Nucleoside Diphosphokinase of *Salmonella typhimurium*

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SUMMARY

Nucleoside diphosphokinase from *Salmonella typhimurium* has been purified and characterized. In many respects, the enzyme is similar to those from higher organisms in that it has a broad specificity for both phosphate donor and recipient and that it functions via a ping-pong mechanism. The enzyme activity of cells is very high at all growth rates but decreases as growth rate increases. Evidence is presented that nucleoside diphosphokinase is sufficient to account for the synthesis of all nucleoside triphosphates required for the polymerization of nucleic acids and that it is probably the only enzyme that catalyzes the synthesis of triphosphates from diphosphates and the γ-phosphate of ATP.

Nucleoside triphosphates, the direct precursors of nucleic acids, are synthesized from the corresponding diphosphates by a reaction catalysed by nucleoside diphosphokinase (ATP: nucleoside diphosphate phosphotransferase EC 2.7.4.6) according to the following reaction:

\[
\text{Nucleoside}_1 \text{ triphosphate} + \text{nucleoside}_2 \text{ diphosphate} \rightarrow \text{nucleoside}_2 \text{ diphosphate} + \text{nucleoside}_1 \text{ triphosphate}. 
\]

Although nucleoside diphosphokinase is well studied in eucaryotes including yeasts (1-5), pea seeds (6), artichokes (7), several beef tissues (8-16), pig tissues (17), and human erythrocytes (17-20), the enzyme from procaryotes has received little attention. Only *Bacillus subtilis* (21) has been examined, and although it is known that *Escherichia coli* contains nucleoside diphosphokinase (22), there are no detailed studies on nucleoside diphosphokinase from enteric bacteria. Because the pathways of biosynthesis of nucleosides and nucleotides and their interconversion are best understood in enteric bacteria (23-25), and the genetic tools necessary for an analysis of nucleoside diphosphokinase are also available with these organisms, an investigation of nucleoside diphosphokinase from *Salmonella typhimurium* was undertaken.

MATERIALS AND METHODS

Media

Basal salts medium 007 (26) was used with glucose (0.2%), or sodium succinate (0.2%) added as carbon source. Casamino Acids-supplemented media contained 0.1% Norit-treated vitamin-free casein hydrolysate (Nutritional Biochemical Corp.). Other supplements, when added, were at the following concentrations: amino acids, 50 μg per ml; nucleic acid bases, 10 μg per ml; vitamins, 1 μg per ml. L broth contained per liter at pH 7.0: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g. Liquid cultures of less than 2 liters were incubated at 37°C on a rotary shaker. Larger batches were sparged with air at 37°C.

Buffers

Two buffers were used. Triethanolamine buffer (Buffer A) contained 100 mM triethanolamine-HCl (pH 8.0), 10 mM MgCl₂, and 2 mM mercaptoethanol; Tris buffer (Buffer B) contained 100 mM Tris-HCl (pH 9.8), 10 mM MgCl₂, and 2 mM mercaptoethanol.

Nucleoside Diphosphokinase Assays

Two methods were used: one employing [2-¹⁴C]UDP and another employing [γ-³²P]ATP.

In the ¹⁴C assay the reaction mixture contained in 40 μl of Buffer A: 6.5 mM ATP, 1 mM UDP, 20 to 50 nCi of [²⁻¹⁴C]UDP, and enzyme. Reactions were initiated by the addition of enzyme, incubated at 20° for 10 min or 37° for 3 min, and stopped by applying 25 μl of the reaction mixture to polyethyleneimine-Avicel cellulose thin layer plates which were prepared by the method of Randerath (27), but substituting 32 g of Avicel-PH-105 for the 22 g of NM 300 cellulose powder. The applied spots were dried immediately and the plates were developed in (a) absolute methanol; (b) 2 M sodium formate (pH 3.4) to 2 cm above the origin; and (c) 4 M sodium formate to 10 cm above the origin. Following drying with hot air, the plates were placed against x-ray film (Kodak Royal Blue) for 8 to 18 hours. The developed radioautographs were used to locate the position of [²⁻¹⁴C]UDP and UDP. These areas were cut from the chromatograms, placed in 7 ml of Bray’s solution (28), and counted in a Nuclear Chicago scintillation spectrometer.

