**Molecular mass, biochemical composition, and physicochemical behavior of the infectious form of the scrapie precursor protein monomer**

(scrapie isoform of prior protein/posttranslational modification/neutralization)


*Laboratory of Central Nervous System Studies, National Institute of Neurological Disorders and Stroke, Laboratory of Pathology, National Cancer Institute, and Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892*

**ABSTRACT** A highly purified fraction obtained from scrapie (263-K strain)-infected hamsters' brains by an alternative procedure without proteinase K treatment contained a protease-resistant form of the scrapie precursor protein (PrPSc) and infectivity of $9.9 \pm 0.7 \log LD_{50}/ml$. Polyclonal antibodies produced against hamster scrapie amyloid protein (PrP27-30) and used in a neutralization test diminished infectivity of the PrPSc preparations by 1.6 log after intracerebral inoculation and by 1 log after intraperitoneal inoculation. PrPSc was subjected to size-exclusion HPLC; ≥60% of the eluted infectious units were recovered from the peak with an apparent mass of 30.4 ± 0.6 kDa. Characterization by UV absorption spectra, SDS/PAGE, immunobLOTS, N-terminal amino acid sequence, and neutral sugar and amino sugar analyses demonstrated homogeneity of the infectious units. The neutral sugar and amino sugar compositional analyses revealed high mannose, glucosamine, fucose, and sialic acid content. This demonstrated an extensive posttranslational modification by the complex type of N-linked glycosylation and glycane core of C-terminal glycolipid of PrPSc. The results correspond to the predicted size, composition, and sequence of PrPSc and indicate that this protein may be the only component of scrapie infectious unit or the infectious form of scrapie precursor.

Scrapie infectivity copurifies with the protease-resistant form (PrPSc) of the normal, host-coded scrapie precursor protein (PrPc) (1-3). The scrapie amyloid protein, PrP27-30, is produced by partial proteolytic cleavage of the protease-resistant form of scrapie precursor PrPSc and also copurifies with infectivity, but its insolubility prohibits further characterization (4-6). It is the protease-resistant, proteolytically cleaved, scrapie amyloid PrP27-30 that has a strong tendency to aggregate and form scrapie-associated fibrils (7) or prion rods with amyloid properties (4, 8). Once formed, these structures are virtually insoluble in conventional buffers and detergents without destroying their infectivity (5, 6, 9, 10). Thus, it is very difficult to directly study the composition, target size, or molecular mass of the infectious unit monomer and the linkage of infectivity with the monomeric and/or polymeric form of PrP27-30.

The initial step of our purification method for PrPSc maintains high levels of infectivity and prevents self-aggregation by using tight association of this protein with endogenous phospholipids and by blocking proteolytic cleavage (11). The separation of PrPc from the infectious form of the scrapie precursor protein PrPSc is based on the different solubility of these two proteins (12). In the present experiments, we directly characterized and determined the molecular mass of the HPLC-purified scrapie infectious unit. UV absorption spectra, SDS/PAGE, immunoblots, neutral sugar and amino sugar analyses, and amino acid sequencing all demonstrate the homogeneity of the infectious unit. The results correspond to the predicted size, composition, and sequence of the PrPSc after proteolytic cleavage of the signal peptide. Thus, the PrPSc appears to be infectious and we did not find any discrepancies that would indicate association with another macromolecule.

**METHODS**

**Materials.** All chemicals were reagent grade and of the highest purity commercially available. Protein and total phospholipid content was determined as described (11).

**Source of Scrapie Agent and Bioassay.** The 263-K strain of scrapie from the sixth intracerebral (i.c.) passage in golden Syrian hamsters (LVG/LAK) was used. Bioassay of infectivity was carried out in groups of four weaning female hamsters inoculated i.c. with 0.03 ml or intraperitoneally (i.p.) with 0.1 ml of serial 1:10 dilutions of test samples in phosphate-buffered saline (PBS) (pH 7.4), containing 0.05% sarcosyl. Endpoint titers were calculated by the method of Reed and Muench (13) and are expressed as log LD_{50}/ml. The clinical diagnosis of scrapie was confirmed by histopathologic evaluation of randomly chosen hamster brains from each dilution of test samples.

