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Agglutination of *Streptococcus mutans* by Low-Molecular-Weight Salivary Components: Effect of β_2-Microglobulin

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Radiolabeled monomeric human β_2-microglobulin (β_2m) was tested for binding to *Streptococcus mutans* strains in buffers containing 1 mM calcium (Ca^{2+}). Binding was seen to strains with a previously established binding capacity of aggregated β_2m. Monomeric β_2m agglutinated β_2m-binding strains when Ca^{2+} was present. At Ca^{2+} concentrations of 1.4 mM, 0.032 μg of monomeric β_2m per ml caused bacterial agglutination. Parotid saliva was gel filtered on a Sephadex G-75 column, and low-molecular-weight fractions containing β_2m could agglutinate *S. mutans* cells. Five of six strains that could bind β_2m were agglutinated by these fractions, but only one of five nonbinding strains was. All strains tested were agglutinated by void volume fractions. A new method for the measurement of turbidity in bacterial agglutination inhibition experiments with parotid saliva was used. Suspensions containing parotid saliva, bacteria, and control serum were directly compared in a spectrophotometer with test suspensions containing goat anti-human β_2m, bacteria, and saliva. Thus, the spectrophotometer directly read the difference in agglutination of the two suspensions, and the result was presented as one curve by the recorder. Agglutination of five β_2m-binding strains of *S. mutans* was inhibited or decreased by the addition of goat anti-human β_2m as compared with control serum. The agglutination of two β_2m-nonbinding strains and one with variable binding was not inhibited. Thus, salivary β_2m may contribute to agglutination of *S. mutans* cells in parotid saliva.

Oral streptococci can be agglutinated by various salivary molecules including high-molecular-weight agglutinins (13, 17–19, 27, 31), secretory immunoglobulin A (IgA) (20, 24, 33), blood group reactive substances (16, 34), and lysozyme (30). Generally, the agglutination phenomenon of bacteria in saliva has been considered to be a host defense system for the disposal of bacteria (32–34). However, the bacterial agglutinating factors can also serve as adherence-promoting factors in the pellicle (23–25), and the formation of small aggregates may increase the number of bacteria binding to saliva-coated hydroxyapatite (23). Even though bacterial binding of a particular molecule does not result in agglutination, the bacterial surface properties may be altered (26) and their colonization abilities may be changed.

It seems unlikely that one bacterial binding or agglutinating component is solely responsible for the clearance or attachment of a specific bacterium; several probably contribute to the resulting effect (15, 32). There have also been reports that indicate interaction between high-molecular-weight agglutinins and secretory IgA, which could both have affinity for the same species (9, 27). This makes the functional picture more complex. Therefore, the importance of bacterial binding by a single isolated factor is not easy to demonstrate. Nevertheless, evaluation of the effect of single factors is necessary.

We have earlier demonstrated that aggregated β_2-microglobulin (β_2m) can interact with strains of oral streptococci (10, 12). β_2m is a low-molecular-weight protein occurring on surfaces of all nucleated cells as the light chain of the major histocompatibility antigens (for a review, see reference 3). β_2m is also present in saliva and other body fluids (2, 3, 11, 28). Previous studies have shown that aggregates, but not monomers, of β_2m bind to bacteria (12, 21). Aggregation of β_2m to high-molecular-weight complexes strongly increases the avidity of the interaction between bacteria and the β_2m complexes. The complexes then can bind to low-affinity receptors (10, 12). The need for a multipoint attachment has been considered significant in the binding of bacteria to cell surfaces (5), where β_2m seems to be present as repetitive units. In saliva, however, high-molecular-weight complexes containing β_2m have not been found, but β_2m is present as monomers (11). One aim of this study was to learn whether binding of β_2m monomers to *Streptococcus mutans* cells can be demonstrated. To do this, the influence of calcium ions (Ca^{2+}) on the binding of the protein to bacteria was analyzed, since some bacteria-binding salivary agglutinins demand Ca^{2+} for interaction with bacteria (9, 17, 31) and others do not (22, 30, 33).