The ³²P assay contained in 40 μl of Buffer A: 1 mM nucleoside diphosphate, 6.25 mM ATP, 0.45 μCi of [γ-³²P]ATP, and enzyme. Reactions were started by the addition of enzyme, incubated at 20° for 10 min and stopped by applying 25 μl to polyethyleneimine-Avicel plates with drying by hot air. The plates were washed with absolute methanol for 20 min, dried, and developed successively in: (a) absolute methanol to the origin; and (b) 0.85 M KH₂PO₄ (pH 3.4) to 15 cm above the origin (29). Radioautography and counting was done as in the ¹⁴C assay except that exposure was only 4 to 8 hours.

Other Assays

Uridine monophosphokinase was assayed at 20° by the method of Ingraham and Neuhard (30), and protein by the method of Lowry et al. (31).

Specific Activities

Unless noted, all specific activities were calculated from the linear portion of plots relating enzyme activity to protein concentration.
Enzymatic Synthesis of [γ-32P]ATP

A method based on that of Glynn and Chappell (32) was used. The reaction mixture contained in 1 ml of Buffer A: 2 mM ATP, 2.5 mM 3-phosphoglycerate, 280 μg of rabbit muscle glyceraldehyde dehydrogenase (Sigma Chemical Co.), 6 nCi of [32P]orthophosphate, and 30 μg of yeast 3-phosphoglycerate phosphokinase (Sigma Chemical Co., type IV). After 25 min at 20°, 84% of the ATP was transferred to [γ-32P]ATP (1.4 X 106 cpm per μmole). Just before use, the [γ-32P]ATP was purified by chromatography on polyethyleneimine-Acid thin layer plates with 2 m sodium formate (pH 3.4) for 2 cm above the origin followed by 4 m sodium formate (pH 3.4) for an additional 8 cm. The areas containing [γ-32P]ATP were cut from the plates and suspended in 0.1 to 0.5 ml of Buffer B.

Preparation of Crude Extracts

For kinase assays the frozen cells were resuspended in Buffer A, suspensions were sonicated 4 X 20 s at 0° using a Bissocoo II oscillator at 90% max, and centrifuged for 20 min at 15,000 X g. The supernatant fluids were treated in 2% streptomycin sulfate and assayed for their content of nucleoside diphosphokinase and UMP kinase.

Purification of Nucleoside Diphosphokinase from Salmonella typhimurium

Step 1: Growth of Cells and Preparation of Extracts—A total of 77 g (wet weight) of S. typhimurium J8386 (paleE181) were harvested from 40 liters of Casamino acid-supplemented basal-glucose medium, washed in 200 ml of 0.9% NaCl, resuspended in Buffer B, sonicated in 50 ml portions for 10 X 30 s, and clarified by centrifugation at 15,000 X g for 10 min.

Step 2: Calcium Phosphate—Four 80- to 100-ml portions of 2% aged calcium phosphate (Calbiochem) in Buffer B were added successively to the crude extract, stirred at 0° for 30 min, and centrifuged at 5000 X g for 10 min.

Step 3: Ammonium Sulfate—Protein in the supernatant fluid from the calcium phosphate treatment was fractionated by ammonium sulfate precipitation; the 40 to 60% saturation fractions were pooled, dissolved in Buffer B, and dialyzed for 5 hours against 20 volumes of the same buffer.

Step 4: DEAE-cellulose Chromatography—The dialyzed solution from Step 3 was applied to a DEAE-cellulose column (2.5 X 40 cm) equilibrated with Buffer B. Activity was eluted at a concave gradient of 0 to 0.375 M KC1 in Buffer B at a flow rate of 18 ml per hour. The fractions from the column (4.8 ml) were assayed, and those containing significant nucleoside diphosphokinase activity (those fractions eluting between 0.25 to 0.57 M KC1) were pooled.

