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Biosynthesis of cardiolipin in liver mitochondria

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SUMMARY

Diphosphatidylglycerol (cardiolipin) biosynthesis from CDP-diglyceride and *sn*-glycerol-3-phosphate has been demonstrated in a preparation of mitochondria from rat liver in a reaction requiring Mg^{2+}. The identity of the diphosphatidylglycerol was established by thin-layer chromatography and mild alkaline hydrolysis. Diphosphatidylglycerol formation from [^{3}H]phosphatidylglycerol was shown to occur only in the presence of CDP-diglyceride. The rate of diphosphatidylglycerol formation appears to be quite slow in mitochondria and may explain why it was not previously observed. Evidence is presented which eliminates the possibility of a bacterial contribution to the biosynthetic process observed.

Stanacev *et al.*[1] showed that cell-free preparations from *Escherichia coli* could synthesize diphosphatidylglycerol (cardiolipin) in the presence of *sn*-glycerol-3-phosphate, CDP-diglyceride, Mg^{2+}, and Triton X-100. They presented good evidence for the following reaction sequence:

(1) *sn*-Glycerol-3-phosphate + CDP-diglyceride → phosphatidylglycerol phosphate + CMP
(2) Phosphatidylglycerol phosphate → phosphatidylglycerol + P_i
(3) Phosphatidylglycerol + CDP-diglyceride → diphosphatidylglycerol + CMP

In view of the predominant localization of diphosphatidylglycerol in the mitochondria of mammalian tissue, attempts have been made to show diphosphatidylglycerol biosynthesis in mitochondria. Thus far, studies in mitochondria from liver[2], brain[3-5], heart[6,7] or ventral prostate[8] did not demonstrate diphosphatidylglycerol biosynthesis, although phosphatidylglycerol, presumed to be the immediate precursor of diphosphatidylglycerol, was formed readily. It has been suggested that the inability to demonstrate diphosphatidylglycerol synthesis in mitochondria may be due to the presence of atypical diphosphatidylglycerol structures[5,9], compartmentation of phospholipid synthesis in the

mitochondria[5], or the presence of a pathway of diphosphatidylglycerol synthesis[5] different from the one previously shown in *E. coli*. We have recently found diphosphatidylglycerol biosynthesis in mitochondria prepared from rat liver.

sn-[2-^{3}H] Glycerol-3-phosphate was prepared from [2-^{3}H] glycerol by the method of Chang and Kennedy[10]. CDP-diglyceride was purchased from Koch-Light Co. or synthesized by the method of Agranoff and Suomi as modified by Prottey and Hawthorne[11]. Mitochondria were isolated from rat liver by the method of Wirtz and Zilversmit[12] and suspended in freshly prepared 0.25 M sucrose–5 mM Tris (pH 7.4). The concentration of mitochondrial protein was determined[13] and the mitochondrial suspension was frozen in small aliquots. Aliquots of the 0.25 M sucrose–5 mM Tris used to suspend the mitochondria were also frozen and used as a control. Mitochondria were also prepared from the liver of a germ-free Wistar rat under sterile conditions. All reagents and equipment were sterilized and sterile techniques were used throughout the procedures. Cultures of the final germ-free mitochondria suspension showed no growth in 5 days on agar plates (peptone–yeast extract medium) at 37°. The incubation conditions are described in the legends of the figures and tables. After the incubation the total lipids were extracted by the method of Bligh and Dyer[14], washed twice with 2 M KCl and once with water. The entire lipid sample was applied to thin-layer chromatograph plates (System A, Table I). Reference diphosphatidylglycerol (*ex* bovine heart, Koch-Light, Co and phosphatidylglycerol (purified from *Micrococcus laidlawii* by the method of Shaw *et al.*[15]) were located using iodine vapors. The ^{3}H-labeled phospholipids were located using a Panax thin-layer chromatography scanner (Panax Equipment Ltd., Redhill Surrey, England) and eluted from the silica gel by the method of Skipski[16]. Radioactivity measurements were made on known aliquots using a Packard TriCarb liquid scintillation spectrometer. Quenching corrections were made using the channels ratio method.

Fig. 1 shows the time course of phosphatidylglycerol and diphosphatidylglycerol synthesis. In this experiment, incorporation of *sn*-[2-^{3}H] glycerol-3-phosphate into phosphatidylglycerol was linear for 1 h, after which no further incorporation of radioactivity occurred. Diphosphatidylglycerol synthesis was linear for 2 h. At 60 min, when phosphatidylglycerol synthesis was maximal, diphosphatidylglycerol formation was only 0.6% of that of phosphatidylglycerol. Thus, diphosphatidylglycerol formation in mitochondria seems to proceed very slowly and may explain why it was not noted in the previous studies which employed low specific activity substrates[2-8]. These results are in agreement with the *in vivo* studies of Taylor *et al.*[17] and McMurray and Dawson[18] who showed very slow turnover of ^{32}P in the cardiolipin of rat liver mitochondria.