Another aim was to investigate whether β_2m monomers, the form present in saliva, could agglutinate strains of *S. mutans* and whether salivary fractions containing β_2m could agglutinate *S. mutans* cells. The effect of addition of anti-β_2m antibodies on the agglutination of *S. mutans* strains in parotid saliva was also studied.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Bacterial strains that were analyzed previously for binding aggregated β_2m were selected (10, 12). Binding of aggregated β_2m was scored as +, −, or +/− (see Table 1, footnote *a*) in the following strains: *S. mutans* AHT (+) and 3720 (+) (serotype a); BHT (−) (serotype b); P8 (−), KPSK 2 (+/−), 1449 (−) and NCTC 10449 (+) (serotype c); ME1 (+) (serotype d); LM7 (−) (serotype e); OMZ 175 (+) (serotype f); K1 (−); and OMZ65 (+) (serotype g). Bacterial cultures were maintained on blood agar plates and transferred to tubes containing 10 ml of dialyzed yeast extract medium (7). After incubation in anaerobic jars overnight in 95% N_2–5% CO_2, the bacteria were washed twice in phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl [pH 7.0]; PBS) and suspended to an optical density (OD) of 1.50 at 700 nm in a Beckman model 35 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Binding studies. Binding of monomeric ^{125}I-labeled human β_2m to bacteria was tested as described earlier, with some exceptions (10, 12). Briefly, 200 μl of a suspension of 10^9

bacteria per ml in KCl buffer was incubated with 25 μl (ca. 0.3 μg, giving a radioactivity of 10,000 cpm) of radiolabeled human β_2m (these mixtures did not contain Tween 20 or human serum albumin [HSA] as in the earlier method). After incubation for 60 min, the suspension was washed with 2 ml of KCl buffer with 0.05% Tween and centrifuged. The radioactivity in the pellet was measured and compared with the radioactivity in 25 μl of labeled protein solution. In another experiment, 0.05% Tween 20 was added to the 25 μl of protein solution before incubation with the bacterial suspensions.

Saliva and gel filtration. Parotid saliva from two subjects was stimulated with citric acid to a flow rate of approximately 0.4 ml/min and collected in modified Lashley cups. Samples were used immediately for gel filtration. A 7-ml sample of saliva was fractioned on a Sephadex G-75 column (Pharmacia Fine Chemicals, Sweden) (2.5 by 42 cm) and eluted with 1 mM phosphate buffer (pH 6.95) (with 0.05 M KCl, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$; KCl buffer) containing 0.02% NaN_3. Fractions (4.9 ml each) were collected at an elution rate of 14.6 ml/h, and the absorbance at 280 nm was measured in the spectrophotometer. The elution positions of blue dextran 2000 (Pharmacia) and hen egg white lysozyme (HEWL) (E. Merck AG, Darmstadt, Federal Republic of Germany) were determined on the G-75 column.

IgA analysis. The concentration of IgA in every second fraction from the Sephadex G-75 column was measured by an immunobead enzyme-linked immunosorbent assay (6).

β_2m analysis. The concentration of β_2m in saliva fractions from the Sephadex G-75 column was determined by a radioimmunoassay, using ^{125}I-labeled purified human β_2m and goat anti-human β_2m (GAβ_2m) (29). Preparation procedures and the properties of GAβ_2m are given extensively elsewhere (4). The elution position of β_2m on the Sephadex G-75 column was also determined by gel filtration of monomeric human ^{125}I-labeled β_2m.

Amylase analysis. The presence of amylase in fractions was assayed by immunodiffusion in agarose gel (1% ME agarose [FMC Corp., Rockland, Me.] in 0.02 M barbital buffer [pH 8.6]) against rabbit anti-human secretory amylase (Nordic, London; batch 9-1177).

Agglutination assays. Fifty μl of gel filtration fractions was mixed with 25 μl of bacterial suspension in a microtiter plate (Dynatech M129A, Dynatech Deutschland, Federal Republic of Germany). Bacterial agglutination was determined after 90 min with a stereomicroscope at 10× magnification by comparing β_2m-containing and β_2m-free suspensions. The degree of agglutination was rated ++, indicating heavy agglutination; +, indicating weak but definite agglutination; or −, indicating no agglutination.

