Solubilization and Purification of \textit{trans}-Farnesyl Pyrophosphate-Squalene Synthetase*

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SUMMARY

A \textit{trans}-farnesyl pyrophosphate-squalene synthetase has been isolated in a soluble form from yeast extracts and purified 45-fold. The molecular weight of the enzyme estimated from sucrose density gradient centrifugation and gel filtration chromatography is 426,000. Solubilization of the squalene synthetase is achieved with deoxycholate. Treatment with the detergent markedly lowers squalene synthetase activity but when deoxycholate is removed by Amberlite XAD-2, the soluble enzyme regains full activity. Such synthetase preparations are relatively labile. They can be stabilized by glycerol and 2-mercaptoethanol. Both TPNH and DPNH serve as electron donors for the squalene synthetase. Their \( k_m \) values are 122 \textmu M and 310 \textmu M, respectively. The two pyridine nucleotides differ somewhat in their effects on the Hill coefficient for the bimolecular condensation of farnesyl pyrophosphate to squalene. With DPNH the Hill slope is 2.0 and with TPNH 1.4. The purified synthetase catalyzes not only the formation of squalene from farnesyl pyrophosphate but also accumulates presqualene pyrophosphate (in the absence of pyridine nucleotide) and converts biosynthetic presqualene pyrophosphate to squalene.

The formation of squalene from \textit{trans}-farnesyl pyrophosphate is catalyzed by particle-bound enzymes. It is associated with light microsomes in mammalian liver (1, 5) and with membrane particles in yeast (2). For this reason, squalene synthetase activity has not been purified or otherwise characterized except that the over-all process is known to require \textit{trans}-farnesyl pyrophosphate (1), reduced pyridine nucleotide, and magnesium both in the yeast (3, 4) and in the liver (1, 5) systems. For mechanistic reasons, the formation of squalene from farnesyl pyrophosphate has been postulated to proceed by way of a stable intermediate (6). Recently, Rilling has isolated and established the structure of such a compound named presqualene pyrophosphate which satisfies the criteria of an intermediate in squalene synthesis from farnesyl pyrophosphate (2, 7, 8). The chemical synthesis of a product apparently identical with biosynthetic presqualene pyrophosphate has also been achieved (6). Independently, two other laboratories have reported the synthesis of presqualene alcohol (10, 11). The available information, therefore, suggests that squalene is synthesized from farnesyl pyrophosphate by two or more discrete steps. To characterize this system at the enzyme level, we have explored procedures for solubilizing and purifying squalene synthetase. A method for obtaining squalene synthetase from yeast in a soluble form is now described. After solubilization the enzyme could be purified 45-fold and its molecular weight determined. At this stage the enzyme behaves as a single entity catalyzing squalene synthesis from either farnesyl pyrophosphate or presqualene pyrophosphate. Furthermore, the ratio of activities of these two substrates remains constant during purification suggesting that the two catalytic activities are associated with the same complex.

EXPERIMENTAL PROCEDURE

\textit{trans}-[1-\textit{\textsuperscript{3}H}]Farnesol—One gram of \textit{trans}-farnesol, kindly given by Professor E. J. Corey, Harvard University, was oxidized to farnesal in n-hexane with 100 g of MnO\textsubscript{2}. The reaction was monitored by chromatographing aliquots on thin layer chromatography plates. The thin layer silica gel plates were developed with ethyl acetate-benzene, 1:19. In this system farnesal had \( R_F = 0.9 \) and farnesol \( R_F = 0.35 \). After 15 hours the aldehyde solution was filtered off and extracted three times with 100 ml of ether. Solvent was evaporated in a nitrogen stream and the oily residue dissolved in 10 ml of ethanol. Sufficient NaB\textsubscript{3}H\textsubscript{4} (specific radioactivity = 140 mCi per mmole) was added for complete reduction of farnesal to farnesol. The reaction was again followed by chromatography of samples as described above. The plates were exposed to iodine vapor and scanned for radioactivity (Packard model 7201). The [1-\textit{\textsuperscript{3}H}]farnesol formed had a specific radioactivity of 35 Ci per mole. It was stored in n-hexane under nitrogen.

