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Activity of Colicin E3-Treated Ribosomes in Initiation and in Chain Elongation

(purified polysomes/immunity substance/streptomycin)

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ABSTRACT **Colicin E3 (called E3 here) is shown to act on polysomal ribosomes as well as on free ribosomes. This treatment severely inhibits subsequent chain elongation, both on preformed polysomes completing their nascent chains and on ribosomes translating poly(U). Elevated Mg^{++} concentration relieves this inhibition. However, elevated Mg^{++} concentration does not relieve the inhibition of ribosomes initiating on natural messenger; hence damage by E3 appears to have an additional, specific effect on some stage in initiation.**

The effect of damage by E3 resembles that of streptomycin in several respects. This similarity suggests that the two agents may act on closely related sites on the ribosome.

Colicin E3 (designated hereafter as E3) inhibits protein synthesis (1) by cleaving a specific "E3 fragment" from the 16S RNA in the ribosome, both in cells (2, 3) and in solution (4, 5). Studies of this process have been largely directed at measuring the effect of various conditions on the interaction of E3 with its substrate (2, 4, 6–8). In addition, the observation that E3 inhibited the translation of phage RNA completely, but that of poly(U) only 50% (4), suggested that ribosomes are especially vulnerable to E3 action during physiological initiation. However, it has not been clearly shown whether this action is specific for various stages of the ribosome cycle; neither is it known whether the altered ribosome is differentially inhibited in initiation and in chain elongation.

In a parallel problem, concerned with defining the action of antibiotics on various stages of the ribosomal cycle, it has been useful to compare ribosomes initiating on viral RNA with ribosomes already complexed in polysomes (9, 10). Accordingly, we have treated polysomes and free ribosomes of *Escherichia coli* with E3, in the absence of components required for protein synthesis, and have then measured the impairment of their activity. Ribosomes in both states are found to be attacked by E3, and the resulting alteration is shown to affect both initiation and chain elongation.

MATERIALS AND METHODS

Initiation-free purified polysomes were prepared from lysates of *E. coli* MRE600 (RNase I^-, ref. 11) by gel filtration on Sepharose 4B, as described (9). Crude initiation factors, NH_4Cl-washed ribosomes, and phage R17 RNA were prepared, and peptide synthesis was assayed with [^{14}C]valine, as described (9). Polymerization of [^{14}C]phenylalanine (180 Ci/mol), directed by poly(U) (200 μg/ml), was assayed similarly except that the other 19 amino acids and the initiation factors were omitted. Ribosomes were incubated with E3, where indicated, in TKMD buffer [Tris·HCl (10 mM) pH 7.6–KCl (50 mM)–$Mg(OAc)_2$ (10 mM)–dithiothreitol (1 mM)]. Radioactivity was measured on Millipore filters in toluene scintillation fluid with a Nuclear Chicago Isocap scintillation counter. As recommended by J. Sidikaro, E3 was handled in plastic tubes.

[^{14}C]Valine and [^{14}C]phenylalanine were obtained from New England Nuclear Corp., and streptomycin sulfate from E. R. Squibb. [^{3}H]Dihydrostreptomycin (750 Ci/mol) was a gift of F. Gros; highly purified E3 and immunity substance were gifts of J. Sidikaro and M. Nomura.

RESULTS

Action of E3 on Polysomal Ribosomes. Elongating (polysomal) ribosomes might conceivably differ from free ribosomes in their ability to be altered enzymatically by colicin E3. In addition, this alteration might selectively affect initiation or chain elongation. The ability to be altered by E3 was tested with free ribosomes and with purified polysomes both in buffer and under conditions permitting protein synthesis.

In the tests for reactivity during protein synthesis a moderate concentration of E3 (10 μg/ml) was added at the start to initiation-free purified polysomes (which complete their round of synthesis in about 10 min: ref. 9), and to free, runoff ribosomes initiating on phage R17 RNA. Both systems showed a gradually increasing inhibition (Fig. 1). Such a delay would be expected if the inhibition depends on enzymatic action of E3.

In the tests for interaction in buffer the polysomes, and the free ribosomes, were preincubated for 30 min with 20 μg/ml of E3 in buffer and then were allowed to synthesize protein at 8 mM Mg^{++}. Both preparations exhibited extensive inhibition (70–80%) without delay (Fig. 2). (In similar experiments with other ribosome preparations inhibition reached 95%). While these data do not precisely compare the kinetics of action of E3 on free and on polysomal ribosomes, they do show that there is no gross difference.

