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THE SYNTHESIS AND UTILIZATION OF dCDP-DIGLYCERIDE BY A MITOCHONDRIAL FRACTION FROM RAT LIVER

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SUMMARY

1. Mitochondrial preparations from rat liver incorporate added dCTP into a compound that is readily extracted with chloroform. This compound was identified as dCDP-diglyceride by co-chromatography with authentic dCDP-diglyceride on silica gels with three different solvent systems and by the absence of an effect of borate on its R_F value.

2. The apparent K_m for dCTP in dCDP-diglyceride synthesis by intact mitochondria was $4 \cdot 10^{-5}$ M; the v_{max} was $1.1 \cdot 10^{-2}$ nmoles/min per mg protein. Synthesis was inhibited by rCTP, whereas the incorporation of the rCDP moiety of rCTP into rCDP-diglyceride was inhibited by dCTP.

3. dCDP-Diglyceride substituted for rCDP-diglyceride in the synthesis of phosphatidylglycerol from *sn*-glycero-3-phosphate by these mitochondrial preparations. The apparent K_m's were $2 \cdot 10^{-5}$ M for dCDP-diglyceride and $7 \cdot 10^{-5}$ M for rCDP-diglyceride. The v_{max} was $0.42 \cdot 10^{-2}$ nmole/min per mg protein with dCDP-diglyceride and $2.5 \cdot 10^{-2}$ nmole/min per mg protein with rCDP-diglyceride as substrate.

INTRODUCTION

During studies of the biosynthesis of mitochondrial DNA by isolated mitochondria it was found (ref. 1; and J. TER SCHEGGET AND P. BORST, unpublished observations) that dCTP was incorporated into acid-insoluble material at a much higher rate than any of the other deoxyribonucleoside triphosphates. About 95% of the acid-insoluble material labelled after incubation with [^{3}H]dCTP could be extracted by chloroform (J. TER SCHEGGET AND P. BORST, unpublished observations). This showed that the dCTP was incorporated into a liponucleotide rather than into DNA.

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The experiments presented in this paper identify this liponucleotide as dCDP-diglyceride.

The essential role of cytidine nucleotides in phospholipid biosynthesis is well documented and it was shown by SCHNEIDER AND ROTHERHAM[2] that dCDP-choline can substitute for rCDP-choline in the biosynthesis of phosphatidylcholine by rat liver preparations. In this paper we demonstrate a similar substitution of rCTP by dCTP in the biosynthesis of phosphatidylglycerol. Mitochondrial preparations from rat liver rapidly incorporate dCTP into dCDP-diglyceride and added dCDP-diglyceride is used by these preparations to convert *sn*-glycero-3-phosphate into phosphatidylglycerol.

MATERIALS AND METHODS

Preparation of mitochondria

For the preparation of chicken-liver and rat-liver mitochondria the procedure of HOGEBOOM[3] was followed, as discribed by MYERS AND SLATER[4].

Protein determination

Protein was determined by a biuret method, as described by CLELAND AND SLATER[5], with crystalline egg albumin as standard.

Mitochondrial DNA

Mitochondrial DNA from chicken-liver mitochondria was extracted and purified according to the method of BORST *et al.*[6].

Incubation of the mitochondria with [^3H]dCTP

The incubation of the mitochondria was carried out at 37° in wide-bore glass tubes (10 cm × 2.5 cm) to ensure good aeration. The standard incubation medium was: 50 mM Tris–HCl buffer, 20 mM sodium phosphate, 20 mM sodium succinate, 4 mM KCl, 0.5 mM ATP, 7 mM $MgCl_2$, 68 mM sucrose, 100 μM [^{3}H]dCTP (in some experiments 4 μM) or 4 μM [^{3}H]dATP or [^{3}H]dTTP, respectively, and 15 μM each of the other dNTP's; final pH, 7.4; final volume, 1 ml. The reaction was started by adding mitochondria and stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid. The precipitate was washed three times with about 5 ml ice-cold 5% trichloroacetic acid, containing 60 mM sodium pyrophosphate. When indicated, two extra washings with chloroform–methanol (2:1, v/v) were performed. To ensure complete precipitation of DNA 1.5 mg yeast RNA was added as carrier in these experiments. Radioactivity was determined in a liquid scintillation counter by dissolving the precipitate in 1 ml hyamine hydroxide (1 M in methanol) and adding 2 ml methanol and 10 ml toluene containing 5.2 g 2,5-diphenyloxazole (PPO) and 65 mg 1,4-bis-(5-phenyloxazolyl-2-)-benzene (POPOP) per l. Efficiencies were determined by the channel-ratio technique (about 18% for ^{3}H).