\textit{trans}-[1-\textit{\textsuperscript{3}H}]Farnesyl Pyrophosphate—The pyrophosphate ester was synthesized according to Popjak et al. (12) and purified by chromatography on 2-mm preparative silica gel plates with 1-propanol-NH\textsubscript{3}-H\textsubscript{2}O (6:3:1) as developing solvent. This step was necessary for separating the farnesyl pyrophosphate from contaminating mono- and polyphosphate esters. In the final step the material was passed over Amberlite XAD-2 resin (13)
and stored in 0.01 M ammonium hydroxide solution. On thin layer silica gel plates developed with 1-propanol-NH₄H₂O (6:3:1), the [1-³H]farnesyl pyrophosphate co-chromatographed with a sample of biosynthetic trans-[⁴C]farnesyl pyrophosphate provided by G. Ponsjak, UCLA, School of Medicine.

[³H]Prequeslène Pyrophosphate—This compound was prepared by incubating [³H]farnesyl pyrophosphate with yeast extracts and purified as described by Kiling (2). The material obtained was stored in 0.01 M ammonium hydroxide solution under nitrogen. Its molar specific radioactivity was assumed to be 1.5 times that of the [³H]farnesyl pyrophosphate from which it was produced on the basis of the known loss of one proton from the C-1 position of one of the two reacting farnesyl pyrophosphate molecules (2). Apoprotein of α₂-lipoprotein was a gift from Dr. A. Seany (University of Chicago). Amberlite XAD-2 was kindly donated by Rohm and Haas, Philadelphia, Pennsylvania. All other materials were obtained from commercial sources.

Assay of Squalene Synthetase—Unless otherwise specified, assay mixtures contained 0.1 M Tris-HCl buffer, pH 7.6, 2 mM TPNH, 4 mM MgCl₂, 6 μM [³H]farnesyl pyrophosphate, and 50 μl of enzyme in a total volume of 0.5 ml. Incubations were started by the addition of enzyme, run at 35° for 2 min, and stopped by addition of 1 ml of acetone. Kinetic studies had shown that the reaction proceeds linearly for at least 5 min. Enzyme concentrations were adjusted so that under the chosen assay conditions approximately 5% of the substrate was converted.

Squalene was extracted from the mixture by three 1-ml portions of benzene-acetone (3:1). The combined extracts were evaporated under nitrogen and the residues applied to thin layer silica gel plates (2 X 20 cm) (Brinkmann F-254). The plates were developed for 10 cm with n-hexane-benzene (4:1). The zone containing squalene (R₂ 0.95) was scraped off the plate into a vial containing scintillation solution and counted (Packard model 2909). Glycerol and 2-mercaptoethanol were present throughout as described in the text.

Cakes of fresh bakers’ yeast (200 g) were suspended in 2 liters of Tris-HCl buffer, pH 7.6. The suspension was passed twice through a French pressure cell at 22,000 p.s.i. Unbroken cells and cell debris were removed by centrifugation at 20,000 x g for 30 min at 4°. The collected supernatant (S₁₀₀) was made 0.02 M with respect to 2-mercaptoethanol, and solid ammonium sulfate was added to the gently stirred solution to 45% saturation. After each 15% increment of ammonium sulfate, the pH of the suspension was adjusted to 7.6 with ammonium hydroxide. The precipitate (10.2 g) was collected by centrifugation at 15,000 x g, dissolved in sufficient Tris-HCl buffer containing 0.02 M mercaptoethanol to give a protein concentration of 20 mg per ml and dialyzed against the same buffer for 24 hours at 4°. Ice-cold glycerol was then added to a final concentration of 30% (v/v) and sodium DOC to a final concentration of 0.3% (w/v). The concentration of protein in this solution was 10 mg per ml. The solution was left standing at 4° for 16 hours. Two columns (5 X 20 cm) were prepared, one packed with DEAE-cellulose (Whatman DE 23) and the other with Amberlite XAD-2. The XAD-2 column was mounted on top of the DEAE-cellulose column and both were equilibrated with 3 column volumes of 0.05 M Tris-HCl buffer (pH 7.6) containing 20% glycerol and 0.02 M mercaptoethanol. The detergent-treated enzyme solution was then applied to the top of the two-column mount, and the flow rate was adjusted to 140 ml per hour by applying a head pressure of 50 cm. This was followed by a wash with 1 bed volume of buffer. No enzyme activity was found in the wash effluent. The Amberlite XAD-2 column was then disconnected and a 2.4 liter linear NaCl gradient (0 to 0.6 M) in buffer (Tris-HCl-glycerol-mercaptoethanol) applied to the DEAE-cellulose column. On collection of 10-ml fractions, Fractions 110 to 158 were found to contain the bulk of squalene synthetase activity. They were combined and concentrated to a volume of 100 ml by passage through a XM-50 Diaflo filter (exclusion limit 50,000) under nitrogen pressure (30 p.s.i.). The resulting enzyme preparation (Fraction 3, Table II) was used for the kinetic studies and molecular weight determinations.

