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Ciliary membrane tubulin and associated proteins: a complex stable to Triton X-114 dissociation

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When either membranes from scallop gill cilia or reconstituted membranes from the same source are solubilized with Triton X-114 and the detergent is condensed by warming, no significant fraction of any major membrane protein partitions into the micellar detergent. Rather, most of the membrane lipids condense with the detergent phase, forming mixed micelles from which nearly pure lipid vesicles may be produced by adsorption of detergent with polystyrene beads. One minor membrane protein, with a molecular weight of about 20000, is associated consistently with these vesicles. The aqueous phase contains a fairly homogeneous protein-Triton X-114 micelle sedimenting at 2.6 S in the analytical ultracentrifuge. Sucrose gradient velocity analysis in a detergent-free gradient indicates moderate size polydispersity but constant polypeptide composition throughout the sedimenting protein zone. Sucrose gradient equilibrium analysis (also in a detergent-free gradient) results in a protein-detergent complex banding at a density of 1.245 g/cm^3 Sedimentation of the protein-detergent complex in the ultracentrifuge, followed by fixation and normal processing for electron microscopy, reveals a fine, reticular material consisting of 5–10-nm granules. These data are consistent with previous evidence that membrane tubulin and most other membrane proteins exist together as a discrete lipid-protein complex in molluscan gill ciliary membranes.

Introduction

The membranes of lamellibranch gill cilia, but not of sperm flagella, contain a tubulin variant as the major protein constituent [1,2] Having a higher percentage of non-polar amino acids than axonemal tubulin, this tubulin, though not that of the 9 + 2 axoneme, can be reconstituted into membranes from a Triton X-100 extract, freed of detergent and subjected to freeze-thaw Under a variety of conditions of reconstitution or extraction and through several cycles of reconstitution, numerous minor membrane proteins remain associated, in constant stoichiometry, with such membranes [3,4]

Detergent-solubilized ciliary membrane constituents, subjected to equilibrium centrifugation on a detergent-free sucrose gradient, band at a density approximating that of the original membrane This basic observation led to the suggestion that ciliary membrane tubulin and associated proteins form a relatively stable structural complex with lipids [4] In fact, the tight association of lipids with membrane proteins is the primary reason that the reconstitution takes place so efficiently after removal of detergent by adsorption to polystyrene beads

Bordier [5] demonstrated a striking separation of integral membrane proteins into a detergent-rich phase when a solution of membrane proteins in Triton X-114 was warmed above the cloud point (22°C) Based on the fact that only amphiphilic integral membrane proteins form mixed micelles with non-ionic detergents, and prompted by the observation of Regula and coworkers [6] that brain

synaptosome tubulin partitions into the Triton X-114-rich phase, I attempted to use Bordier's detergent condensation method to determine which ciliary membrane proteins were integral Somewhat unexpectedly, I found that the bulk of the lipids condense with the detergent micelles while essentially all of the membrane proteins remain in the aqueous phase, evidently as a large protein-detergent complex

Materials and Methods

Cilia isolation and membrane reconstitution Cilia were isolated from excised gills of the bay scallop, *Aequipecten irradians,* by hypertonic deciliation, followed by differential centrifugation at normal tonicity [3] For reconstitution, membranes were solubilized with 0 5% Triton X-100, the detergent was removed by treatment with SM-2 Bio-Beads (Bio-Rad Laboratories), and the resulting cloudy solution was frozen with liquid nitrogen, upon thawing, reconstituted membranes were recovered by high-speed centrifugation [3,4]

Triton X-114-solubilization and condensation Isolated whole cilia or reconstituted membranes were treated with 5–10 vol of a solution containing 1% Triton X-114, 30 mM Tris-HCl (pH 8 0), 3 mM $MgCl_2$, and 1 mM dithiothreitol for 30 min at 0°C Insoluble material (axonemes and mucus in the first case, mucus and denatured protein in the second) was removed by centrifugation for 15 min at 25 000 × *g* The supernatant was warmed to 30°C for 5 min and the detergent-rich phase was separated from the aqueous phase by low-speed centrifugation through a detergent-saturated sucrose cushion [5] The aqueous phase was again made 1% in Triton X-114 and the condensation was repeated, the first and second condensates being combined A third condensation was sometimes done but the condensate was discarded [5] These moderate-scale detergent condensations were most accurately and conveniently carried out using a 10-ml Kolmer urine sediment tube (Pyrex brand, No 8360)

