The Influence of Incorporation of Octadecenoic Acid on the Cell-associated Fructosyltransferase and the Extracellular Glucosyltransferase Activities of Streptococcus salivarius

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The rate of expression of the cell-associated fructosyltransferase (FTF<sub>m</sub>) activity of Streptococcus salivarius ATCC 25975 grown in continuous culture was linearly related to the rate of octadecenoic acid (C<sub>18:1</sub>) incorporation into the membrane lipids irrespective of the presence or absence of Tween 80 in the growth medium. This observation was confirmed with data obtained from cells grown in the presence of a series of n-alkanols. The results suggested that co-synthesis of lipids containing C<sub>18:1</sub> residues was necessary for FTF<sub>m</sub> expression and accounted for the slight stimulation of enzyme expression by Tween 80 at all growth rates. In contrast, addition of Tween 80 to the growth medium resulted in several-fold increases in extracellular glucosyltransferase (GTF<sub>e</sub>) production irrespective of the growth rate. Following the addition of the surfactant to the growth medium, an exponential relation between the increased rate of GTF<sub>e</sub> production and the concomitant net increase in the rate of C<sub>18:1</sub> incorporation was noted. The results obtained in continuous culture emphasized the underlying effect growth rate had on GTF<sub>e</sub> production, especially when Tween 80 was added to the growth medium. In the presence of n-alkanols, the rate of GTF<sub>e</sub> production plotted as a single 'U'-shaped curve with respect to the rate of C<sub>18:1</sub> incorporation irrespective of the chain length of the n-alkanol studied. Rapid analyses of the extracellular proteins by SDS-PAGE suggested that hexan-1-ol and Tween 80 specifically stimulated the synthesis and secretion of GTF<sub>e</sub> and no other extracellular protein. The combined results emphasized the dissimilarity between amphiphilic modulation of FTF<sub>m</sub> and GTF<sub>e</sub> production as well as the apparent unique stimulation of the synthesis and secretion of the latter enzyme(s).

INTRODUCTION

A number of observations have led us to believe that the lipid composition is an important parameter in controlling the expression of glycosyltransferases in Streptococcus salivarius. In particular, the apparent co-ordinated synthesis and secretion of glucosyltransferase activity (GTF<sub>e</sub>) can be enhanced by a variety of amphiphilic compounds including Tween 80 (polyoxyethylene sorbitan mono-oleate) (Wittenberger et al., 1978; Jacques et al., 1985), octyl β-D-glucopyranoside (Jacques, 1985) and hexan-1-ol (Markevics et al., 1987) or by changing the environmental concentration of K+ (Markevics & Jacques, 1985). While all of these conditions give rise to an adaptive change in the fatty acid composition of the membrane lipids, particularly the C<sub>18</sub>-fatty acids, there appears to be no consensus as to the direction or degree of change that occurs within the fatty acids of the membrane lipids leading to enhanced GTF<sub>e</sub> expression. In contrast, all available data indicate that cell-associated fructosyltransferase (FTF<sub>m</sub>) activity decreases with an increase in saturation of the membrane lipids (Jacques, 1985; Markevics et al., 1987).

Abbreviations: GTF<sub>e</sub>, extracellular glucosyltransferase; FTF<sub>m</sub>, cell-associated fructosyltransferase; FTF<sub>e</sub>, extracellular fructosyltransferase; U/S ratio, ratio of unsaturated to saturated fatty acids.
In the present communication we have analysed the rates of incorporation of octadecenoic acid in continuous culture in the presence or absence of Tween 80. The results have led us to propose that the expression of FTF<sub>m</sub> activity is dependent upon the rate of incorporation of octadecenoic acid (C<sub>18:1</sub>) into the cell membrane while reaffirming that the enhancement of GTF<sub>c</sub> expression is far more complex.