Human β_2m was isolated from urine samples as previously described (2) and was aggregated with glutaraldehyde (1). Monomeric and aggregated β_2m were diluted in KCl buffer, and 50-μl samples were used for agglutination assays as described above, except that agglutination was determined after 3 to 5 h. Different volumes of 10 mM solutions of $CaCl_2$ in distilled water were added to incubation mixtures in some experiments to determine whether the divalent cation Ca^{2+} would influence agglutination. Lysozyme has a molecular weight somewhat higher than that of β_2m and has been reported to agglutinate oral streptococci (30). HEWL was therefore included as a control in agglutination assays.

Agglutination inhibition experiments. The five strains of *S. mutans* capable of binding aggregated β_2m were tested for agglutination by freshly collected parotid saliva mixed with GAβ_2m or preimmune control serum (CS) from the same animal. Two nonbinding strains and one with a variable binding were also tested. First, 10 μl of GAβ_2m or CS diluted 1:10 in PBS was added to 150 μl of parotid saliva diluted 1:2 in PBS, in Hellma Suprasil Quartz microcells (Hellma, Müllheim/Baden, Federal Republic of Germany) at 37°C. After 50 min, 250 μl of bacterial suspension was added. Agglutination was followed at 700 nm in a Beckman model 35 spectrophotometer with a four-position manual sample changer at 37°C. Positive controls contained 150 μl of parotid saliva diluted 1:2 in PBS and a 250-μl bacterial suspension. Negative controls contained PBS instead of saliva.

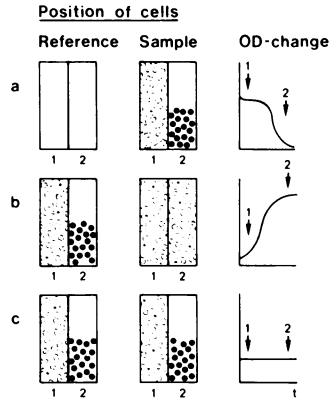

FIG. 1. Placement of cells for studying agglutination of bacteria in the spectrophotometer. (a) If bacteria agglutinate in the sample position cell, a decrease in OD at $t = 2$ will be seen as compared with the buffer-containing cell in the reference position. (b) If bacteria agglutinate in the reference position cell, but not in the sample position cell, the OD will increase from 0 at $t = 1$ (if both cells had the same OD) to a value showing the difference in OD between the cells at $t = 2$. (c) No change in OD will be seen if bacteria behave the same in sample and reference positions.

Cells containing positive control or CS were placed in reference positions, and cells containing negative control or GAβ_2m were placed in sample positions in the spectrophotometer (Fig. 1b). Agglutination in the positive control but not the negative control was read as an increase in absorbance. Similarly, when GAβ_2m-containing cells were read against CS-containing cells, a faster agglutination rate in the CS-containing cells was read as an increase of absorbance. If the agglutination rate was identical in the cells, no increase of absorbance was seen (Fig. 1c). For comparison, three of the strains were assayed according to Ericson et al. (13), using cells containing PBS in reference position (Fig. 1a, 2a, and 3a).

RESULTS

Binding experiments. Radiolabeled monomeric β_2m bound to three strains that had also bound aggregated β_2m and to one strain that had shown a variable affinity for aggregated β_2m (Table 1). The addition of 0.05% Tween 20 totally inhibited binding of all strains tested (Fig. 4).

Parotid saliva fractioned on a Sephadex G-75 column eluted as four peaks (Fig. 5). Salivary IgA eluted in the void volume and β_2m eluted in peak 3. The maximum concentration of β_2m was 4.7 μg/l. In another control column run, radiolabeled human monomeric β_2m eluted at the same position. The molecular weight marker blue dextran 2000 eluted with void volume, and HEWL eluted in fractions 32 to 39. Amylase was detected in fractions 34 to 49 (peaks 2 and