Step 5: Ammonium Sulfate—The pooled fractions from Step 4 were again fractionated by ammonium sulfate. The protein that precipitated between 50 and 85% saturation was dissolved in Buffer B, and dialyzed for 4 hours against 20 volumes of the same buffer.

Step 6: DEAE-Sephadex G-200—The concentrated protein solution was applied to a Sephadex G-200 column (1.8 X 100 cm) and eluted with Buffer B. The elution volume for nucleoside diphosphokinase was 112 ml.

Step 7: DEAE-Sephadex—The pooled active fractions were applied to a DEAE-Sephadex column (2.6 X 25 cm) and eluted with a concave gradient of 0 to 0.25 M KC1 in Buffer B. The peak of activity eluted at 0.25 M KC1.

Results

Effect of Growth Rate on Cellular Content of Nucleoside Diphosphokinase—Cultures of S. typhimurium were grown in media supporting growth at various rates, and assayed for their content of nucleoside diphosphokinase and UMP kinase (Table I). As growth rate increases the specific activity of UMP kinase remains approximately constant, but surprisingly the specific activity of nucleoside diphosphokinase decreases. This occurs in spite of the fact that the cellular requirements for the products of the enzyme are increased.

Purification of Nucleoside Diphosphokinase—The scheme of purification of nucleoside diphosphokinase resulting in a 686-fold increase in specific activity is summarized in Table II. The purified enzyme can be held at -20° for at least 2 months without detectable loss of activity, but at higher temperatures it is quite unstable. At 25° the half-life in the absence of substrates is only 30 min.

Properties of Purified Enzyme—The addition of mercaptoethanol stabilized the enzyme, suggesting that a thiol group is required for maximal activity. Treatment with p-chloromercuribenzenesulfonyl rapidly and reversibly inactivates the enzyme, and ATP, ADP, and AMP decrease the sensitivity to inactivation (Table III). Taken together these data indicate that a thiol group is required for nucleoside diphosphokinase activity.

Nucleoside diphosphokinase has a broad pH optimum being essentially equally active between pH 6.5 and 9.0; we selected pH 8.0 for subsequent assays.
Table III

Sensitivity of nucleoside diphosphokinase to thiol group reagents under various conditions

Purified nucleoside diphosphokinase was treated as indicated for 1 hour at 20°C. Assays were done by the 3H method, except mercaptoethanol was deleted from the assay buffer when the treatment did not contain mercaptoethanol.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleoside diphosphokinase activity remaining (μmoles/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1000</td>
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<tr>
<td>5 mM mercaptoethanol</td>
<td>2150</td>
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<tr>
<td>0.1 mM PMS</td>
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<td>5 mM ATP + 0.1 mM PMS</td>
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</tr>
<tr>
<td>5 mM ADP + 0.1 mM PMS</td>
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</tr>
<tr>
<td>5 mM AMP + 0.1 mM PMS</td>
<td>860</td>
</tr>
<tr>
<td>5 mM mercaptoethanol + 0.1 mM PMS</td>
<td>1030</td>
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</table>

* p-Chloromercurisulfonate.

Fig. 1. Relationship between K_a of certain proteins of known molecular weight (O) and their molecular weight. The K_a for nucleoside diphosphokinase (+) is located on the curve as an estimate of its molecular weight. Purified nucleoside diphosphokinase (3 ml containing 0.02 mg at specific activity of 2500 μmoles mg⁻¹ min⁻¹ mg⁻¹ of protein) was added to a Sephadex G-200 column (1.6 X 95 cm) along with the following standards: ribonuclease (10 mg), chymotrypsinogen A (5 mg), ovalbumin (10 mg), aldolase (10 mg), and blue dextran (5 mg). Samples of eluate (2 ml) were assayed for nucleoside diphosphokinase activity and absorbance was measured at 280 nm. The value of K_a were calculated from the formula: K_a = (V_e - V_o)/(V_e - V_i) where V_e is the volume of eluate necessary to remove the enzyme, V_o is the void volume, and V_i is the total column volume.

