New studies on the heat resistance of hamster-adapted scrapie agent: Threshold survival after ashing at 600°C suggests an inorganic template of replication

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One-gram samples from a pool of crude brain tissue from hamsters infected with the 263K strain of hamster-adapted scrapie agent were placed in covered quartz-glass crucibles and exposed for either 5 or 15 min to dry heat at temperatures ranging from 150°C to 1,000°C. Residual infectivity in the treated samples was assayed by the intracerebral inoculation of dilution series into healthy weanling hamsters, which were observed for 10 months; disease transmissions were verified by Western blot testing for proteinase-resistant protein in brains from clinically positive hamsters. Unheated control tissue contained 9.9 $\log_{10}LD_{50}$/g tissue; after exposure to 150°C, titers equaled or exceeded 6 $\log_{10}LD_{50}$/g, and after exposure to 300°C, titers equaled or exceeded 4 $\log_{10}LD_{50}$/g. Exposure to 600°C completely ashed the brain samples, which, when reconstituted with saline to their original weights, transmitted disease to 5 of 35 inoculated hamsters. No transmissions occurred after exposure to 1,000°C. These results suggest that an inorganic molecular template with a decomposition point near 600°C is capable of nucleating the biological replication of the scrapie agent.

transmissible spongiform encephalopathy | scrapie | prion | medical waste | incineration

The infectious agents responsible for transmissible spongiform encephalopathy (TSE) are notoriously resistant to most physical and chemical methods used for inactivating pathogens, including heat. It has long been recognized, for example, that boiling is ineffective and that higher temperatures are most efficient when combined with steam under pressure (i.e., autoclaving). As a means of decontamination, dry heat is used only at the extremely high temperatures achieved during incineration, usually in excess of 600°C. It has been assumed, without proof, that incineration totally inactivates the agents of TSE, whether of human or animal origin. It also has been assumed that the replication of these agents is a strictly biological process (1), although the notion of a "virus" nucleant of an inorganic molecular cast of the infectious β-pleated peptide also has been advanced (2). In this paper, we address these issues by means of dry heat inactivation studies.

Materials and Methods

Scrapie Agent and Sample Preparation. A single high-passage brain pool of the 263K strain of hamster-adapted scrapie agent was used in all experiments in this study (3). Twenty brains that had been stored at −70°C were partially thawed and minced into fine fragments and then mixed to homogeneity before distribution into a series of 1-g samples that were transferred to 10-ml quartz-glass crucibles (Fisher Scientific) and refrozen until used. A portion of the pool was fixed overnight in 10% formol-saline, followed by repeated saline washings to remove the formalin before transfer to the crucibles.

Exposure to Dry Heat. The heating source was a bench-top type F1500 Muffle Furnace with inside dimensions of 10 cm × 10 cm × 23 cm (Barnstad/Thermolyne). For each test run, loosely covered quartz-glass crucibles containing 1 g of fresh or fixed thawed brain tissue were placed on each end of a small, metal platform that in its center held an identical covered crucible containing a thermocouple (model 8528–20; Cole–Parmer). The platform was attached to a metal holder for inserting and removing the specimens from the oven. To minimize the lag times for heating and recooling specimens, the oven was heated to approximately 100°C above the desired temperature, and once the specimens had reached temperature, the platform was repeatedly moved in and out of the oven such that the thermocouple temperature was maintained within 10°C of the chosen test temperature. Upon completion of heating at the test temperature for either 5 or 15 min, the crucibles were placed in dry ice. This procedure resulted in a heating-up period of 1–2 min (depending on the test temperature) and a cooling-down period of less than 30 sec.

Infectivity Bioassays. PBS, pH 7.4, was added to the specimen residues to bring them to their original 1-g weights. Specimens then were pulverized by manual grinding in a Tenbroeck homogenizer, followed by sonication for 15 sec. Weanling 4- to 6-week-old female Golden Syrian hamsters (Charles River Breeding Laboratories) were inoculated intracerebrally with 0.03 ml of undiluted or serial 10-fold-diluted specimens.