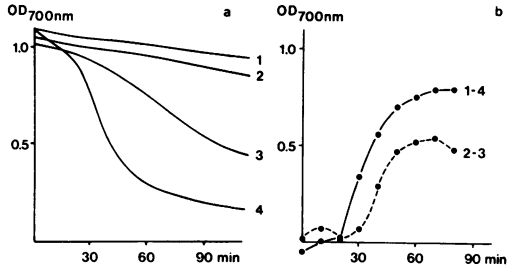

FIG. 2. Agglutination of *S. mutans* ME1 (a β_2m-binding strain) in parotid saliva and buffer. (a) Curves: 1, bacteria and buffer (negative control); 2, bacteria, saliva, and GAβ_2m; 3, bacteria, saliva, and CS; 4, bacteria and saliva (positive control). (b) According to Fig. 1b, the increasing OD value shows that agglutination is taking place in cells in control position (as compared with in the cell in the sample position). Curves: 1 − 4, the sample-positioned cell contains ME1 and buffer, and the reference-positioned cell contains ME1 and saliva; 2 − 3, the sample-positioned cell contains bacteria, saliva, and GAβ_2m; the reference-positioned cell contains bacteria, saliva, and CS. The increase in the OD value indicates that GAβ_2m inhibits or decreases agglutination as compared with CS.

TABLE 1. Binding of aggregated radiolabeled human β_2m by *S. mutans* in relation to agglutination by β_2m, salivary fractions, and HEWL, and in relation to uptake of radiolabeled monomeric β_2m

Parameter	Rate of binding of aggregated radiolabeled β_2m in previous studies[a]		
	+	−	+/−
Agglutination by peak 3 from Sephadex G-75	5/6	1[b]/5	0/1
Uptake of radiolabeled monomeric β_2m	3/3	0/2	1/1
Agglutination by monomeric β_2m	3/3	0/3	1/1
Agglutination by aggregated β_2m	3/3	0/3	1/1
Agglutination by lysozyme	3/3	3/3	1/1

[a] Expressed as number of reactive strains/total number of strains tested. +, Binding of aggregated β_2m; −, no binding of aggregated β_2m; +/−, variable binding of aggregated β_2m.
[b] Strain BHT.

3). All strains tested were agglutinated by fractions from peak 1. Of six strains that bound aggregated β_2m, five strains were agglutinated by fractions from peak 3, and of five negative strains, only *S. mutans* BHT was agglutinated in one of two assays (Table 1). Agglutination by void volume fractions was mostly seen after 30 min, but in low-molecular-weight fractions it was not seen until after 60 to 90 min.

Preparations of aggregated human β_2m agglutinated all three strains that had bound aggregated human β_2m; bacteria were also agglutinated by aggregated β_2m in PBS free of Ca^{2+}. For *S. mutans* ME1, showing the highest affinity for aggregated β_2m, the lowest agglutinating concentration was 0.02 μg/ml. Three nonbinding controls were also not agglutinated. Monomeric human β_2m agglutinated strains that bound the aggregated form of the protein. *S. mutans* ME1 was agglutinated at a concentration of 0.032 μg/ml.

HEWL agglutinated all six strains tested at concentrations from 39 to 200 μg/ml (Table 1). *S. mutans* BHT agglutinated at the lowest concentration. None of the bacteria tested were agglutinated by G-75 fractions at the elution position of HEWL.

The agglutination of eight *S. mutans* strains in parotid saliva with the addition of GAβ_2m or CS was followed spectrophotometrically. Figure 2a illustrates the agglutination of the β_2m-binding strain ME1. GAβ_2m totally inhibited agglutination (curve 2), and CS gave a minor inhibition (curve 3). Figure 2b shows the same events as in Fig. 2a, but the assay was performed by the method for Fig. 1b. In this assay, only the difference between the agglutinations in Fig. 2a was measured. The numbers (1 to 4) in Fig. 2a correspond to the same numbers in Fig. 2b. The curve 1 − 4 expresses the change of the absolute value of the difference in OD between curve 1 and 4 in Fig. 2a. Thus, the suspension that contained saliva and bacteria agglutinated faster than did the suspension with PBS and bacteria. Similarly, the curve 2 − 3 increases, showing that GAβ_2m inhibited agglutination as compared with CS.

