Crude brain homogenates of terminally diseased hamsters infected with the 263K strain of scrapie (PrPSc) and purified prion fibrils were heated or pressurized at 800 megapascals and 60 °C for 2 h in different buffers and in water. Prion proteins (PrP) were analyzed for their proteinase K resistance in immunoblots and for their infectivity in hamster bioassays. A notable decrease in the proteinase K resistance of unpurified prion proteins, probably because of pressure-induced changes in the protein conformation of native PrPSc or the N-truncated PrP-(27–30), could be demonstrated when pressurized at initially neutral conditions in several buffers and in water but not in a slightly acidic pH. A subsequent 6–7 log10 reduction of infectious units/g in phosphate-buffered saline buffer, pH 7.4, was found. The proteinase K-resistant core was also not detectable after purification of prions extracted from pressurized samples, confirming pressure effects at the level of the secondary structure of prion proteins. However, opposite results were found after pressurizing purified prions, arguing for the existence of pressure-sensitive β-structures (PrPSc Δprres) and extremely pressure-resistant β-structures (PrPSc Δprres). Remarkably, after the first centrifugation step at 540,000 × g during isolation, prions remained proteinase K-resistant when pressurized in all tested buffers and in water. It is known that purified fibrils retain infectivity, but the isolated protein (full and N-truncated) behaved differently from native PrPSc under pressure, suggesting a kind of semicrystalline polymer structure.

Transmissible spongiform encephalopathies (TSEs) are associated with the accumulation of an isoform of the cellular prion protein, designated PrPSc in brain. Evidence indicates that misfolding of the cellular isoform into a β-sheet-rich aggregated pathogenic self-propagating multimer is the main step of infection (1). Even though detailed studies on the structure of the cellular prion protein (PrPSc) have been performed (e.g. with NMR (2)) and the amino acid sequence is known to be preserved in both isoforms, many features of prions are intriguing because PrPSc, causing TSE is known to be insoluble. Up to now, studies of the PrPSc structure after expression of recombinant cellular prions (2), the purification of native PrPSc after immunoaffinity chromatography and elution at basic pH (3), or the isolation of proteinase K-resistant materials (iPrPres) (4) have been prerequisites for performing studies on the structure of infectious prions. Thus, existent models are mainly derived from spectroscopic data from studies of such structures. Moreover, treatments with heat, salts, denaturants, and extreme pH revealed new conformational states of prions that were different from native. And so far, when a conformational change leads to a reduction in infectivity, inactivation is irreversible (5).

The β-rich secondary structure most likely provides infectious prions with a tremendous resistance to conventional autoclaving, which leads to the need for severe inactivation strategies, such as autoclaving at 133 °C for at least 20 min, the combination of alkali and heat, or the use of hypochlorite solutions. Those strategies, although efficient, are excessively aggressive, cause a consequent loss in quality and texture in the treated tissues, and are not applicable to foodstuffs. Consequently, our interest in assessing the effects of unconventional milder technologies on prion stability and prion infectivity arises from the necessity of finding milder techniques that provide alternative sterilization procedures for risk materials.

High hydrostatic pressure is a mild processing technology with a promising potential in food pasteurization and sterilization. By avoiding heat, undesirable alterations in foods such as vitamin loss and changes in taste and color can be minimized (6, 7). Moreover, the use of high pressure to assist thermal food processing, exploiting the unique effect of adiabatic heating and cooling, has already been reported effective in the inactivation of prion proteins when the temperature is shifted up to 135–142 °C (8). Furthermore, high hydrostatic pressure is a thermodynamic parameter providing useful information when studying protein denaturation and reaction mechanisms (9). Protein response to pressure is driven by the volume change of the protein solvent system, which is associated with conformational changes, protein folding-unfolding, and aggregation. Pressure shifts the conformational transitions of a protein toward the state with the lower volume; therefore, unexpected effects that are different from temperature or chemical denaturation can be seen under pressure (10). The largest contribution of pressure is at the level of weak (non-covalent) interactions; it is accepted that hydrogen bonds are slightly stabilized under pressure and that pressure disfavors hydrophobic and electrostatic interactions, which are mainly responsible for protein aggregation (11). For example, mild high pres-
sures treatments induce the conversion of the non-infectious cellular prion protein into a protease K-resistant form (12, 13). Additionally, an impressive reduction in the infectivity of native hamster prion proteins has been reported in PBS buffer (14) at relatively low pressures and with temperatures below customary sterilization parameters. An equivalent reduction in the protease K resistance was observed at pressure-holding times that would still be realistic (e.g., 30 min) (15) when trying to employ this technology at the industrial level. However, this irreversible effect is astonishing because usually only extremely high pressures are able to rearrange secondary structures of proteins, and the pressure stability of hydrogen bridges and \( \beta \)-sheets should remain resistant to such high pressure treatments.