Incubation of the mitochondria with [2-^3H]-sn-glycero-3-phosphate

The incubation mixture consisted of [2-^{3}H]-*sn*-glycero-3-phosphate (concentrations as indicated), 50 mM Tris–HCl buffer (pH 7.5), 15 mM 2-mercaptoethanol, 0.1 mM of either CDP-diglyceride or dCDP-diglyceride and 1.5 mg mitochondrial protein in a total volume of 0.5 ml. The mixture was incubated for 60 min at 37°.

Phosphatidylglycerol synthesis was measured by the filter-paper disc method described by GOLDFINE[7].

Extraction of lipid from the mitochondrial preparation

Lipids were extracted by the method of BLIGH AND DYER[8] or that of ROUSER AND FLEISCHER[9].

Analysis of lipid extracts by thin-layer chromatography

1. Aliquots of the lipid extracts from the mitochondria after incubation with [^{3}H]dCTP were spotted on thin-layer plates prepared from silica gel G. Chromatograms were developed with one of the following solvent mixtures: I, chloroform–methanol–water–acetic acid (50:28:8:4, by vol.); II, diisobutylketone–acetic acid–water (80:80:21, by vol.); III, chloroform–methanol–water–ammonia (70:38:2:8, by vol.).

2. The ^{3}H-labelled product after incubation of mitochondria with [2-^{3}H]-*sn*-glycero-3-phosphate was analysed in the following systems: IV, 0.4 M boric acid-impregnated silica gel G plates with chloroform–methanol–water–ammonia (70:30:3:2, by vol.) as developing system; V, silica gel H impregnated with 0.001 M Na_2CO_3, developed with chloroform–methanol–acetic acid–water (50:25:7:3, by vol.); VI, silica gel H impregnated with 0.1 M Na_2CO_3, developed with chloroform–methanol–1 M ammonia (80:36:2, by vol.).

After drying, the lipid spots were located by exposing the plates to iodine vapour. Phospholipids were subsequently visualized by spraying the plates with a molybdate reagent as described by DITTMER AND LESTER[10]. Radioactivity in the silica gel plates was located using a Panex-TLC scanner.

Water-soluble products were identified by paper chromatography on Whatman No. 1 paper with propanol–ammonia–water (6:3:1, by vol.), as descending developing system. Compounds were made visible with a periodate–Schiff staining reagent[11].

Synthesis and characterization of dCDP-diglyceride

For the synthesis of dCDP-diglyceride the method of AGRANOFF AND SUOMI[12] was followed. Due to the presence of unsaturated fatty acids in the phosphatidic acid used, the dCDP-diglyceride was isolated and purified from the reaction mixture as recommended by PROTTEY AND HAWTHORNE[13].

The purified preparation gave one spot on thin-layer chromatography in solvent systems I–III mentioned above. The dCDP-diglyceride showed phosphorus: deoxycytidine: acyl ester ratios of 2.0:1.08:1.94 and an $A_{280\,nm}:A_{260\,nm}$ ratio of 2.38 as compared to 2.23 for the commercial CDP-diglyceride. Deoxycytidine content was determined spectrophotometrically at 280 nm in methanol–chloroform–water–0.1 M HCl (2:1.2:0.1:0.7, by vol.) with dCTP in the same solvent mixture as a standard. Phosphorus was analysed by the method of JAMES AND DUBIN[14]. Acyl ester values were determined according to the method of SHAPIRO[15] with triglyceride as a standard.

