Influence of Water, Fat, and Glycerol on the Mechanism of Thermal Prion Inactivation*

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Extending the recent analysis of the safety of industrial bovine fat-derived products for human consumption (Müller, H., Stitz, L., and Riesner, D. (2006) Eur. J. Lip. Sci. Technol. 108, 812–826), we investigated systematically the effects of fat, fatty acids, and glycerol on the heat destruction of prions. Prion destruction was quantitatively evaluated in PrP 27–30, or prion rods, by the inactivation of infectivity as well as by the degradation of the polypeptide backbone. Under all conditions analyzed, inactivation of prion infectivity was achieved more efficiently than backbone degradation by several orders of magnitude. The presence of fat enhanced prion inactivation and offers a mild treatment for prion decontamination. In contrast, the presence of fat, fatty acids, and especially glycerol protected the PrP 27–30 backbone against heat-induced degradation. Glycerol also protected against heat-induced inactivation of prion infectivity. A phase distribution analysis demonstrated that prions migrated to the interphase of a fat/water mixture at room temperature and accumulated in the water phase at higher temperatures. In a systematic study of the mechanism of prion destruction, we found an intermediate structure of PrP that has fewer fibrils in β-sheet formation, lower resistance to protease digestion, greater aggregation, and reduced solubility compared with PrP 27–30 but retains residual infectivity. These findings suggest that prion infectivity depends on β-sheet-rich fibrillar structure and that inactivation proceeds in a stepwise manner, which explains the tailing effect frequently observed during inactivation.

The prion diseases include bovine spongiform encephalopathy (BSE),3 scrapie in goat and sheep, chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease in humans. These diseases can be acquired by transmission of an infectious agent, occur sporadically, or be inherited. In contrast, other fatal neurodegenerative disorders like Alzheimer disease, Parkinson disease, and Huntington disease are exclusively characterized by a sporadic or genetic etiology.

The entity causing these diseases is called a prion, derived from “proteinaceous infectious particle” (2). Prions are composed primarily, if not exclusively, of an abnormal isoform, denoted PrPSc, of the normal, cellular prion protein (PrPC), which is a hydrophobic glycoprotein expressed in all mammals studied to date. During disease pathogenesis, PrPC is converted into PrPSc with the assistance of one or more cofactors (3–5). Although the mechanism of conversion remains unclear, the “protein-only” hypothesis posits that PrPSc acts as template for the post-translational conversion of PrPC into PrPSc. The recent demonstration that infectious PrPSc fibrils can be formed solely from recombinant PrP offers strong support for the protein-only hypothesis (6).

The conformational transition of PrPC to PrPSc changes the physicochemical properties of the protein. PrPC is soluble in mild detergents, exhibits a mainly α-helical secondary structure, and is sensitive to degradation by proteases, e.g. proteinase K (PK). In contrast, PrPSc is partially PK-resistant and forms insoluble, β-sheet-rich multimeric aggregates that accumulate most prominently in the central nervous system. Upon purification using detergents and limited digestion by PK, the N terminus of PrPSc is truncated to yield a still infectious fragment of 27–30 kDa, designated PrP 27–30. As a result of hydrophobic interactions, PrP 27–30 forms rod-shaped fibrils, also called prion rods (7). Small amounts of two sphingolipids attach to PrP in prion rods (8), and 10% (w/w) of a polyglucose scaffold has been associated with PrP 27–30 (9).

Prions are extraordinarily resistant to decontamination by physical or chemical procedures that inactivate conventional pathogens (10). The unintentional application of inappropriate inactivation methods during rendering of offal to prepare meat and bone meal may have selected for the most resistant prions, which may have perpetuated the BSE epidemic in Europe as dairy cattle consumed this infected dietary supplement (11, 12). Compelling evidence indicates that Creutzfeldt-Jakob disease can be transmitted by improperly decontaminated surgical equipment (13). Even though the chemical nature of prions is not fully characterized, effective methods for a significant reduction of prion infectivity titers have been described. Most
Mechanism of Thermal Prion Inactivation

### TABLE 1

Summary of experimental set ups for testing the influence of heat on the stability of PrP 27–30 in the presence of water, fat, and glycerol

| Experimental setup | A          | B          | C          | D          | E          | F          |
|--------------------|------------|------------|------------|------------|------------|------------|
| Deionized water    | 100%       |            |            |            |            |            |
| Tallow             | 100%       | 10%        | 90%        | 100%       |            | 87.75%     |
| Oleic acid         |            |            |            |            |            | 2.5%       |
| Glycerol           |            |            |            |            |            |            |
| Highest temperature examined (Western blot/bioassay) | 140/170°C | 155/200°C | 140/200°C | 150°C/ND* | 100%       | 165/170°C  |
| Time to reach highest temperature (Western blot/bioassay) | 21/28 min | 24/45 min | 11/30 min | 22 min/ND | 22/47 min | 27/28 min |

*ND indicates not determined.

Many factors affect prion inactivation, including the prion strain, method of sample preparation (e.g. undiluted tissue, tissue homogenate, and tissue macerate), the fixation and dehydration state of the agent, and surface effects (smearing, particularly on steel surfaces). For instance, for 263K prions, a Syrian hamster-adapted scrapie strain, reductions of infectivity declines rapidly followed by slow inactivation with time proceeds in an exponential fashion. How- ever, when milder, partially inactivating conditions are applied, a tailing phenomenon is observed (15, 21, 22). In this case, infectivity titers.

| Prion Protein Samples—PrP 27–30 from Syrian hamsters infected with scrapie strain 263K was prepared as described earlier (24). Centrifugation of a 50% sucrose gradient at 4 °C for 1 h at 100,000 × g in a TL45 rotor yielded the final PrP 27–30 pellet, which was stored at −70 °C until use. | | | | | | |

| Bovine Edible Tallow, Oleic Acid, and Glycerol—Bovine edible tallow was provided by the European Oleochemicals and Allied Products Group. The fat had been recovered from adipose tissue and bones by dry melting at 100 °C followed by purification of the lipid fraction from tissues and proteinaceous matter by filtration through a diatomaceous earth bed. | | | | | |

Heat Treatment of Prions and Recovery from Mixtures Containing Fat, Fatty Acids, and Glycerol—As described in detail earlier (1, 25), an inactivation system was utilized including a pressure reactor with external electric heating, digital pressure and temperature displays, and a magnetic stirrer (Parr Instruments Micro Bench Reactor 4591). PrP 27–30 was loaded into the reactor vessel containing 30-ml mixtures of raw materials (Table 1). Depending upon the reactor content, temperatures of up to 200 °C were reached within 47 min (Table 1). For the duration of the reaction, the reactor content was stirred at 150 rpm to permit an even temperature distribution within the reactor vessel as well as rapid heating and cooling.