Polyacrylamide gel electrophoresis Slab gels (1 5 mm) with 5–15% linear polyacrylamide gradients were run using the discontinuous, SDS-containing ionic system of Laemmli [7] Staining with Coomassie (Serva) Blue was by the sensitive equilibrium method of Fairbanks et al [8] Silver staining was performed by the method of Wray and coworkers [9]

Gradient centrifugation Linear sucrose gradients, 20–60% (w/w) buffered with 10 mM Tris-HCl (pH 8 0) but containing no detergent, were centrifuged at 4°C in a Beckman SW 41 rotor at 35 000 rpm for periods of time dictated by the experimental protocol The gradients were pumped into 20 equal fractions, using a fine needle inserted through the gradient to the tube bottom [4]

Analytical ultracentrifugation Detergent-solubilized constituents were analyzed at 20°C with a Beckman Model E ultracentrifuge, using Schlieren optics and 12-mm double-sector cells The solvent sector contained the experimental buffer, subjected to the same condensation protocol as the sample, in order to equalize aqueous-phase detergent concentrations

Electron microscopy Thin pellets (< 1 mm) were fixed for 1 h with 2 5% glutaraldehyde in the experimental buffer, rinsed, and post-fixed for 1 h with Karnovsky's osmium-ferrocyanide [10] The material was stained en bloc overnight with 1% uranyl acetate, dehydrated with ethanol and embedded in Epon-Araldite resin Ultrathin sections were observed and photographed with a Zeiss EM-10C electron microscope

Results

When ciliary membranes were dissolved with Triton X-114 and the detergent was condensed by warming, most of the solubilized protein remained in the aqueous phase One minor protein, having a molecular weight of about 20 000, specifically partitioned into the detergent-rich phase at pH 8 0 A trace of membrane tubulin and a 42 kDa protein were also generally found When the solubilization was carried out using a microtubule polymerization buffer at pH 6 4 [cf 3], a greater proportion of the 42 kDa protein was found in the detergent-rich phase The same basic results were obtained when reconstituted ciliary membranes were used, the only difference being that fewer minor proteins were present, since soluble proteins of the ciliary matrix, chiefly calmodulin, were discarded as a consequence of the reconstitution but, of course, were present in the Triton X-114 solubilized ciliary

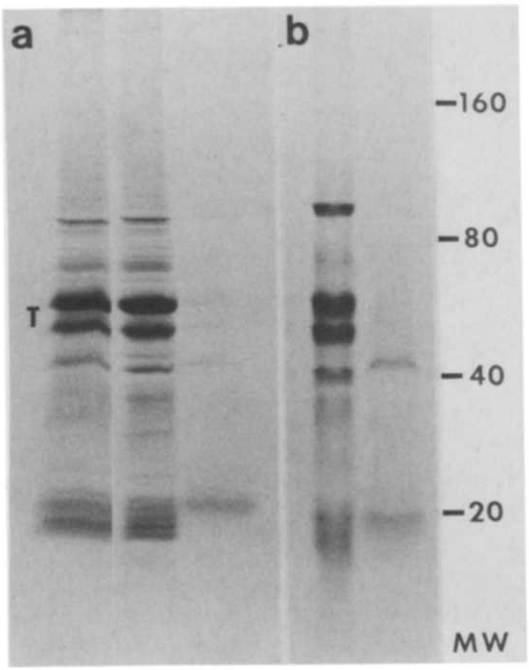

Fig 1 Partitioning of ciliary membrane proteins into Triton X-114

(a) Ciliary membrane plus matrix the first lane is the initial extract of isolated cilia in 1% Triton X-114, T designates membrane tubulin subunits The second lane is the aqueous phase (supernatant), containing most of the protein, while the third lane is the detergent-rich phase (condensate), containing mainly a protein with a molecular weight of about 20000, a trace of tubulin subunits and a protein with a molecular weight of about 42000 are also visible The condensate and supernatant were diluted to the same volume as the initial extract to achieve stoichiometric loading Band broadening is the result of the high concentration of Triton X-114 present in the extract and diluted condensate Coomassie Blue stain