Other chemicals and reagents

CDP-Diglyceride was purchased from Koch and Light. 4-Morpholidine-*N*,*N*′-dicyclohexylcarboxamidium deoxycytidine-5′-phosphomorpholidate and phospho-

lipase D were obtained from Sigma. Egg lecithin was isolated and purified as described by SINGLETON *et al.*[16] and converted into phosphatidic acid by treatment with phospholipase D according to the method of DAVIDSON AND LONG[17]. [2-^{3}H]Glycerol was a product of the Radiochemical Centre, Amersham, England. PPO and POPOP were obtained from New England Nuclear, Dreieichenhain, Germany. Silica gel was obtained from A.G. Merck, Darmstadt, Germany. All other chemicals were purchased from British Drug Houses Chemicals Ltd., Poole, England (Analar). [2-^{3}H]-*sn*-Glycero-3-phosphate was synthesized enzymatically from [2-^{3}H]glycerol and ATP (ref. 18).

[^{3}H]dCTP and [^{3}H]CTP were obtained from Schwarz BioResearch, New York, U.S.A. dATP, dGTP, dTTP and dCTP were purchased from Sigma Chemicals Co., St. Louis, U.S.A. Yeast RNA, type XI, was also obtained from Sigma Chemicals Co. Hyamine hydroxide was a product of Nuclear Chicago Corp.

RESULTS

Table I presents experiments on the incorporation of dNTP's into acid-insoluble material by mitochondrial preparations from chicken liver. Although dCTP is incorporated much faster than the other dNTP's very little of the incorporated material is recovered in purified mitochondrial DNA and nearly all of it is retained in the chloroform extract.

TABLE I

RATE OF INCORPORATION OF dNTP'S INTO ACID-INSOLUBLE MATERIAL BY CHICKEN-LIVER MITOCHONDRIA AND RECOVERY OF RADIOACTIVITY AFTER CHLOROFORM EXTRACTION AND IN THE PURIFIED MITOCHONDRIAL DNA

All incubations to determine the incorporation rate were performed under standard conditions (see MATERIALS AND METHODS). The mitochondria were extracted with chloroform and the DNA was purified as described in MATERIALS AND METHODS. The recovery in the water layer after the first chloroform extraction and in the purified DNA is presented (whole mitochondria = 100%). Most values are averages of several experiments.

Substrate	*Incorporation rate (pmoles/10 min per mg protein)*	*Recovery (%) in*	
		H_2O layer	*DNA*
dATP (4 μM)	0.5	49	19
dTTP (4 μM)	0.4	—	—
dCTP (4 μM)	4.3	2.3	0.3
dCTP (15 μM)	6.6	7	0.8

This anomalously high incorporation rate of dCTP was further studied with rat-liver mitochondria. Incorporation was linear with protein concentrations up to at least 23 mg mitochondrial protein per ml. The time course is shown in Fig. 1. From these data we calculate an apparent K_m value for dCTP of 0.04 mM. This is much higher than the apparent K_m of about 2 μM found for dATP incorporation. Incorporation of dCTP was inhibited by rCTP (Fig. 2A) and the incorporation of rCTP by dCTP (Fig. 2B). At a substrate concentration of 100 μM the rate of incorporation of rCTP was only slightly higher than that of dCTP (Fig. 2). As with dCTP as substrate, most of the acid-insoluble material, labelled after incubation of the mitochondria with rCTP was extracted with chloroform.