To quantify the amount of prions remaining undegraded after heat treatment in a large excess of fat and glycerol, a quantitative purification method based on a methanol/chloroform precipitation was applied (1, 25, 26). A minimum PrP recovery rate of 95% was achieved. Methanol/chloroform precipitation of PrP 27–30 subjected to 40 °C for 20 min did not decrease infectivity titers.

**Quantification of Heat-treated Prions by Immunoblotting and Incubation Time Interval Assay**—After heat treatment, degraded PrP was separated by PAGE (27). The amount of

widely used are treatments with 1 M sodium hydroxide (NaOH) or 2.5% sodium hypochlorite (NaOCl). Partial inactivation can also be achieved by heat, i.e. steam autoclaving at 134 °C for 20 min. For complete inactivation, either steam autoclaving at ≥121 °C after treatment with 1 M NaOH or boiling in 1 M NaOH is required (10).

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An acceptable minimum. To establish a mechanistic model for the mechanism of thermal prion inactivation, we provide qualitative and quantitative data on the inactivation of infectivity as well as the degradation of the PrP 27–30 polypeptide backbone under continuous variation of conditions, particularly in the presence of different concentrations of fat, fatty acids, and glycerol.

**EXPERIMENTAL PROCEDURES**

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**Quantification of Heat-treated Prions by Immunoblotting and Incubation Time Interval Assay**—After heat treatment, degraded PrP was separated by PAGE (27). The amount of...
undegraded PrP was quantitatively detected by an improved immunoblot comprising staining with a mixture of the monoclonal antibodies 3F4 and R1, enhanced chemiluminescence, and densitometry as described earlier (1, 25). The sensitivity threshold of the method was 1 ng of PrP as determined by a dilution series of PrP 27–30.

Bioassays of prion infectivity were performed by inoculation of weanling female Syrian gold hamsters (1). Bioassays were terminated 390 days after inoculation. Prion titers were calculated by measuring the time intervals from inoculation to onset of clinical symptoms (2). The dose-response curve from these titers was utilized for incubation times up to 140 days. For incubation times longer than 140 days, a bioassay detection limit of 2 log_{10} ID_{50} was used. For hamsters remaining healthy after 390 days, brain homogenates were prepared and subjected to PK digestion followed by Western blotting.

Quantification of prion levels was performed as described (20). Briefly, the heat destruction of prions can be described by first-order decay kinetics with a rate constant \( k \) (20, 28). Therefore, the reduction factor \( RF_i \) can be defined as the ratio between the concentration of PrP or prion infectivity, respectively, before and after heat treatment at a certain temperature for time \( t \) as shown in Equation 1,

\[
\ln\left(\frac{C_{PrP}(t)}{C_{PrP}(0)}\right) = -k \times t \quad \text{(Eq. 1)}
\]

or with the reduction factor after heating for 20 min as shown in Equation 2,

\[
\log_{10}RF_{20} = k \times 1200/2.303 \quad \text{(Eq. 2)}
\]

The rate constant \( k \) depends on the temperature according to the Arrhenius Equation 3,

\[
\delta \ln k/\delta 1/T = -E_\delta/R \quad \text{(Eq. 3)}
\]

where \( E_\delta \) indicates the activation energy, and \( R \) is the gas constant. Combining both equations leads to a linear relationship of \( \ln \log_{10} RF_{20} \) as a function of \( 1/T \). The mean values of all data points fall within the experimental error and are in accordance with the linear dependence (Fig. 1). Because the error limits of the Western blot densitometry were 10–15%, the majority of degradation experiments was repeated until reproducible. The mean standard error of all degradation data points in Fig. 1 was \( \log_{10} 0.24 \).

**Distribution of PrP 27–30 between Fat and Water Phases**—Different amounts of PrP 27–30 were suspended in 15-ml polymer tubes containing 1:1 mixtures of bovine edible tallow and water preheated to a temperature of 40 °C. After agitation for 30 s, the specimens were incubated in a water bath at temperatures of 20, 40, 60, 80, or 100 °C until clear phases were achieved (usually for 30 min). The still-liquid fat phase was carefully separated using a needle attached to a glass syringe. Subsequently, the polymer tube was broached at the bottom, and the water phase was extracted. The protein in all three phases, i.e. fat, water, and remaining interphase of about 2.5-ml solution, was purified by methanol/chloroform precipitation as described above. The amount of PrP 27–30 in each phase was determined by Western blotting and densitometric quantification as described above.

**PK Assay**—PK digestions were performed in 1.5-ml Eppendorf tubes or 96-well, black-bottom plates (Nunc, Wiesbaden, Germany). Specimens were incubated in 50 \( \mu l \) of 50 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, and 2.5 mM EDTA with PK at a final concentration of 50 \( \mu g/ml \) at 37 °C for incubation times between 15 min and 60 h. The reactions were terminated by the addition of SDS-PAGE loading buffer and boiling for 6 min. The degree of digestion was determined by real time fluorescence (thioflavin T assay) or by comparison with an undigested control on the same SDS gel (Western blotting).

**Determination of Solubility by Differential Centrifugation**—According to the standards of Hjelmeland and Chrambach (29), particles remaining in the supernatant after centrifugation at 100,000 \( \times g \) for 1 h are regarded as soluble. To test whether heat treatment affected solubility, PrP 27–30 was or was not subjected to temperatures of up to 125 °C for 20 min as described above. Solubilized PrP 27–30 was obtained by sonication with a Bandelin HD 2070 sonicator (Bandelin Electronic, Berlin, Germany) in 10 mM sodium phosphate buffer (pH 7.2) and 0.3% SDS. The specimens were sonicated 10 times at 15-s bursts at 65% power on ice followed by a 15-s cooling pause. Subsequent centrifugation in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA) with a TLA-45 rotor for 1 h at 100,000 \( \times g \) and 4 °C separated soluble PrP 27–30 from the insoluble pellet fraction. The amounts of PrP 27–30 in the supernatant and pellet fractions were determined by Western blotting and densitometric quantification as described above.