This work was a phenomenological study using the protease K resistance of native prion proteins as an analytical measure for the effects of pressure, contrasting data with the remaining infectivity and aiming at a first estimation of the effects of pH on the structure of prion proteins under pressure. Additionally, pressure effects on brain homogenates containing \( N \)-truncated prions were analyzed to verify the recently postulated pressure-induced protease K sensitivity of prion proteins after treatments at 60 °C (14). Furthermore, this work also focused on the assay of the protease K resistance of \( N \)-truncated purified prions after pressure treatments in different matrices, aiming at establishing whether those are acceptable models for explaining pressure effects on native prions. Results were expected to be useful as a basis for the application of high pressure as an alternative technology for the mild inactivation of infectious prions at pressures below the known denaturing conditions.

**EXPERIMENTAL PROCEDURES**

**Native PrPSc**—Experiments on native PrP\textsuperscript{Sc} were performed with several brain pools of the 263K strain of the hamster-adapted scrapie agent. Brains from terminally diseased hamsters containing PrP\textsuperscript{Sc} were homogenized (10\textsuperscript{1} w/v in) in phosphate-buffered saline (8 mM phosphate buffer, 120 mM NaCl), pH 7.4, 50 mM Tris/HCl buffer, pH 7.0, PBS buffer, pH 5.6, or biddistilled sterile water in a FastPrep cell disrupter (Qiagen). Sets of duplicate samples were each heated at 60 °C or pressurized at 800 MPa and 60 °C independently.

**PrP-(27-30)**—Another set of experiments was performed on the \( N \)-truncated prion protein (PrP-(27-30)) after digestion with protease K (73 \textmu g/ml for 60 min at 37 °C) of hamster brain homogenates (10\textsuperscript{1} w/v) in PBS) infected with the 263K strain of scrapie. Duplicate samples were heated at 60 °C or pressurized at 800 MPa and 60 °C independently after inactivation of the protease at 95 °C for 10 min.

**Purification of iPPrP**—Isolation of iPPrP was performed following a slightly modified version of the method proposed in Ref. 16. 1.0 ml of brain homogenate (10\textsuperscript{1} w/v) in PBS buffer, pH 7.4, pressurized at 800 MPa at 60 °C for 120 min or one unpressurized brain (1 g) was mixed in a solution containing 10% \( N \)-lauroyl sarcosine sodium salt, pH 7.4, and centrifuged for 10 min at 22,000 \( \times \) g. The supernatant was centrifuged again for 20 min at 54,000 \( \times \) g and the pellet was mixed with a solution containing 1% \( N \)-lauroyl sarcosine sodium salt and 10% sodium chloride, pH 7.2, and centrifuged for 25 min at 540,000 \( \times \) g. The pellet was digested during 60 min with a protease K solution (10 \mu g/ml final concentration) to remove non-resistant proteins. Afterward, the supernatant was centrifuged again for 10 min at 22,000 \( \times \) g and resuspended in 500 \mu l of PBS, pH 7.4, 50 mM Tris/HCl, pH 7.0, or distilled water. The protease was then inactivated with heat at 95 °C for 10 min. Sets of duplicate samples were each heated at 60 °C or pressurized at 800 MPa and 60 °C independently after protease K digestion of the isolates or, in a second approach, after every centrifugation step.