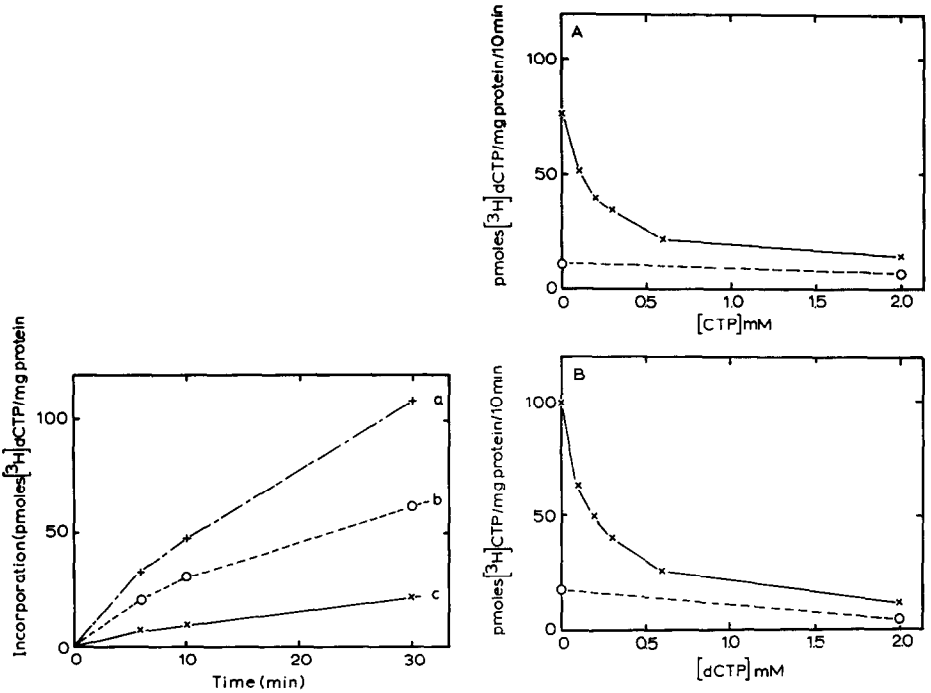


Fig. 1. The incorporation of [^{3}H]dCTP into acid-insoluble material by rat-liver mitochondria. a, 30.83 μM dCTP; b, 15.42 μM dCTP; c, 3.85 μM dCTP. The incorporation of [^{3}H]dCTP was measured as described in MATERIALS AND METHODS.

Fig. 2. A. Effect of unlabelled CTP on the incorporation of [^{3}H]dCTP into acid-insoluble material by rat-liver mitochondria. [^{3}H]dCTP incorporation was measured as described in MATERIALS AND METHODS. The dCTP concentration was 0.1 mM. ×——×, Standard washing procedure with trichloroacetic acid; ○----○, standard washing procedure and two additional washings with chloroform–methanol (2:1, v/v). B. Effect of unlabelled dCTP on the incorporation of [^{3}H]CTP into acid-insoluble material by rat-liver mitochondria. [^{3}H]CTP incorporation was measured as described for the [^{3}H]dCTP incorporation in MATERIALS AND METHODS. ×——×, Standard washing procedure with trichloroacetic acid; ○----○, standard washing procedure and two additional washings with chloroform–methanol (2:1, v/v).

The lipid-soluble material synthesized from dCTP was further analysed by thin-layer chromatography. In preliminary experiments mitochondria were extracted with a chloroform–methanol mixture as described by BLIGH AND DYER[8], but this led to losses of up to 90% of the acid-insoluble radioactivity into the water wash. In a later experiment we therefore used the extraction procedure of ROUSER AND FLEISCHER[9] and removed hydrophilic contaminants by reversed-phase Sephadex chromatography. In the purified extract 84% of the acid-insoluble radioactive material, present in the mitochondria after the incubation, was recovered. Samples of this extract were spotted on silica gel G plates and developed with the use of the solvent system described in MATERIALS AND METHODS. In all three solvent systems the radioactivity was present in one spot that coincided with the reference dCDP-diglyceride obtained by chemical synthesis. In solvent systems I and II, dCDP-diglyceride ran only slightly ahead of CDP-diglyceride, but in solvent system III dCDP-diglyceride was completely resolved from CDP-diglyceride and the radioactive material from mitochondria co-chromatographed with dCDP-diglyceride (Fig. 3).

