

## BBA Report

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BBA 51152

### The mechanism of cardiolipin biosynthesis in liver mitochondria

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(Received February 14th, 1972)

#### SUMMARY

1. When added exogenously, both CDP- and dCDP-diglyceride supported diphosphatidylglycerol synthesis from phosphatidyl[1'-<sup>14</sup>C]glycerol in intact mitochondria. The maximum rate observed for CDP-diglyceride was about 2-fold greater than for dCDP-diglyceride. At optimal concentrations of CDP-diglyceride a 30-fold stimulation of diphosphatidylglycerol synthesis was observed.

2. [<sup>14</sup>C]Glycerol was not formed in significant amounts during mitochondrial conversion of phosphatidyl[1'-<sup>14</sup>C]glycerol to diphosphatidylglycerol.

3. In mitochondria, [2-<sup>3</sup>H]phosphatidyl[1'-<sup>14</sup>C]glycerol was converted in the presence of unlabeled CDP-diglyceride to a diphosphatidylglycerol having a nearly identical <sup>3</sup>H/<sup>14</sup>C ratio. The above evidence confirms that diphosphatidylglycerol is formed from phosphatidylglycerol and CDP-diglyceride in mitochondria.

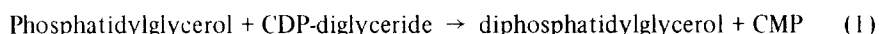
4. In contrast, in preparations of membranes from *Escherichia coli* K12, the diphosphatidylglycerol formed at low concentrations of CDP-diglyceride had a <sup>3</sup>H/<sup>14</sup>C ratio of 2.0 relative to its precursor [2-<sup>3</sup>H]phosphatidyl[1'-<sup>14</sup>C]glycerol. Furthermore, significant amounts of [<sup>14</sup>C]glycerol were isolated after incubation of the *E. coli* membranes with phosphatidyl[1'-<sup>14</sup>C]glycerol. These findings indicate that in *E. coli* diphosphatidylglycerol is formed from two molecules of phosphatidylglycerol. However, evidence is presented which suggests the operation of the CDP-diglyceride pathway in *E. coli* at higher concentrations of CDP-diglyceride.

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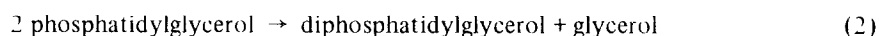
Previous reports from this laboratory described diphosphatidylglycerol (cardiolipin) biosynthesis in normal and germ-free preparations of rat liver mitochondria<sup>1</sup> and indicated that diphosphatidylglycerol synthesis is localized in the mitochondrial inner membrane<sup>2</sup>. Other investigators have reported incorporation of radioactive-labeled glycerol-3-phosphate into diphosphatidylglycerol in liver mitochondria in the presence of glycerol-

3-phosphate-acylating and CDP-diglyceride-generating systems<sup>3,4</sup>. Neither our studies<sup>1,2</sup> nor those of Davidson and Stanacev<sup>3,4</sup> have indicated the actual mechanism of diphosphatidylglycerol formation.

The following reaction mechanism was suggested by Stanacev *et al.*<sup>5</sup> in their study of diphosphatidylglycerol synthesis in *Escherichia coli*.



Evidence supporting this reaction has been provided by our studies in liver mitochondria which showed a 20-fold stimulation of diphosphatidylglycerol synthesis by CDP-diglyceride<sup>2</sup> whereas this stimulation in *E. coli* was less than 3-fold<sup>5</sup>. Another reaction was proposed by Rampini *et al.*<sup>6</sup> after they observed diphosphatidylglycerol formation from phosphatidylglycerol under conditions where *de novo* synthesis of phospholipids (e.g. CDP-diglyceride) presumably could not occur.



Stanacev and Stuhne-Sekalec<sup>7</sup> demonstrated that Reaction 2 occurs to a small degree during phospholipase D action on phosphatidylglycerol. Recently, Desiervo and Salton<sup>8</sup> have also suggested that Reaction 2 is operative in preparation of *Micrococcus lysodeikticus*.

This report presents evidence which indicates that diphosphatidylglycerol synthesis occurs by Reaction 1 in mitochondria.

Intact mitochondria and inner membranes were isolated as described previously<sup>2</sup>. *E. coli* (strain K12) were grown in a synthetic medium containing 80 mM phosphate buffer (pH 7.0); 38 mM NH<sub>4</sub>Cl; 2 μM MgSO<sub>4</sub>; 1.8 μM FeSO<sub>4</sub> and 110 mM glucose. The bacteria were harvested during the mid-logarithmic phase; the pellet was suspended in 0.05 M Tris buffer (pH 7.4) and the cells disrupted by sonication. A particulate fraction was obtained essentially as described by Stanacev *et al.*<sup>5</sup>. Both the mitochondrial and *E. coli* preparations were resuspended in 0.25 M sucrose–5 mM Tris (pH 7.4) and, after protein determination by the method of Lowry<sup>9</sup>, were stored at –18 °C in small aliquots.