(b) Reconstituted membranes when pure membranes are dissolved in microtubule polymerization buffer (0 1 M 4-morpholineethanesulfonic acid, pH 6 4, 1 mM EGTA and 0 5 mM $MgCl_2$), most proteins remain in the aqueous phase (first lane) but more of the 42 kDa protein and less of the tubulin subunits partition into the detergent-rich phase (second lane) Similar results were obtained when ciliary membranes were dissolved directly in this pH 6 4 buffer Band distortion is a result of the Mes buffer present during electrophoretic stacking Coomassie Blue stain The molecular weight scale is expressed in kilodaltons

membrane plus matrix extract Examples of these partitioning results are illustrated in Fig 1

When the Triton X-114 of the aqueous-phase membrane extract (supernatant) was adsorbed with SM-2 Bio-Beads, a mildly turbid suspension resulted, becoming more turbid upon standing on ice or after freeze-thaw When the detergent-rich phase (condensate) was diluted and treated with Bio-Beads, a highly turbid suspension resulted, changing little upon standing or after freeze-thaw When these two Bio-Bead-treated fractions were analyzed by sucrose gradient equilibrium centrifugation, they had dramatically different equilibrium densities The material derived from the condensate had a density range consistent with nearly pure lipid ($d = 1\,132 \pm 0\,012$ g/cm^3), contained less than 5% protein (mainly consisting of one polypeptide with a molecular weight of approx 20000), and was vesicular The material derived from the supernatant had a density range consistent with a much higher protein content ($d = 1\,245 \pm 0\,005$ g/cm^3), contained ciliary membrane tubulin and its associated proteins, and was mainly granular but contained some membranous material Overnight dialysis of the dense-band material to remove sucrose, followed by precipitation of the protein with trichloroacetic acid, revealed the presence of 3 8–4 2% Triton X-114 (w/w of protein), as estimated from the ultraviolet absorption spectrum of the supernatant, assuming no loss of bound Triton X-114 during the short-term dialysis Chloroform-methanol extraction of the material precipitated by trichloroacetic acid showed no detectable lipid, but as much as 5% lipid by weight could have been present but not detected, since the amount of material was very limited The original reconstituted membrane contained more than 55% lipid by weight [3] Single condensation steps yielded intermediate and variable equilibrium densities, indicating partial delipidation Fig 2 illustrates the equilibrium gradient centrifugation of the two Triton X-114 fractions, the electrophoretic analysis thereof, and electron micrographs of low- and high-density material recovered from the gradients

Since the protein composition of the Bio-Bead-treated aqueous-phase material, at isopycnic equilibrium, closely approximated that of the original membrane, it was of interest to determine whether there was size or composition heterogeneity as the material sedimented toward equilibrium To minimize initial aggregation upon detergent removal, the aqueous phase was applied directly (i e without Bio-Bead treatment and freeze-thaw) to a detergent-free sucrose gradient and replicates were