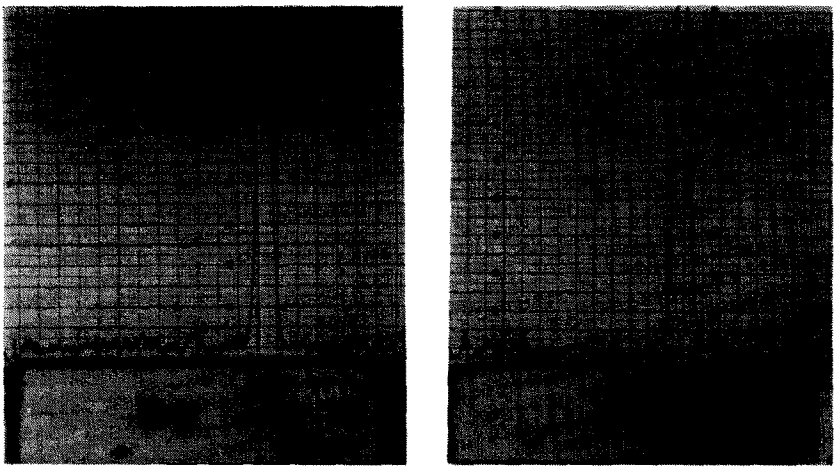


Fig. 3. Analysis of a mitochondrial lipid extract by thin-layer chromatography. A. Purification of labelled product from mitochondrial lipid extracts after addition of marker CDP-diglyceride and dCDP-diglyceride. Lane 1, CDP-diglyceride; Lane 2, mitochondrial extract; Lane 3, dCDP-diglyceride. B. Co-chromatography of purified product (extracted from plate A). Lanes 1 and 3, as in A; Lane 2, purified labelled product *plus* marker CDP-diglyceride and dCDP-diglyceride. Chromatograms in A and B were developed in solvent system III (see MATERIALS AND METHODS). Radioactivity tracings obtained from Lane 2 are shown.

The absence of CDP-diglyceride in the unknown material was confirmed by repeating the chromatography in the presence of borate. This complexes with vicinal hydroxyl groups and hence retards compounds that contain such groups on thin-layer plates developed with organic solvents. When the labelled product was chromatographed in borate-impregnated silica gel G plates, using solvent system III, CDP-diglyceride hardly moved from the origin whereas the radioactivity still coincided with dCDP-diglyceride, the R_F value of which is not affected by borate.

The synthesis of dCDP-diglyceride by rat-liver mitochondria prompted us to investigate whether this liponucleotide could substitute for CDP-diglyceride in the mitochondrial biosynthesis of phosphatidylglycerol[19]. Therefore, the synthesis of phosphatidylglycerol from liponucleotide and [2-^{3}H]-*sn*-glycero-3-phosphate was investigated. Phosphatidylglycerol synthesis was completely dependent on the presence of either CDP- or dCDP-diglyceride (Table II). The ^{3}H-labelled product gave one spot on thin-layer chromatography in the systems IV–VI mentioned in MATERIALS

TABLE II

DEPENDENCY OF PHOSPHATIDYLGLYCEROL SYNTHESIS ON CDP-DIGLYCERIDE OR dCDP-DIGLYCERIDE

The standard incubation mixture (see MATERIALS AND METHODS) contained 0.21 mM [2-^{3}H]-*sn*-glycero-3-phosphate, 50 mM Tris–HCl buffer (pH 7.5), 3 mg mitochondrial protein and additions as specified. After the incubation lipids were extracted[8], the extracts were washed twice with 2 M KCl and once with water and chromatographed as described in MATERIALS AND METHODS (solvent system V). Zero time controls were <100 disint./min.

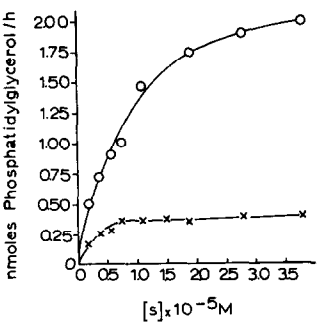
Additions	[^{3}H]*Phosphatidylglycerol synthesized (disint./min)*
None	<100
dCDP-diglyceride	7040
CDP-diglyceride	32000

AND METHODS. The R_F values were 0.26 (System IV), 0.71 (System V) (ref. 20) and 0.30 (System VI) (ref. 21). Radioactivity coincided in all these systems with authentic phosphatidylglycerol. Alkaline hydrolysis according to the method of CHANG AND KENNEDY[22] yielded glycerophosphorylglycerol and degradation with phospholipase D gave glycerol as the only water-soluble product, indicating that the labelled phospholipid was phosphatidylglycerol. Phosphatidylglycerolphosphate apparently did not accumulate in detectable quantities in inbubations with mitochondria. Water-soluble products were identified by paper chromatography as described in MATERIALS AND METHODS.