**High Pressure Experiments**—Total volumes of 250 ml of hamster brain homogenate (10\textsuperscript{1} w/v) or iPPrP\textsuperscript{Sc} pressurized in a hydraulic press U 101 (Polish Academy of Sciences, Warsaw, Poland). U 101 is a manually operated twin piston hydraulic press (100-mm piston length, 80-mm piston movement). The vessel is a cylinder made of steel with an 16-mm inside diameter and a 150-mm height. The piston position is monitored with a linear transformer transducer, and the pressure measuring unit is an in-vessel manganese pressure gauge; both are digitally displayed. The pressure-transmitting medium was a 7:3 mix-ture of petroleum ether (boiling point 80–100 °C) and hydraulic oil (viscosity 32). Polyethylene caps were perfectly isolated in heat-sealed polyethylene-coated aluminum bags prior to pressurization. The effects of adiabatic heating were minimized because of the long pressure rise necessary to achieve the highest pressures (at least 150 s above 700 MPa) and by continuous control of the temperature in the vessel using a thermostat (Polystat, Huber, Germany) coupled to the cylinder. Holding time was always 2 h.

Detection of Hamster-Prion Proteins on Immunoblots—After pressure treatments, samples (15 μl of iPPrP\textsuperscript{Sc} or brain homogenates containing PrP\textsuperscript{Sc} or PrP-(27-30)) were digested with protease K (73 \mu g/ml final concentration in brain homogenates and 46 \mu g/ml in iPPrP\textsuperscript{Sc}, Sigma) for 60 min at 37 °C. Triplicates of each sample were examined. Positive controls were untreated samples. After denaturation at 95 °C, the samples were separated by electrophoresis on 10% polyacrylamide gels. Separated proteins were electrotransferred to polyvinylidene difluoride membranes (0.2-μm pore, Bio-Rad). Surplus binding sites were blocked by incubating the membranes in 5% nonfat dry milk and 5% bovine serum albumin in PBS with 0.1% Triton. Membranes were then incubated with the anti-hamster PrP\textsuperscript{Sc} 3F4 monoclonal antibody (Signet Laboratories) diluted to 1:5000 in blocking solution. After incubation with antibodies, membranes were washed extensively with PBS with 0.1% Triton and incubated with peroxidase-labeled goat anti-mouse IgG (1:3000 (Oncogene) in PBS with 0.1% Triton. After further washing, the membrane was visualized on a high-intensity hyperfilm\textsuperscript{TM} ECL\textsuperscript{TM} (Amersham Biosciences) using the enhanced chemiluminescence detection system.

**Infectivity Bioassays**—Infectivity bioassays were performed with the temperature and/or pressure-treated materials following an incubation time interval protocol (17). After pressurization, 10-fold dilutions were intracerebrally inoculated into groups of four or five weanling hamsters. The lowest amount inoculated was 1.5 mg of brain homogenates or 15 \mu l of isolated resuspended iPPrP\textsuperscript{Sc}. Untreated or heated samples (2 h at 60 °C) of the same brain homogenates were examined as controls. Hamsters were observed for clinical signs of scrapie during a period of 270 days and sacrificed immediately after showing disease symptoms.

**RESULTS**

**Proteinase K Sensitivity of Native Prion Proteins**

Proteinase K Sensitivity and Infectivity of Native PrP\textsuperscript{Sc} Pressurized in Different Buffers—Crude brain homogenates positive for the 263K strain of scrapie were submitted to 800 MPa at 60 °C for 2 h in different buffers or in water. After treatment, the samples were incubated with (Fig. 1, \( K \)) or without proteinase K to determine the remaining amount of PrP\textsuperscript{Sc} to proteolytic digestion. Immunoblots with the specific antibody 3F4 are reported in Fig 1. Blots of heated samples only were all positive for PrP\textsuperscript{Sc} after 60 °C for 2 h and showed no difference from the untreated controls (results not shown).

The accumulation of \( \beta \)-rich proteinase K-resistant aggregates is associated with pathological prions and used for TSE diagnostics. Results here prove that the native structure of PrP\textsuperscript{Sc} was not fully retained when pressurized in the neutral pH range, for example in Tris/HCl, pH 7.0 (Fig. 1a, PK), a pressure stable buffer, but also not in pressure non-stable matrices such as PBS buffer, pH 7.4 (Fig. 1b, PK), or biddistilled water (Fig. 1c, PK). The sensitivity of the prion structure to pressure is evident because the proteinase-resistant core of native PrP\textsuperscript{Sc} was not detected on immunoblots after pressure treatment in any of the mentioned matrices. In opposition to this observation, an extensive fraction of native PrP\textsuperscript{Sc} remained proteinase K-resistant after pressurization in PBS, pH 5.6 (Fig. 1d, PK), because the resistant prion protein was detected, illustrating a protective effect because of the acidic pH, which is not reverted during pressurization. The remarkable phenomena found in homogenates at initially neutral pH permit the postulation of effects on the proteinase-resistant core of
prion proteins, although these are not easy to explain because of the more likely pressure-inaccessible hydrogen bridges (change in volume nearly zero) stabilizing the protein secondary structure (10).

