Mechanism of the Enzymatic Synthesis of Cardiolipin in Escherichia coli
(phosphatidylglycerol/glycerol/CDP-diglyceride/isotope distribution)

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ABSTRACT In previous studies, the enzymatic conversion of phosphatidylglycerol to cardiolipin (diphasphatidylglycerol) in cell-free preparations from E. coli was shown to be stimulated by the addition of CDP-dipalmitin, suggesting the participation of the cytidine coenzyme as phosphatidyl donor. The present communication, however, presents three lines of evidence supporting the following mechanism for the synthesis of cardiolipin in E. coli:

2 Phosphatidylglycerol → cardiolipin + glycerol

When CDP-dipalmitin labeled with 32P in the phosphatidyl moiety was incubated with phosphatidyl[2-3H]glycerol, the cardiolipin produced in the enzymatic reaction was labeled with tritium, but not with 32P. Thus, CDP-diglyceride stimulates the reaction but does not participate as phosphatidyl donor. When [32P]phosphatidyl[2-3H]glycerol was used as substrate, the ratio of tritium to 32P in the cardiolipin product was only half of that in the starting phosphatidylglycerol, consistent with the elimination of 1 mol of glycerol during conversion to cardiolipin. Finally, free glycerol produced during the reaction has been unambiguously identified by phosphorylation with ATP in a reaction catalyzed by glycerol kinase (EC 2.7.1.30), followed by chromatographic isolation of labeled sn-3-glycerol-3-phosphate.

In a previous communication from this laboratory, Stanacev et al. (1) described a cell-free enzyme preparation from Escherichia coli that catalyzed the formation of cardiolipin (diphasphatidylglycerol) from phosphatidyl[2-3H]glycerol.

The addition of unlabeled CDP-dipalmitin stimulated the conversion of phosphatidylglycerol to cardiolipin 2- to 3-fold. These workers suggested that the synthesis of cardiolipin in this system proceeded according to the following equation:

Phosphatidylglycerol + CDP-diglyceride → cardiolipin + CMP [1]

Considerable evidence, however, has recently been obtained in several laboratories suggesting that the biosynthesis of cardiolipin may proceed by the following reaction:

2 Phosphatidylglycerol → cardiolipin + glycerol. [2]

In this laboratory, Lusk studied the metabolism of phosphatidylglycerol in living cells of E. coli by means of pulse-chase experiments with [2-3H]glycerol (2, 3), and found that a major pathway for the metabolism of phosphatidylglycerol in vivo must involve the elimination of free glycerol. Rampini et al. (4) observed the continued formation of cardiolipin from phosphatidylglycerol in E. coli under conditions in which the supply of metabolic energy was limited, leading them to suggest that the conversion may proceed via reaction [2]. These workers also made somewhat similar observations in Staphylococcus aureus (5). In an important contribution, De Siervo and Salton (6) found that the synthesis of cardiolipin from [32P]phosphatidylglycerol took place in enzyme preparations from Micrococcus lysodeikticus without added CDP-diglyceride. These workers, however, did not test the effect of added CDP-diglyceride, nor do these result rule out the possibility that equilibration of phosphatidylglycerol with endogenous cardiolipin in the enzyme preparation might be taking place via reaction [1]. If a phosphatidyl enzyme complex can be generated from cardiolipin in the reverse of reaction [1], equilibration of phosphatidylglycerol and cardiolipin might be expected in the absence of cytidine nucleotides.

We now report three lines of evidence that the biosynthesis of cardiolipin in cell-free preparations of E. coli takes place by reaction [2] rather than reaction [1]. Experiments with CDP-dipalmitin labeled with 32P in the phosphatidyl moiety have revealed that, under conditions in which the formation of cardiolipin from phosphatidylglycerol is stimulated by CDP-diglyceride, there is no transfer of 32P to the product. Thus, while CDP-dipalmitin stimulates the reaction, it does not participate as phosphatidyl donor. Further, when phosphatidylglycerol labeled with tritium in the unacylated glycerol moiety, and also with 32P, was used as substrate, the cardiolipin formed had a H/32P ratio that was one-half of that of phosphatidylglycerol, consistent with the elimination of a mole of glycerol in reaction [2]. Finally, labeled glycerol eliminated from phosphatidyl[2-3H]glycerol during conversion to cardiolipin has been unambiguously identified by enzymatic phosphorylation and subsequent isolation as sn-glycerol-3-P.