**METHODS**

*Organism, media and growth conditions.* *S. salivarius* ATCC 25975 was used throughout these studies. All batch cultures were of 11 ml volume and were grown to late exponential–early stationary phase in semi-defined medium (Jacques, 1983) containing 1 mM-CaCl<sub>2</sub>. Glucose (25 mM) served as the fermentable carbon source. Continuous culture employed semi-defined medium without added CaCl<sub>2</sub> and was carried out at a constant pH 6.5 in a Bio-Flo chemostat of 325 ml capacity (model C30, New Brunswick Scientific) with 25 mM-glucose as the limiting nutrient. All media used in continuous culture were filter sterilized irrespective of whether or not they contained Tween 80 (0.5 µl ml<sup>-1</sup>) (Jacques et al., 1979).

*Assay of enzyme activities and analysis of fatty acids.* Cell-associated and extracellular glycosyltransferase activities (Jacques, 1985) and the fatty acid constituents of the membrane lipids (Markevics & Jacques, 1985) were measured as previously described. One unit of enzyme activity (U) was defined as the amount of glycosyltransferase or fructosyltransferase that catalysed the incorporation of 1 pmol of the glucose or the fructose moiety of sucrose, respectively, into 75% (v/v) ethanol-insoluble polysaccharide min<sup>-1</sup>. Activities (Jacques et al., 1985) and the fatty acid constituents of the membrane lipids (Markevics & Jacques, 1985) were measured as previously described. One unit of enzyme activity (U) was defined as the amount of glycosyltransferase or fructosyltransferase that catalysed the incorporation of 1 pmol of the glucose or the fructose moiety of sucrose, respectively, into 75% (v/v) ethanol-insoluble polysaccharide min<sup>-1</sup>

**Analysis of extracellular protein components.** Culture fluids obtained from batch cultures by centrifugation (10000 g, 4 °C, 10 min). were either unconcentrated or concentrated fivefold at 4 °C by dialysis against Aquacide III (Calbiochem–Behring). No significant loss of GTF<sub>c</sub> or extracellular fructosyltransferase (FTF<sub>c</sub>) activity was observed following the concentration step. SDS-PAGE slab gel electrophoresis was carried out in the buffer system devised by Laemmli (1970) as described by Russell (1976) using 9% (w/v) acrylamide in the separating gel and a constant voltage of 60 V. Culture supernatant samples (100 µl) were solubilized in SDS sample buffer for 2 h at room temperature and electrophoresis of the samples commenced within 7 h of inoculating the culture medium. By so doing the breakdown and/or aggregation of the glycosyltransferases was minimized. Molecular mass standards (Pharmacia) were ferritin (220 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa). Separated proteins were fixed in methanol/acetic acid/water (40 : 10 : 50, by vol.) and stained with Gradipure electrophoresis gel stain (Gradipore). The limit of detection for this stain is 100 ng protein. Each condition was repeated at least three times to confirm the reproducibility of the results.

Zones of polymer-synthesizing ability in gels were detected using a periodic acid-Schiff stain at room temperature (Konat et al., 1984) after incubation of the gels in sucrose. Following electrophoresis, gels were washed in three changes of 50 mM-Tris/HCl buffer pH 7.5 containing 1 mM-CaCl<sub>2</sub> and then incubated for 2 h at 37 °C in 50 mM-sodium phosphate buffer pH 6.5 containing 1% (w/v) sucrose, 1 mM-CaCl<sub>2</sub> 20 µM-dextran T10 (Sigma) plus 0.01% (w/v) thiomersal (BDH) and 1% (v/v) Triton X-100 (Sigma) (Russell, 1979a).

The protein profiles of the concentrated samples were more readily quantified. This was done by scanning at 590 nm with a Gelman ACT scanning microdensitometer attached to a digital integrator.

**Analyses of data.** All data points represent results from individual experiments unless otherwise indicated. All continuous culture experiments were repeated at least once.

In batch culture, the specific rate of product formation, q<sub>p</sub>, is given by:

\[
q_p = \frac{(p - p_0) \mu}{X_0 (e^{\mu t_1} - 1)}
\]

where \(p\) is the final product concentration; \(p_0\) is the initial product concentration; \(X_0\) is the initial number of bacteria inoculated into the growth vessel; \(\mu\) is the specific growth rate and \(t_1\) is the time interval between measuring \(p_0\) and \(p\) (Pirt, 1975). This equation can be directly applied to GTF<sub>c</sub> and FTF<sub>m</sub> production where the expression of that constituent that is required to maintain the final product concentration. No estimate of a more rapid turnover can be made.