Conditions that reduce β-sheet structure are accompanied by diminution of infectivity. Consequently, if only samples containing proteinase K-resistant prions are considered pathological, the homogenates pressurized in Tris or PBS buffer or in water should have lost potential infectivity. To test this hypothesis, bioassays were performed in hamsters by intracerebral inoculation of the treated PBS homogenates. Typically, signs of scrapie become prominent 70–90 days postinoculation. Animals in this experiment were observed for up to 270 days. Untreated crude homogenates and those treated at 60 °C without pressure led to terminal TSE disease after 75–93 days (Table I). Typical incubation times, however, were considerably increased after inoculation of homogenates treated at 800 MPa (Table I). These results confirm previous results of our group (14). This means an increased after inoculation of homogenates treated at 800 MPa (Table I). These results confirm previous results of our group (14).

The quantified titer reduction and the decrease in the signal on the immunoblots reported above. All analyzed samples of terminally diseased animals at the end of the reported bioassay were positive for scrapie, independent of the applied treatment (data not shown).

**Proteinase K Sensitivity of PrP-(27–30) Pressurized at Initial Neutral Conditions**—Fig. 2 shows the results after pressurization of the N-truncated PrPSc. Concordant with previous experiments, native infectious prions were found to be pressure-sensitive at initial neutral conditions, and no spot could be detected by specific antibodies after digestion with proteinase K (data not shown). However, pressure is able to modify the quaternary structure of proteins provoking protein aggregation/deaggregation (18, 19), which may form conglomerates not reachable by SDS, leading to misinterpretation during electrophoresis. However, we point out that immunoblots of the N-truncated form of PrPSc (PrP-(27–30)) were positive, showing the typical glycoforms (Fig. 2, KP), arguing that prion proteins were not further aggregated after adiabatic treatments because they were able to enter the gels. Furthermore, a subtle change in the protein structure leading to a diminished affinity of the specific antibodies, and therefore to false negatives in Fig. 1, could also be discarded after viewing the results in Fig. 2. Contrary to this, no proteinase K-resistant PrP-(27–30) was detected on the immunoblots after additional digestion with proteinase K (Fig. 2, KPK), confirming the postulated proteinase sensitivity.

**Proteinase K Sensitivity and Infectivity of iPrP** Pressurized from Pressurized Native PrPSc—N-Truncated prion proteins were purified from high-pressure-treated brain homogenates obtained during the first experiment and diluted in PBS buffer, pH 7.4, after extraction following the method in Ref. 16. The comparison of immunoblots from crude brain homogenates and iPrP hK was reported in Fig. 3. Materials purified from non-pressurized samples were resistant to proteolytic digestion and positive for PrPSc because the typical glycoforms were detected (Fig. 3b, CiKK). In contrast, results of iPK hK purified from pressurized samples agreed with the pressurized crude brain homogenates (Fig. 3a, PK) and iPK hK was almost undetectable on the immunoblots after isolation and renewed proteinase K digestion (Fig. 3b, PPK). These results confirm the already discussed pressure-induced sensitivity to proteinase K that could only be explained by changes in the protein conformation because false negatives due to protein aggregation could already be discarded (see “Proteinase K Sensitivity of PrP-(27–30) Pressurized at Initial Neutral Conditions”).

Infectivity bioassays were performed in hamsters by intracerebral inoculation of the purified iPK hK materials. Animals in this experiment showed the first signs of disease after 105 days (∼3; log10 ID50 = 4.98) following inoculation with untreated controls. The mean incubation by controls was significantly prolonged (∼30; log10 ID50 = 3.05) from pressurized samples showing an acceptable concordance with the reduction in the detectable core resistant to proteolysis reported above.