Hamsters were observed for clinical signs of scrapie during a period of 10 months, and the brains from all dying animals were removed and frozen at −70°C. Brains from all dead hamsters in the 600°C and 1,000°C exposure groups and a random sampling of brains from dead animals in the highest dilution groups exposed to lower temperatures were tested for the presence of proteinase-resistant protein (PrP) by Western blot. Brain tissue (50–100 mg) was extracted by using the method of Parchi *et al.* (4), and after routine PAGE and transfer to nitrocellulose paper, PrP was visualized by incubation with a 1:2,000 dilution of the anti-hamster scrapie mAb 3F4 (R. Rubenstein, Institute for Basic Research, Staten Island, NY) and a 1:1,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG/IgM (Bio-

Abbreviations: TSE, transmissible spongiform encephalopathy; PrP, proteinase-resistant protein.

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Table 1. Observations during and after heating fresh (unfixed), 1-g aliquots of a 263K strain scrapie-infected hamster brain macerate for 5 or 15 min in covered quartz-glass crucibles at various temperatures

Temperature	Combustion	Crucible appearance	Tissue appearance	Weight loss, %
150°C	No flame	Transparent	Dehydrated, buff-colored	48–52
300°C	No flame	Transparent	Gummy, amber-colored	81–87
600°C	Flame, black smoke	Blackened	Black ash	98–99
1,000°C	Flame, black smoke	Blackened, then transparent	Gray-black ash	98

Source International, Camarillo, CA). Some specimen duplicates also were extracted by using a modification of the micro-Diringer purification method (5), and tested as above, with identical positive or negative results.

Infectivity levels are expressed as logarithms of the LD_{50} ($\log_{10}LD_{50}$), calculated according to the method of Reed and Muench (6). For titrations in which infectivity levels equaled or exceeded the highest-tested dilutions, approximations of end points were made by comparing the mean incubation times of groups of hamsters inoculated with dilutions of the heated specimens with those of animals inoculated with the unheated controls.

Results

Fresh Brain Tissue. Observations of the tissue specimens during and after heating at the various temperatures are summarized in Table 1, and infectivity assays of the specimens are shown in Table 2. Unheated brain tissue had an infectivity titer of 8.4 $\log_{10}LD_{50}$/0.03 ml inoculum (equal to 9.9 $\log_{10}LD_{50}$/g). Specimens heated for either 5 or 15 min at 150°C (which yielded severely dehydrated but still recognizable tissue) or 300°C (which converted the tissue into an amber gummy mass) transmitted disease to 100% of the hamsters at all tested dilution groups. Thus, the levels of residual infectivity equaled or exceeded the highest-tested dilutions of 10^{-4} (after 150°C) and 10^{-2} (after 300°C).

Comparisons of the incubation periods in hamsters inoculated with either unheated control specimens or specimens heated for 5 min suggested negligible inactivation at both 150°C and 300°C (Table 3). Specimens heated for 15 min appeared to have lost 2–3 $\log_{10}LD_{50}$ at 150°C and about 6 $\log_{10}LD_{50}$ at 300°C.

Exposure of undiluted inocula to 600°C (which completely ashed the tissue) almost completely destroyed infectivity: no transmissions occurred in 15 hamsters inoculated with the 5-min specimen, but five transmissions occurred in 18 animals inoculated with the 15-min specimen. This apparent inconsistency and the prolonged and variable incubation periods in the five positive hamsters (174, 186, 193, 205, and 313 days) suggest that infectivity in these inocula was near the point of extinction. Heating at 1,000°C for 5 min produced total inactivation.

Fixed Brain Tissue. Unheated fixed control tissue had an infectivity titer of 6.2 $\log_{10}LD_{50}$/0.03 ml inoculum (equal to 7.7 $\log_{10}LD_{50}$/g). Heating for 5 min at either 150°C or 300°C produced the same 100% mortality rates at all tested dilutions as seen for fresh unfixed tissue, and analysis of incubation period data suggested that about 2 $\log_{10}LD_{50}$ were lost at 150°C and about 4 $\log_{10}LD_{50}$ were lost at 300°C. In the 600°C exposure group, transmission to 1 of 24 inoculated hamsters occurred after an incubation period of 231 days.

Discussion

The transmissible amyloid-forming agents of spongiform encephalopathy have been the subject of inactivation studies beginning shortly after their discovery more than 60 years ago. They have been shown to resist inactivation by a wide array of physical and chemical treatments, including heat and formaldehyde (7). Different agent strains show different degrees of heat resistance, and the hamster-adapted 263K strain of scrapie used in the present experiments ranks among the most resistant strains studied in comparative testing[‖]. A small fraction of infectivity in wet brain tissue can withstand autoclaving at 134°C (10), or exposure to dry heat at 200°C[¶], and if the tissue has been freeze-dried under partial vacuum, some survival is detectable after exposure to 360°C (11). Formaldehyde fixation increases resistance to autoclave inactivation (11, 12).