The phosphatidylglycerol synthesis as described in Table II was linear with time and protein concentration up to at least 1 h and 2.5 mg, respectively. With both CDP- and dCDP-diglyceride the reaction proceeded optimally at about pH 7.5. The substrate concentration curve for *sn*-glycero-3-phosphate in the presence of optimal amounts (0.1 mM) of either liponucleotide indicated that maximum synthesis of phosphatidylglycerol occurred at about $1 \cdot 10^{-5}$ M dCDP-diglyceride and $4 \cdot 10^{-5}$ M with CDP-diglyceride (Fig. 4). A kinetic analysis of the dependency of phosphatidylglycerol synthesis on CDP- or dCDP-diglyceride was made in the presence of $3.8 \cdot 10^{-5}$ M *sn*-glycero-3-phosphate. Apparent K_m values of $7 \cdot 10^{-5}$ and $2 \cdot 10^{-5}$ M for CDP- and dCDP-diglyceride, respectively, were determined from Lineweaver–Burk plots of the dependency of the reaction rates on liponucleotide concentrations (Fig. 5). Maximum velocities of $2.5 \cdot 10^{-2}$ and $0.42 \cdot 10^{-2}$ nmole/min per mg protein for CDP- and dCDP-diglyceride, respectively, were observed.

DISCUSSION

The experiments presented in this paper show that mitochondrial preparations from rat liver catalyse the synthesis of dCDP-diglyceride from added dCTP and endogenous phosphatidic acid and that added dCDP diglyceride can be used for the synthesis of phosphatidylglycerol.



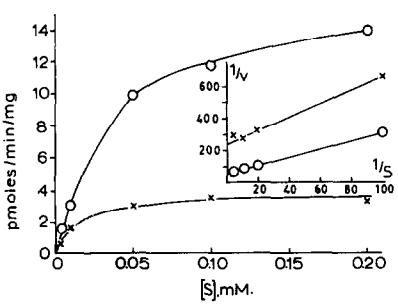


Fig. 4. Effect of *sn*-glycero-3-phosphate concentration on the rate of phosphatidylglycerol synthesis by rat-liver mitochondria. The standard reaction mixture, described in MATERIALS AND METHODS, was used with 1.5 mg mitochondrial protein and the [2-^{3}H]-*sn*-glycero-3-phosphate (specific activity, 10000 disint./nmole) concentrations as specified. Total volume, 0.5 ml. ×——×, dCDP-diglyceride present; ○——○, CDP-diglyceride present.

Fig. 5. Effect of liponucleotide concentrations on phosphatidylglycerol biosynthesis. Conditions as in Fig. 4, except that the concentration of [2-^{3}H]-*sn*-glycero-3-phosphate was kept constant at $3.8 \cdot 10^{-5}$ M and that the liponucleotide concentrations were varied as indicated. ×——×, dCDP-diglyceride; ○——○, CDP-diglyceride.

Synthesis of phosphatidylglycerol from CDP-diglyceride has previously been shown by KIYASU *et al.*[19] with isolated chicken-liver mitochondria, whereas VORBECK AND MARTIN[23] have recently described an enzyme from mitochondria of bovine liver and heart that catalyzes the synthesis of CDP-diglyceride from CTP and phosphatidate. From distribution studies the authors concluded that the enzyme is exclusively localized in the mitochondria. We have not verified this point for the process utilizing dCTP.

The virtually complete inhibition of the incorporation of labelled dCTP into dCDP-diglyceride by unlabelled rCTP and of labelled rCTP into CDP-diglyceride by unlabelled dCTP is compatible with the idea that the synthesis of CDP-diglyceride and dCDP-diglyceride is catalysed by the same enzyme. Since all experiments were done with intact mitochondria we cannot exclude, however, that the inhibition is due to competition by CTP and dCTP for entry into the mitochondria or by competition of two different enzymes for a limiting supply of endogenous phosphatidic acid. This can be synthesized by isolated mitochondria[24–26].

Although the experiments reported in this paper establish that rat-liver mitochondria can use dCTP instead of CTP for the biosynthesis of phosphatidylglycerol, the physiological significance of dCTP in lipid biosynthesis remains to be evaluated. This will require a kinetic analysis of the isolated enzymes involved in the biosynthesis for CTP and CDP-diglyceride and their deoxy analogues, and the determination of the CTP and dCTP concentrations in mitochondria.

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