MATERIALS AND METHODS
CDP-dipalmitin labeled with 32P in the phosphatidyl moiety was prepared from 1,2-dipalmitoyl-sn-glycerol-3-32P by an adaptation of the method of Agranoff and Suomi (7). It was purified by chromatography on DEAE-cellulose in chloroform–methanol–water (1), and was shown to be completely reactive enzymatically in an assay with CDP-diglyceride: l-serine phosphatidyltransferase. The labeled phosphatidic acid used in this synthesis was prepared by the enzymatic phosphorylation of 1,2-dipalmitoyl-sn-glycerol with [γ-32P]-ATP, by the use of a partially purified preparation of diglyceride kinase from E. coli kindly provided by E. Gayle Reed. Phosphatidyl[2-3H]glycerol was prepared essentially by the method of Chang and Kennedy (8). [32P]Phosphatidyl-[2-3H]glycerol was similarly prepared with the 32P-labeled CDP-dipalmitin described above.
Cells of \textit{E. coli} strain ML 308 used in this investigation were grown on medium 63 (9) with succinate as carbon source and harvested in mid-logarithmic phase. Cell-free preparations were made by sonic irradiation of the cell suspension (in 0.1 M Tris·HCl buffer (pH 7.0)–10 mM 2-mercaptoethanol) in an ice-bath for four 30-sec bursts with an MSE apparatus. Intact cells were removed by centrifugation at 5000 \( \times g \) for 15 min. A particulate, membrane-containing fraction was obtained by further centrifugation of the sonic extract at 40,000 \( \times g \) for 1 hr.

**RESULTS**

**Experiments with cytidine-P-\(^{32}\)P-dipalmitin**

If the synthesis of cardiolipin from phosphatidyl[2-\(^{3}H\)]glycerol takes place according to reaction [1] with cytidine-P-\(^{32}\)P-dipalmitin as phosphatidyl donor, the cardiolipin produced should contain \(^{32}\)P as well as \(^{3}H\). Fig. 1 reveals the results of such an experiment. The newly synthesized cardiolipin, isolated by silicic acid-impregnated paper chromatography, contained \(^{3}H\), but no significant amounts of \(^{32}\)P. To verify further this conclusion, the labeled products of the enzymatic reaction were subjected to mild alkaline hydrolysis, followed by chromatography of the water-soluble products essentially as described by Stanacev \textit{et al.} (1). The hydrolysis product of cardiolipin (glycerol-P-glycerol-P-glycerol) contained \(^{3}H\), but no significant \(^{32}\)P (data not shown).

In control incubations, it was found that the conversion of phosphatidylglycerol to cardiolipin in the experiment of Fig. 1 was doubled by the addition of CDP-dipalmitin. In experiments with other enzyme preparations, stimulations as high as 5- to 10-fold have been observed. Thus, CDP-dipalmitin stimulates the conversion of phosphatidylglycerol to cardiolipin, but does not participate as a phosphatidyl donor.

**Conversion of doubly labeled phosphatidylglycerol to cardiolipin**

Phosphatidylglycerol labeled with \(^{3}H\) in the free glycerol moiety and with \(^{32}\)P was used as substrate for the synthesis of cardiolipin in the experiment of Table 1. The ratio of \(^{3}H/\(^{32}\)P in the cardiolipin produced was about one-half that of the starting phosphatidylglycerol, consistent with reaction [2], which requires the elimination of 1 mol of glycerol during the conversion of phosphatidylglycerol to cardiolipin.