Determination of K_a values of nucleoside diphosphokinase and various known proteins on a Sephadex G-200 column (Fig. 1) indicates the molecular weight of the enzyme is approximately 85,000. This value is slightly smaller than that reported for nucleoside diphosphokinase from other sources; molecular weights of the enzyme from yeast (2), B. subtilis (21), and human erythrocytes (33) have been reported to be about 100,000.

Table IV

Activity of crude extract and purified preparation of nucleoside diphosphokinase in phosphotransferase reaction between ATP and various nucleoside diphosphates

The details of the assay techniques are described under "Materials and Methods."

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<tr>
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</tr>
<tr>
<td>ADP⁺</td>
<td>3.1</td>
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</tr>
<tr>
<td>UDP</td>
<td>3.0</td>
<td>1790</td>
</tr>
<tr>
<td>GDP</td>
<td>2.5</td>
<td>1110</td>
</tr>
<tr>
<td>CDP</td>
<td>2.2</td>
<td>1050</td>
</tr>
<tr>
<td>dADP⁺</td>
<td>2.2</td>
<td>1050</td>
</tr>
<tr>
<td>dGDP⁺</td>
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<td>980</td>
</tr>
<tr>
<td>dCDP</td>
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<tr>
<td>dUDP</td>
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<td>640</td>
</tr>
<tr>
<td>dTDP</td>
<td>0.8</td>
<td>480</td>
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* These assays were made with 3H-labeled diphosphate rather than [γ-3H]ATP. All other conditions and concentrations are the same.

In the chromatographic system employed there was some overlap between these triphosphates and ATP. As a result these values are low.

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The enzyme is the nucleotide-Mg²⁺ complex (9). Measurements of the effect of ATP concentration on the velocity of the reaction indicated normal saturation kinetics (Fig. 2). In the case of UDP (Fig. 3) inhibition occurs at high substrate concentrations.

Double reciprocal plots of ATP concentration against velocity at various concentrations of UDP resulted in a family of parallel curves (Fig. 4) which are typical of a ping-pong mechanism (3). However, at higher concentrations of UDP (above 1 mM), UDP becomes a competitive inhibitor of ATP. By replotted the various values of K_m for ATP against the concentrations of UDP one can determine the true K_m for ATP. From our data this

Inhibitor Studies—UTP, ADP, UMP, AMP, NAD, NADH, NADP, NADPH, FAD, and FMN at 5 mM, 0.5 mM, and 0.05 mM were tested for activation or inhibition of the reaction between ATP and UDP (data not shown). ADP and UTP were competitive inhibitors of the reaction; however, the other compounds had no effect on the reaction. This was surprising in that AMP protects the enzyme from inactivation by p-choloromercurisulfonate (Table III).
FIG. 2. Relationship between the reciprocal of the concentration of ATP and the reciprocal of the velocity of the reaction between ATP and UDP. The inset shows the same data in the form of a saturation curve. UDP was kept constant at 0.3 mm.

FIG. 3. Relationship between the reciprocal of the concentration of UDP and the reciprocal of the velocity of the reaction between ATP and UDP. The inset shows the same data in the form of a saturation curve. ATP was kept constant at 6.25 mm.

value was determined to be 0.23 mm. Similar replots of these data revealed the true $K_m$ for UDP to be 0.09 mm. These results are similar to those previously reported for human erythrocytes (19) and yeast (3).

The above kinetic evidence for a ping-pong mechanism suggests the existence of a phosphorylated enzyme intermediate. This encouraged us to attempt to isolate the intermediate as has been done with nucleoside diphosphokinase from other sources (3, 6, 7, 12, 16, 18), by reacting purified enzyme with [γ-$^{32}$P]ATP in the absence of nucleoside diphosphate. The reaction mixture was applied to a Sephadex G-75 column and developed with Buffer B. Fractions collected from the column were assayed for radioactivity and nucleoside diphosphokinase activity. The peaks of these two activities were coincident (Fig. 5), and the ratio of the activities was constant throughout the peak indicating that a phosphorylated enzyme had been formed. The purified [γ-$^{32}$P]nucleoside diphosphokinase contained

FIG. 4. Relationship between the reciprocal of the concentration of ATP and the reciprocal of the velocity of the reaction between ATP and UDP at concentrations of UDP of: (■) 3.05 mm, (△) 2.05 mm, (●) 1.05 mm, (□) 0.55 mm, (▲) 0.35 mm, (●) 0.25 mm, and (●) 0.15 mm.