For testing the effect of lipids on squalene synthetase activity,
TABLE II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/min/mg protein</td>
<td>nmoles/min</td>
<td>g</td>
</tr>
<tr>
<td>1. p20</td>
<td>0.0373</td>
<td>376.0</td>
<td>0.24</td>
</tr>
<tr>
<td>2. Dialyzed p20</td>
<td>0.0514</td>
<td>711.0</td>
<td>13.8</td>
</tr>
<tr>
<td>3. Enzyme from DEAE-cellulose</td>
<td>1.540</td>
<td>376.0</td>
<td>0.22</td>
</tr>
<tr>
<td>4. Enzyme from Sephadex G-200</td>
<td>0.270</td>
<td>61.7</td>
<td>0.22</td>
</tr>
</tbody>
</table>

2 ml of an enzyme preparation were extracted with chloroform-methanol according to Folch et al. (14). The extract was evaporated under nitrogen to a volume of 2 ml. A butanol extract of an enzyme preparation was similarly prepared. In some assays for squalene formation 50 μl of one of these extracts were added to the reaction tubes and the organic solvent was removed by a stream of nitrogen. Tris-HCl buffer, 0.2 ml, was then added and the tubes shaken vigorously. Incubation and analysis for squalene were then carried out as described above.

An attempt to purify squalene synthetase further by gel filtration on Sephadex G-200 yielded enzyme with greatly reduced specific activity (Table II).

peak after centrifugation was determined by the standard assay with either presqualene pyrophosphate or farnesyl pyrophosphate as substrates. The thyroglobulin and catalase peaks were located by absorbance measurements at 280 nm and 280 and 415 nm, respectively.

The values for V_{max} were determined from reciprocal plots of

![Figure 1. Chromatography of DOC-treated enzyme on DEAE-cellulose. The enzyme eluted from an Amberlite XAD-2 column was passed directly onto a DEAE-cellulose column (5 x 20 cm) and eluted with 2.4 liters of a linear NaCl gradient in buffer (Tris-HCl-glycerol-mercaptoethanol). Fractions of 10 ml were collected. Absorbance of the various fractions at 280 nm (O-O), squalene synthetase activity (C-O), and (---) NaCl gradient.](image)

![Figure 2. Effect of glycerol and mercaptoethanol on the stability of squalene synthetase. The three samples were diluted to give the same squalene synthetase activity from farnesyl pyrophosphate (0.05 nmoles per min). Aliquots from each sample were taken for assay. Decay in enzymatic activity of p20 fraction (O-O), DEAE-enzyme (A-A), and DEAE-enzyme in the presence of 20% (v/v) glycerol and 0.02 M mercaptoethanol (O-O).](image)
FIG. 3. Determination of Stokes radius of squalene synthetase by chromatography on Sepharose 4B. \( R_g \) was determined with blue dextran. The position of each of the protein markers was determined in separate runs by measuring absorption at 280 nm. The position of squalene synthetase was determined by assaying 0.4-ml aliquots from the various fractions. Samples were eluted with 0.15 M Tris-HCl buffer containing 2% (v/v) glycerol and 0.05 M mercaptoethanol.