**Identification of labeled glycerol released from phosphatidyl[2-\(^{3}H\)]glycerol**

The enzymatic conversion of phosphatidyl[2-\(^{3}H\)]glycerol to cardiolipin was carried out in the presence of \([1,3,\text{\textsuperscript{14}C}]\)glycerol of high specific-activity in the experiment described in Fig. 2. When the products of the reaction were partitioned between chloroform and aqueous methanol, all of the \(^{14}C\) was recovered in the aqueous phase, along with significant amounts of \(^{3}H\) that had been released during the course of the enzymatic reaction, as revealed by “zero time” controls. Upon paper chromatography of the water-soluble products in ethanol–1 M ammonium acetate (pH 7.5) 68:32, the tritiated product ran exactly coincident with the \([1,3,\text{\textsuperscript{14}C}]\)glycerol. For further identification, the water-soluble product was phosphorylated with ATP in the presence of glycerol kinase (EC 2.7.1.30).

After phosphorylation, chromatography on Dowex-1 formate led to the recovery of exactly coincident peaks of \(^{14}C\) and of \(^{3}H\) in the position expected for \textit{sn}-glycerol-3-P (Fig. 2). The recovery of both \(^{3}H\) and of \(^{14}C\) as glycerophosphate is almost quantitative, indicating that tritiated glycerol is virtually the sole labeled water-soluble product. From the specific activity of the starting phosphatidylglycerol, we calculated that 82 nmol of glycerol had been released. Analysis of the lipid phase by thin-layer chromatography revealed that 88 nmol of cardiolipin had been formed. The stoichiometry is thus in good agreement with that expected from reaction [2], but this result may have been fortuitous because equilibration of labeled lipids with unlabeled endogenous lipids could take place if reaction [2] is reversible.

**DISCUSSION**

The results presented here strongly support the view that the enzymatic synthesis of cardiolipin in \textit{E. coli} takes place ac-
Table 1. Ratio of isotopes in cardiolipin derived from

\[ ^{32}P \text{phosphatidyl}[^2\,^3H] \text{glycerol} \]

<table>
<thead>
<tr>
<th>Exp.</th>
<th>(a) Phosphatidylglycerol</th>
<th>(b) Cardiolipin</th>
<th>( b/a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.108</td>
<td>0.214</td>
<td>1.97</td>
</tr>
<tr>
<td>2</td>
<td>0.117</td>
<td>0.242</td>
<td>2.06</td>
</tr>
</tbody>
</table>

In Exp. 1, the incubation mixture contained 0.1 M Tris-HCl (pH 7.0)–2 mM 2-mercaptoethanol–2 mM MgCl\(_2\)–0.4 mM \[^{32}P\]phosphatidyl[2\,-^3H]glycerol (6.08 \times 10^4 \text{ cpm of } ^{32}P \text{ per mol} and 77,500 \text{ cpm of } ^{32}P \text{ per mol}–0.4 mM CDP-diglyceride–2 mg/ml of Triton X-100–0.1 ml of a sonic extract of E. coli strain ML 308 (1.3 mg of protein), in a final volume of 0.5 ml. The reaction was stopped after 1 hr of incubation at 37°C by the addition of chloroform–methanol 2:1, 12 ml containing 0.01 M HCl, 2 mg of carrier phosphatidylglycerol and 2 mg of cardiolipin. The chloroform-soluble lipids were washed with 20 ml of 2 M KCl; after centrifugation at 3000 \times g for 5 min, the upper layer was removed. The lower layer was discarded, and a cardiolipin-enriched fraction was obtained by DEAE-cellulose chromatography, as described in Fig. 1. A portion of this fraction was applied to silicic acid-impregnated paper and the lipids were separated as described in Fig. 1. Over 98% of the radioactivity had the same mobility as cardiolipin. Over 90% of the radioactivity was solubilized from the paper strips after 1 hr in a scintillation vial containing 10 ml of Patterson-Green counting fluid (5). The paper could be removed and the \[^{32}P\] to \[^{3}H\] ratio was determined. Corrections for quenching were performed with an internal standard (\(^{3}H\)) of toluene.