The action of E3 on polysomes does not cause (or depend on) their breakdown. Thus, after treatment of polysomes with E3 in buffer the profile observed in zonal centrifugation was not altered, and in a protein-synthesizing system the inhibited polysomes persisted for a long time (at 8 mM Mg^{++}) compared with the runoff of untreated polysomes. Moreover, as we shall see below, the activity of the treated polysomes could be restored under certain test conditions.

In the preceding experiments the E3 might conceivably continue to act on the ribosomes during the test for activity after the pretreatment. In subsequent experiments this complication was avoided by adding "immunity substance" from

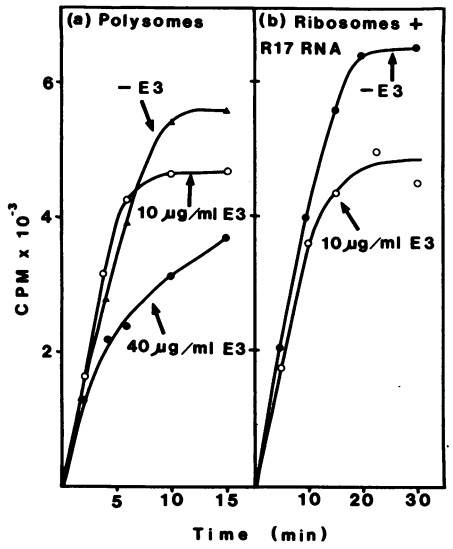

FIG. 1. Delayed effect of E3 on activity of purified polysomes and initiating ribosomes. (*a*) Protein-synthesizing mixtures containing 600 μg/ml of purified polysomes, [^{14}C]valine, and E3 as indicated were incubated at 35°; E3 was added before the polysomes. At the times indicated, 30 μl was removed for measurement of amino-acid incorporation. (*b*) Purified polysomes (600 μg/ml) were allowed to run off by being incubated with all the required reagents for 15 min at 35°. Phage R17 RNA, crude initiation factors, and [^{14}C]valine were added (see *Methods*), together with E3 where indicated; incubation and sampling were as in (*a*). Values on ordinate have been multiplied by 10^{-3}.

colicinogenic *E. coli* (5) at the end of the pretreatment. This material has been shown to form an inactive complex with E3 (12). In our experiments (see Figs. 3 and 5) the amount of

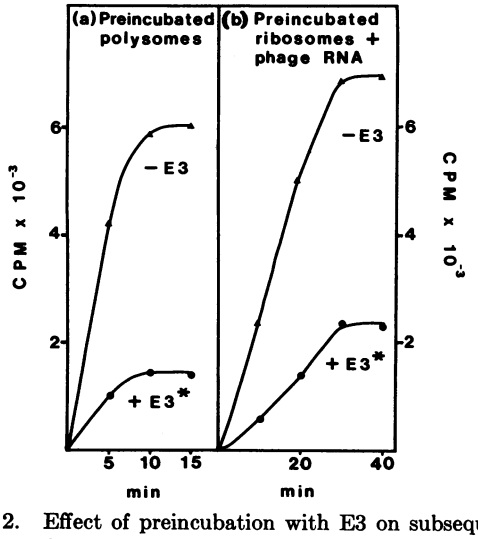

FIG. 2. Effect of preincubation with E3 on subsequent peptide elongation at 8 mM Mg^{++}. (*a*) Purified polysomes (60 μg) were preincubated with or without 2 μg of E3 in 50 μl of TKMD buffer at 36° for 30 min. All the additional components required for protein synthesis (including [^{14}C]valine) were then added, in an equal volume of buffer. At intervals, 25-μl samples were analyzed for incorporation. (*b*) NH_4Cl-washed ribosomes were preincubated as in (*a*) and then tested for incorporation of [^{14}C]valine in the presence of phage R17 RNA and initiation factors.