**Negative Staining and Electron Microscopy**—Negative staining was performed as described previously (33). In brief, negative staining was done on Formvar/carbon-coated, 200-mesh copper grids (Ted Pella, Inc., Redding, CA), which were glow-discharged prior to staining. Five-\( \mu l \) samples were adsorbed for 30 s, and the grids were each washed with 50 \( \mu l \) of 0.1 and 0.01 M ammonium acetate (pH 7.4). Afterward, the grids were stained with 50 \( \mu l \) of freshly filtered stain, 2% ammonium molybdate. After drying, the samples were viewed in a FEI Tecnai F20 electron microscope (Eindhoven, The Netherlands) at 80 kV and a standard magnification of 25,000. Electron micrographs were recorded with a Gatan Ultrascan CCD camera. The magnification was calibrated using negatively stained catalase crystals and ferritin.

**Congo Red Birefringence**—Protein solutions diluted to 150 \( \mu g/ml \) were air-dried on a glass microscope slide and incubated for 10 min in 0.5% Congo red, 80% ethanol. After successive washing with 90% ethanol and 96% ethanol, the specimens were viewed at \( \times 400 \) magnification with a Zeiss III polarizing microscope.

**Thioflavin T (ThT) Assay**—Thioflavin T binds specifically to amyloid fibrils such that its long axis is parallel to the long axis of the fibril, i.e. in neat rows running along the length of the \( \beta \)-sheets between the amino acid side chains, perpendicular to the strands (30). In the absence of amyloid fibrils, ThT exhibits fluorescence at the excitation and emission maxima of 342 and 343 nm, respectively. Binding to PrP 27–30, however, results in new excitation and emission maxima of 442 and 482 nm, respectively. This change depends on the aggregated state of PrP (31, 32). Because of the irregular arrangement of \( \beta \)-sheets
Within amorphous aggregates, the ThT assay is capable of discriminating between amorphous and fibrillar structures.

To test for fibril formation, fluorescence measurements were performed in 96-well, black-bottom plates (Nunc, Wiesbaden, Germany) in a microplate reader Safire (Tecan Trading AG, Switzerland). All measurements were carried out at 37 °C in 50 μM Tris-HCl (pH 8.0), 0.1 mM NaCl, 2.5 mM EDTA, and 5 μM ThT. The recording parameters were as follows: excitation wavelength of 445 nm, emission range of 470–550 nm, step resolution of 2 nm, bandwidth of 2.5 nm, gain of 180, z-position of 4400 ± 400 μm. Kinetics were recorded for up to 240 cycles with intervals of 15 min. For analysis, the integral of the intensity measured from 470 to 550 nm was calculated.

Circular Dichroism—CD spectra were recorded with a Jasco spectropolarimeter, model 715 (Jasco Inc.) and are depicted as ellipticity (θ). All measurements were performed at room temperature in 10 mM sodium phosphate (pH 7.2) with or without addition of 10% glycerol. Ten spectra were recorded over the spectral range of 185–260 nm at a scan speed of 50 nm/min and a step resolution of 1 nm. Below a wavelength of 185 nm, excessive absorption by the buffer impeded further readings. The presence of glycerol limited the spectral range to wavelengths higher than 191 nm. A blank spectrum for cuvette and buffer was subtracted from each spectrum. Satisfactory CD spectra were only obtained with sonicated PrP 27–30 at a concentration of 1 μg/μl. Sonication was carried out with a Labsonic U cup sonicator (B. Braun Diessel Biotech) at 180 watts for 5 min at <25 °C. The specimens were contained in a screw-cap tube immersed 1 cm into the water bath of the sonicator. Thermostating of the sonication bath was established by a water cooling system.

RESULTS

All experiments were carried out with prion rods purified from hamster brains infected with the hamster-adapted scrapie strain 263K. Heat treatment of prion samples was performed in a lab-scale autoclave appropriate for high pressure (1), and analysis was accomplished by gel electrophoresis followed by Western blotting as well as by bioassays in Syrian golden hamsters.

Degradation of PrP 27–30—From the quantitative analysis of Western blots, reduction factors (RF$_{20}$) for degradation, indicated by hydrolysis of the peptide bonds, of PrP 27–30 in different fat/water mixtures were determined after heat treatment for 20 min at temperatures up to 165 °C (Fig. 1A). No degradation experiments were conducted at temperatures >165 °C because unacceptably large amounts of PrP 27–30 would have
been required. Comparing degradation experiments with and without tallow, the presence of fat increases the backbone stability of PrP 27–30 by 1 order of magnitude. However, in the presence of water, the protective effect of fat decreases with increasing temperature (Fig. 1A). At temperatures ≥110 °C, no significant difference was detected when degradation in 90% fat and 10% water was compared with that in pure water. Heat treatment in pure tallow in comparison with pure oleic acid did not exhibit significant differences over the whole temperature range.

The slopes of the regression curves yield the activation energy of the degradation processes. The activation energy of heat-mediated degradation in the presence of fat or fatty acids was ∼31 (±4.4) kJ/mol, whereas in pure water, an activation energy of 20 (±5.3) kJ/mol was obtained.

Compared with the presence of fat, the presence of glycerol resulted in greater stability of PrP 27–30 against heat degradation (Fig. 1B). The heat stability of PrP 27–30 in pure glycerol was raised by 2 orders of magnitude compared with that of PrP 27–30 in pure water. In contrast to fat, the presence of water in the mixtures did not affect the protective effect of glycerol; we found that 10% glycerol offered as much protection against degradation as 100% glycerol at high temperatures. The activation energies in 100 and 10% glycerol were 51 (±9.8) and 29 (±2.2) kJ/mol, respectively. The latter value should be considered with some care because the data suggest a biphasic degradation.

At higher temperatures, in addition to the PrP triplet bands reflecting different levels of glycosylation, increased amounts of aggregate mixtures of higher molecular mass were noticed. Besides the formation of those large aggregates, signals at 60 kDa indicative of PrP dimers were detected as described earlier (20, 34). Weak PrP-specific signals at 60 kDa were already present in untreated PrP 27–30 and particularly noticeable after heat treatment at medium temperatures. At further increased temperatures they disappeared in favor of aggregates of higher molecular mass. Higher amounts of oligomers after heat exposure were always paralleled by lower concentrations of monomer bands (data not shown).