One aim of the present study was to examine the effect of formaldehyde fixation on tissues exposed to dry heat. Although we did not determine a precise dilution end point, a comparison of incubation times in groups of hamsters inoculated with heated and unheated specimens indicates that prior fixation with formaldehyde did not increase (and may have decreased) resistance to dry heat inactivation.

A second aim of the study was to investigate the survival of the infective agent at temperatures in the range of those used in the primary (600°C) and secondary (1,000°C) chambers of medical waste incinerators. The survival of infectivity at 600°C, even at the very low levels observed in this study, suggests that the agent may not be fully inactivated in the residual ash from the first chamber, and ash formed in this chamber usually is removed without being exposed to the 1,000°C temperature used to treat the gaseous and particulate emissions that enter the second chamber before discharge to the atmosphere. There is also no assurance that any combustion emissions containing the agent that enter the secondary chamber would be inactivated because the residence time there is at most only a few seconds. Moreover, we assayed only the ashed residue for infectivity and not the combustion emissions. Further experiments are in progress, including chemical, immunological, and electron microscopic studies, to assess inactivation of the agent in ash and emissions under conditions simulating those of medical waste incinerators.

The third aim of our study was to define and interpret the extraordinary heat resistance of TSE infectivity in terms of possible mechanisms of replication. Life forms able to replicate in unconventional ecosystems have been categorized as ''extremophiles,'' of which the greatest number of studied examples are resistant to heat, the so-called ''hyperthermophiles'' (13, 14). Numerous archaeal species have been discovered in proximity to terrestrial volcanic fields and deep-sea hydrothermal vents with optimal growth temperatures hovering around 100° (14). The current record for maximum temperature supporting propagation is 113°C, held by the archaeal species *Pyrococcus furiosus* (15), and various enzymes and proteins isolated from several different thermophilic genera have been found to retain biological activity within the same temperature range (16, 17).

However, these temperatures refer to conditions under which growth and replication can occur, and not resistance to thermal destruction, which is an entirely different matter. The upper

[¶]Steele, P. J., Taylor, D. M., Fernie, K. & McConnell, I., Conference on Characterization and Diagnosis of Prion Diseases in Animals and Man, September 23–25, 1999, Tübingen, Germany.

[‖]Taylor, D. M., Fernie, K., Steele, P. J. & McConnell, I., Conference of Characterization and Diagnosis of Prion Diseases in Animals and Man, September 23–25, 1999, Tübingen, Germany.

Table 2. Proportions of disease transmissions to hamsters after intracerebral inoculation of 0.03 ml of fresh or formalin-fixed scrapie-infected brain tissue, either unheated or exposed to dry heat at various times and temperatures

		Log_{10} dilution										
Specimen	Undiluted	−0.7	−1	−2	−3	−4	−5	−6	−7	−8	−9	−10
Fresh tissue												
Unheated control				4/4		7/7		12/12	11/11	10/12	0/12	0/8
Heated 5 min												
150°C			8/8	8/8	8/8	8/8						
300°C		34/34	12/12	8/8								
600°C	0/15											
1,000°C	0/17											
Heated 15 min												
150°C			8/8	8/8	8/8	8/8						
300°C		33/33	12/12	7/8								
600°C	5/18											
Formalin-fixed tissue												
Unheated control						7/8		5/12	3/12	1/12	0/12	0/8
Heated 5 min												
150°C			8/8	8/8	8/8	7/8						
300°C		34/34	12/12	8/8								
600°C	1/24											

thermal stability limit for biological macromolecules *in vitro* is 150–160°C (18); denaturation of proteins and nucleic acids occurs at temperatures well below their decomposition temperatures because of thermal disruption of hydrogen and hydrophobic noncovalent bonds (19, 20). The best-studied examples of microbial heat resistance are found among bacterial spores, which typically are used to test the efficiency of autoclaves and incinerators: wet spores of *Bacillus subtilis* and *Serratia marcescens* have been shown to resist brief exposures to temperatures between 270°C and 340°C, and some viability of dry spores persists after exposure to 370°C (21).

None of these organic molecules or microorganisms has been shown to retain biological or replicating activity after exposures to temperatures above 400°C, and certainly none would be expected to survive 600°C. How could the amyloidogenic agents of TSE not only withstand chemical decomposition, but also retain the power to nucleate their own replication (even if only as a few infectious particles from suspensions in which up to 10^{10} infectious particles were initially present) after exposure to temperatures that should decompose or volatilize organic molecules?