**Purification of PrPSc.** The first steps of the purification procedure are described in detail elsewhere (11). Briefly, the brains of terminally ill scrapie-infected hamsters were homogenized at 0°C in 0.32 M sucrose, containing 0.5 mM KCl, 1 mM MgCl₂, 1 mM NaHCO₃, 5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol by 40 gentle strokes in a glass Dounce homogenizer. The homogenate (10%, wt/vol) was centrifuged at 3000 x g (Sorvall SS34 rotor) for 10 min at 4°C and the resulting pellet was rehomogenized in sucrose by 50 gentle strokes in a glass Dounce homogenizer, adjusted to the original volume, and centrifuged again at 3000 x g (Sorvall SS34 rotor) for 10 min at 4°C. Pooled supernatant fluids 1 and 2 were centrifuged at 100,000 x g (Beckman 50.2 Ti rotor) for 1 hr at 4°C. Triton X-114 was then added at 0°C to the pellet obtained from the previous step with a final detergent concentration of 2% (wt/vol) and proteins at 5 mg/ml in TBS (10 mM Tris-Cl/0.1 NaCl/1 mM EDTA/5 mM phenylmethylsulfonyl fluoride, pH 7.4). After 30 min on ice, the resulting solution was centrifuged at 100,000 x g (Beckman 50.2 Ti rotor) for 30 min at 0°C. The pellet from this step (designated the phospholipid-rich phase) (11) was

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Abbreviations: PrPc, cellular isoform of prion protein; PrPSc, scrapie isoform of prion protein.

†To whom reprint requests should be addressed.
washed twice at 0°C in TBS (pH 7.4), first in the presence of 2% Triton X-114 and then in its absence. The phospholipid-rich phase was then resuspended in a solution in PBS (pH 7.4) containing 1% (wt/vol) sarcosyl and 5 mM phenylmethylsulfonyl fluoride and stirred for 2 hr at 37°C. The resulting opalescent solution was centrifuged at 0.32 M sucrose at 100,000 x g (Beckman 50.2 Ti rotor) for 60 min at 10°C. The pellet was washed twice, first in PBS (pH 7.4) containing 10% NaCl and 1% (wt/vol) sarcosyl and, finally, in 0.1% sarcosyl in H2O. This pellet containing PrPSc was resuspended in PBS (pH 7.4), assayed for infectivity and used throughout the subsequent steps of this study.

**In Vito Neutralization Test.** The above PrPSc preparation was sonicated by three 30-sec bursts at 50 W on ice using a BraunSonic 2000 sonicator. Serial 1:10 dilutions of PrPSc were prepared in PBS (pH 7.4) and mixed with preimmune or postimmune rabbit serum produced against hamster PrP27-30 (CG14) to obtain a constant final 1:10 dilution of serum. Samples were incubated for 1 hr at 37°C and immediately thereafter were assayed for infectivity by i.p. or i.c. inoculation into hamsters as described.

**Size-Exclusion HPLC.** PrPSc was further purified on size-exclusion HPLC by using three TSK 3000 SW (300 x 7.8 mm) columns connected in tandem and calibrated by a mixture of low molecular weight standard proteins (Bio-Rad). Samples of PrPSc containing 2–5 mg of total protein were resuspended in 50 µl of 150 mM sodium phosphate buffer (pH 7.2) containing 10% (vol/vol) glycerol and SDS to obtain an SDS/protein ratio of 1.2–1.4 (wt/wt); the mixture was heated for 3 min at 100°C and injected into a Beckman 421A HPLC system with UV detection at 280 nm. Columns were developed by 150 mM sodium phosphate buffer (pH 7.2) containing 0.1% (wt/vol) SDS at a flow rate of 0.25 ml/min at room temperature. Fractions eluted between 50 and 120 min were collected and concentrated by Centricon 30 (Amicon), and the protein was determined by BCA assay. Samples collected between 80 and 90 min were used for sequencing and immunoblotting. Amino acid composition in aliquots of each fraction was determined by SDS/PAGE, followed by silver staining. For amino acid composition analysis, HPLC-purified PrPSc was precipitated by 4 vol of acetone at -20°C in the presence of 0.1 M NaCl. Reconstitution in Normal Hamster Phospholipids. For bioassay, the volume and free SDS content in aliquots of the SDS-boiled PrPSc and in fractions from HPLC were reduced 50 times by ultrafiltration in Centricon 30 (Amicon). The resulting concentrated was diluted 1:10 in PBS (pH 7.4), and 0.5 ml was sonicated for 20 min in a sonication bath with 2.5 mg of dry normal hamster phospholipids. Homologous hamster phospholipids were obtained from the normal hamster phospholipid-rich phase (prepared as described for scrapie) after extraction by 20 vol of chloroform/methanol (2:1, vol/vol) and dried under an N2 stream.