**Inactivation of Prion Infectivity**—In addition to PrP 27–30 degradation, inactivation of prion infectivity was studied after heat treatment at temperatures of up to 200 °C under conditions otherwise identical to the degradation experiments. Compared with untreated samples, infectivity of heat-treated samples in the presence of different fat/glycerol/water mixtures varied considerably (Table 2 and Fig. 2). The different molecular environments induced differences in inactivation of up to 5 orders of magnitude. Assuming that the curves relating incubation time and titer also apply to heat inactivation in the presence of fat and glycerol (cf. below), log_{10} RF_{20} values can also be derived for inactivation of infectivity (Fig. 3). Comparing degradation and inactivation data, inactivation of prion infectivity was much more efficient than the degradation of PrP 27–30. Under all conditions examined, we found a roughly linear temperature dependence on the inactivation efficiency (Fig. 3). In some experiments, such as those in fat-containing mixtures, not all inoculated animals became sick. In these cases, mean values could not be calculated, resulting in imprecise interpolation, and significant values for activation energy could not be

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**TABLE 2**

| Treatment | No. infected / No. injected | Mean incubation period ± S.E. | Infectivity titer ± S.E. | log_{10} ID_{50} / sample |
|-----------|----------------------------|------------------------------|-------------------------|--------------------------|
| Negative control | 50% tallow/50% glycerol, 200 °C, 20 min | 0/5 | 74 ± 1 | 7.9 ± 0.5 |
| Positive controls | Starting titer | 10/10 | 68 ± 3 | 8.9 ± 1.4 |
| | After MeOH/CHCl₃ precipitation | 5/5 | 148 ± 32 | <2.0 |
| 100% fat, 20 min | 110 °C | 5/5 | 130 ± 4 | 2.3 ± 0.3 |
| | 140 °C | 5/5 | 126 ± 8 | 2.6 ± 0.6 |
| | 170 °C | 4/4 | 270 ± 31 | <2.0 |
| | 200 °C | 8/10 | 270 ± 31 | <2.0 |
| 90% fat/10% water, 20 min | 70 °C | 5/5 | 94 ± 4 | 5.2 ± 0.4 |
| | 80 °C | 5/5 | 93 ± 4 | 5.3 ± 0.2 |
| | 90 °C | 4/5 | 108 ± 2 | 3.9 ± 0.2 |
| | 100 °C | 5/5 | 118 ± 10 | 3.2 ± 0.7 |
| | 110 °C | 5/5 | 124 ± 8 | 2.7 ± 0.6 |
| | 120 °C | 5/5 | 208 ± 67 | <2.0 |
| | 140 °C | 5/5 | 207 ± 38 | <2.0 |
| | 170 °C | 5/5 | 116 ± 6 | 3.3 ± 0.3 |
| 90% fat/10% water, 90 °C | 60 min | 5/5 | 104 ± 1 | 4.2 ± 0.1 |
| | 180 min | 5/5 | 120 ± 6 | 3.0 ± 0.4 |
| | 540 min | 5/5 | 108 ± 4 | 3.9 ± 0.3 |
| 90% fat/10% water, 140 °C, 1 min | 5/5 | 102 ± 4 | 4.4 ± 0.4 |
| 90% fat/10% water, 90 °C, 20 min | 10⁻¹ | 3/4 | 151 ± 16 | 4.2 ± 0.0 |
| | 10⁻² | 3/5 | 182 ± 23 | 4.2 ± 0.0 |
| | 10⁻³ | 0/5 | 195 ± 40 |
| | 10⁻⁴ | 2/5 | |
| | 10⁻⁵ | 0/5 | |
| | 10⁻⁶ | 1/5 | |
| 75% fat/25% water, 90 °C, 20 min | 2.25 M urea, 0.5 M dithiothreitol | 5/5 | 93 ± 3 | 5.3 ± 0.4 |
| | 5/5 | 141 ± 3 | <2.0 |
| 50% fat/50% water, 90 °C, 20 min | 4/4 | 110 ± 10 | 3.8 ± 0.1 |
| 100% water, 20 min | 80 °C | 5/5 | 78 ± 4 | 7 ± 0.7 |
| | 90 °C | 5/5 | 85 ± 1 | 6.2 ± 0.2 |
| | 110 °C | 5/5 | 97 ± 1 | 5.0 ± 0.1 |
| | 140 °C | 5/5 | 141 ± 3 | <2.0 |
| | 170 °C | 5/5 | 231 ± 33 | <2.0 |
| 90% water/10% glycerol, 20 min | 80 °C | 5/5 | 80 ± 2 | 7.0 ± 0.3 |
| | 110 °C | 5/5 | 94 ± 2 | 5.3 ± 0.3 |
| | 140 °C | 5/5 | 114 ± 4 | 3.5 ± 0.3 |
| | 170 °C | 5/5 | 130 ± 7 | 2.4 ± 0.4 |
| 90% water/10% glycerol, 140 °C, 1 min | 5/5 | 124 ± 70 | 2.7 ± 0.5 |
| 100% glycerol, 20 min | 110 °C | 5/5 | 81 ± 2 | 6.8 ± 0.3 |
| | 140 °C | 5/5 | 91 ± 2 | 5.5 ± 0.2 |
| | 170 °C | 5/5 | 103 ± 3 | 4.3 ± 0.3 |
| | 200 °C | 4/5 | 145 ± 11 | <2.0 |

* Data were partly from Müller et al. (1).
* One hamster was found dead without clinical signs or PK-resistant PrP.
* Hamsters remaining healthy after 390 days were negative for PK-resistant PrP[sc] in Western blots.
* One hamster remaining healthy after 390 days was positive for PK-resistant PrP[sc] in Western blots and has therefore to be considered to be diseased.
Mechanism of Thermal Prion Inactivation

Heat inactivation of prion infectivity in different fat/glycerol/water mixtures. PrP 27–30 was heated at the indicated temperatures in a 50-ml pressure steel reactor under stirring at 150 rpm for 20 min in the presence of 90% fat and 10% water (C, dotted line); pure fat (■, dashed line); pure water (●, solid line); 90% water and 10% glycerol (△, dashed line); and pure glycerol (▲, dotted line). Samples were then inoculated into hamsters, and mean incubation times were calculated from diseased hamsters only. If data from animals not developing disease were included, incubation times for these samples (indicated by arrows) would be substantially higher. For reasons of clarity, the means ± S.E. were omitted here but are presented in Table 2.