It is helpful in thinking about this question to correlate the physical characteristics of the brain tissue with residual infectivity over the range of tested temperatures. Exposure to 150°C visibly dehydrated the tissue, which lost approximately 50% of its initial weight, but did not change color, and retained its morphological appearance. Moreover, even the dehydration was incomplete, because normal brain tissue contains approximately 77% water (22). Infectivity was only slightly affected.

Exposure to 300°C yielded a decomposed gummy mass that had lost approximately 85% of its initial weight. Thus, dehydration was complete, and some organic pyrolysis products probably also were formed and vaporized. It seems likely that this temperature must have damaged or destroyed the integrity of much of the molecular and macromolecular structure, and, yet, despite the loss of most of the infectivity, a significant amount remained (several $log_{10}LD_{50}$).

Table 3. Mean incubation periods (in days) of the same groups of hamsters shown in Table 2

		Log_{10} dilution										
Specimen	Undiluted	−0.7	−1	−2	−3	−4	−5	−6	−7	−8	−9	−10
Fresh tissue												
Unheated control				75		86		90	116	182	ND	ND
Heated 5 min												
150°C			75	75	76	82						
300°C		75	75	75								
600°C	ND											
1,000°C	ND											
Heated 15 min												
150°C			82	82	84	127						
300°C		105	118	142								
600°C	214											
Formalin-fixed tissue												
Unheated control						142		163	184	(234)	ND	ND
Heated 5 min												
150°C			85	85	97	119						
300°C		91	101	119								
600°C	(231)											

ND, no deaths in group.

Exposure to 600°C or 1,000°C produced a flaming tissue combustion that lasted several seconds and yielded a residue of glowing black ash that had lost 98–99% of its initial weight. Under these conditions it is difficult to imagine the survival of any replicative (or even nonreplicative) biological structure composed of proteins or other organic compounds. Yet, combustion is a series of pyrolysis and oxidation reactions that proceed rapidly but incompletely. At the comparatively low combustion temperature of 600°C and with limited oxygen penetration into the crucibles, it is remotely possible that some of the organic molecules originally present might have escaped destruction. Incomplete combustion of organic compounds also would be likely to introduce pyrolysis products and elemental carbon into the combustion residues, which probably was responsible for their black color. Carbon has been reported to partially protect TSE infectivity at autoclave temperatures (23). If even a few PrP lattice "ghosts" survived combustion, perhaps protected by carbon, they might be sufficient to account for the trace amount of infectivity in tissue combusted at 600°C, whereas at 1,000°C, the thermal degradation and oxidation processes would have been pushed beyond the point at which any survival was possible. The clearing of the black residue and smoke from crucibles at 1,000°C suggests that most of the carbon and other dark-colored pyrolysis products had been oxidized.

An alternative explanation is that an inorganic replica of the necessary molecular geometry was made, which nucleated the conformational change of the PrP precursor protein to its infectious, β-pleated isoform (1, 24, 25), similar to the heteronucleation by minerals of many protein crystallizations (26, 27). Engineers in material science have succeeded in directing the morphological pattern of inorganic crystals and noncrystalline solids by the interaction of organic templates with inorganic minerals to produce high-order coorganization states of consolidated matter (28–30). They are making micromolecular sieves of zeolites, silica, and aluminum silicates, as well as of other minerals with pore size and connectivity determined by the organic templates. The organic molecules are later removed from these organic–inorganic complexes by calcination or heating to 600°C, leaving a microporous or mesoporous replica in silica or alumina silicates (8, 31). Biomineralization of single-cell organisms and bacterial fibrils has been achieved in what is essentially a molecular tectonics of macromolecular organization (8, 9).

Even enzyme-mimicking inorganic materials can be made by template catalysts such as amorphous metal oxides and zeolites. Such templates are chemically coded surfaces transcribed at the molecular level into inorganic nuclei with highly specific structural, orientational, and positional properties. Inorganic self-assembly of the organic architecture results from repetition of these processes in association with outgrowth along and within the organic template, creating inorganic–organic hybrids with nano-, micro-, and macroscale patterns. The low levels of infectivity found to survive long exposures to temperatures over 300°C after rapid reduction of infectivity titers by many orders of magnitude may be accounted for by a similar formation of inorganic "fossil templates" of organic amyloid nucleants.

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