In Exp. 2, the incubation mixture was the same as in Exp. 1, but the final volume was 0.05 ml. The reaction was stopped by the addition of 1.5 ml of chloroform–methanol 2:1, which was 0.01 M in HCl. 2 M KCl (2.5 ml) was added and the mixture was centrifuged at 3000 \times g for 5 min. The upper layer was discarded and an aliquot of the lower layer was applied directly to silica-gel sheets (Eastman Kodak). The lipids were separated in chloroform–methanol–water 70:23:3. Phosphatidylglycerol and cardiolipin were well separated. The isotope ratio across each peak was determined and found to be constant. Correction for quenching was not possible, since not all of the radioactivity was soluble, but it is likely that the degree of quenching of both phosphatidylglycerol and cardiolipin is similar.

cording to reaction [2], but of course do not eliminate the possible occurrence of reaction [1] in this organism or elsewhere. The experiments of Lusk and Kennedy (2), however, which reveal that the elimination of free glycerol from phosphatidylglycerol is a major reaction during \( in vitro \) metabolism, suggest that reaction [2] may represent the principal, if not the sole, pathway in living cells.

The striking stimulation of reaction [2] by added CDP-diglyceride, even though it does not participate directly in the reaction, deserves some attention. A possible role of CDP-diglyceride as an allosteric effector must be considered, since plausible regulatory mechanisms can be envisaged in which the rate of formation of cardiolipin, one of the end products of phospholipid biosynthesis, is regulated by the concentration of CDP-diglyceride in the cell. Alternatively, CDP-diglyceride may be functioning simply as a surfactant or, since this nucleotide is also a glycerophosphatide, fulfilling some phospholipid requirement for the enzyme. Further work is needed to explore these possibilities.

Fig. 2. Isolation of labeled glycerol and conversion to ang
glycerol-3-P.

The incubation mixture contained Tris-HCl pH 7.0 (0.1 M); 2-mercaptoethanol (2 mM), MgCl\(_2\) (2 mM); phosphatidyl[2\,-^3H]glycerol of specific activity 1.8 \times 10^5 \text{ cpm per mol} (0.4 mM); CDP-dipalmatin (0.4 mM); [1,3\,-^3H]glycerol (a negligible amount; 9100 cpm); Triton X-100 (2 mg/ml); and 0.2 ml of membranes (1.4 mg of protein). The final volume was 1.0 ml. After 1 hr at 37°C, the reaction was stopped by the addition of 4.5 ml of 0.01 M HCl in chloroform–methanol 2:1. The chloroform-soluble lipids were washed with water (2.0 ml) and the mixture was centrifuged at 3000 \times g for 10 min. Most of the upper layer (4.0 ml) was removed. The lower layer was washed twice more with water (4.5 ml). Over 98% of the \(^{13}C\) label was recovered in the combined aqueous extracts. An aliquot of this material, containing 13,400 cpm of \(^{13}C\) and 6000 cpm of \(^{14}C\), was dried and phosphorylated with ATP and glycerol kinase as described (8). After phosphorylation, the sample was applied to a Dowex-1 (formate) column (16 \times 1.4 cm), and the column was eluted with a few bed-volumes of water. No radioactivity was eluted. Gradient elution was then begun with an apparatus similar to that of Hurlbert et al. (16), with 250 ml of water in the lower chamber and 4 M formic acid in the upper reservoir. Fractions (10 ml each) were collected and assayed for \(^{14}C\) and for \(^{3}H\) in a scintillation counter.

Stanacev (10) was the first to show that the synthesis of cardiolipin can proceed according to reaction [2], catalyzed by phospholipase D from cabbage. However, the efficiency of conversion in the phospholipase D-catalyzed reaction was exceedingly low. It seems doubtful that enzymes that efficiently hydrolyze cardiolipin, such as the cabbage enzyme studied by Stanacev (10) or the enzyme from \( Haemophilus parainfuenzae \) described by Ono and White (11), may play significant biosynthetic roles.

Reaction [2], where one phospholipid (phosphatidylglycerol) is the phosphatidyl donor in the biosynthesis of another phospholipid (cardiolipin), has some resemblance to the formation of phosphatidylserine in rat liver (12) and \( Tetrahymena pyriformis \) (13). In these species, the phosphatidyl moiety of phosphatidylethanolamine is transferred to L-serine to yield phosphatidylserine in a reaction catalyzed by phosphatidyl-ethanolamine:L-serine phosphatidyl transferase. Studies of this transformation in \( Tetrahymena \) (13) have revealed that this "exchange reaction" is catalyzed by an enzyme that is not primarily a hydrolase.

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