The inactivation energy values ranged from 18 to 21 kJ/mol for pure glycerol, aqueous glycerol, and pure water.

In contrast to its protective role in PrP degradation, the presence of fat helped to destroy PrP infectivity. However, heat treatment at 200 °C in 100 or 90% fat resulted in shorter incubation times, reflecting decreased prion inactivation, compared with similar treatment at lower temperatures (Fig. 2). This phenomenon is most likely a consequence of the production of appreciable amounts of glycerol because of fat hydrolysis at 200 °C. According to this assumption, values for inactivation in fat and in glycerol converge for experiments run at 200 °C. Consequently, the protective effect of glycerol seems to dominate not only the influence of water but also that of fat. Glycerol shows qualitatively similar protection against PrP degradation and inactivation as notable at temperatures ≥170 °C (Fig. 1B and Fig. 3, B and D).

Because of concerns about the reliability of incubation time interval assays after physical or chemical treatment (35–38), we conducted an end point dilution titration for heat treatment at 90 °C for 20 min in a 90% fat and 10% water mixture, which yielded an infectivity titer of 4.2 log ID50/ml (Table 2). An incubation time interval assay after heat treatment under identical conditions resulted in an infectivity titer of 3.9 log ID50/ml. Because no significant difference exists between these two titers, we conclude that the calculation of infectivity titers under these or similar conditions from incubation times is reliable.

Optimization of Low Temperature Inactivation of Prions by Fat/Water Mixtures and Other Ingredients—From our observations on the enhancing effect of fat on prion inactivation, we wished to determine whether different amounts of fat would yield the same results. We studied inactivation in the presence of 50, 75, or 90% fat but did not find substantial variations in mean incubation times (Table 2). After heat treatment for 20 min at ≤90 °C, reductions in infectivity titer of up to 5 log10 ID50 were achieved in presence of different amounts of fat. In pure water, the infectivity titer was reduced by 3 log10 ID50 only. When we combined the inactivating effect of fat with denaturants, we found that an aqueous mixture of fat, urea, and dithiothreitol reduced the infectivity titer by up to 7 log10 ID50 (Table 2).

Kinetics of PrP 27–30 Degradation and Prion Inactivation—To determine the kinetics of prion degradation and inactivation, we measured PrP levels at different time points during heat treatment. For degradation experiments, we treated samples at 120 °C in the presence of 90% glycerol and 10% water. We found that the amount of PrP declined rapidly in the first 20 min of incubation, which was followed by gradual degradation over the remaining time (Fig. 4, 60, 80, and 540 min). Of the initial PrP levels, 75–95% was degraded during heating and cooling phases (Fig. 4).

For prion inactivation studies, we treated samples at 90 °C in the presence of 90% fat and 10% water with incubation times of 20, 60, 180, or 540 min. Similar to the kinetics of degradation, we found a biphasic inactivation; infectivity rapidly decreased after 20 min, with residual infectivity diminishing very gradually over the remaining time points (Fig. 4). The results from other solution conditions also exhibited similar biphasic kinetics (Table 2). These findings indicate that heat destruction of prions includes two distinguishable phases as follows: a fast phase during which the majority of prions is destroyed followed by a slow phase of reduction.

Distribution of PrP 27–30 between Fatty and Aqueous Phases—Because fat and water are immiscible and both affect degradation of PrP 27–30 as well as inactivation of prion infectivity, the distribution of PrP between the phases is of particular relevance. The amount of PrP 27–30 in each phase of a thoroughly stirred mixture of equal volumes of bovine edible tallow and water was analyzed at different temperatures after phase separation. Between both phases, an interphase of insoluble protein was detected, which was analyzed separately. At room temperature, nearly all PrP 27–30 was detected within the interphase (Table 3). Only at higher temperatures were progressively increased amounts of PrP 27–30 found within the water phase. At 100 °C, about 25% of the total PrP 27–30 content was enriched in the water phase. In contrast, less than 1% of PrP 27–30 was detected within the fat phase at all temperatures examined. We conclude that PrP 27–30 tends to accumulate in the interphase rather than in either the lipophilic or the aqueous phase. Notable amounts of PrP 27–30 were found in the water phase only when salt or SDS was added (Table 3).
Solubility and Aggregation of PrP 27–30 after Heat Treatment—
To determine the effect of heat treatment on the solubility of PrP 27–30, differential ultracentrifugation was performed. Samples sonicated in the presence of 0.3% SDS yielded ~80% of total PrP in the supernatant after ultracentrifugation, which reflects soluble PrP (Table 4). However, an additional heat treatment (100–
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FIGURE 4. Inactivation of prion infectivity and degradation of PrP 27–30 are biphasic processes. Degradation of PrP 27–30 was determined by gel electrophoretic analysis after exposure to 120 °C in 90% water and 10% glycerol for the incubation times indicated (A). The value for degradation after heating and cooling without remaining at target temperature is depicted at 1 min. Inactivation of prion infectivity was determined by bioassays after exposure to 90 °C in 90% fat and 10% water for the incubation times indicated (B).

TABLE 3
Distribution of PrP 27–30 in fat, water, and the fat-water interphase

| Treatment | Fat phase | Fat-water interphase | Water phase |
|-----------|-----------|----------------------|------------|
| 25 °C     | 0         | 100                  | 0          |
| 40 °C     | 0         | 92.5 ± 0.5           | 2.5 ± 0.5  |
| 60 °C     | 0         | 92.0 ± 0.5           | 8.0 ± 0.5  |
| 80 °C     | 1.1 ± 1.0 | 93.1 ± 2.8           | 5.8 ± 2.4  |
| 100 °C    | 0         | 73.2 ± 1.1           | 26.8 ± 1.1 |
| 40 °C, 250 mM NaCl | 6.1 ± 5.2 | 46.6 ± 3.0           | 47.3 ± 3.1 |
| 40 °C, 1% SDS | 3.4 ± 3.4 | 13.0 ± 9.4           | 83.6 ± 8.4 |

TABLE 4
Effect of heat on the solubility of PrP 27–30

The solubility of PrP 27–30 after heat treatment was determined by differential centrifugation at 100,000 × g for 1 h followed by gel electrophoresis, Western blotting, and densitometric quantification. Heat treatment was performed at <125 °C for 20 min. Insoluble and soluble PrP were determined as the percentages of total PrP found in the pellet and supernatant, respectively, after ultracentrifugation (29).

| Treatment                        | Insoluble PrP | Soluble PrP |
|----------------------------------|--------------|-------------|
| None                             | 100          | 0           |
| Heat exposure                     | 100          | 0           |
| Sonication without SDS           | 100          | 0           |
| Heat exposure, sonication without SDS | 100          | 0           |
| Sonication in 0.3% SDS           | 20.7 ± 2.8   | 79.3 ± 2.7  |
| Heat exposure, sonication in 0.3% SDS | 76.7 ± 9.4   | 23.3 ± 9.4  |

125 °C for 20 min) prior to sonication in 0.3% SDS resulted in a majority of insoluble PrP 27–30. Heat treatment impaired the solubilizing effect of SDS and increased the insolubility of PrP.