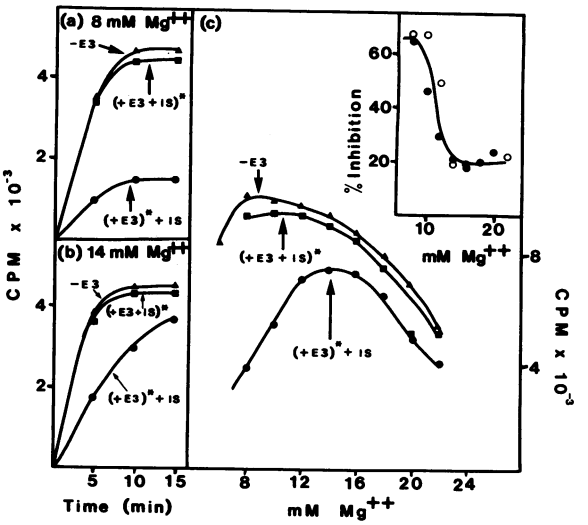

FIG. 3. Retention of activity of E3-treated polysomes at high Mg^{++} concentration. Purified polysomes were preincubated in buffer with E3 where indicated (2 μg of E3 per A_{260} unit of ribosomes per 20 μl; molar ratio 1.5) at 36° for 30 min. (▲) No E3; (●) E3 present, and after preincubation 1.25 μg of immunity substance (*IS*) was added (3.5 mol/mol of E3); (■) E3 and IS added together [(E3 + IS)*] before the preincubation. The activity of the variously treated polysomes in complete protein-synthesizing systems, at various Mg^{++} concentrations, was then determined. (*a* and *b*) During the incubation at the indicated Mg^{++} concentration, 25-μl samples were removed at intervals for analysis of [^{14}C]valine incorporation. (*c*) Samples (50-μl) were analyzed after incubation at various Mg^{++} concentrations for 15 min. *Inset:* inhibition of incorporation at various Mg^{++} concentrations (●), calculated from data in (*c*); *open circles* were from another set of similar experiments. (+*E3* + *IS*)*: preincubation of ribosomes with E3 and IS. (+*E3*)* + *IS:* preincubation of ribosomes with E3, IS then added.

immunity substance added was sufficient to inactivate all the E3, and the mixture had no effect on protein synthesis by either purified polysomes or initiating ribosomes.

Increased Activity of E3-Treated Polysomes at Elevated Mg^{++}. E3-treated polysomes were unexpectedly found to carry out considerably more synthesis at 14 mM Mg^{++} (though at a reduced rate) than at 8 mM Mg^{++} (Fig. 3a and b). Further studies showed that the treated polysomes had an optimum Mg^{++} concentration of 12–16 mM (Fig. 3*c*) and, indeed, were nearly as active as untreated polysomes under these conditions (Fig. 3*c*, *inset*). It thus appears that elevated Mg^{++} concentration restores a conformation to E3-damaged ribosomes that permits them to be active in chain elongation, though they are inactive at Mg^{++} concentrations optimal for undamaged ribosomes.

Effect of Mg^{++} on Inhibition of Translation of Poly(U). The effect of E3 and Mg^{++} was also studied with the poly(U)-directed polymerization of phenylalanine, which, like the activity of purified polysomes, involves chain elongation but not physiological initiation. As Fig. 4 shows, pretreatment of NH_4Cl-washed ribosomes with E3 markedly reduced their subsequent activity at 10 mM Mg^{++}, as previously observed (5). However, the inhibition disappeared at elevated Mg^{++} concentration, even more completely than had been observed with preformed polysomes: at 18 mM Mg^{++} the rate as well