*sn*-[2-<sup>3</sup>H] Glycerol-3-phosphate and *sn*-[1-<sup>14</sup>C] glycerol-3-phosphate were prepared as described previously<sup>2</sup>. 1,2-Dioleoyl-*sn*-[2-<sup>3</sup>H] glycerol-3-phosphate and its unlabeled equivalent were synthesized by the method of Lapidot *et al.*<sup>10</sup>. 3-Cytidine diphosphate-1,2-dioleoyl[2-<sup>3</sup>H] glycerol (source: 1,2-dioleoyl-*sn*-[2-<sup>3</sup>H] glycerol-3-phosphate); (dioleoyl)CDP-diglyceride (source: (dioleoyl)phosphatidic acid); CDP- and dDCP-diglycerides (source: phosphatidic acid derived from egg lecithin) were prepared by the method of Agranoff and Suomi<sup>11</sup> as modified by Prottey and Hawthorne<sup>12</sup>. All CDP-diglycerides were found to be pure by thin-layer chromatography as described in ref. 13.

1,2-Dioleoyl-*sn*-glycero-3-phosphoryl-1'-*sn*-[1-<sup>14</sup>C] glycerol was obtained biosynthetically from *sn*-[1-<sup>14</sup>C] glycerol-3-phosphate and (dioleoyl)CDP-diglyceride and purified as previously described<sup>2</sup>. 1,2-Dioleoyl-*sn*-[2-<sup>3</sup>H] glycero-3-phosphoryl-1'-*sn*-glycerol was obtained similarly from 3-cytidine diphosphate-1,2-dioleoyl-*sn*-[2-<sup>3</sup>H] glycerol and unlabeled glycerol-3-phosphate. These two synthesized compounds were mixed to give

suitable  $^3\text{H}/^{14}\text{C}$  ratios (see Table III); the fatty acid composition was assumed to be similar since a dioleoyl CDP-diglyceride was the phosphatidyl donor in each case. For simplicity this substrate is hereafter referred to as  $[2\text{-}^3\text{H}]$  phosphatidyl $[1\text{-}^{14}\text{C}]$  glycerol. Phosphatidyl $[1\text{-}^{14}\text{C}]$  glycerol was similarly prepared from *sn*- $[1\text{-}^{14}\text{C}]$  glycerol-3-phosphate and CDP-diglyceride (source: phosphatidic acid derived from egg lecithin) and purified as above. Phosphorus was determined by the method of Ames and Dubin<sup>14</sup>.

Diphosphatidylglycerol synthesis was assayed as described previously<sup>2</sup>.

Experimental details are given in the respective legends. Total lipid extractions, thin-layer chromatography and scanning, elution of phospholipids from the silica gel and radioactivity measurements were made as previously described<sup>2</sup>.

Glycerol was isolated from the aqueous phase of the total lipid extraction by passage over a mixed-bed ion-exchange column [Amberlite IRC 50 ( $\text{H}^+$ ) and Amberlite IR 45 ( $\text{OH}^-$ )]. The eluate was concentrated to a small volume *in vacuo*; the entire sample was applied to Whatman 3 MM paper and the chromatogram developed with *n*-propanol-conc. ammonia-water (6:3:1, by vol.; descending). Reference glycerol was identified by periodate-Schiff staining; the areas corresponding to glycerol were cut out, placed in scintillation vials and counted.

Fig. 1 shows the relationship between the concentration of CDP- or dCDP-diglyceride and the rate of diphosphatidylglycerol synthesis using intact mitochondria. CDP-diglyceride was more active than dCDP-diglyceride in supporting diphosphatidylglycerol synthesis. The observed rate for diphosphatidylglycerol synthesis with CDP-diglyceride was about 2-fold greater than the rate with dCDP-diglyceride and approximately 30 times greater than the control which contained no added CDP-

