinetics with characteristic D- and z-values cannot therefore be answered on the basis of the data obtained.

One of the basic limitations of this type of experiment is not the scale on which they are carried out, but the injection material

used. The 'natural' hosts were selected as providers of the source material, and the spike material was mixed with rendering material, to reduce the uncertainties in interpreting the results. It would have been possible to work with strains of TSE agents that had already been passaged in the same experimental animals as used in the bioassay, in which case the initial titre would have been much higher and thus the measuring range larger. The agents' thermoresistance could, however, have changed during this process of adaptation, and it was therefore decided to work directly with bovine BSE and ovine scrapie-infected materials.

Primary inoculations of scrapie and BSE in the mice using 0·02 and 0·03 ml intracerebral inoculations only, in general show titres approximately only 1·0 log higher than the spiking material used in this study (Hadlow and others 1979, Fraser and others 1992). This difference can be explained by differences in the route and size of the inoculum; if only 0·02 ml intracerebrally and 0·1 ml intraperitoneally had been used (doses which are known to be as efficient as 0·02 ml intracerebrally combined with 0·5 ml intraperitoneally) (Taylor and others 1994), the titres per ml would have been about 0·6 log higher. Compared with the rendering studies of Taylor and others (1995, 1997), the titres of the spike material were approximately 0·5 log lower in the case of scrapie when measured in C57BL mice, whereas the level of infectivity of BSE measured in the same strain of mice (RIII) was about 1·0 log higher in the authors' material. The titre of BSE-infectivity was also of the same order of magnitude as the results of other inactivation studies have indicated (Taylor and others 1994).

Although it could be argued that the increase in the titre of BSE after process A (Tables 2 and 3) indicates a technical problem, the authors consider that the difference in titres was within acceptable errors of measurement. A second possible problem, especially relevant to the scrapie material, is that there may have been several strains in the spike material, which could have had different levels of thermostability. This would have interfered with the calculation of the titres of infectivity.

When the inoculations of the heat-treated materials began, the C57BL mice used for the scrapie materials started to give

problems immediately after the intracerebral injections. By inoculating the various components of the inoculum separately, it was concluded that a hypersensitivity associated with one of the components of the antibiotic solution used to dilute the inoculum was responsible. The same problem occurred later at the NPU in Edinburgh (D. Taylor, personal communication). Because of the problems encountered with the C57BL mice, it was necessary to change to RIII mice for the heat-treated scrapie materials in series 1. Unfortunately, no reference titre was available for the original spike material in this line of mice. The titre in the lowest heat treatment cycle (process A) in RIII was therefore used as a reference. It was considered that this was justified because the material was being prepared in one batch in each series of experiments, which may be considered more comparable than the undiluted spike material. In addition, it was not expected that process A would have any detectable inactivating effect. For reasons of homology, similar procedures were used in calculating the results of the BSE materials in series 1 and for both scrapie and BSE materials in series 2.

An additional advantage of using the titre in the lowest heat treatment cycle (process A) as a reference titre was that, because ruminant material was not specifically excluded, any accidental contamination with TSE infectivity from the raw material used for diluting the spike material would be accounted for. The chance that any clinically positive animals, in particular BSE-infected animals, might have been included in the waste material used for the experiments, is considered to have been remote.

The presently EU-endorsed process E (20 minutes at 133°C at 3 bar), gave a reduction of infectivity for scrapie of rather more than 2·0 log, taking the average of both series of experiments. The results with undiluted scrapie brain material suggest that the inactivation was more than 3·1 log. No residual infectivity was found in these scrapie experiments.

For BSE in series 1 process E gave a reduction of infectivity of about 2·2 log, and in series 2 it gave a reduction of more than 2·0 log. With undiluted brain material (code P) the inactivation was about 3 log. This is a refinement of the results of the pilot scale experiments done in the UK by Taylor and others (1995) which indicated for hyperbaric processes an inactivation of at least 80-fold for BSE. The reductions in titre of BSE and scrapie infectivity by processes that were considered the theoretical worst case scenarios for the two plants in the Netherlands in the early 1990s were 0·5 and 1·5 logs smaller. The BSE agent consistently appeared to be more resistant to heat inactivation procedures than the scrapie agent, particularly in processes involving lower temperatures for shorter times.

A possible explanation for the relatively low efficacy of process E, at least in series 1, may be that within the mixed material, prions were partitioned partially in the fat component, and were thereby protected from the exposure to wet heat. Heat is less effective in inactivating TSE agents in a lipid environment than in conditions with saturated steam. Stirring might improve the efficacy of inactivation for such a process. However, in other rendering studies, tallow has not been shown to contain any infectivity (Taylor and others 1995, 1997). Another possible explanation could be protective aggregation, in which some of the agent survives owing to the influence of its molecular micro-environment (Rohwer 1984).

Comparative titrations of the BSE agent in mice and cattle have now indicated that cattle can be as much as 1000 times more susceptible to intracerebral exposure to the BSE agent than mice (R. Bradley, personal communication, G. A. H. Wells and S. A. C. Hawkins, unpublished observations). This implies that even after process E, the residual level of BSE infectivity could be considerable, notwithstanding that the above experiments involved intracerebral inoculations rather than oral exposure. The efficacy of exposure by the oral route is generally assumed to be about 10^5 times less than exposure by the intracerebral route (Kimberlin 1994). These factors could, together with the results of inactivation studies, form the basis of risk assessment studies or experimental transmission studies with cattle as the target animals.