The β_2m-nonbinding LM7 was tested (Fig. 3a and b). GAβ_2m did not inhibit agglutination as compared with CS (Fig. 3a). In Fig. 3b, the curve 2 − 3 does not increase, which shows that bacterial suspensions with parotid saliva and CS or GAβ_2m agglutinated almost identically. The curve 1 − 4

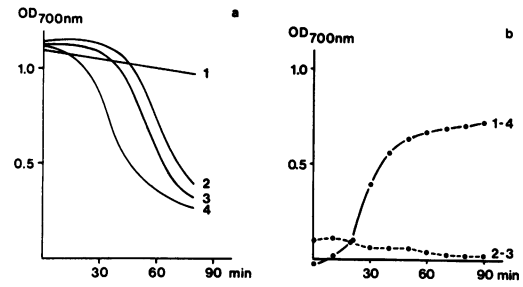

FIG. 3. Agglutination of *S. mutans* LM7 (a β_2m-nonbinding strain). Symbols are the same as in Fig. 2.

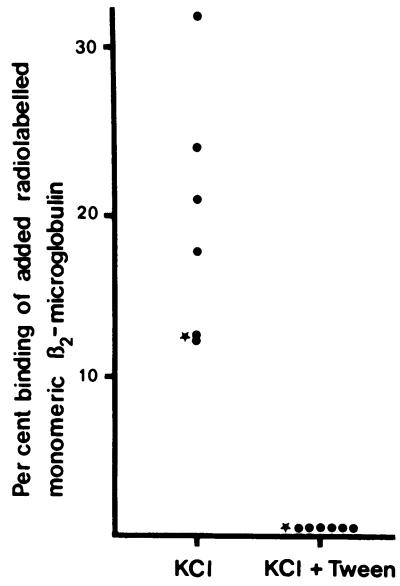

FIG. 4. Binding of ^{125}I-radiolabeled monomeric β_2m to strains of *S. mutans*, expressed as the percentage of added β_2m with and without 0.05% Tween 20 in reaction mixtures. Symbols: ●, binding to bacterial strains; ★, binding to control tubes.

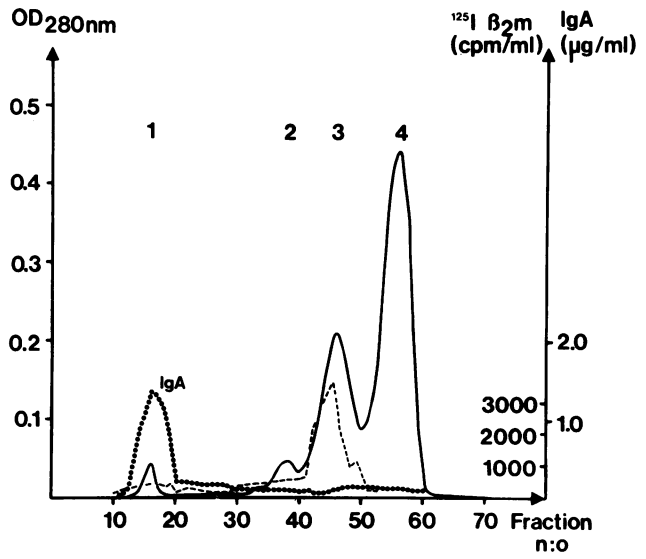

FIG. 5. Elution profile of 7 ml of parotid saliva separated on a Sephadex G-75 column. Symbols: —, absorbance at 280 mm; – – –, radioactivity corresponding to ^{125}I-labeled human monomeric β_2m; · · ·, IgA concentrations as measured by immunobead assay.

increases, which shows that the strain LM7 was agglutinated by parotid saliva but not by PBS.

The agglutination of the five strains tested that could bind aggregated β_2m was inhibited by GAβ_2m. ME1 was inhibited the most. The nonbinding and the variably binding strains were not affected differently by GAβ_2m or CS.

DISCUSSION

It was demonstrated that β_2m monomers also can bind to *S. mutans* strains, and not only in the aggregated form as earlier assumed (10, 12, 21). As with some other salivary molecules (9, 17, 31), binding of β_2m to bacteria seemed to require the presence of Ca^{2+}. Previous β_2m-binding studies were performed with Ca^{2+}-free buffers containing Tween 20 or HSA. When a KCl buffer with 1 mM Ca^{2+} and without Tween 20 or HSA was used, monomeric β_2m was bound to the four strains that also bound aggregated β_2m, but not to the negative strains. HSA or Tween is often used to prevent binding to reaction vessels and might interfere with low-affinity interactions. Even though a higher adsorption of β_2m to tubes and negative control bacteria was found in this study, there was a clear difference between controls and β_2m-binding strains. The presence of Tween or HSA might mask the presence of low-affinity receptors, but it does not seem to interfere with the higher avidity reactions between β_2m aggregates and bacteria (10, 12, 21).