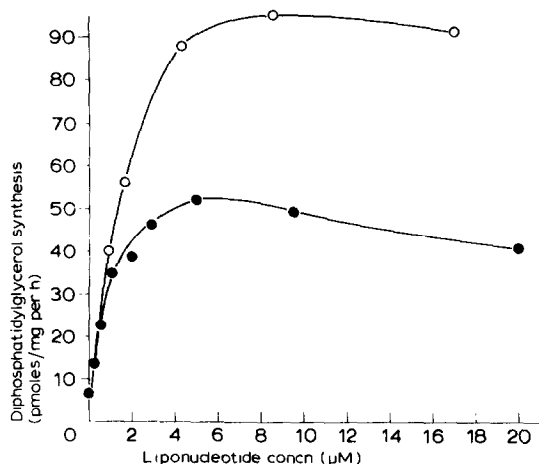


Fig. 1. Effect of CDP-diglyceride or dCDP-diglyceride concentration on diphosphatidylglycerol synthesis. Incubation mixtures contained liponucleotides at indicated concentrations; 50 mM Tris (pH 7.5);  $7\text{ }\mu\text{M}$  phosphatidyl $[1\text{-}^{14}\text{C}]$  glycerol (specific activity  $2.5 \cdot 10^7$  dpm/ $\mu\text{mole}$ ); 1.07 mg mitochondrial protein; 20 mM  $\beta$ -mercaptoethanol; 2 mM EDTA; 10 mM  $\text{MgCl}_2$  was the last addition. Final volume was 0.250 ml and incubation was for 1 h at  $37^\circ\text{C}$ .

diglyceride (3 pmoles/mg per h). Higher concentrations (above 10  $\mu$ M) of both liponucleotides were somewhat inhibitory; a similar phenomenon has been noted in phosphatidylglycerol synthesis<sup>13,15</sup>. In subsequent experiments the optimal concentration of CDP-diglyceride (10  $\mu$ M) was used.

Table I shows results of incubation of intact mitochondria with *sn*-[2-<sup>3</sup>H]glycerol-3-[<sup>32</sup>P]phosphate and unlabeled CDP-diglyceride. Phosphatidylglycerol phosphate was the only compound labeled with <sup>32</sup>P while all three compounds contained <sup>3</sup>H. This suggests that CDP-diglyceride rather than glycerol-3-phosphate is the source of the phosphate of diphosphatidylglycerol and appears to exclude the possibility suggested by Lecocq and Ballou<sup>16</sup> that phosphatidylglycerol phosphate could react with 1,2-diacylglycerol to form diphosphatidylglycerol.

A key feature of Reaction 2 above, as compared with Reaction 1, is that 1 mole of free glycerol is formed for each mole of diphosphatidylglycerol; this does not occur in

TABLE I

INCORPORATION OF *sn*-[2-<sup>3</sup>H]GLYCEROL-3-[<sup>32</sup>P]PHOSPHATE INTO PHOSPHATIDYL GLYCEROL PHOSPHATE, PHOSPHATIDYLGLYCEROL AND DIPHOSPHATIDYLGLYCEROL

The incubation vessel contained 1.6 mM *sn*-[2-<sup>3</sup>H]glycerol-3-[<sup>32</sup>P]phosphate; 20 mM  $\beta$ -mercaptoethanol; 2 mM EDTA; 50 mM Tris (pH 8.0); 80  $\mu$ M CDP-diglyceride; 1.84 mg of mitochondrial protein; and 10 mM MgCl<sub>2</sub> in a final volume of 0.5 ml. The specific activity of *sn*-glycerol-3-phosphate was  $1.6 \cdot 10^6$  cpm/mole for <sup>3</sup>H and  $1.4 \cdot 10^6$  cpm/mole for <sup>32</sup>P. Incubation was for 4 h at 37 °C.

Compound	cpm	
	<sup>3</sup> H	<sup>32</sup> P
Phosphatidylglycerol phosphate	6 900	4300
Phosphatidylglycerol	60 000	0
Diphosphatidylglycerol	3 700	0

TABLE II

PRODUCTION OF [<sup>14</sup>C]GLYCEROL DURING DIPHOSPHATIDYLGLYCEROL FORMATION FROM PHOSPHATIDYL[1-<sup>14</sup>C]GLYCEROL: COMPARISON OF MITOCHONDRIAL INNER MEMBRANES AND *E. coli* MEMBRANES

The incubation mixture for *E. coli* membranes contained 1.2 mg protein, 50 mM Tris (pH 8.0); 0.01 mM CDP-diglyceride; 1  $\mu$ M phosphatidyl[1-<sup>14</sup>C]glycerol (specific activity  $2.5 \cdot 10^7$  dpm/ $\mu$ mole); and 2 mM EDTA. 10 mM MgCl<sub>2</sub> and 2 mM MnCl<sub>2</sub> were added last. For mitochondria the mixture contained 4.0 mg protein, 0.01 mM CDP-diglyceride, 4  $\mu$ M phosphatidyl[1-<sup>14</sup>C]glycerol. Other additions were present as above; the final volume was 1.0 ml. Incubation was for 3 h at 37 °C.