In the chemostat, changing from one condition to another results initially in non-steady-state conditions for which it can be shown that:

\[
q_p = \frac{D (p - p_0) e^{\mu t_1}}{e^{\mu t_1} - 1}
\]

where \(q_p\), \(p_0\), \(p\) and \(t_1\) are as defined above and \(D\) is the dilution rate.
For equation 2, as $t_1 \to \infty$, $q_p \to D_p$. For the purpose of this paper, it was assumed that $t_1 \to \infty$ after cultures were allowed to equilibrate for seven mean generation times (five volume changes) after changing from one $D$ to another (Jacques et al., 1979). Following the addition of Tween 80 to a culture growing at $D = 0.05$ h$^{-1}$, $C_{18:1}$ began to accumulate in the cells after 18 h at the rate equivalent to the final steady-state rate. This gave rise to a slow but steady increase in the amount of $C_{18:1}$ in the membrane lipids until the maximum was achieved some 100 h after the addition of Tween 80 to the medium. The result implied that at $D = 0.05$ h$^{-1}$ the rate of $C_{18:1}$ incorporation – given by $0.05 \times [C_{18:1}]$ at steady state – was most likely the maximum rate of incorporation of the fatty acid into the membrane lipids. Whether this was true for each $D$ studied was not determined.

RESULTS

Effect of the dilution rate on membrane fatty acid composition, FTF$_m$ activity and GTF$_e$ production

As the dilution rate, $D$, was increased from 0·05 h$^{-1}$ to 0·8 h$^{-1}$, the degree of unsaturation of the membrane lipids increased. Octadecenoic and eicosenoic acid (C$_{20:1}$) levels rose essentially at the expense of tetradecanoic (C$_{14:0}$) and hexadecanoic (C$_{16:0}$) acids (Table 1).

When cells were grown in the presence of Tween 80, the oleic acid residue of the surfactant appeared to be incorporated into the cells (Jacques et al., 1985). This resulted in an increase in the amount of octadecenoic acid in the membrane lipids and a significant rise in the unsaturated to saturated fatty acid (U/S) ratio for each $D$ (Table 1).

In the absence of Tween 80, the amount of GTF$_e$ and FTF$_m$ activity increased with increasing $D$. There was, however, no simple (linear) relation between either of these two enzyme activities and $D$, or between the expression of either enzyme and the amount of octadecanoic acid in the membrane. This conclusion was confirmed by growth in the presence of Tween 80. Although the levels of both enzymes increased when grown in the presence of the surfactant, the condition giving rise to the highest incorporation of octadecanoic acid ($D = 0.1$ h$^{-1}$) did not maximize the degree of expression of either FTF$_m$ or GTF$_e$ activity (Table 1).

Dependence of FTF$_m$ production on the rate of octadecanoic acid incorporation into the membrane lipids

Although the degree of FTF$_m$ expression was not dependent upon the absolute amount of octadecanoic acid present in the cells for any given $D$, the rate of FTF$_m$ expression was directly related to the minimum rate of $C_{18:1}$ accumulation in the cell membrane, irrespective of the presence or absence of Tween 80 in the growth medium (Fig. 1a). The data were consistent with

Table 1. Dilution-rate-dependent alterations in the membrane fatty acids and GTF$_e$ and FTF$_m$ expression in S. salivarius in the presence or absence of Tween 80