Thin-layer chromatography of the synthesized phospholipids showed the presence of radioactivity which coincided with R_F values of reference phosphatidylglycerol and diphosphatidylglycerol, respectively (Table I, System A). Both radioactive compounds were eluted from the silica gel and shown to co-chromatograph with reference phosphatidylglycerol and diphosphatidylglycerol in two other systems (Table I, Systems B and C). The ^{3}H-labeled products were further identified using mild alkaline hydrolysis by the method of Chang and Kennedy[19]. Reference bis(glyceryl phosphoryl) glycerol and glyceryl phosphoryl glycerol were prepared from diphosphatidylglycerol and phosphatidylglycerol standards on the same day that the ^{3}H-labeled compounds were treated by alkaline hydrolysis. The water-soluble products were immediately spotted on Whatman I paper and chromatographed in two solvent systems. The ^{3}H-labeled water-soluble alkaline hydrolysis

TABLE I

APPROXIMATE R_F VALUES OF ^{3}H-LABELED PHOSPHOLIPIDS FROM MITOCHONDRIA AND THEIR ALKALINE HYDROLYSIS PRODUCTS

System	*Support*	*Solvent (by vol.)*	*Thin-layer chromatography of intact ^{3}H-labeled phospholipid*		*Alkaline hydrolysis product from:*		*Reference compounds*		
			PG	*DPG*	*PG*	*DPG*	*GP*	*GPG*	*GPGPG*
A	Silica gel G, 0.4 M boric acid	Chloroform–methanol–water–conc. NH_3 (70:30:3:2)	0.22	0.51	–	–	–	–	–
B	Silica gel H, 1 mM Na_2CO_3	Chloroform–methanol–acetic acid–water (50:25:7:3)	0.72	front	–	–	–	–	–
C	Silica gel H, 0.1 M $NaHCO_3$	Chloroform–methanol–1 M NH_3 (80:36:2)	0.36	0.56	–	–	–	–	–
D	Whatman I paper	Propan-1-ol–conc. NH_3–water (6:3:1)	–	–	0.43	0.30	0.20	0.43	0.31
E	Whatman I paper	1 M NH_3 acetate–abs. ethanol (35:65)	–	–	–	0.58	0.31	–	0.61

Abbreviations: PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; GP, glycerol phosphate; GPG, glyceryl phosphoryl glycerol; GPGPG, bis(glyceryl phosphoryl) glycerol.

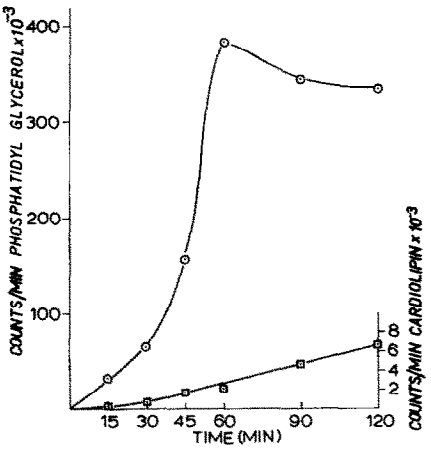

Fig. 1. Time course of phosphatidylglycerol and diphosphatidylglycerol formation. The incubation medium contained $3.4 \cdot 10^{-5}$ M *sn*-[2-^{3}H]glycerol-3-phosphate (specific activity 0.25 C/mmole; $1.34 \cdot 10^7$ counts/min); $5 \cdot 10^{-5}$ M CDP-diglyceride; 27 mg mitochondrial protein; 50 mM Tris– HCl (pH 7.4); 8 mM β-mercaptoethanol; 2 mM EDTA; and 75 mM sucrose. 10 mM $MgCl_2$ was added last after the contents had been thoroughly mixed. The final volume was 3.00 ml. The mixture was incubated with shaking at 37° and 0.5-ml aliquots were removed at the times indicated in the figure for phospholipid analysis.

products were located using a thin-layer chromatography scanner. Bis(glyceryl phosphoryl) glycerol and glyceryl phosphoryl glycerol were located using the phosphorus spray of Hanes and Isherwood[20]. In both solvent systems the water-soluble products of alkaline hydrolysis showed R_F values corresponding to the reference glycerol phosphoryl glycerol and bis(glyceryl phosphoryl) glycerol, respectively, thus confirming the identity of the synthesized phospholipids as phosphatidylglycerol and diphosphatidylglycerol.