	Mitochondrial inner membranes	<i>E. coli</i> membranes
dpm diphosphatidylglycerol	20 400	3170
dpm glycerol	150	1850
Ratio glycerol/diphosphatidylglycerol	0.008	0.58

**TABLE III**  
**CONVERSION OF DOUBLY-LABELED PHOSPHATIDYLGLYCEROL TO DIPHOSPHATIDYLGLYCEROL WITH UNLABELED CDP-  
 DIGLYCERIDE: MITOCHONDRIAL INNER MEMBRANES *versus E. coli* MEMBRANES**

Incubation mixture contained 50 mM Tris (pH 8.0); 1,2-dioleoyl-*sn*-[2-<sup>3</sup>H]glycero-3-phosphoryl-1'-*sn*-[1'-<sup>14</sup>C]glycerol (specific activity 6.10 · 10<sup>5</sup> dpm <sup>14</sup>C and 1.68 · 10<sup>6</sup> dpm <sup>3</sup>H per mmole) and unlabeled CDP-diglyceride as indicated; 2 mM EDTA; and either 2.8 mg mitochondrial inner membrane protein or 0.4 mg *E. coli* membrane protein; 10 mM MgCl<sub>2</sub> and 2 mM MnCl<sub>2</sub> were added last. Final volume was 0.5 ml; incubation was for 2 h at 37 °C.

Sample	Phosphatidyl- glycerol concn (μM)	CDP-diglyceride concn (μM)	dpm <sup>3</sup> H/ <sup>14</sup> C phosphatidyl- glycerol	dpm <sup>3</sup> H/ <sup>14</sup> C diphosphatidyl- glycerol	Ratio diphosphatidyl- glycerol phosphatidyl- glycerol
Mitochondrial inner membrane	5	10	2.48	2.34	0.94
	10.7	10	2.46	2.04	0.83
	16.7	10	3.06	3.52	1.15
<i>E. coli</i> membrane	5	10	2.44; 2.54★	4.90; 5.50★	2.01; 2.16★
	10.7	1000	2.86	5.00	1.75
	16.7	1000	2.88	4.50	1.56

★ This incubation contained 2.0 mg/ml Triton X-100.

Reaction 1. Table II indicates that incubation of phosphatidyl[1'- $^{14}\text{C}$ ]glycerol with mitochondrial inner membranes did not result in the production of significant amounts of [ $^{14}\text{C}$ ]glycerol by mitochondria. This was in contrast to *E. coli* where the amount of [ $^{14}\text{C}$ ]glycerol recovered was almost 60% of the [ $^{14}\text{C}$ ]diphosphatidylglycerol formed. [ $^{14}\text{C}$ ]Glycerol recovery was not quantitative in these experiments, but assuming that losses during purification were similar, there is a clear difference between mitochondria and *E. coli* (0.8% versus 58%, respectively).

In order to elucidate this difference in a more quantitative manner, we have made use of a second feature which distinguishes Reaction 1 from Reaction 2. Namely, when [2- $^3\text{H}$ ]phosphatidyl[1'- $^{14}\text{C}$ ]glycerol is converted to diphosphatidylglycerol by Reaction 1 in the presence of unlabeled CDP-diglyceride, there should be no change in the ratio of  $^3\text{H}/^{14}\text{C}$ . In contrast, Reaction 2 would be expected to give diphosphatidylglycerol with a  $^3\text{H}/^{14}\text{C}$  ratio twice that of the phosphatidylglycerol.

Table III shows the results of these experiments. The diphosphatidylglycerol isolated from mitochondrial inner membranes showed a  $^3\text{H}/^{14}\text{C}$  ratio close to 1.0 in every instance relative to phosphatidylglycerol, while *E. coli*, when examined at 10  $\mu\text{M}$  CDP-diglyceride concentrations, showed a ratio close to 2.0. These results confirm that the CDP-diglyceride pathway (Reaction 1) is operative in mitochondria, whereas in *E. coli* the results confirm the operation of Reaction 2. Stanacev *et al.*<sup>5</sup> originally reported the presence of Reaction 1 in *E. coli* using a much higher concentration of CDP-diglyceride (1000  $\mu\text{M}$ ) than that which is optimal for mitochondria (10  $\mu\text{M}$ ). We therefore repeated the experiments in *E. coli* using 1000  $\mu\text{M}$  CDP-diglyceride; under these circumstances the  $^3\text{H}/^{14}\text{C}$  ratio in diphosphatidylglycerol was found to be less than 2.0 (1.5 and 1.75) at least suggesting that Reaction 1 may also be present in *E. coli* at high concentrations of CDP-diglyceride as originally proposed by Stanacev *et al.*<sup>5</sup>. However, further investigations will be necessary to establish this possibility conclusively.

This work was supported by funds from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

K.Y.H. is a postdoctoral fellow of the Department of Medicine, Case Western Reserve University, Cleveland, Ohio, U.S.A. (U.S. Public Health Service, Grant No. AM-1005).

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