FIG. 4. Effect of TPNH concentration on the rate of formation of squalene from farnesyl pyrophosphate. Assay mixtures contained 0.1 M Tris-HCl buffer, pH 7.6; MgCl₂, 4 mM; \(^{3}H\)farnesyl pyrophosphate, 6 \( \mu \)M; enzyme solution, 26 \( \mu \)g of protein and TPNH as indicated in a total volume of 0.5 ml. The mixtures were incubated at 38° for 2 min and assayed as described in the text. ○—○, rate of squalene formation = \( v \); ●—●, \([S]/v\).

rates against substrate concentrations or, when appropriate, of rates against the second power of substrate concentrations. Both extrapolations gave nearly identical \( V_{\text{max}} \) values.

RESULTS

In extracts prepared by French pressure cell disruption of bakers' yeast, squalene synthetase activity is partitioned between the 105,000 \( \times g \) sediment and supernatant fractions. The lower zone of the supernatant containing a thick fluffy layer is particularly rich in enzyme activity. It is likely, therefore, that at this stage most of the squalene synthetase activity is associated with membrane fragments. The fact that over 70% of the enzyme precipitates between 0 and 35% (NH₄)₂SO₄ also points to this conclusion. Moreover, enzyme activity was irreversibly lost when active (NH₄)₂SO₄ fractions were chromatographed on DEAE-cellulose.

Attempts were, therefore, made to obtain a truly "soluble" preparation by treating the presumably membrane-associated synthetase fraction with detergent. Such experiments are illustrated in Table I. DOC clarified the enzyme solutions but detergent concentrations above 0.1% led to substantial losses of enzyme activity. These losses could be markedly reduced by carrying out the detergent treatment in the presence of 30% glycerol and 0.02 M mercaptoethanol. The optimal DOC concentration for solubilization was 0.3% and under these conditions approximately one-third of the enzyme activity survived. A period of 16 hours was arbitrarily chosen for the detergent treatment. When DOC-treated enzyme was next passed over Amberlite XAD-2, a resin that effectively absorbs hydrophobic substances including deoxycholate, the effluent was enzymatically active and to a degree which depended critically on the DOC concentration during solubilization. Under optimal conditions (0.3% DOC) squalene synthetase activity was fully recovered from the XAD-2 column. In some cases this activity exceeded that of the preparations not treated with detergent. It is of interest that enzyme not treated with DOC is recovered from the XAD-2 resin in very low yield, presumably because it is tightly associated with hydrophobic membrane fragments which are bound to the resin.

Detergent-treated enzyme freed subsequently of DOC by XAD-2 was successfully chromatographed on DEAE-cellulose (Fig. 1). Synthetase activity was eluted as a single peak at approximately 0.3 M NaCl. The recovery was about 55% with a 30-fold increase in specific activity.

Attempts to purify the DEAE-enzyme further, either by gel filtration on Sephadex G-200 or by sucrose density gradient centrifugation, not only failed to raise the specific activity of
the synthetase but led to substantial losses. Higher glycerol concentrations during gel filtration on Sephadex G-200 might have improved the recovery of enzymic activity. However, the high viscosity of such solutions excessively retarded the flow rate through the Sephadex column. Supplementation with phosphatidic acid, lecithin, or with the lipid fractions obtained by extracting S20, enzyme with butanol or methanol-chloroform extract (14) failed to enhance or restore enzyme activity. The apoprotein component of apoprotein which has been claimed to serve as a carrier protein for intermediates of sterol biosynthesis (20) was also without effect.

Table II summarizes the results of the purification procedure for squalene synthetase. The existence of presqualene pyrophosphate as an intermediate in squalene formation from farnesyl pyrophosphate (8, 9) suggests that more than one enzymic reaction occurs in the over-all process. Our results thus far indicate that these reactions, whatever their number, are carried for periods up to 6 months. On the other hand, enzyme preparations that had been treated with 0.3% DOC and then with Amberlite XAD-2 decayed very rapidly but could be markedly stabilized by 30% glycerol and 0.02 M 2-mercaptoethanol (Table II). Fig. 2 demonstrates the relative stability of crude enzyme and the stabilizing effect of glycerol and 2-mercaptoethanol on the DOC-treated preparation. Once lost, activity could not be restored by adding glycerol and mercaptoethanol.

The sedimentation coefficient (s20,w) of squalene synthetase as determined by sucrose gradient centrifugation with the aid of catalase and thyroglobulin as standards (15) was found to be 14.5. Chromatography on Sepharose 4B as shown in Fig. 3 yielded a Stokes radius of 71.2 A. From these data and assuming a partial specific volume ∈ = 0.725 the molecular weight of squalene synthetase is estimated (16) to be 428,000.