The results of these experiments indicate that with the EU recommended process E (20 minutes at 133°C at 3 bar), BSE infectivity was reduced by approximately 150 and 1000-fold, when measured in the diluted and undiluted form, respectively. These results would not guarantee an absolute absence of any risk for ruminants which may be fed material which contained high levels of BSE-infectivity when it entered the rendering process. This residual risk has also been indicated by Taylor (1997). The residual risk from material containing high levels of BSE infectivity is expected to be blocked by the ban on rendering specified risk material from ruminants (Commission Decision 97/534/EC implementation postponed until April 1, 1998). Follow-up studies covering the entire rendering process, together with risk assessment studies, could indicate whether residual infectivity would constitute a tangible risk of propagating an epidemic of BSE.

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Acute phase toxoplasma abortions in sheep

M. R. Owen, M. J. Clarkson, A. J. Trees

Within 13 days of the experimental infection of 15 oestrus-synchronised ewes with 2000 sporulated oocysts of *Toxoplasma gondii* at 80 to 90 days of gestation 11 had aborted. The infection induced pyrexia and specific antibody in all the ewes. One ewe resorbed its fetus, 11 ewes aborted and three delivered, at full term, live congenitally infected lambs whose pre-colostral serum was antibody-positive. Tissues from the aborted fetuses and placentae from the live lambs were examined for toxoplasma infection by polymerase chain reaction (PCR) amplification of the B1 gene and by mouse inoculation. The live lambs were all shown to be infected by both methods, but there was no evidence of infection in any of the tissues from the acute phase abortions, suggesting that these fatalities occurred before the placenta or the fetus had been invaded by *T gondii*. Such toxoplasma-induced, acute-phase abortions are likely to be impossible to diagnose from fetal tissues. These results have implications not only for the diagnosis of naturally occurring ovine abortions but also for the understanding of the pathogenesis of toxoplasma-induced abortion.

TOXOPLASMOSIS is a significant cause of abortion, neonatal mortality and reproductive losses in sheep in Britain (Blewett and Watson 1984, Blewett and Trees 1987), and many other countries (Dubey and Towle 1986). Reproductive losses typically occur only when a ewe contracts a primary infection during pregnancy. Infection during early pregnancy may lead to fetal death and resorption so that the ewe appears barren, whereas infection between approximately 50 and 120 days gestation leads to abortion late in gestation, or the birth of stillborn or weakly lambs which may be accompanied by a mummified fetus (Beverley 1976, Buxton 1989). Placental cotyledons from these infected lambs and aborted fetuses may show characteristic small white necrotic foci while the intercotyledonary membranes are normal. In such cases, the characteristic gross pathology and specific antibody to *Toxoplasma gondii* in fetal fluids, detectable three weeks after infection of the ewe, are diagnostic of toxoplasmosis (Buxton and Finlayson 1986). However, experimental infections of pregnant ewes with *T gondii* oocysts indicate that a proportion of ewes abort as early as seven to 12 days after infection (Dubey

and others 1987, Trees and others 1989), presumably owing to the pyrexia induced by the infection (McColgan and others 1988). The placentae from such abortions do not show the characteristic lesions of focal necrosis typical of toxoplasmosis (McColgan and others 1988) and fetal fluids from such abortions are negative for toxoplasma antibodies (Dubey and others 1987, Trees and others 1989).

The extent to which acute phase abortions occur in natural cases of ovine toxoplasmosis is unknown and may well be underestimated (Trees and others 1989), owing to diagnostic difficulties. Using a nested polymerase chain reaction (PCR) technique for the detection of *T gondii*, tissues from acute phase abortions resulting from experimental infections have been examined for evidence of *T gondii* infection. This PCR technique has been shown to be equal in sensitivity to mouse inoculation (Owen and others 1998).

Materials and methods

Experimental infections

Eighteen Welsh half-bred ewes, without detectable *T gondii*-specific antibody (see below), were oestrus-synchronised, mated and confirmed pregnant at 60 to 70 days gestation by ultrasound examination. Between 80 to 90 days of gestation, 15 ewes were dosed with 2000 sporulated toxoplasma oocysts of the M1 isolate (Buxton and others 1979). Three ewes were kept with the infected groups as sentinel control animals to allow any adventitious toxoplasma infection to be detected. The animals' rectal temperatures were measured from two days before infection to 14 days after infection. The infections were confirmed by postinfection pyrexias and an eight-fold increase in *T gondii* antibody titre after 21 days (Blewett and others 1983). Tissues from the aborted fetuses and fetal membranes from the live lambs were collected and tested for toxoplasma infection by mouse inoculation and PCR. Samples of thoracic fluid from the aborted fetuses, pre-colostral serum from the live lambs and serum from the ewes taken before and seven, 14 and 21 days after infection were tested for toxoplasma-specific antibody.

Serology

Toxoplasma antibody in the sheep and live lambs was assayed by the latex agglutination test (Eiken Chemical Company) (Trees and others 1989), using a dilution of 1/64, and in mice and aborted lambs by the modified agglutination test (Biomerieux) which is more sensitive than the latex agglutination test for aborted lambs (Dubey and others 1987). For the modified agglutination test sera were assayed at dilutions of 1/40 and 1/4000, the latter to detect false negative results which can occur when testing serum with a high antibody titre at low dilutions.

M. R. Owen, PhD, BSc, MRCVS, **A. J. Trees,** PhD, BVM&S, MRCVS, Department of Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool L3 5QA

M. J. Clarkson, PhD, DVSc, BSc, DSHP, MRCVS, Department of Veterinary Clinical Science and Animal Husbandry, Veterinary Teaching Hospital, University of Liverpool, Leahurst, Chester High Road, Neston, South Wirral L64 7TE

Studies on the efficacy of hyperbaric rendering procedures in inactivating bovine spongiform encephalopathy (BSE) and scrapie agents

B. E. C. Schreuder, R. E. Geertsma, L. J. M. van Keulen, et al.

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