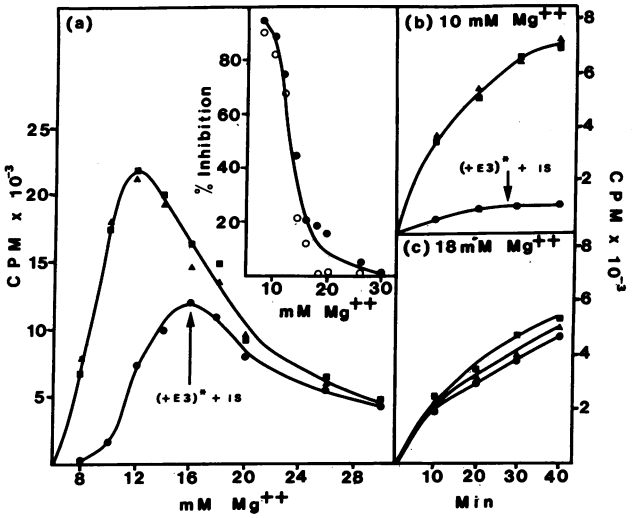

FIG. 4. Retention of activity of E3-treated ribosomes on poly(U) at high Mg^{++} concentration. Incubations and symbols as in legend of Fig. 3, except that NH_4Cl-washed ribosomes were used and the preincubation was for 60 min. Poly(U)-directed incorporation of [^{14}C]phenylalanine was then measured (see *Methods*). (*a*) Samples (50-μl) were removed after incorporation for 30 min. *Inset: closed circles,* data from (*a*); *open circles,* data from a set of similar experiments, except that preincubation with E3 was for 30 min. (*b* and *c*) Samples (25-μl) were removed for analysis after incorporation for various periods.

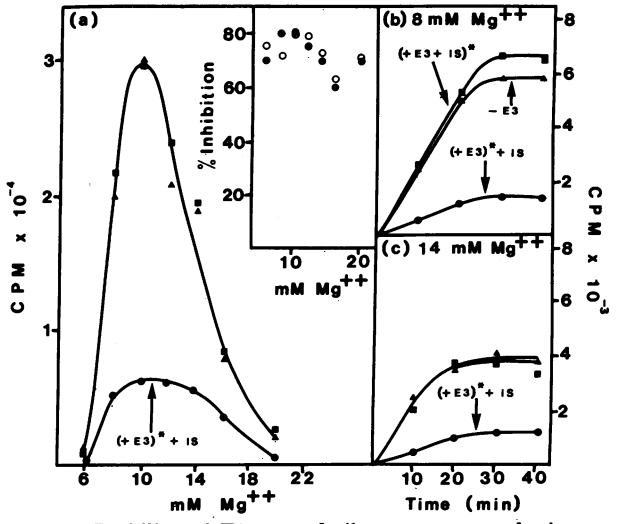

FIG. 5. Inability of E3-treated ribosomes to synthesize protein on phage RNA. Preincubations and symbols as in legend of Fig. 4. After the preincubation, the ribosomes were assayed for [^{14}C]valine incorporation directed by R17 RNA, with initiation factors added.

as the extent of translation of poly(U) by treated ribosomes was virtually identical with that observed with untreated ribosomes (Fig. 4*c*).

Inability of E3-Treated Ribosomes to Function in an Initiating System. E3-treated ribosomes are also blocked in the translation of phage RNA at the usual Mg^{++} concentrations, as would be expected since that translation involves the same mechanism of chain elongation as the systems described above. However, in contrast to its effect on these systems, elevated Mg^{++} concentration did not restore the activity of E3-treated ribosomes initiating on phage RNA. Indeed, as Fig. 5 shows, the inhibition, relative to the activity of untreated ribosomes, varied only slightly over the whole effective range of Mg^{++} concentration.

It thus appears that the cleavage of the "E3 fragment" impairs both a step in chain elongation and a step in initiation; and while elevated Mg^{++} concentration can correct the former block, it has little effect on the latter.

Similarity in Mechanism of Inhibition by E3 Damage and by Streptomycin. Like E3 damage, streptomycin also causes Mg^{++}-dependent inhibition of polysomes and Mg^{++}-independent inhibition of initiating ribosomes (10). Moreover, Dahlberg *et al.* (13) have shown that it antagonizes the enzymatic action of E3 on ribosomes. We have therefore compared the two agents in other respects.

The antagonism of streptomycin to E3 action was found to be complemented by an effect of E3 on the binding of the antibiotic. Thus, pretreatment of free ribosomes with E3 decreased the binding of dihydrostreptomycin about 50% (Table 1). This loss is not as great as the loss of activity in protein synthesis; hence the affinity for streptomycin in E3-damaged ribosomes, though reduced, may be sufficient to permit some retention of bound antibiotic under the conditions used. The loss of binding evidently depends on the enzymatic action of E3, since immunity substances added with the E3 protected against the loss (Table 1).