FIG. 5. Purification of the products of a reaction mixture between [γ-$^{32}$P]ATP and purified nucleoside diphosphokinase. A reaction mixture containing in 2.5 ml of Buffer A: 0.026 mg of nucleoside diphosphokinase (specific activity of 2500 μmoles per min per mg of protein), and 0.014 mM [γ-$^{32}$P]ATP (1.2 x $10^7$ cpm), was incubated at 20° for 10 min and then applied to a Sephadex G-75 column (1.6 x 96 cm) and developed with Buffer A at 8 ml per hour. Each fraction (2 ml) was assayed for nucleoside diphosphokinase (○) and for radioactivity (○). Fractions 42 to 44 containing both radioactivity and enzyme activity were pooled (total activity of 23 μmoles per min and 5 x $10^7$ cpm). The fractions from the column were also assayed for [γ-$^{32}$P]ATP by applying 50 μl of the sample to a polyethyleneimine-Avicel plate and chromatographing it with formate solvents. The dried chromatograms were exposed to x-ray film for 3 days and developed. No [γ-$^{32}$P]ATP could be detected in the pooled sample.

in Fractions 42 to 44 was pooled and allowed to react with unlabeled CDP. This second reaction mixture was also passed over a Sephadex G-75 column and the eluate was again assayed for nucleoside diphosphokinase activity and radioactivity (Fig.
of the enzyme. We found no evidence for other physiologically significant regulation from the mechanism of the reaction and the loose specificity of phosphates are competitive inhibitors as would be expected whereby the donor triphosphate transfers its \( \gamma \)-phosphate to Salmonella is rather like nucleoside diphosphokinase from other sources. The reaction is mediated by a ping-pong mechanism and a sulfhydryl group for maximum activity. Di- and tri-

present investigation represents the first study of this enzyme from these bacteria. Using a 680-fold purified enzyme we have shown that in many respects nucleoside diphosphokinase from \( S. \) *lyphimurium* has two distinct sequences, namely: (a) \( \text{ATP} + \text{Ndk} = \text{ADP} + \text{P-Ndk} \), and (b) \( \text{CDP} + \text{P-Ndk} = \text{CTP} + \text{Ndk} \).

**FIG. 6.** Purification of the products of the reaction between \( ^{32}\text{P} \)-phosphorylated nucleoside diphosphokinase and CDP. The reaction mixture contained in 5 ml of Buffer A: 1 mm CDP and \( [\text{P}] \) nucleoside diphosphokinase \( (4 \times 10^4 \text{ cpm with a total activity of 17 amoles per min}) \). After 20 min at 20\(^{\circ}\) the mixture was applied to a Sephadex G-75 column and eluted with Buffer A. Fractions (2 ml) were collected and assayed for nucleoside diphosphokinase (●) and for radioactivity (○). Samples from the peak of radioactivity were chromatographed on polyethyleneimine-Avicel thin layer plates to determine the distribution of radioactivity between CDP, CTP, and inorganic phosphate.

6). In the second reaction virtually all label is removed from the enzyme, the \( ^{32}\text{P} \) label forms a second well defined peak. The chemical identity of the \( ^{32}\text{P} \) in the second peak was determined by thin layer chromatography. On the basis of comigration with known standards the composition was: 60% CTP (3244 cpm), 0% CDP, and 40% inorganic phosphate (2293 cpm).