As described above, heat exposure induced the formation of aggregates, indicated by the high molecular mass bands in SDS gels. To confirm this observation, we used negative-stain electron microscopy to characterize the ultrastructure of samples after heat treatment in the presence of pure water, 10% aqueous glycerol, and 90% aqueous fat (Fig. 5). In all cases, we observed that the fibrillar ultrastructure of PrP 27–30 was converted to amorphous aggregates. The loss of ordered, fibrillar substructure correlated with increasing temperature, indicating that the transition was temperature-dependent. In the presence of 90% fat, the ultrastructure of PrP 27–30 was more readily converted to amorphous aggregates (Fig. 5K), compared with samples treated at the same temperature in pure water (Fig. 5D). Additionally, lower temperatures were sufficient to form amorphous aggregates in the presence of fat (Fig. 5J). In contrast, glycerol appeared to protect partially the ultrastructure of prion rods against heat denaturation (Fig. 5, G and H), confirming the electronegativity and solubility results.

In another method to visualize the ultrastructure of the fibrils, we stained samples with Congo red dye, which binds to amyloid and exhibits green-orange birefringence under cross-polarized light. For samples treated at <100 °C, we observed amyloid structure (Fig. 6). At higher temperatures (≥100 °C), we observed protein areas with dark appearance in cross-polarized light, i.e. without birefringence, indicating β-sheet-poor regions, which coincided with increased aggregate size (Fig. 6). Heat treatment at 160 °C resulted in only a few small aggregates and no birefringence. From these observations, we conclude that heat treatment induced a transition from highly structured prion rods to amorphous aggregates with a less ordered substructure.

Using another technique to analyze the fibrillar structure of PrP 27–30, we monitored the presence of amyloid fibrils using the ThT fluorescence assay (Fig. 7). At temperatures of ≥80 °C, a significant reduction in the ThT fluorescence of fibrils was observed. In contrast, the amount of degraded PrP, indicating an intact PrP 27–30 backbone, remained stable until temperatures exceeded 100 °C. This difference between the fibrillar structure and PrP backbone integrity confirmed the presence of an intermediate PrP 27–30 form (i.e. amorphous aggregate), which has less fibrillar structure but an intact protein backbone.

High Temperatures Decrease β-Sheet-like Secondary Structure—To verify whether the heat-induced changes of fibrillar substructure are accompanied by alterations in secondary structure, CD spectra were recorded. PrP 27–30 samples were heated in 10 mM sodium phosphate (pH 7.2) to temperatures of 25, 100, 120, 140, or 160 °C (Fig. 8). To avoid degradation, samples were incubated for only 30 s at the final temper-
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Heat Treatment Decreases PK Resistance—To determine the effect of heat treatment on the protease resistance of PrP 27–30, we compared the effect of PK digestion on the intensities of SDS gel bands and ThT fluorescence with and without prior heat treatment. For unheated PrP 27–30, the intensity of PrP bands and the ThT fluorescence intensity decreased simultaneously upon PK digestion, suggesting that fibrillar substructure and PrP backbone integrity are reduced concurrently. After incubation for several hours, both the PrP band intensity in SDS gels and the ThT fluorescence intensity were reduced to less than 25% of the initial signal. During digestion for further prolonged incubation times, the intensities decreased much more slowly, reflecting the tailing phenomenon of prion destruction as described above (data not shown).

After exposure to heat up to 100 °C, SDS gel band intensities demonstrated that the PK resistance of PrP 27–30 was reduced by 20–30% in comparison with unheated samples. In contrast, ThT fluorescence intensities of PrP 27–30 during PK digestion did not differ in heated and not heated samples (data not shown). From these observations, we conclude that the PrP 27–30 fraction in fibrillar structure is also the PK-resistant portion.

DISCUSSION

More insight into the molecular mechanism of in vivo prion amplification has become available only recently (6, 39, 40), and the components of the living cell involved in this process are unknown. Also, methods of prion decontamination are based on empirical grounds rather than on a molecular interpretation. Prions are unusually resistant to heat, irradiation, chemical disinfectants, and enzymatic digestion. Furthermore, the inactivation process is heterogeneous and depends upon the hydration of prions. Prion strains, the existence of which is difficult to interpret, have been characterized by different resistances to inactivation (22, 41). Additionally, particularly resistant subpopulations of prions exist. The molecular environment, such as the presence of lipids or a polyglucose scaffold, can have a profound influence on inactivation (9, 20).

We initiated this study to analyze prion inactivation during hydrolytic fat splitting, a basic industrial process, and to deliver a risk assessment for BSE contamination (1). Toward this end, we characterized systematically the thermal inactivation of pri-
ons and assessed the effect of fat, fatty acids, and glycerol in this process to understand the mechanism of inactivation.

**Methodological Aspects**—We chose to use PrP 27–30 for our studies because it is the most stable fraction of infectious PrPSc (8, 42–45) and therefore represents the worst case scenario with respect to decontamination of prions. We isolated PrP 27–30 from Syrian golden hamsters infected with the hamster-adapted, 263K scrapie strain (7, 24, 42). These animals yield short incubation times and high infectivity titers in the brain, which make them a widely accepted, well validated, and highly sensitive model for prion infection. Most importantly, PrP 27–30 isolated from these animals is highly resistant to decontamination methods (46–52).