<table>
<thead>
<tr>
<th>$D$ (h$^{-1}$)</th>
<th>Fatty acid composition (% w/w)</th>
<th>GTF$_e$ [mU (mg dry wt)$^{-1}$]</th>
<th>FTF$_m$ [mU (mg dry wt)$^{-1}$]</th>
<th>U/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>No added Tween 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0·05</td>
<td>17·7 51·4 10·6 2·8 17·0 0·6</td>
<td>4 16</td>
<td>0·39</td>
<td></td>
</tr>
<tr>
<td>0·1</td>
<td>20·9 49·8 10·9 2·3 15·2 0·9</td>
<td>5 80</td>
<td>0·41</td>
<td></td>
</tr>
<tr>
<td>0·2</td>
<td>15·2 44·5 13·8 4·8 20·4 1·2</td>
<td>6 108</td>
<td>0·55</td>
<td></td>
</tr>
<tr>
<td>0·4</td>
<td>10·0 45·7 9·9 3·7 25·8 5·0</td>
<td>13 136</td>
<td>0·68</td>
<td></td>
</tr>
<tr>
<td>0·6</td>
<td>4·1 45·3 10·1 5·2 29·4 6·0</td>
<td>21 272</td>
<td>0·84</td>
<td></td>
</tr>
<tr>
<td>0·8</td>
<td>1·4 39·7 9·0 7·6 31·9 10·5</td>
<td>38 262</td>
<td>1·02</td>
<td></td>
</tr>
<tr>
<td>+ Tween 80*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0·05</td>
<td>5·9 23·4 8·0 5·4 54·1 3·2</td>
<td>674 36</td>
<td>1·88</td>
<td></td>
</tr>
<tr>
<td>0·1</td>
<td>6·6 18·7 8·1 4·3 60·2 2·2</td>
<td>495 184</td>
<td>2·38</td>
<td></td>
</tr>
<tr>
<td>0·2</td>
<td>7·2 25·1 9·1 3·4 49·7 5·4</td>
<td>562 251</td>
<td>1·80</td>
<td></td>
</tr>
<tr>
<td>0·4</td>
<td>5·3 24·8 9·0 4·1 47·4 9·3</td>
<td>1180 339</td>
<td>1·92</td>
<td></td>
</tr>
<tr>
<td>0·6</td>
<td>3·1 33·7 9·3 3·8 42·3 7·9</td>
<td>477 406</td>
<td>1·47</td>
<td></td>
</tr>
<tr>
<td>0·8</td>
<td>2·9 33·5 9·5 3·3 42·8 8·2</td>
<td>644 350</td>
<td>1·53</td>
<td></td>
</tr>
</tbody>
</table>

* Concentration, 0·5 μl ml$^{-1}$. 

a biphasic linear relation between the two variables, a more rapid expression of FTF\textsubscript{m} activity occurring once the rate of C\textsubscript{18:1} accumulation exceeded 6–8\% w/w h\textsuperscript{-1}.

When \textit{S. salivarius} was grown in batch culture in the presence of a series of n-alkanols, FTF\textsubscript{m} expression decreased with increasing concentrations of each alkanol studied (Markevics \textit{et al.}, 1987). Normalized data from each of the series of n-alkanols plotted in the same biphasic fashion, confirming the relation between the rate of FTF\textsubscript{m} expression and the rate of C\textsubscript{18:1} incorporation into the membrane lipids (Fig. 1b).

\textbf{Stimulation of GTF\textsubscript{e} production}

The rate of GTF\textsubscript{e} production increased in a non-linear manner with respect to the rate of C\textsubscript{18:1} incorporation into the membrane lipids when cells were grown in continuous culture in the absence of added Tween 80. The rate of GTF\textsubscript{e} production was exponential, but biphasic when plotted against the rate of C\textsubscript{18:1} incorporation (Fig. 2).

In the presence of Tween 80, no relation between the rate of GTF\textsubscript{e} production and rate of C\textsubscript{18:1} incorporation was apparent (data not shown). However, analysis of the data for each $D$ indicated an exponential relation between the increased rate of GTF\textsubscript{e} production following the addition of Tween 80 to the culture medium and the concomitant net increase in the rate of C\textsubscript{18:1} incorporation (Fig. 3). These observations on chemostat-grown organisms emphasized that $D$
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Fig. 2. Relation between the rate of GTF₆ production and rate of C₁₈:₁ incorporation in continuous culture in the absence of added Tween 80.

Fig. 3. Relation between the increase in the rate of GTF₆ production and the increase in rate of C₁₈:₁ incorporation for each D following the addition of Tween 80 (0.5 μl ml⁻¹) to the continuous culture medium. Values were calculated by subtracting the rates obtained in the absence of Tween 80 from those obtained in the presence of Tween 80 for each growth condition (D).

and hence growth rate had an underlying effect on the expression of GTF₆ activity independent of the effect of Tween 80 and unlike that for the expression of FTF₆ activity. Removal of Tween 80 from the inflowing medium resulted in a complete reversal both in the rate of GTF₆ production and the rate of C₁₈:₁ incorporation into the membrane lipids (data not shown).