Purified preparations of human monomeric and aggregated β_2m agglutinated the strains that bound the protein but not the negative strains. As little as 0.032 μg of monomeric β_2m per ml induced agglutination. However, in gel-filtered saliva fractions causing agglutination, the maximum concentration of β_2m was 0.0047 μg/ml. Purification procedures and storage might have decreased the affinity of the purified β_2m to bacteria as compared with freshly tested salivary β_2m. The concentration of β_2m in unfractioned saliva is 0.2 to 0.9 μg/ml (11), which is several times the concentration needed for agglutination.

Amylase, having a molecular weight of ca. 5.5×10^5, eluted in fractions with lower molecular weights. This has been ascribed to interaction between the Sephadex gel and amylase (14), and might explain the lower absorption at 280 nm by higher-molecular-weight fractions.

Among the Sephadex G-75 fractions, all bacteria were agglutinated by void volume fractions. The lower-molecular-weight fractions not containing IgA or high-molecular-weight agglutinins, but containing β_2m, could agglutinate five of six strains that readily bound β_2m. The low concentration of β_2m in the fractions may explain the failure of one strain to agglutinate. The slower agglutination seen in the lower-molecular-weight fractions indicates that agglutinating factors are either not as strong or not as concentrated as those seen in the void volume.

Strain BHT was the only β_2m-nonbinding strain that was variably agglutinated by fractions containing β_2m. This strain has been reported to show a high affinity for lysozyme (30). However, since HEWL eluted in fractions just before peak 2 on the G-75 column, and these fractions never agglutinated bacteria, lysozyme here is probably not responsible for the agglutination of strain BHT. Also, binding and agglutination by β_2m did not correlate with agglutination by lysozyme for the strains tested.

The method presented in this paper had some advantages as compared with previously described methods (13) for turbidimetrical measurements of bacterial agglutination inhibition experiments. In measuring the effect of added antiserum against a specific salivary molecule, a CS often also interferes with agglutination. This effect is often not interesting to study per se, but it has to be subtracted from the phenomenon one wishes to study. In measuring only the differences between agglutination in saliva treated with antiserum and with CS, the effect of CS was subtracted in the spectrophotometer. Thus, small differences in agglutination could be detected, and specific inhibition by the antiserum was directly revealed. Also, as the agglutinations in control and test cuvettes were measured against each other, and not against a buffer control, only half the number of cuvettes was used as compared with the method described for Fig. 1a, 2a, and 3a. The results obtained with this method correlate with those obtained by other methods (13) (Fig. 2a and b, Fig. 3a and b).

For the highest affinity strain, *S. mutans* ME1, agglutination was inhibited almost completely when GAβ_2m was added (Fig. 2a and b). Agglutination of other β_2m-binding strains tested was not so strongly affected by GAβ_2m. Since agglutination of bacteria can be caused by several factors in saliva, one would expect that interfering with one factor with antibodies would not totally inhibit agglutination. However, this was seen with strain ME1. As ME1 showed the highest affinity of the strains tested, it might have been possible that complexes between antibody and β_2m were absorbed onto the bacteria without causing agglutination. Receptors for other bacterial agglutinating factors may then have been blocked sterically.

The fact that several factors in saliva interact with the indigenous flora is perhaps an important feature of the bacteria. Maybe it is important for the indigenous flora to have several ways to interact with host substances, instead of one strong interaction that might be more vulnerable. Microheterogeneities among salivary molecules and thus many interaction sites have been suggested to provide "zip codes" for disposal or attachment of microorganisms (32). A favorable combination of surface receptors on the bacteria then would be the key to attachment. It is therefore important to clarify all types of interactions between bacteria and salivary factors to be able to understand better the oral

ecology. β_2m interacts with hydroxapatite (8) and with some strains of oral bacteria (10, 12). β_2m also participates in the saliva-induced agglutination of these strains. Even though *S. mutans* can agglutinate in salivary fractions without β_2m, binding of β_2m seems to be of significance for agglutination of these strains.

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