**Proteinase K Resistance of Purified Prion Proteins Pressurized in Different Buffers**

Purified prion proteins (iPrP hK) were pressurized by dissolving in neutral Tris/HCl and PBS buffers, or bidistilled water. The results with (K) or without proteinase K digestion before separation with electrophoresis are summarized in Fig. 4. Contrary to the described pressure effects on the proteinase K resistance and the infectivity of native PrPSc (Fig. 1), the typical glycoforms of infectious prions were detected after pressurization of the iPrP hK core in water (Fig. 4a, KP and KPK) and Tris/HCl buffer, pH 7.0 (Fig. 4b, KPK and KPK), showing a preserved proteinase K resistance after pressure treatment.

### Table I

| Disease transmission | Incubation (days) | log10 ID50 units/g |
|----------------------|-------------------|--------------------|
| Untreated control    | 5/5               | 82 ± 7             |
| 60 °C for 120 min    | 5/5               | 87 ± 6             |
| 800 MPa at 60 °C     | 5/5               | 169 ± 37           |
| for 120 min          |                   | 1.7                |

**Fig. 1. Immunoblot detection of native hamster PrPSc with the antibody 3F4.** a, pressurized in Tris/HCl buffer, pH 7.0. b, pressurized in water, c, pressurized in water, d, pressurized in PBS buffer, pH 5.6. C, non-pressurized controls; P, treated at 800 MPa at 60 °C for 120 min; K, incubated with proteinase K. The order of characters denotes the order of treatments.
The same proteinase K resistance was stated for samples pressurized in PBS buffer, pH 7.4 (Fig. 4e, KPK), although before carrying out intense denaturation (sonication and long boiling in SDS of the pressurized proteinase K-digested iPrPSCO), no signal could be detected on the immunoblots in the absence of proteinase digestion or in the presence of proteinase K (Fig. 4e, KP and KPK, respectively). After denaturation, however, the typical glycosylation pattern of prion proteins was recovered, showing a distinct pressure effect on the protein structure in PBS buffer, which was probably caused by protein aggregation. PBS ionic strength and its pH shift in the acidic region during pressure might initiate the aggregation of purified prion rods, which is already known to be the case for treatments of native PrPSCO with acids at atmospheric pressure.

Identical results on the resistance to digestion with proteinase K were stated when iPrPSCO was mixed with a scrapie negative brain homogenized 10⁻³ w/v in PBS buffer, pH 7.4 (Fig. 4d, KP and KPK). In that case, other brain components acted as stabilizers, and no changes leading to aggregation were induced by pressure because the typical glycosylation bands of prion proteins were detected on the immunoblots without the denaturing procedure.

Discrepancies between native and purified PrPSCO led to a more profound study of the pressure effects on the protein structure during the isolation procedure. For this, samples were collected and pressurized after every centrifugation step during isolation. Results with (K) and without proteinase K are reported in Fig. 5. As expected, the high concentration of lauryl sarcosine did not induce changes in the prion protein structure, which could have led to an increased pressure resistance, and indeed no difference could be established with native PrPSCO. After digestion with proteinase K and the last centrifugation step at 22,000 × g, the pressure-induced aggregation of iPrPSCO was evident, and again prions were not visible on immunoblots of non-digested/digested samples, concordant with the observation already discussed in Fig. 4.

**DISCUSSION**

Possibilities of high pressure for the inactivation of pathogenic microorganisms have been intensively explored (20). Bacterial spores have been found to be the most persistent species, and only combinations of heat and extreme high pressures are able to inactivate them at a satisfactory rate. Prions are even more resistant than spores to conventional autoclaving. Nevertheless, the pure proteinaceous nature of prions motivated our expectations for a suitable inactivation when subjected to high pressures at mild temperatures. In a recent paper (14), we reported an increase in the time necessary for the onset of disease and postulated a decrease in proteinase K resistance in view of the negative immunoblots after pressure treatments of brain homogenates.