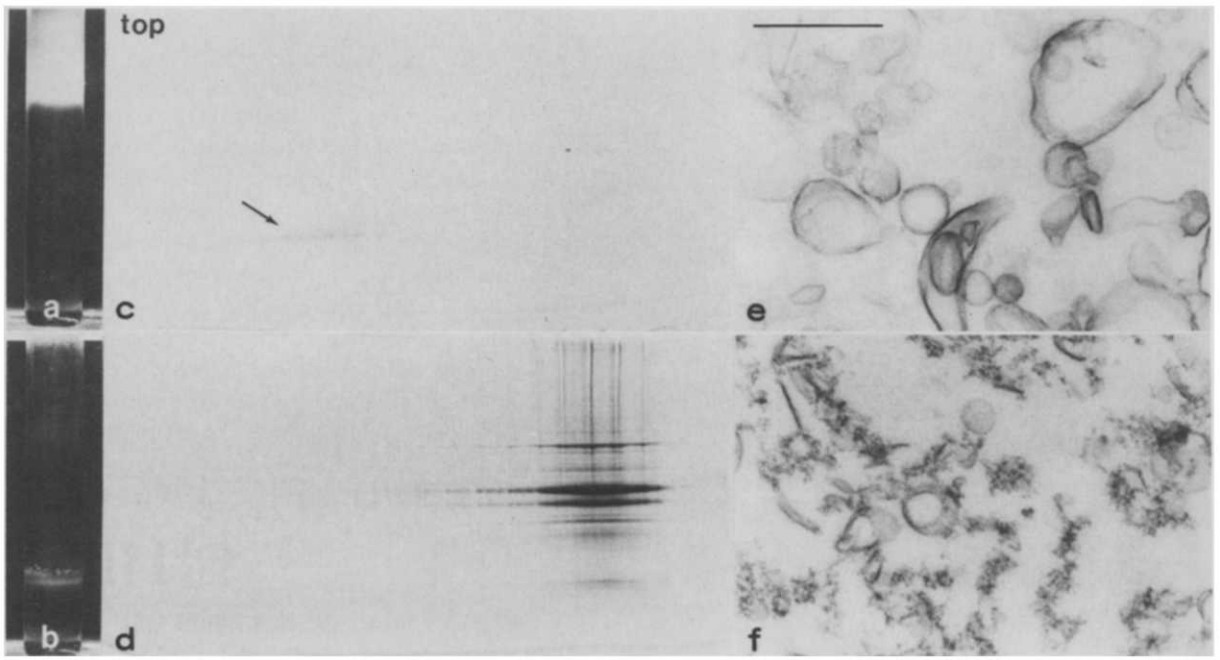

Fig 2 Characteristics of the aqueous and detergent-rich phases from Triton X-114 condensation after removal of excess detergent by adsorption, followed by freeze-thaw (a, b) Sucrose gradient equilibrium analysis (linear 20–60% sucrose w/w for 12 h at 150000 × *g*) of the condensate (a) and the supernatant (b) fractions of reconstituted membranes initially dissolved in 1% Triton X-114 at pH 8 0 (c, d) Polyacrylamide gel analysis of 20 fractions obtained from the two respective gradients The arrow in (c) designates the 20 kDa polypeptide that selectively partitions into the detergent-rich phase The equilibrated polypeptides from the aqueous fraction (d) are distributed with relatively constant stoichiometry near the bottom of the gradient Coomassie Blue stain (e f) Electron micrographs of the material recovered from the low- and high density bands of the respective gradients The low-density material consists of convoluted membrane vesicles of quite variable size (e) while the high-density material is mainly granular containing some membrane fragments The bar indicates 0 5 μm Lead citrate post-stain

centrifuged for various periods of time, excess detergent being lost as the proteins sedimented The results of one such run, silver stained to detect minor components, is illustrated in Fig 3 Although polydisperse in terms of size, the protein composition of the sedimenting material was relatively constant throughout the gradient

The results obtained above are consistent with a membrane protein complex of uniform composition, essentially devoid of lipid as a consequence of condensation with Triton X-114, but still 'soluble' by virtue of associated detergent Ultracentrifugal analysis of the Triton X-114 aqueous-phase revealed the presence of a particle (protein-detergent mixed micelle?) with a sedimentation rate of 2 6 S at 1–2 mg/ml, showing little concentration-dependence of sedimentation rate (Fig 4a) However, attempts to prepare solutions more concentrated than 2–3 mg/ml (e g by ultrafiltration followed by recondensation) resulted in aggregation of protein so polydisperse that it was not observed during rotor acceleration (although still no additional protein partitioned into the detergent-rich phase during the recondensation) In the ultracentrifuge, the sedimenting peak of soluble protein remained relatively constant in area after such a concentration-condensation step, suggesting a critical concentration for aggregation in the absence of excess detergent At lower protein concentrations, where initial concentration-induced aggregation was not a problem, the sedimenting material (as expected) accumulated at the cell sector bottom This material was not readily dispersed at the end of the run, allowing removal and fixation for electron microscopy Fig 4b indicates that this concentrated material is reticular in nature, consisting of 5–10-nm granules In contrast to Fig 2f, the sample for which was derived by