**Fat Stabilizes PrP 27–30 against Degradation but Destabilizes Prion Infectivity against Inactivation**—At least four types of interactions compete during heat treatment, including hydrophobic intra- and intermolecular interactions of PrP aggregates, hydrophobic PrP-fat interactions, hydrophilic intra- and intermolecular interactions of PrP aggregates, and hydrophilic PrP-water interactions, including hydrolytic activity. These interactions are characterized by different temperature dependences. The enhanced stability of the PrP 27–30 backbone in the presence of fat may be due to additional hydrophobic interactions saturating the PrP surface. The hydrophobic interactions between PrP and fat molecules increase with temperature, which maintains the protective effect even after structural denaturation. The hydrolytic activity of water exhibits the strongest increase with temperature. Fat restricts hydrolytic activity by excluding water molecules. However, with increasing temperature, hydrolytic activity overcomes the protective effect of fat in water/fat mixtures.

In contrast to its stabilizing effect against PrP 27–30 degradation, fat destabilizes prion infectivity over the whole temperature range analyzed (Fig. 2). Consequently, fat appears to compete with the intra- and intermolecular hydrophobic interactions of prions, which are responsible for the infectious structure. The effect might be similar to that of SDS, which is known to impose hydrophobic interactions and, at the same time, to reduce infectivity (53). Because hydrophobic interactions increase with temperature, denaturation of PrP 27–30 by fat molecules is gradually facilitated, whereas the PrP 27–30 backbone is concurrently protected, at least at temperatures below 110 °C. At temperatures ≥110 °C, the hydrolytic activity of water dominates the backbone-protecting effect of fat, which results in degradation efficiencies similar to those from pure water or a fat/water mixture.

The competition between hydrophobic and hydrophilic interactions, particularly at room temperature, became obvious from the finding that all PrP 27–30 molecules were concentrated in the fat-water interphase and tend to migrate to the water phase only at higher temperatures (Table 4). The PrP

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**FIGURE 7.** Lower treatment temperatures induce the loss of PrP fibrillar structure, whereas higher temperatures are needed to degrade PrP. Fibrillar structure (dotted line) decreases at lower treatment temperatures compared with PrP degradation (solid line). Undegraded PrP was determined by densitometric analysis of Western blots; PrP retaining fibrillar structure was determined by ThT assays.

**FIGURE 8.** Heat-induced conformational transitions of PrP 27–30 as determined by CD spectroscopy. Samples of 1 μg/μl PrP 27–30 were treated in 1.5-ml Eppendorf tubes without stirring for 30 s at 25 °C (solid line), 100 °C (◇), 120 °C (●), 140 °C (△), and 160 °C (○) followed by sonication at 180 watts for 5 min. As a control, PrP 27–30 was sonicated at 180 watts for 5 min in the presence of 0.3% SDS without prior heat exposure (dotted line). CD spectra were then recorded in 10 mM sodium phosphate (pH 7.2) at room temperature.
27–30 fraction in the water phase increased also in the presence of salt or detergents, which is probably because of denaturation of the PrP molecule. The phase distribution observed is in accordance with water molecules displacing fat molecules at higher temperature as well as with findings reported by others, assuming that prion infectivity exists in a water phase rather than in a fat phase (1, 54).

**Fat as a Disinfectant**—Unexpectedly, pure fat and fat/water mixtures offer mild prion inactivation. When prion inactivation was determined at temperatures below 100 °C, fat/water mixtures reduced prion infectivity by 5.1 log$_{10}$ ID$_{50}$. In contrast, an inactivation factor of only 2.7 log$_{10}$ ID$_{50}$ was achieved for water. The addition of 2.25M urea and 0.5M dithiothreitol further inactivated prion infectivity, resulting in a reduction of 6.9 log$_{10}$ ID$_{50}$ (Table 2). These observations can be explained by structural denaturation rather than degradation. In contrast to standard prion disinfectants, a mixture of water, fat, and urea at temperatures below 100 °C can be used as a mild prion disinfectant, which can be used to sterilize medical and manufacturing equipment. Because of the need for alternative sterilization procedures, further assessment of the effects of this mild protocol on prion infectivity will be worthwhile.

Partial PK resistance is used as surrogate marker for prion infectivity. Both PK resistance and insolubility of PrP 27–30 result from multimeric aggregation, which is associated with an increased β-sheet-rich secondary structure. In prion rods, a fibrillar substructure based on β-sheets correlates with prion infectivity (5). Treatments altering the content of the β-sheet-rich structure and multimeric aggregation ought to be accompanied by a reduction of infectivity (53). In complete accordance with this expectation, our experiments demonstrate prion destruction occurring in at least two steps as follows: (i) denaturation of PrP to an intermediate form, which is characterized by a loss of structure responsible for prion infectivity, and (ii) the complete loss of structure and backbone degradation of PrP (Fig. 9). The first step occurs at lower temperatures, whereas the second step is observed at higher temperatures. Most but not all prion infectivity is destroyed during the first step.

For prion destruction, the following three effects have to be considered: (i) displacement of solvent molecules, which stabilize or destabilize prions; (ii) denaturation of prion structure; and (iii) degradation of the PrP backbone. Each effect adds to an activation energy that must be overcome for complete prion destruction. According to the literature, the total stability of

**Glycerol Stabilizes PrP 27–30 against Both Degradation and Inactivation**—Glycerol exerts a protective effect on both the polypeptide backbone integrity of PrP 27–30 as well as prion infectivity, which was observed at all temperatures examined. Glycerol is a known reagent for stabilization of bacteria and proteins. For long term storage at low temperatures, 20 and 66% glycerol are added to *Escherichia coli* cells and proteins, respectively. Because of its ability to act as both a hydrogen bond acceptor and donor, glycerol replaces water at least partially in its structure-forming function. It drastically increases viscosity and thus decelerates unfolding reactions, although its effect on the folding rate constant of proteins is small (53). Furthermore, hydrolysis of the peptide bonds is diminished in the presence of glycerol. Glycerol might replace water also in the cage structure or destroy the cage on hydrophobic areas of the protein surface, leading to a stabilizing entropy gain. In addition to protection against thermal impact, glycerol has been shown to protect PrP 27–30 infectivity against inactivation by hexafluoroisopropanol, a known α-helix-inducing solvent (33).

**Mechanism of Heat Inactivation**—Partial PK resistance is used as surrogate marker for prion infectivity. Both PK resistance and insolubility of PrP 27–30 result from multimeric aggregation, which is associated with an increased β-sheet-rich secondary structure. In prion rods, a fibrillar substructure based on β-sheets correlates with prion infectivity (5). Treatments altering the content of the β-sheet-rich structure and multimeric aggregation ought to be accompanied by a reduction of infectivity (53).