At present, the pressure instability of native prion proteins has been additionally proven in several buffers and in water. The initial neutrality of brain homogenates was sufficient to induce changes in the protein structure during the slow pressure build up and the prolonged holding time. This initiated a loss of the detectable PrPSCO core resistant to proteolytic digestion, generally leading to negative immunoblots. The pH of pressure-unstable media, such as PBS containing phosphate buffer and water, shifts during pressurization into the acidic region (0.3 units/100 MPa for phosphate buffer) (21). However, the effect of pressure on pH must have happened simultaneously with the effect of pressure on the prion conformation, which leads to proteinase K sensitivity. If pressure effects on proteinase K sensitivity were almost immediate, the use of high pressure processing as a mild decontaminating technology of risk materials could be feasible. Indeed, a remarkable level of inactivation has been achieved at shorter holding times (30 min) (15), which is more relevant for an industrial application, and the optimized parameters remain to be investigated.

The decrease in detectable proteinase K-resistant core correlated well with a remarkable drop in the amount of infectious prion units, and a reduction of 6–7 log₁₀ infectious units/g in different experiments undertaken with native prion proteins pressurized at 800 MPa at 60 °C for 120 min is reported in Table I. However, a residual infectious fraction (~1–2 log₁₀ ID₅₀ units/g) remained in the pressurized brain homogenates after 2 h holding time. This fraction was assumed resistant to digestion with proteinase K but was not detectable on immunoblots. (Bioassays of proteinase K-digested pressurized samples showed a good concordance with biosassays of non-digested samples, data not shown.) In fact, residual fractions are already known to survive autoclaving and re-autoclaving (22).

The strong reduction in infectivity in PBS reported here is comparable with the (already accepted as safe) decontamination of samples by porous load autoclaving at 135 °C for 20 min (23) or by soaking in 1 M sodium hydroxide for 1 h (24). However, this method is probably still not equivalent to the most effective methods, such as sodium hydroxide and autoclaving at 121 °C (25) or sodium hypochlorite solutions (23). However,
First centrifugation at 540,000 \(g\) samples were pressurized (\(P\)) in bi-distilled water (\(\mathrm{H}_2\mathrm{O}\)), in Tris/HCl, pH 7.0 (\(b\)), in PBS buffer, pH 7.4 (\(c\)), or diluted in a brain homogenate (in PBS buffer, pH 7.4 (\(d\)) negative for scrapie (with PrP\(^{\mathrm{Sc}}\)). c, iPrP\(^{\mathrm{Sc}}\) was pressurized in PBS buffer, pH 7.4, proteinase K-digested, and denatured (30 min sonication; 120 min 2% SDS at 95 °C) before electrophoresis. All samples were pressurized (\(P\)), K, results of iPrP\(^{\mathrm{Sc}}\) incubated with proteinase K (50 \(\mu\)g/ml for 1 h at 37 °C). The order of characters denotes the order of treatments.

The achieved reduction should be acceptable for a high pressure treatment of food because an oral infection is 10\(^9\)-fold less effective than intracerebral inoculation (26).

Opposite to the results at initially neutral conditions, the scrapie prions pressurized in slightly acidic buffers remained proteinase K-resistant, and it may be assumed that the protein structure was mostly preserved during pressurization. A protective effect that may be interpreted as a pH-induced aggregation of prion proteins was enough to inhibit the effects of pressure. Indeed, the folding of the prion protein in acidic pH into an aggregated state that is able to precipitate has been reported previously (27, 28), and the enhanced \(\beta\)-sheet content of PrP-(106–126) under several conditions, namely low pH (29) and in the presence of lipids (30), has been concluded. Those aggregated states induced by low pH remained infectious.

Accumulating evidence supports the "protein only theory" and the fact that a conformational change of cellular prion proteins is the main feature in prion propagation and infectivity (1), but previous findings insist on the high improbability of an irreversible pressure-induced denaturation of \(\beta\)-sheets (31) and on the relative pressure sensitivity of the quaternary structures of proteins, which are stabilized by hydrophobic interactions (11). In principle, the unexpected results described here on the reduction in the amount of proteinase K-resistant protein detected on immunoblots and the parallel diminution in the infectivity of brain homogenates would be more easily explained at the structural quaternary level (destabilization of aggregated fibrils at relatively low pressures) than at the secondary level (destabilization of \(\beta\)-sheets).