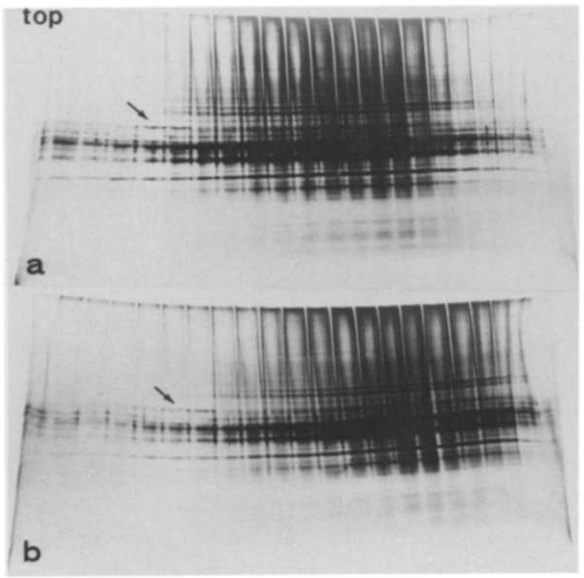

Fig 3 Sedimentation of aqueous-phase protein on a detergent-free sucrose gradient Without prior detergent-removal or freeze-thaw the proteins solubilized from reconstituted ciliary membranes achieve density equilibrium more slowly, but sediment with relatively constant composition when centrifuged at 150000 × g for 24 h (a) or 48 h (b) One protein, designated by the arrow, plus other polypeptides in the tubulin region remain near the top of the gradient Silver stain

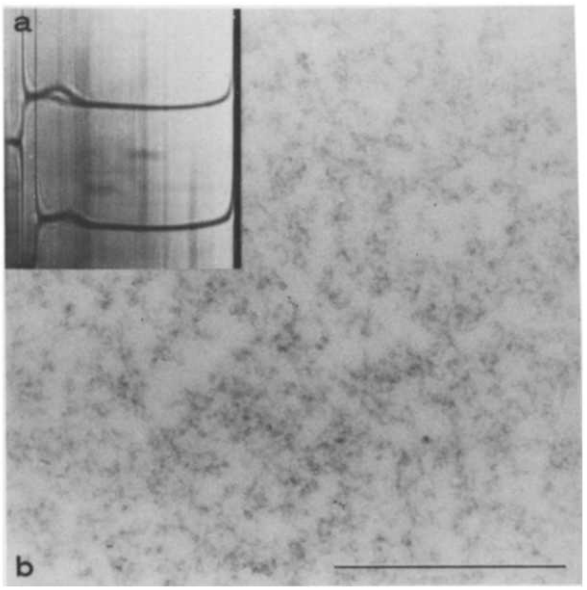

Fig 4 Velocity sedimentation and electron microscopy of aqueous-phase ciliary membrane protein (a) Analytical ultracentrifugation, 80 min after reaching a speed of 42 040 rpm Upper pattern approx 2 mg/ml, lower one-half this concentration Solvent sectors contain buffer alone saturated with Triton X-114 by condensation In these cases the initial buffer used for the solubilization and condensation (3 mM $MgCl_2$, 30 mM Tris-HCl pH 8 0) was supplemented with 0 1 M NaCl to minimize charge interactions Temperature = 20°C, bar angle = 50° (b) Electron microscopy of the material sedimenting during analytical ultracentrifugation The run above was terminated at 120 min (about 30% sedimentation) and the pellet was removed from the dismantled cell, then fixed embedded and sectioned Any detergent present should be lost during the fixation-embedment procedure Regardless of position within the thickness of the pellet, a uniform granulo-reticular material was seen Uranyl acetate en bloc stain Bar = 0 5 μm

freeze-thaw from a twice-condensed preparation, little or no membranous material is seen after ultracentrifugation of thrice-condensed material, suggesting even more complete delipidation

Attempts were made to induce microtubules or tubulin polymorphs from the ciliary membrane proteins solubilized with Triton X-114, using microtubule polymerization buffer in the initial extraction of ciliary membranes (cf Ref 3) Experiments were carried out either with detergent at or above the critical micelle concentration (i e after condensation) or only with bound detergent (i e after Bio-Bead treatment) Preparations were incubated with (a) 25 μM taxol for taxol-induced microtubules [11], (b) 0 1 mM vinblastine sulfate for vinblastine-tubulin crystals [12], and (c) 0 5 mM Zn^{2+} for zinc-induced tubulin protofilament sheets [13], all at pH 6 4 in the presence of 1 mM GTP and 0 1 mM dithiothreitol Incubations were for 1 h at 35°C or overnight at 0°C After high-speed centrifugation, no ordered structures were observed in pelleted material from any of these cases Only the granulo-reticular material noted above was seen electron microscopically Also, none of these treatments resulted in any increase in sedimentable protein in comparison with untreated controls incubated for comparable times Very high divalent cation concentrations (20 mM Mg^{2+} or Ca^{2+}) caused substantial unselective protein precipitation but induced no ordered structure