We also compared E3 and streptomycin to see whether their inhibitory effects on protein synthesis are additive, synergistic, or mutually exclusive. Polysomes were tested at 13 mM Mg^{++} (an intermediate concentration), which permits considerable activity after E3 damage (Fig. 3) and also in the presence of streptomycin (10). As Fig. 6 shows, polysomes pretreated with E3 were not further inhibited by streptomycin.

Though E3 and streptomycin have a similar effect on the rate of chain elongation, they do not have a similar effect on its accuracy. As Table 2 shows, pretreatment of ribosomes with E3 did not increase the misreading of poly(U), measured by the ratio of (isoleucine plus leucine) to phenylalanine incorporated. Streptomycin, however, caused a marked increase in misreading, as expected (14, 15).

TABLE 1. *Inhibitory effect of E3 pretreatment on binding of dihydrostreptomycin to ribosomes*

Ribosome treatment	Dihydrostreptomycin binding	% Inhibition
−E3 − IS	11,239	—
(+E3)* + IS	5,319	52
(+E3 + IS)*	11,367	−1

Binding of dihydrostreptomycin was measured by the Millipore assay of Chang and Flaks (26). Reaction mixtures for binding contained 0.1 ml of $TKM_{10}D$ buffer with 5 μM [^{3}H]dihydrostreptomycin (750 Ci/mol) and 60 μg of NH_4Cl-washed ribosomes, pretreated as in legend of Fig. 4 (*asterisk* denotes addition at start rather than at end of pretreatment). After incubation at 34° for 5 min, 2 ml of cold buffer was added and the mixtures were filtered through Millipore filters, which were washed with cold buffer, dried, and counted for ^{3}H. Background binding without ribosomes (280 cpm) has been subtracted.

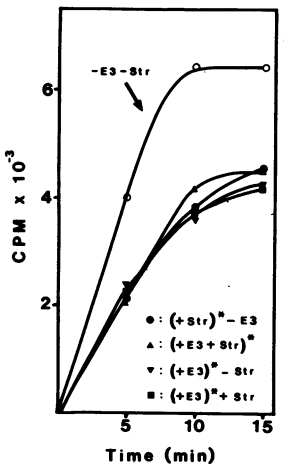

FIG. 6. Failure of streptomycin to inhibit E3-treated polysomes. Polysomes were preincubated, without (○) or with (▼) E3, as in Fig. 3, and were then tested for incorporation of [^{14}C]valine. In addition, streptomycin was added to a mixture before (▲) or after (■) preincubation with E3, and to an incubation mixture without E3 (●). Final concentrations: polysomes, 600 μg/ml; streptomycin, 20 μg/ml.

DISCUSSION

Effects on Initiation and Chain Elongation. It has seemed likely that colicin E3 can attack polysomal ribosomes, since in cells it inhibits the release of nascent peptides (16), which suggested inhibition of elongation or termination; such treated cells also maintain a substantial level of polysomes (A. Dahlberg, personal communication). The present paper presents more direct, *in vitro* evidence that E3 can attack polysomal ribosomes, for it inhibits protein synthesis by purified, initiation-free polysomes (Figs 2 and 3) without causing their breakdown. We could not confirm the reported instability of E3-treated polysomes in solution (16), even in sucrose gradients with 1 mM Mg^{++} (unpublished observations).

The degree of inhibition of polysomes was found to decrease with increasing Mg^{++} concentration (Fig. 3). A very similar Mg^{++}-sensitive inhibition was seen when free ribosomes were treated with E3 and then used to translate poly(U) (Fig. 4) (which also involves chain elongation without physiological initiation). This similarity supports the reasonable assumption that E3 causes the same alteration in polysomal ribosomes as that already demonstrated with free (or unspecified) ribosomes, i.e., cleavage of the "E3 fragment" from 16S RNA. We do not know precisely which step(s) in chain elongation is blocked as a consequence of E3 damage.

The effect of Mg^{++} on the translation of poly(U) by ribosomes treated with E3 reconciles the apparent discrepancy between the report that this treatment reduced the translation of this messenger only 50% (4) and the report that the reduction was almost complete (5, 8). The former study tested for synthesis at 17 mM Mg^{++} and the latter at 10 mM.