**Mechanism of Thermal Prion Inactivation**

![Proposed mechanism for thermal destruction of prions](image-url)
Mechanism of Thermal Prion Inactivation

Monomeric proteins amounts to a $\Delta G^0$ value of $\pm 15$ to $\pm 50$ kJ/mol. This stability increases for a polymeric substrate, such as with the fibrillar assembly of PrP $27–30$, adding to the activation energy barrier for prion destruction. All activation energies obtained during inactivation of the majority of prion infectivity, i.e. the first step, were $<21$ kJ/mol (Fig. 3). Such a low value indicates that a denaturation process featuring a loss of mainly noncovalent bonds has occurred. During the second step, we obtained higher values from $20 \pm 5$ to $51 \pm 10$ kJ/mol, which suggests that a cooperative reaction featuring the breaking of covalent bonds has occurred. It should be noted that a superimposition of several effects contributes to the activation enthalpies obtained.

A quantitative comparison of the loss of $\beta$-sheet-based fibrillar structure with the degradation of the PrP backbone indicated clearly that heat treatment alters the structure of PrP first (Figs. 3 and 7). This analysis demonstrated a temperature difference between $\beta$-sheet reduction and backbone degradation of about 30 K (Fig. 8). This loss of fibrillar structure was characterized by additional biophysical techniques. Gel electrophoresis and differential ultracentrifugation after sonication in SDS demonstrated that solubilization of PrP aggregates became less effective. Electron microscopy confirmed a tendency to form larger aggregates. In correlation with electron microscopy, Congo red birefringence and ThT fluorescence confirmed the loss of fibrillar structure during the first transition.

From CD spectra, we conclude that the secondary structure was altered drastically during the first transition (Fig. 8). At temperatures $>100$ °C, a strong negative peak at 188 nm and two smaller negative peaks at 210–212 and 229–231 nm appeared, respectively. The spectra cannot be interpreted by superimposition of known spectra from the literature. The negative peak at 188 nm in addition to a slight red shift of the positive peak by 2.5 nm might indicate the emergence of one or several $\beta$-turn motifs. The additional negative bands at 210–212 and 229–231 nm, however, cannot be correlated to known secondary structure elements. Because CD spectroscopy can be evaluated quantitatively only for soluble proteins, an experimental artifact because of light scattering cannot be excluded for our highly insoluble samples. Artifacts, however, should be more pronounced in the range below 210 nm because scattering increases with decreasing wavelength, and protein absorption is much higher below 210 nm. Consequently, we conclude that the appearance of the two negative peaks at 210–212 and 229–231 nm indicates a new, as-yet unknown structure.

Concomitant to the loss of fibrillar structure, prion infectivity is reduced drastically but not completely abolished; residual infectivity remains. This clearly suggests that infectivity of prion rods depends on an intact fibrillar structure containing $\beta$-rich subunits. At higher temperatures, PrP $27–30$ aggregates dissociate, which facilitates the cleavage of the covalent PrP backbone and inactivates prion infectivity completely (Fig. 9). In the presence of glycerol, degradation is impaired over the whole temperature range because of a displacement of water molecules that are necessary for hydrolysis.

**Multistep Inactivation or Subpopulations with Different Stabilities?**—The prion inactivation curve (Fig. 4) exhibits a rapid decline in prion titer followed by an extremely subtle and slow decline. Similar tailing effects have been observed earlier (14), which have been suggested to result from a population of infectious agents with heterogeneous properties, in particular, a subpopulation of higher heat resistance. Although the existence of in vivo heterogeneity cannot be excluded, we consider it more likely that the heat-resistant infectivity results from the first transition as described above. The rapid decline in prion infectivity appears to result from a comparatively fast denaturation during the first step, which yields an intermediate state with a more highly aggregated and therefore even more heat-resistant core. Thus, partially denatured and more highly aggregated PrP molecules are assumed to protect core prion particles responsible for residual infectivity. In Western blots, PK resistance was reduced slightly after exposure to heat. This finding is in accordance with Garcia et al. (55), who claim that preheated PrPSc is digested more efficiently. When infectivity and PK resistance were related to the degree of fibrillization, we found that the loss of fibrillar organization, based on $\beta$-sheet-rich secondary structure, decreased PK resistance. However, amorphous aggregates also exhibited substantial residual PK resistance, and $\beta$-sheet structure and PK resistance have been induced in other studies without acquiring infectivity (56). Thus, infectious and noninfectious fibrillar forms of PrPSc clearly exist (57).

In summary, heat treatment induced an intermediate form of amorphous PrP particles that are characterized by reduced solubility, increased aggregation, reduction in $\beta$-sheet and fibril contents, lower PK resistance, and diminished but residual infectivity. This residual infectivity was destroyed by increased treatment temperatures, which caused the disassembly of aggregates and PrP backbone hydrolysis (Fig. 9). This intermediate state observed in our studies might explain the phenomenon of resistant subpopulations.

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**REFERENCES**

1. Müller, H., Stitz, L., and Riesner, D. (2006) *Eur. J. Lip. Sci. Technol.* **108**, 812–826.
2. Prusiner, S. B. (1982) *Science* **216**, 136–144.
3. Prusiner, S. B., Tremblay, P., Safar, J., Torchia, M., and DeArmond, S. J. (1999) *Prion Biology and Diseases*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
4. Cohen, F. E. (1999) *J. Mol. Biol.* **293**, 313–320.
5. Riesner, D. (2007) in *Prions in Humans and Animals* (Hörmann, B., Riesner, D., and Kretzschmar, H., eds) pp. 104–118, Walter de Gruyter, Berlin.
6. Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (2004) *Science* **305**, 673–676.
7. McKinley, M. P., Meyer, R. K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A., and Prusiner, S. B. (1991) *J. Virol.* **65**, 1340–1351.
8. Klein, T. R., Kirsch, D., Kaufmann, R., and Riesner, D. (1998) *Biol. Chem.* **379**, 655–666.
9. Dumpitak, C., Beekes, M., Weinnmann, N., Metzger, S., Winkhofer, K. F., Tatzelt, J., and Riesner, D. (2005) *Biol. Chem.* **386**, 1149–1155.
10. Taylor, D. M. (2004) *Contrib. Microbiol.* **11**, 136–145.
11. Wilesmith, J. W., Ryan, J. B. M., and Atkinson, M. J. (1991) *Vet. Rec.* **128**, 199–203.