From this experiment we conclude that the reaction between ATP and CDP catalyzed by nucleoside diphosphokinase (Ndk) from \( S. \) *typhimurium* has two distinct sequences, namely: (a) \( \text{ATP} + \text{Ndk} = \text{ADP} + \text{P-Ndk} \), and (b) \( \text{CDP} + \text{P-Ndk} = \text{CTP} + \text{Ndk} \).

**DISCUSSION**

Nucleoside diphosphokinase clearly plays a central role in the biosynthesis of the precursors of nucleic acids, the study of which has been done largely in enteric bacteria. Surprisingly the present investigation represents the first study of this enzyme from these bacteria. Using a 680-fold purified enzyme we have shown that in many respects nucleoside diphosphokinase from *Salmonella* is rather like nucleoside diphosphokinase from other sources. The reaction is mediated by a ping-pong mechanism whereby the donor triphosphate transfers its \( \gamma \)-phosphate to the enzyme and in turn to the recipient diphosphate. The enzyme has a rather broad pH optimum and requires both \( \text{Mg}^{2+} \) and a sulfhydryl group for maximum activity. Di- and tri-

phosphates are competitive inhibitors as would be expected from the mechanism of the reaction and the loose specificity of the enzyme; but in our survey of inhibitors or activators we found no evidence for other physiologically significant regulation of the enzyme.

The specificity of the enzyme is quite broad with respect to triphosphates as donors (data not shown) and diphosphates as recipients. However, believing that the physiological role of the enzyme is largely concerned with the synthesis of various triphosphates using ATP as a donor, we concentrated our attention on this series of reactions and were able to show that all ribo- and deoxyribonucleoside diphosphates which are precursors of nucleic acid can be phosphorylated to the triphosphate level by this reaction. In addition XDP, IDP, and UDP are good substrates for the reaction. Other data indicate that nucleoside diphosphokinase is also able to phosphorylate hydroxymethyldeoxyuridine diphosphate (22). Certain interesting conclusions can be drawn from these results. Firstly, the fact that enteric bacteria do not contain detectable pools of XTP and ITP must depend exclusively on the specificity of the monophosphokinases. Also, it is clear that nucleoside diphosphokinase is sufficient to account for the synthesis of all required triphosphates. Activities for the various diphosphates copurify proportionally (Table IV), and thus it appears that there are no other enzymes in *Salmonella* with significant activity for transferring the \( \gamma \)-phosphate of ATP to other diphosphates. An unequivocal establishment of this presumption must await the availability of mutant strains deficient in nucleoside diphosphokinase. The lesser activity toward the deoxynucleotides as compared with the corresponding ribonucleotides is consistent with the tentative conclusion that this single enzyme has evolved to synthesize all necessary triphosphates.

The levels of nucleoside diphosphokinase in *Salmonella* and the effect of growth rate on these levels raises a number of interesting questions. Nucleoside diphosphokinase concentration falls significantly as the growth rate of the culture is increased although the demands for triphosphates must increase dramatically at faster growth rates because both the rate of synthesis of nucleic acids and the fraction of the mass which is nucleic acid increase (34). However, at all growth rates the activity of the enzyme is surprisingly high. Crude extracts of cells grown even at high growth rates contain about 44 times as much nucleoside diphosphokinase as UMP kinase and at slow growth rates this ratio increases to almost 200-fold (Table I). However, there are other monophosphokinases. In enteric bacteria there are probably a total of 5; 1 for UMP (30), 1 for AMP and dAMP (35), 1 for CMP and dCMP (35), 1 for GMP and dGMP (37), and 1 for dTMP (38). Limited data (30) indicate that they are present at about the same specific activities. Thus, the very high ratio of nucleoside diphosphokinase to monophosphokinase activity most certainly indicates that nucleoside diphosphokinase is not rate-limiting even at the fastest growth rates. However, it is reasonable to expect that the ratio of tri- to diphosphates decreases with growth rate and that this change might in turn have very important effects on the regulation of cellular processes.

The precise physiological role of nucleoside diphosphokinase and the impact of changes in the ratio of tri- and diphosphates can only be answered when proper mutants are available.

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