In contrast to its effect on systems engaged in chain elongation without physiological initiation, elevated Mg^{++} concentration had little effect on the block observed with ribosomes initiating on natural messenger (Fig. 5*a*, *inset*). It thus appears that damage by E3 specifically affects some step in initiation in addition to affecting some step in chain elongation. It should be emphasized that such a block in an initiating system does not necessarily imply inhibition *of* initiation, i.e., a block in the formation of an initiation complex. Thus several antibiotics, including streptomycin (17, 18), permit formation of an initiation complex but block the transition into chain elongation and destabilize the attachment of the blocked complex to messenger RNA. We have found (unpublished) that treatment of free ribosomes with E3 markedly decreases their subsequent net formation of initiation complexes with fMet-tRNA and phage messenger, but further work will be required to determine whether the actual formation or the stability of the complex is altered.

TABLE 2. *Lack of misreading by E3-treated ribosomes*

Treatment	Relative incorporation Phe	Relative incorporation Ile + Leu	(Ile + Leu)/Phe × 100*
None	100	9	9 (8–9)
E3	28	2	7 (7–8)
Str	25	20	80 (80–95)

Twenty-five A_{260} units of NH_4Cl-washed ribosomes in 50 μl of buffer were incubated with or without 2 μg of E3 at 37° for 60 min. These ribosomes were assayed for their poly(U)-directed incorporation (30 min at 37°) at 10 mM Mg^{++} in the presence of 0.03 mM each of [^{14}C]phenylalanine (180 Ci/mol), [^{3}H]isoleucine, and [^{3}H]leucine (720 Ci/mol each). Untreated ribosomes were also assayed in the presence of 4 μg/ml of streptomycin (*Str*). Relative incorporation of 100 = 225 pmol of phenylalanine incorporated per 0.1 ml of reaction mixture. Background and cross-channel radioactivity has been corrected.

* Numbers in parentheses represent the ratio of (Ile + Leu)/Phe after 10 and 20 min of incubation, respectively.

Relation to the Action of Streptomycin. A resemblance between the actions of E3 and streptomycin was noted earlier by Konisky and Nomura (19), and Dahlberg *et al.* (13) have shown that the enzymatic action of E3 on ribosomes is antagonized by streptomycin or tetracycline. While this effect could be due to a specific link between the actions of the antibiotic and of E3, with streptomycin it could also be due to the more general effect of decreasing the conformational flexibility of the ribosome (20). Our findings support the former interpretation. Thus, elevated Mg^{++} concentration and ethanol, which resemble streptomycin in antagonizing dissociation of ribosomes (21), did not interfere with the action of E3 (unpublished observations). Even more significant is the observation that damage to ribosomes by E3 decreases their binding of dihydrostreptomycin (Table 1). Moreover, at an intermediate Mg^{++} concentration, at which E3-damaged polysomes exhibit only partial inhibition of activity, streptomycin causes no additional inhibition (Fig. 6). Finally, the binding of streptomycin, like E3 damage, causes a Mg^{++}-sensitive inhibition of chain elongation (10, 22) and a relatively Mg^{++}-insensitive inhibition of some aspect of initiation (22).

In one important respect, however, the two agents differ in their effect on ribosome function: E3-damaged ribosomes do not exhibit increased misreading (Table 2). Thus, the effects of E3 damage are less pleiotropic than those of streptomycin and do not affect the specificity of codon–anticodon interaction.

The similarities in the effects of E3 and streptomycin noted here support the suggestion (13) that the RNA fragment removed from the 30S moiety of the ribosome by E3 may be located close to, or may have a strong conformational link to,

a site of action of streptomycin. The relation is of particular interest because the inhibitory action of streptomycin requires a sensitive allele of a known protein, S12 (23); moreover, absence of this protein in reconstituted particles (23) or its alteration at a nonpermissive temperature in a temperature-sensitive mutant (24, 25) prevents synthesis that requires physiological initiation on viral RNA but has little effect on the translation of poly(U). Since the action of E3 is related to a known portion of the 16S RNA in the same subunit, the similarity of several effects of E3 and streptomycin may provide a probe into the interactions between ribosomal RNA and proteins.

Note Added in Proof. Recently Kaufmann and Zamir (27) reported that ribosomes of *E. coli* complexed with Phe-tRNA and poly(U) are resistant to inactivation by E3. It is not obvious why the complexed ribosomes in these experiments should differ so strikingly from those in endogenous polysomes, described in the present paper.

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