**Amino Acid Sequence.** Samples were sequenced with an Applied Biosystems 475A System: 470A sequencer, 120A PTH analyzer, 900 control/data analysis module under the standard program RUn 470-1. Before applying the sample, the glass fiber filter was treated with 30 µl of Polybrene (Applied Biosystems) and then subjected to three cycles of treatment with sequencing reagents and solvents. Samples were dissolved in 70% formic acid and applied in 30-µl aliquots to the treated filter.

**Neutral and Amino Sugar Composition Analysis.** HPLC-purified PrPSc (~0.3 nM) was transferred to a clean reaction vial, lyophilized, and resuspended in 200 µl of 5.5 M trifluoroacetic acid in a vial closed with a Teflon-lined septum cap. Hydrolysis was carried out for 4 hr at 100°C. The sample was dried under a stream of N2 at 37°C, dissolved in 100 µl of deionized water, and injected into a Dionex 400i HPLC system with an amperometric detector and a Spectro-Physics 4270 integrator. An anion-exchange AS6 column was developed by 25 mM NaOH at a flow rate of 0.5 ml/min with detector settings E1 = 0.1 V, E2 = 0.6 V, E3 = -0.8 V, V1 = 300 mS, V2 = 120 mS, and V3 = 300 mS. Sulfonic acid determination was similar except that hydrolysis was carried out in 0.1 M trifluoroacetic acid for 1 hr at 80°C, and Dionex anion-exchange columns AS6 and AS6A connected in tandem were eluted with 50 mM NaOH containing 50 mM NaOAc.

**SDS/PAGE.** SDS/PAGE (formula, T 12.5/C 0.3), silver staining of electrophoresed proteins, immunoblotting, preparation, characterization, and specificity of PrP antibodies used in these procedures are described elsewhere (14). The polyclonal antibodies against synthetic peptides corresponding to the N-terminal and C-terminal sequence of hamster PrPSc were provided by R. A. Barry (15).

**RESULTS**

**Purification of PrPSc.** The first steps of the purification procedure were based on temperature-dependent separation of scrapie-infected synaptosomal/microsomal membranes (Fig. 1, lane 2) in Triton X-114 (11) followed by separation of PrPSc from PrPSc by differential solubilization in sarcosyl. PrPSc together with PrPSc were quantitatively recovered in the liposome-forming phospholipid-rich phase (11) (lane 3). In the next step, PrPSc was separated from PrPSc by sarcosyl.

**Table 1. Yields of the infectivity of PrPSc preparations, and recovery of infectious units after SDS treatment and HPLC followed by reconstitution in normal hamster phospholipids**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein content, mg/ml</th>
<th>Infectivity, log LD50/ml</th>
<th>Specific infectivity, log LD50/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrPSc (n = 8)</td>
<td>5.2 ± 0.4</td>
<td>9.9 ± 0.7</td>
<td>9.2 ± 0.9</td>
</tr>
<tr>
<td>PrPSc/SDS/boiling (n = 5)</td>
<td>5.1 ± 0.3</td>
<td>8.1 ± 0.7</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>Size-exclusion HPLC fraction F3 (n = 4)</td>
<td>0.8 ± 0.2</td>
<td>7.7 ± 1.4</td>
<td>7.7 ± 1.5</td>
</tr>
</tbody>
</table>