When batch cultures were grown in the presence of a series of n-alkanols, the rate of GTF₆ production decreased with decreasing rate of C₁₈:₁ incorporation until a minimum was reached after which the rate of GTF₆ production increased. High concentrations of hexan-1-ol stimulated GTF₆ production in this manner more than any other n-alkanol tested (Markevics et al., 1987). Despite this, a single ‘U’-shaped curve for the relative rate of GTF₆ production versus the relative rate of C₁₈:₁ incorporation could be drawn. All data points plotted about this curve regardless of the chain length of the n-alkanol studied (Fig. 4). The data obtained from continuous culture in the absence of Tween 80 (Fig. 2) plotted along the right hand branch of the ‘U’-shaped curve shown in Fig. 4. After the minimum was reached, the continuous culture data approached zero at low relative rates of C₁₈:₁ incorporation.

Effect of Tween 80 on the extracellular protein profile of S. salivarius

The addition of 0.05 μl Tween 80 ml⁻¹ to batch cultures of S. salivarius resulted in major increases in the two protein bands of 145 and 160 kDa that possessed the majority of the GTF₆ activity. Smaller increases were noted within the intervening region above 90 kDa where some glycosyltransferase activity was detected using the periodic acid-Schiff stain (data not shown). The existence of multiple activity bands is characteristic of the instability of these enzymes (Russell, 1979b). In fact, storage of the culture fluids for 7 d at 4 °C led to significant degradation of the two major protein bands and an increase in activity within the intervening region (data not shown). The data obtained from these electrophoretic protein profiles suggested that
Fig. 4. Increase in the rate of GTF₂ production at low rates of C₁₈:₁ incorporation in batch cultures grown in the presence of n-alkanols. Data are normalized with respect to the appropriate batch culture grown in the absence of added n-alkanol (100%). Cells grown in the presence of ethanol (■), pentan-1-ol (□), hexan-1-ol (●), heptan-1-ol (○) or octan-1-ol (▲).

Fig. 5. Microdensitometry scans following SDS-PAGE of concentrated culture fluid obtained from cells grown in batch culture in the presence (a) and absence (b) of 0.05 µl Tween 80 ml⁻¹. Migration distances for proteins of known molecular mass are also shown. No apparent increase in any protein band was detected in the region below 43 kDa when 0.05 µl Tween 80 ml⁻¹ was added to the culture fluid.
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Tween 80 uniquely stimulated GTF<sub>e</sub> production as no concomitant net increase in other extracellular proteins was observed (Fig. 5). As further evidence for this conclusion, the preparations from cultures grown in the presence of Tween 80 and analysed by electrophoresis (Fig. 5) possessed 7.5-fold greater GTF<sub>e</sub> activities compared with those grown in the absence of the surfactant. The same preparations showed no significant increase in FTF<sub>m</sub> activity. Similar electrophoretic protein profiles with unique stimulation of the two major GTF<sub>e</sub> protein bands have also been obtained by growth in the presence of 12.5 mM-hexan-1-ol (data not shown).

**DISCUSSION**

Analyses of the data obtained by modulating the fatty acid profile of the cell membrane by the three separate procedures of altered growth rate, addition of Tween 80 or the addition of a series of n-alkanols gave rise to the same biphasic linear relation between the rate of FTF<sub>m</sub> expression and the rate of C<sub>18:1</sub> incorporation into the membrane. This biphasic relation, in fact, existed for both FTF<sub>m</sub> and GTF<sub>e</sub> expression in the presence or absence of Tween 80 once the rate of C<sub>18:1</sub> incorporation exceeded 8% w/w h<sup>-1</sup>. In the absence of surfactant, this change occurred at a U/S ratio of about 0.65, and has been observed in other studies where K<sup>+</sup> concentration affects GTF<sub>e</sub> expression (L. J. Pitty & N. A. Jacques, unpublished observations). Whether alterations in membrane structure such as changes in the lipid species are occurring at this point is not known. The data for FTF<sub>m</sub> expression, however, were consistent with one of two alternative models for the mode of expression of FTF<sub>m</sub> in *S. salivarius*. Either FTF<sub>m</sub> could not be inserted into the membrane without concomitant net synthesis of lipids containing C<sub>18:1</sub> fatty acids (Nesmeyanova, 1982), or FTF<sub>m</sub> may need a covalently linked lipid moiety containing the C<sub>18:1</sub> fatty acid or simply the C<sub>18:1</sub> fatty acid covalently linked to the protein itself for activity to be expressed on the surface of the cell (Neilsen & Lampen, 1982; Pugsley & Schwartz, 1985; Fujiyama & Tamanoi, 1986; Low et al., 1986; Wold, 1986).