Table II gives the results of incubations where various components of the complete incubation medium were omitted. No radioactive lipid was synthesized when the mitochondria were omitted. Very small amounts of diphosphatidylglycerol and phosphatidylglycerol were formed when CDP-diglyceride was omitted. The small amount of phospholipid synthesized (less than 2% of the complete system) is thought to reflect the presence of endogenous CDP-diglyceride which is synthesized predominantly in the mitochondria of rat liver[21]. Phosphatidylglycerol synthesis was only slightly lower (about 80% of complete system) when Mg^{2+} was omitted; in contrast, very little diphosphatidylglycerol was formed without Mg^{2+} (less than 1% of complete system). Thus diphosphatidylglycerol synthesis appears to have a definite requirement for Mg^{2+}. While this ion is not required for phosphatidylglycerol synthesis, Davidson and Stanacev[5] have shown that phosphatidylglycerol synthesis occurs somewhat faster in brain mitochondria if Mg^{2+} is present. Omission of EDTA resulted in a large decrease in diphosphatidylglycerol synthesis but had little effect on phosphatidylglycerol synthesis. The reasons for this are not known, but are currently under investigation. Omission of β-mercaptoethanol resulted in a slight decrease in phosphatidylglycerol synthesis while diphosphatidylglycerol synthesis was decreased to a somewhat greater degree. Results similar to those in Table II were also obtained with a preparation of mitochondria from beef heart.

TABLE II

CONVERSION OF *sn*-[2-^{3}H] GLYCEROL-3-PHOSPHATE TO PHOSPHATIDYLGLYCEROL AND DIPHOSPHATIDYLGLYCEROL BY RAT LIVER MITOCHONDRIA

The incubation medium contained $1 \cdot 10^{-4}$ M CDP-diglyceride; other additions were present as in Fig. 1 in a final volume of 0.500 ml. The incubation was for 2 h at 37°.

	Diphosphatidylglycerol (pmoles/mg per h)	*Phosphatidylglycerol (pmoles/mg per h)*
Complete system	6.1	560
No mitochondria	0	0
No CDP-diglyceride	0.1	5
No Mg^{2+}	0.6	380
No EDTA	1.9	424
No β-mercaptoethanol	3.7	502

^{3}H-labeled phosphatidylglycerol formed in the incubations of mitochondria with *sn*-[2-^{3}H] glycerol-3-phosphate and CDP-diglyceride was recovered from the silica gel and characterized as noted above (Table I). After quantitative measurements were made on aliquots, the radioactive phosphatidylglycerol was pooled and further purified by two passages on preparative thin-layer chromatography plates (System C, Table I). After removal of the organic solvent from an aliquot *in vacuo*, this material was suspended in water by brief sonication using a Branson Sonifier with microtip and was used in the incubation shown in Fig. 2. Mitochondria were incubated with [^{3}H] phosphatidylglycerol with and without CDP-diglyceride. The radioactive products were isolated and characterized as shown above (Table I). Although there is a small amount of diphosphatidylglycerol synthesis in the absence of CDP-diglyceride (1.6% of recovered counts) it is apparent from Fig. 2 that diphosphatidylglycerol synthesis is greatly enhanced by added CDP-diglyceride (19% of recovered counts). This strongly suggests that mitochondrial diphosphatidylglycerol synthesis proceeds by the same last step (Eqn. 3 above) as that previously shown in *E. coli*[1], although the overall rate in mitochondria is apparently much slower.

In view of the very slow rate of diphosphatidylglycerol formation, experiments were carried out to determine if bacterial contamination of the mitochondria preparation could account for the observed synthetic activity. Table III shows the results of incubations comparing "normal" *versus* germ-free mitochondria. There was no consistent difference between rates of phosphatidylglycerol and diphosphatidylglycerol formation in the "normal" *versus* germ-free mitochondria. Cultures taken from the germ-free mitochondria before and at the end of incubation showed no bacterial growth. In contrast, there was a moderate amount of bacterial contamination (about 400 colonies/mg mitochondrial protein) in the "normal" mitochondria. It can also be seen that preincubation with antibiotics had no significant effect on either preparation. Based on the similarity of the rates of [^{3}H] phospholipid synthesis in the "normal" *versus* germ-free mitochondria and on the lack of effect of antibiotics, a significant contribution of bacteria to the events observed can be excluded. Finally, the fact that diphosphatidylglycerol formation was found in sterile, germ-free mitochondria indicates that we have observed a process occurring in the mitochondrial preparations from rat liver.