Values represent means ± SD determined by independent experiments, and specific infectivity is expressed as infectivity per mg of total protein.
(1%, wt/vol) in PBS (pH 7.4) at 37°C. Under these conditions, PrPSc is solubilized, whereas PrPSc precipitates (12). After final high-speed sucrose centrifugation, the pellet contained PrPSc with its characteristic heterogeneous pattern on SDS/PAGE (see Fig. 4, lane 1) and infectivity of 9.9 ± 0.7 log LD50/ml (Table 1). In contrast to PrPSc, all bands were relatively protease resistant and were shifted by 3–5 kDa after protease K treatment toward the lower molecular mass, thereby forming the PrP27-30 complex (Fig. 1, lane 7).

The fractions obtained after final high-speed centrifugation in sucrose contained 15 ± 0.7 µg of PrPSc and 8.3 log LD50/g of scrapie-infected hamster brain. After 3 min of boiling in the presence of SDS followed by reconstitution in normal hamster phospholipids, 8.1 ± 0.7 log LD50/g of infectivity was recovered (Table 1). As determined by HPLC followed by SDS/PAGE loaded without boiling in Laemmli buffer, at a SDS/protein ratio of 1.2–1.4 (wt/wt), the majority of the PrPSc was solubilized in a monomeric form (see Fig. 4).

To test whether the larger molecular mass bands of the immunoreactive PrPSc are products of in vivo or in vitro proteolysis, three other polyclonal antibodies were used on immunoblots: CG5, produced against a synthetic peptide corresponding to the N-terminal sequence of originally described PrP27-30 (14); P2, produced against a synthetic peptide corresponding to the N-terminal sequence of PrPSc after cleavage of the signal peptide (15); and P3, developed against a synthetic peptide corresponding to the C-terminal sequence of PrPSc after cleavage of the hydrophobic domain (15). All three antibodies reacted with large and small molecular mass bands, and the patterns of CG5 and P2 immunoreactivity were identical (Fig. 1, lanes 4–6).

In Vivo Neutralization Test. Polyclonal antibodies produced against hamster PrP27-30 (CG14) in rabbits diminished the infectivity of PrPSc by 1.6 log after i.c. inoculation and by 1 log after i.p. inoculation (Table 2). The incubation of samples with preimmune sera did not change the infectivity titers of the PrPSc preparation (Tables 1 and 2).

Size-Exclusion HPLC. The calibration of size-exclusion HPLC columns demonstrated a linear relationship between log of molecular mass and retention time with correlation coefficient −0.9971 in the size range 92.5–14.4 kDa. The specific resolution coefficient (16) for ovalbumin (45.0 kDa) and carbonanhydrase (31.0 kDa) was 2.37. Samples of PrPSc containing 2–5 mg of total protein and, after SDS treatment, were loaded onto a column of Superose 12 (Amicon). The UV absorption spectrum of the fraction F3 with an A280/A260 ratio of 1.64 ± 0.09 (mean ± SD; n = 3) is consistent with that of a protein.

Partial N-Terminal Amino Acid Sequence. A sample corresponding to a single peak on HPLC (F3) was analyzed by gas-phase sequencing from the amino terminus by automated Edman degradation. A sequence matching the translated

Table 3. Fractions obtained after size-exclusion HPLC, size range, recovery of infectivity, and specific infectivity of each fraction

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Retention time, min</th>
<th>Size, kDa</th>
<th>Total protein, mg</th>
<th>Infectivity, log LD50/ml</th>
<th>Specific infectivity, IU × 10⁴ per mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>50–67</td>
<td>197.2–86.9</td>
<td>0.8</td>
<td>6.0</td>
<td>125.0</td>
</tr>
<tr>
<td>F2</td>
<td>67–80</td>
<td>86.9–38.3</td>
<td>1.3</td>
<td>4.3</td>
<td>1.5</td>
</tr>
<tr>
<td>F3</td>
<td>80–90</td>
<td>38.3–22.2</td>
<td>0.9</td>
<td>6.2</td>
<td>176.1</td>
</tr>
<tr>
<td>F4</td>
<td>90–110</td>
<td>22.2–8.8</td>
<td>0.1</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>F3-R</td>
<td>84.4</td>
<td>30.1</td>
<td>0.7</td>
<td>5.8</td>
<td>90.1</td>
</tr>
</tbody>
</table>

Protein content was determined after concentration in Amicon 30 by BCA assay. IU, infectious units.
FIG. 3. Rechromatography of PrPSc (fraction F3) and recovery of infectivity. Infectivity is expressed as infectious units \( \times 10^6 \) per ml.