Effect of Pyridine Nucleotides—Figs. 4 and 5 show the response of squalene synthetase to varying concentrations of DPNH and TPNH. The Km values calculated according to the method of Lineweaver and Burk (21) employing a weight factor of 1/[S] for the various points (22), were 122 μM for TPNH and 310 μM for DPNH. The observed Vmax values with TPNH is 1.4 times that observed with DPNH. The two reduced pyridine nucleotides differ also in their effect on the Hill coefficients calculated from plots of log v/(V - v) against log farnesyl pyrophosphate concentrations (23) (Figs. 6 and 7). A slope of approximately 2 was obtained for DPNH as expected for the bimolecular reaction involving 2 moles of farnesyl pyrophosphate. On the other hand, when TPNH was the reductant, a Hill slope of approximately 1.4 was consistently observed. This rather unexpected result was also obtained with crude S20, as determined by sucrose gradient centrifugation with the aid of catalase and thyroglobulin as standards (15) was found to be 14.5. Chromatography on Sepharose 4B as shown in Fig. 3 yielded a Stokes radius of 71.2 A. From these data and assuming a partial specific volume ∈ = 0.725 the molecular weight of squalene synthetase is estimated (16) to be 428,000.

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Squalene formation from either farnesyl pyrophosphate or presqualene pyrophosphate catalyzed by purified enzyme has an
absolute requirement for magnesium (Table III). The $K_{M}^{i+} (\text{MgCl}_2)$ in the presence of either TPNH or DPNH was found to be 1.13 mm.

**DISCUSSION**

Detergents are widely used to disrupt membranes in order to solubilize its components prior to protein purification. In some instances solubilized membrane-derived enzymes remain active in the presence of detergent and may actually require it for activity. 2,3-Oxidosqualene-lanosterol cyclase solubilized from liver microsomes by DOC is one such enzyme (24). On the other hand, squalene synthetase prepared from yeast particles is strongly inhibited by DOC concentration which are needed for obtaining this enzyme in a soluble form. Effective removal of the detergent was therefore deemed necessary before purification of the enzyme could be attempted. Our early efforts to remove DOC by dialysis, by DEAE-cellulose chromatography, or by gel filtration on Sephadex G-200 resulted in complete loss of enzyme activity. We then turned to Amberlite XAD-2, a highly hydrophobic polystyrene resin, which has been used with success for the adsorption of a wide variety of lipid substances (13) including those containing ionizable groups. For example, farnesyl pyrophosphate is quantitatively adsorbed to this resin from aqueous solution. DOC is also an amphipathic molecule and XAD-2 therefore seemed promising as a reagent for its removal. When DOC (0.3%)-treated, partially inhibited squalene synthetase was passed through XAD-2, activity was fully recovered. Presumably, the resin was effective in removing DOC and as a result, the inhibition of squalene synthetase by DOC was reversed. However, other explanations for the recovery of enzyme activity following DOC treatments are not excluded.

Since little if any of the DOC-treated enzyme is retained by the resin, the solubilized synthetase is probably not a lipoprotein nor does it appear to require a lipid for activity. By contrast enzyme not exposed to DOC is recovered from XAD-2 in poor yield suggesting that prior to detergent treatment squalene synthetase is tightly associated with hydrophobic membranes.

As mentioned earlier, the total activity recovered from the XAD-2 resin occasionally exceeded that in the $S_{20}$ fraction, on one occasion by a factor of 3. Possibly, at the $S_{20}$ stage the enzyme is still partially imbedded in the membrane and, therefore, not accessible to the aqueous phase.

The solubilized preparation (DOC-treatment followed by XAD-2) differs markedly from the crude $S_{20}$ enzyme in stability and chromatographic behavior. It is totally inactivated by freezing and thawing and its stability at $0^\circ$ is quite low. Glycerol (20 to 30%) and 2-mercaptoethanol protect the enzyme somewhat but even in the presence of these agents it does not survive freezing and thawing. The presence of glycerol and 2-mercaptoethanol is also essential for protecting squalene synthetase during solubilization by DOC. Evidently the enzyme becomes very labile when removed from the native (membrane) environment.