The situation with GTF<sub>e</sub> production was far more complex. GTF<sub>e</sub> synthesis and secretion in the presence or absence of Tween 80 was not simply (linearly) related to the rate of C<sub>18:1</sub> incorporation into the plasma membrane as was the rate of FTF<sub>m</sub> expression. This fact was exemplified by the observation that a similar rate of GTF<sub>e</sub> expression occurred at D = 0.8 h<sup>-1</sup> in the absence of Tween 80 and at D = 0.05 h<sup>-1</sup> in the presence of Tween 80. The rate of C<sub>18:1</sub> incorporation, however, was ninefold less in the latter case. This observation emphasized the underlying effect that growth rate in continuous culture had on the expression of GTF<sub>e</sub> activity before any superimposed effect of the surfactant. Despite this, the addition of Tween 80 to the medium resulted in significant increases in the U/S ratio for each D, such that at low D more than 30% of the lipid species would have to contain di-unsaturated fatty acids. Such changes are consistent with the formation of the inverted hexagonal (H<sub>II</sub>) phase within the plasma membrane, for a number of phospho- and glycolipids are capable of forming these phases as the degree of unsaturation increases (Cullis & de Kruijff, 1979; Israelachvili et al., 1980; Brenner, 1984, Gruner et al., 1985). A role for non-bilayer phases, including the formation of the H<sub>II</sub> phase, has been suggested in various models for the general translocation of proteins across membranes (Di Rienzo & Inouye, 1979; Nesmeyanova, 1982; Pugsley & Schwartz, 1985; Martinek et al., 1986). However, such phases play a role in the Tween 80 stimulation of GTF<sub>e</sub> production, that role would have to be unique to this enzyme(s) as other extracellular proteins of *S. salivarius* are not stimulated in the same manner. Furthermore, with hexan-1-ol (Markevics et al., 1987) and octyl β-D-glucopyranoside (Jacques, 1985) such a model is far more difficult to perceive since the degree of unsaturation of the membrane decreases with increasing concentrations of these amphiphiles (Markevics et al., 1987). However, theoretical considerations suggest that hexan-1-ol, at least, may self-associate and form alkanol-rich domains or non-bilayer clusters within the membrane at concentrations which lead to a stimulation of GTF<sub>e</sub> synthesis and secretion (Brasseur et al., 1985).

Irrespective of whether or not the various methods previously reported for the enhancement of GTF<sub>e</sub> underlie a unified mechanism for the control of the synthesis and secretion of the enzyme(s) by *S. salivarius*, the present study indicates that with decreasing D (and therefore with
increasing generation times), the bacterium readily adapts its membrane to a more ordered state consistent with a lowering of the phase transition temperature (Table 1). This observation may have a number of important ramifications with respect to streptococcal physiology not the least of which is the one seen here – that of the apparent control over the expression of the two major glycosyltransferase enzyme systems used for polymerizing sucrose. Such changes in the membranes may represent a simple feed-back control to prevent wastage in the formation of unnecessary enzymes under unfavourable (non-ingestion of food) conditions in the natural oral environment. This notion is supported by the results obtained previously with the addition of Tween 80 or the changing of K+ concentration, both of which may play a role in vivo in man in controlling glycosyltransferase expression with food intake since Tween 80 is listed as a surfactant that can safely be used in food processing (Fisher & Parker, 1985) while the K+ concentration alters with salivary flow (Shannon et al., 1974).

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REFERENCES


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