Discussion

In contrast to the results of Bordier [5] with several well-known integral membrane proteins,

only one minor protein from ciliary membranes selectively partitions into Triton X-114 micelles One must conclude either that none of the other proteins is integral or that, as a group, they behave anomalously The latter appears to be the case Condensation with Triton X-114 results in delipidation of the proteins and the subsequent formation of a soluble detergent-protein complex of relatively uniform size and composition

A similar anomaly was recently reported by Maher and Singer [14] for the acetylcholine receptor, a pentameric complex of four non-identical polypeptides with a molecular weight of about 250000 This complex remained intact in the aqueous phase after Triton X-114 condensation, still able to bind ligands normally These authors suggested that this channel-forming integral membrane protein complex differed from other kinds of integral protein in the exterior surface of its hydrophobic domains, unable to form detergent-protein micelles, apparently for steric reasons In support of this, they noted that mixed micelles of Triton X-114 and linoleic acid carried the acetylcholine receptor into the detergent-rich phase

Earlier, Glenney and Glenney [15] utilized Triton X-114 to demonstrate that a 110 kDa protein of the microvillus was integral They noted that either SDS or lauric acid was required for the initial solubilization by Triton X-100, after further purification, the protein was shown to partition into the detergent-rich phase of Triton X-114 However, Verner and Bretscher [16] found this protein to be water-soluble, given that ATP was present continually, and they suggested that the Triton X-114 solubility noted by the above authors may have been the consequence of SDS denaturation, exposing hydrophobic regions Alternatively, it could be argued that bound SDS (or lauric acid) facilitates micelle formation with Triton X-114, as in the case of the acetylcholine receptor

Regula and coworkers [6] purified synaptosomal membranes, dissolved them in Triton X-100, taxol-precipitated the tubulin therein, and then extracted with Triton X-114 Upon condensation, about half of the tubulin and some of the microtubule-associated proteins partitioned into the micellar detergent phase This preliminary report noted that soluble tubulin differed chemically from hydrophobic tubulin and that certain microtubule-associated proteins were uniquely present in the hydrophobic phase, suggesting that membrane-specific tubulin and microtubule-associated proteins might be involved in membrane function As noted above, taxol did not precipitate ciliary membrane tubulin from Triton X-114 solutions, so a direct comparison with synaptosome tubulin partitioning is not possible In fact, taxol will induce vesicle formation from Triton X-100 solutions of ciliary membrane proteins [17], possibly a consequence of taxol-induced lipid-protein separation from the mixed detergent-lipid-protein micelles formed during Triton X-100 (or Nonidet P-40) solubilization [4]

How much lipid still remains bound to integral membrane proteins after Triton X-114 extraction and condensation has not been reported, but residual lipid may influence the partitioning of specific integral membrane proteins or complexes thereof into Triton X-114 micelles Similarly, displacement of lipid by detergent may result in a soluble entity, having insufficient bound detergent to form large (i e condensable) micelles

That even cytoplasmic tubulin can interact with non-denaturing detergents was demonstrated clearly by Andreu [18] Brain tubulin was shown to bind about 1/4 and 1/2 of its weight of deoxycholate and octyl glucoside, respectively, and probably a comparable amount of Triton X-100 Deoxycholate promoted microtubule assembly, octyl glucoside prevented it, and Triton X-100 moderately inhibited assembly, each case indicating some degree of detergent interaction with the hydrophobic sites involved in microtubule assembly

It thus seems most likely that when ciliary membrane tubulin and associated proteins are solubilized with Triton X-114, a mixed detergent-lipid-protein micelle results, as is the case with Triton X-100 [4] But unlike the situation with Triton X-100 where treatment with polystyrene beads adsorbs only the detergent, condensation with Triton X-114 must involve selective removal of lipid by the condensing micellar detergent, evidently leaving the tightly-associated membrane proteins as a soluble, non-condensing complex stabilized by bound Triton X-114

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