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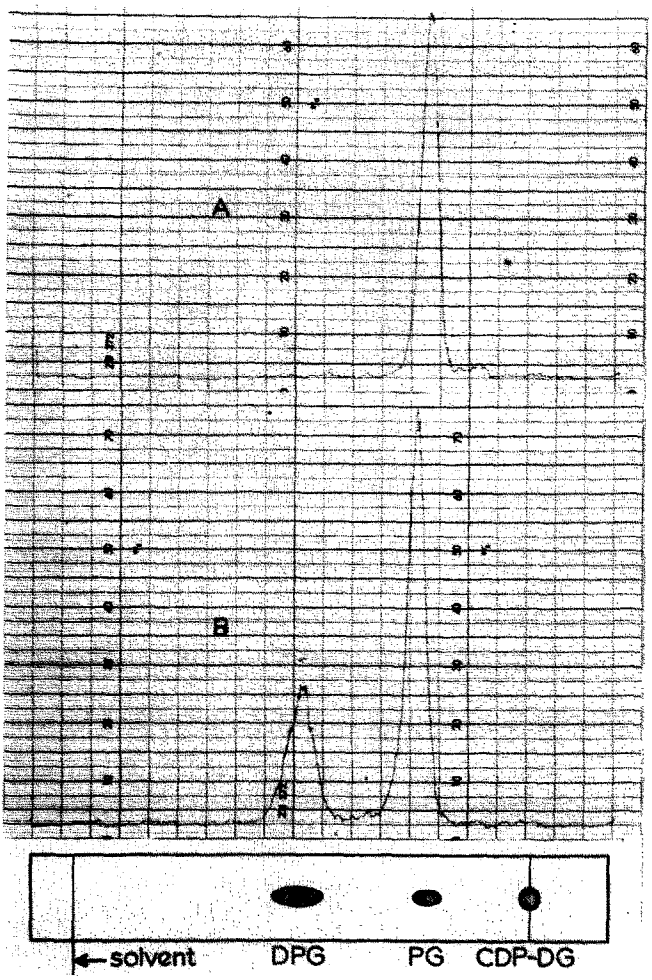

Fig. 2. Formation of [^{3}H] diphosphatidylglycerol from [^{3}H] phosphatidylglycerol. Incubation mixtures contained $3 \cdot 10^5$ counts/min [^{3}H] phosphatidylglycerol in place of *sn*-[2-^{3}H] glycerol-3-phosphate. Mixture A contained no CDP-diglyceride, and Mixture B contained $1 \cdot 10^{-4}$ M CDP-diglyceride. 4.5 mg mitochondrial protein was present in a final volume of 0.5 ml. Other additions were the same as in Fig. 1. After a 2-h incubation at 37°, total lipid extracts were prepared and chromatographed on thin-layer chromatography plates (System A, Table I). The plates were scanned for ^{3}H activity and the tracings are shown above. The bottom strip shows the positions of reference compounds relative to the radioactivity peaks. Abbreviations: see Table I; CDP-DG, CDP-diglyceride.

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TABLE III

SYNTHESIS OF PHOSPHATIDYLGLYCEROL AND DIPHOSPHATIDYLGLYCEROL BY "NORMAL" AND GERM-FREE MITOCHONDRIA AND EFFECT OF PREINCUBATION WITH ANTIBIOTICS

The incubation medium contained $6 \cdot 10^{-6}$ M *sn*-[2-^{3}H] glycerol-3-phosphate (specific activity 0.5 C/mmole; $7.87 \cdot 10^5$ counts/min); $1 \cdot 10^{-4}$ M CDP-diglyceride; 50 mM Tris–HCl (pH 7.4); 20 mM β-mercaptoethanol; 2 mM EDTA; 75 mM sucrose; 200 μg streptomycin and 200 units/ml penicillin G when indicated; 3.0 mg mitochondrial protein. 10 mM $MgCl_2$ was added last after thorough mixing of the other contents. Substrates were added after a 30-min preincubation at 37°. The final mixture (0.50 ml) was incubated for 2 h at 37°.

	Total counts/min	
	Phosphatidylglycerol	*Diphosphatidylglycerol*
"Normal" mitochondria	$1.73 \cdot 10^5$	$2.88 \cdot 10^3$
"Normal" *plus* antibiotics	$1.72 \cdot 10^5$	$2.86 \cdot 10^3$
Germ-free mitochondria	$1.75 \cdot 10^5$	$3.78 \cdot 10^3$
Germ-free *plus* antibiotics	$1.35 \cdot 10^5$	$4.54 \cdot 10^3$

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