These premises made it necessary to confirm the proteinase K sensitivity through two approaches. First, immunoblots confirmed the proteinase sensitivity of the high pressure-treated N-truncated native prion protein (PrP-(27–30)). Changes on protein structure induced by pressure had not altered the affinity of the antibody 3F4 to bind to PrP-(27–30), and furthermore, PrP-(27–30) was not aggregated after pressure because it was detectable on immunoblots before proteolytic digestion took place. However, the same protein was not detected after proteinase K digestion, showing that certain features of native prion proteins, namely the basic characteristics of the \(\beta\)-rich aggregated pathogenic multimer, were not preserved after pressurization at initial neutral conditions.

Second, pressure-induced changes in the conformation of the prion protein at mild temperatures could also be verified after purification of the proteinase K-resistant core of pressurized brain homogenates. In fact, native prions presented the expected three characteristic bands after isolation, and pressurized samples indicated a change in the structure of prions leading to the proteinase K sensitivity of the purified materials. Again, a reasonable agreement between immunoblots and infectivity bioassays allowed us to conclude that prions purified from samples pressurized in a buffer at neutral pH are less infectious and more proteinase K-sensitive than prions purified from non-pressurized samples. Data on reduced prion infectivity have usually been related to an alteration in the secondary structure of the protein, namely a decrease in the content of \(\beta\)-sheets (32). This thesis would clarify the results of immunoblots and the reported proteinase K sensitivity, but then the unlikely pressure-induced modification of the secondary structure of prion proteins, and therefore of the \(\beta\)-sheet backbone, would have to be assumed.

Contradictory results are evident when analyzing the effects of pressure on purified proteinase-resistant brain materials. The conformation of purified prion proteins did not seem to be altered when submitted to high pressures because the main feature related to native infectious prions, namely the proteinase K resistance of the \(\beta\)-sheet-aggregated multimer, is preserved after pressure. In the case of purified pressurized prions, results do not definitively disagree with existing models. Several factors during isolation should be considered separately. On one hand, centrifugation at 540,000 \(g\) originated several hundred MPa at the bottom of small centrifugation tubes. Indeed, extremely high pressures generated during centrifugation might have helped the inactivation of infectious prions. However, a second aspect involved during centrifugation is that the density of proteins is higher than water, and the agglomeration of hydrophobic proteins might lead to the formation of a hydrophobic core at high speeds, where proteins are highly aggregated and not hydrated, forming a semicrystalline
structure. In fact, a crystalline structure has been reported after isolation of PrP(27–30) (33) and was the basis for the analysis performed on electron crystallography, which determines the most recent accepted model on the structure of prion proteins. Also not to be overlooked is the fact that an extrastabilizing factor, theorized by other authors to be in the form of a chaperone (see Ref. 34 for review), could become displaced during centrifugation.

Our results suggest that PrPSc adopts a structure different from native very soon during purification. The displacement of a stabilizing factor or the high aggregation and the lack of hydration are probably the factors that originate a protein conformation that is not yet pressure-sensitive and therefore not accessible to proteolytic digestion. Those proteins would still be infectious but would behave differently from the native PrPSc and would probably have a distinct structure. This would mean that available models on prion structure, having been constructed based on a non-native protein structure, could fail when trying to represent the real native prion features. To take the structure of iPrPres as a basis for model building and for understanding prion characteristics might lead to misinterpretations. Actually, the discrepancy between native and dehydrated prions (32) has already been discussed. Those authors presented an increasing β-sheet content during the loss of free water, which is parallel with an abnormal resistance to heat in isolated prions in the solid state that could correlate to the extreme pressure resistance reported here.

Indeed, another recent work (35) points out the important role of hydration and aggregation on the pressure sensitivity of the secondary structure of recombinant prions. Also, our results probably argue for the existence of pressure-sensitive β-structures (PrPScΔProne) and extremely pressure-resistant β-structures (PrPScΔPres) because of the high aggregation and low hydration in centrifuged purified prions. We conclude that native prion proteins do not necessarily have the same structure as purified prions in the same buffer. Bioassays from samples in other buffers different from PBS and in food matrices at neutral pH are under way and will clarify the realistic possibilities of this technology in decontaminating risk materials below the customary denaturation temperature.

Acknowledgment—We thank Prof. Dr. Horst Ludwig (University of Heidelberg) for very helpful discussions during the preparation of this manuscript.

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