The specific activity of squalene synthetase increases about 30-fold during DEAE-cellulose chromatography. On the other hand, subsequent gel filtration on Sephadex G-200 partially inactivates the enzyme, despite the fact that the activity peak coincides with a distinct peak of protein. That this relatively mild treatment would inactivate the synthetase seemed unlikely. We have, therefore, considered the possibility that Sephadex filtration separates the enzyme from a protein that serves as an acceptor for squalene. For highly hydrophobic reaction products such as squalene, dissociation from the active enzyme site into the aqueous medium is an unfavorable process and to facilitate it and to allow turnover of the enzyme, a protein carrier may be necessary. Evidence for proteins serving in the role of carriers for squalene and other intermediates in sterol biosynthesis in rat liver has recently been reported (20, 25, 26). In one instance the apoprotein portion of serum apolipoprotein has been shown to duplicate or mimic the carrier function of purified sterol carrier protein from liver (20). However, this apoprotein did not enhance the activity of squalene synthetase which had been chromatographed on Sephadex G-200. The combination of this enzyme with other Sephadex fractions also failed to reactivate. We, nevertheless, consider it possible that a carrier molecule may be involved in the squalene synthetase reaction. If this assumption is correct, the observed specific activity would not be a true measure of the degree of purification achieved.

In our experiments to date the enzymic activities for the conversion of farnesyl pyrophosphate to presqualene pyrophosphate and for the formation of squalene from the latter have not been separable. Both are associated with an entity having the relatively high molecular weight of 426,000. These facts must be considered in context with the evidence that in crude yeast and liver systems and with the purified synthetase described here, squalene formation can clearly take place in two consecutive steps. On omission of TPNH, presqualene pyrophosphate accumulates (2) and this substance, made either biosynthetically or by chemical synthesis can serve as a squalene precursor in the same enzyme systems (9). The existence of presqualene pyrophosphate as a stabilized intermediate is, therefore, not in doubt. Nevertheless, some of its properties do not obey the criteria of a free, dissociable intermediate. Thus, the rate of the formation of squalene from farnesyl pyrophosphate is twice as fast as its rate of formation from presqualene pyrophosphate (Table III). Although we did not measure initial velocities for presqualene pyrophosphate, the brief incubation period (2 mm) and the slow rate of conversion of this intermediate (total conversion of 10% to 5%) suggest that initial velocities are being observed. We have also observed that at the same concentrations of presqualene pyrophosphate and TPNH, the enzyme rate is at least three times faster than the rate of formation of presqualene pyrophosphate in the absence of TPNH. These observations suggest that presqualene pyrophosphate, even though capable of existence is not an intermediate in the sense that it freely dissociates from the enzyme. Once formed it may transfer directly from one enzyme site to another without ever dissociating from the protein. The possibility that presqualene pyrophosphate is formed in a reversible side reaction in the formation of squalene must also be considered.

The $K_{M}$ values for TPNH (122 $\mu$m) and for DPNH (310 $\mu$m) are higher than for most other enzymic reactions which require reduced pyridine nucleotides. These values have been obtained with farnesyl pyrophosphate as substrate but have not been determined with presqualene pyrophosphate.

The condensation of 2 farnesyl pyrophosphate molecules to squalene is a bimolecular reaction. A Hill coefficient (23) of 2 for this reaction was, indeed, found when DPNH was the reductant. Unexpectedly, however, the Hill slope was 1.4 with

\[ 2 \text{farnesyl pyrophosphate} \rightarrow \text{squalene} \]

$^3$ Unpublished data.
TPNH. Two explanations can be offered for this low value. (a) In the presence of TPNH the formation of squalene from presqualene pyrophosphate is the rate-limiting step or (b) TPNH affects the formation of the enzyme-farnesyl pyrophosphate complex so as to cause the second farnesyl pyrophosphate molecule to bind more readily to the enzyme than the first. Since the $K_m$ value for TPNH is lower and $V_{max}$ is slightly higher than for DPNH, it is not likely that the reduction of presqualene pyrophosphate becomes the rate-limiting step in the squalene synthetase reaction only when TPNH serves as the electron donor. Explanation $b$ remains a possibility but there is no obvious way for testing it experimentally.

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