The hamster PrP gene open-reading frame beginning at the signal peptide cleavage site was obtained: Ser-Lys-Arg-Pro-Lys-Pro-Gly-Gly-Trp-Asn . . . .

Nine of the 10 N-terminal amino acids corresponded to the DNA sequence. Position 1 was identified as serine (machine call) and we identified position 3 as arginine. The small amount of protein available for gas-phase sequencing precluded detecting a reproducible signal beyond cycle 10.

Neutral and Amino Sugar Composition. After rechromatography and acid hydrolysis, fraction F3 was directly analyzed for neutral and amino sugar composition by HPLC with amperometric detection; similar conditions were used for the sialic acid determination (Table 4). The results demonstrated a high molar ratio for mannose, glucosamine, and neumamic acid with a relatively high proportion of fucose. There was no peak associated with 0.1 nmol or more of deoxyribose and/or ribose per nmol of PrPSc in HPLC fraction F3. However, we cannot exclude the possibility that they were destroyed under the conditions used for the acid hydrolysis.

**DISCUSSION**

The exact composition, molecular mass, and characteristics of the scrapie (and Creutzfeldt–Jakob disease) infectious unit remained uncertain despite indirect evidence about linkage between PrP27-30, PrPSc, and infectivity (2, 3, 17, 18). Scrapie amyloid protein PrP27-30, first described in highly infectious fractions obtained from scrapie-infected brains after limited proteolysis K and sarcosyl treatment, resisted further analysis because of its high tendency to aggregate and form scrapie-associated fibrils (7) or morphologically related prion rods with amyloid properties (4, 8, 18). This structure prevents solubilization, which is obligatory when studying subunit composition, direct molecular mass determination, the role of monomers or polymers in infectivity, or determination of hypothetical nucleic acid content. Moreover, a few compounds able to disrupt scrapie-associated fibrils or prion rods simultaneously destroy infectivity (5, 9, 10, 18).

To characterize the infectious unit of scrapie, we purified the PrPSc precursor protein without protease, while still maintaining high levels of infectivity (11). Separation of PrPSc by differential solubilization (12) and final sucrose centrifugation yielded the fraction containing high specific infectivity and the protease-resistant scrapie precursor protein PrPSc. Indeed, incubation with proteolysin K (before SDS treatment) produced PrP27-30 and no change in the intensity of bands.

**FIG. 4.** Direct SDS/PAGE analysis of the fractions collected from size-exclusion HPLC: silver staining (a) and Western blot detection (b) by polyclonal antibodies produced against hamster PrP27-30. Samples containing 1–5 \( \mu \)g of total protein correspond to the PrPSc preparation (lane 1), first peak of HPLC (fraction F1, lane 2), second peak (fraction F2, lane 3), third peak (fraction F3, lane 4), and fourth peak (fraction F4, lane 5).

This provides further evidence that purified material obtained by differential solubilization contained PrPSc and probably little or no PrP isoform.

Our *in vivo* neutralization tests using polyclonal antibodies produced against hamster PrP27-30 (14) indicated limited neutralizing activity on scrapie infectivity in the fraction containing PrPSc. The effects are limited to relatively low antibody dilution, which might be explained by the low neutralizing capacity of the antibodies or high PrPSc/infectious unit ratios (19). Our results with PrPSc following both inoculation routes extend the recent observation that polyclonal antibodies may neutralize scrapie infectivity in PrP27-30 preparations after i.c. inoculation (17). However, the precise determination of the neutralizing mechanism remains to be established (19).

SDS treatment of the fraction containing PrPSc with a low SDS/protein ratio, followed by size-exclusion HPLC and reconstitution in normal hamster phospholipids yielded 35.5% of loaded infectious units. The molecular mass cut-off for what are probably infectious unit monomers was \( \approx 22 \) kDa; \( \approx 60\% \) were recovered from the fraction with apparent size ranging from 22 to 38 kDa. After rechromatography, the material had a residual infectivity of 5.8 \( \log LD_{50}/ml \). According to UV absorption spectra, silver staining, and immunoblotting, only PrPSc monomers were detectable. The predicted molecular mass of the PrPSc monomer, 28.7–31.1 kDa (based on the assumption that both signal and C-terminal...

**Table 4.** Neutral, amino sugar, and sialic acid compositional analyses of infectious HPLC fraction F3 containing PrPSc

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Fucose</th>
<th>Galactosamine</th>
<th>Glucosamine</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Sialic acid</th>
<th>Glycosyl, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.75</td>
<td>1.32</td>
<td>9.21</td>
<td>4.22</td>
<td>1.38</td>
<td>6.90</td>
<td>4.74</td>
<td>5.28</td>
</tr>
<tr>
<td>2</td>
<td>3.02</td>
<td>1.15</td>
<td>9.78</td>
<td>4.61</td>
<td>1.46</td>
<td>7.82</td>
<td>4.74</td>
<td>5.55</td>
</tr>
<tr>
<td>Molar ratio</td>
<td>3</td>
<td>1</td>
<td>9–10</td>
<td>4–5</td>
<td>&lt;1</td>
<td>7</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
peptides are cleaved with each molecule of PrP containing one or two glycosyl chains and one glycolipid), corresponded to that calculated from retention times, and we did not observe any anomaly that would indicate the hydrophobic interaction or ion-exchange effect and resulting differences in size. However, such differences cannot be completely excluded.

The ratio between infectivity of the void volume and detectable PrP was greater than in the fraction containing PrP monomers. This may reflect the fact that most PrP monomers probably exist in a random coil configuration and only a portion were partially resistant to the detergent or underwent refolding after reconstitution in homologous phospholipids (20–23). The precise mechanism is not known; preliminary data indicate partial SDS resistance of scrapie infectivity at a low SDS/protein (wt/wt) ratio.

Partial N-terminal amino acid sequencing of the infectious HPLC fraction F3 together with the results of immunoblotting with various anti-synthetic antibodies demonstrated that the major sequence in the PrPSc immunoreactive complex in vivo is a full-length protein with an intact N terminus. The signal of the first position obtained during gas-phase sequencing was serine. However, according to the translated hamster PrP gene open reading frame beginning at the signal peptide cleavage site, this should have been lysine (1). Either free amino acid contamination in the first cycle or an allelic polymorphism is a possible explanation. Since amino acid determination in the same material did not yield higher serine values (data not shown), the second explanation is more likely. Posttranslational modification of arginine at the third position has also been suggested (24). Since we had no difficulties with determination of this amino acid, the finding is apparently not consistent in different lines of animals inoculated with the same strain of scrapie (263-K).

Complete neutral sugar, amino sugar, and sialic acid analyses of infectious HPLC fraction F3 containing PrPSc showed high molar ratios of glucosamine, galactose, mannose, fucose, and neuraminic acid. After subtracting the possible common components of the C-terminal glycolipid (one non-acetylated glucosamine, three mannose) (25, 26), the composition resembled the N-linked glycosylation. Asparagine-linked glycosylation was predicted for this protein by DNA sequence data. The high neuraminic acid, galactosamine, and fucose levels are usually found in the heterogenous, extensively processed, complex type of glycosyl sequences (27). This finding explains the extreme charge heterogeneity of the PrPSc observed in isoelectric focusing (28) and its complex pattern and microheterogeneity on PAGE. It also implies extensive processing in the Golgi apparatus (27). The role of this highly complex posttranslational processing on infectivity remains to be established.

In conclusion, the data indicate that both PrPSc oligomers and monomers may be the only components of the scrapie infectious unit. We detected no discrepancies indicating that another macromolecule was associated with PrPSc. This conclusion is further supported by data obtained in our laboratory in which a different experimental approach was used (29).

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