GLYCEROPHOSPHATIDE BIOGENESIS: I. SUBCELLULAR LOCALIZATION OF CYTIDINE TRIPHOSPHATE: PHOSPHATIDIC ACID CYTIDYL TRANSFERASE

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SUMMARY

The subcellular localization of cytidine triphosphate: phosphatidic acid cytidyl transferase (CTP: PA cytidyl transferase) has been investigated. Rat liver and bovine heart homogenates were fractionated by differential centrifugation and the composition of the fractions evaluated by marker enzymes and electron microscopy. In both rat liver and bovine heart preparations, the highest relative specific activity was associated with the mitochondrial fraction and closely paralleled the distribution pattern for succinoxidase, a mitochondrial marker.

INTRODUCTION

Cytidine triphosphate: phosphatidic acid cytidyl transferase (CTP: PA cytidyl transferase) catalyzes the reaction:

CTP + Phosphatidic acid $\rightleftharpoons$ CDP-Diglyceride + PP (1).

CDP-diglycerides are lipid-soluble nucleotides which can be considered activated forms of phosphatidic acids. These liponucleotides have been identified as precursors of phosphatidyl inositol (2), phosphatidyl glycerol (3,4) and phosphatidyl glycerol phosphate (3,4) in animal tissues, and phosphatidyl serine (5), phosphatidyl ethanolamine (5), phosphatidyl glycerol (6), phosphatidyl glycerol phosphate (6), and cardiolipin (7) in Escherichia coli. In E. coli and possibly other biological systems, CDP-diglycerides may constitute the sole nucleotide intermediate for the biogenesis of glycerophosphatides.

In view of the central role of CDP-diglycerides in glycerophosphatide biosynthesis, the present investigation was undertaken to determine its intracellular site of formation. Marker enzymes and electron microscopy were used to evaluate the actual composition of the subcellular fractions.

MATERIALS AND METHODS

[5-^{3}H] Cytidine 5'-triphosphate, tetralithium (13.5 Ci/mmole) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y.; cytidine 5'-triphosphate, disodium, from Sigma Chemical Corp., St. Louis, Mo., and Nagarse proteinase from Enzyme Development Corp., New York, N.Y. Phosphatidic acid, sodium salt, was obtained from Pierce Chemical Corp., Rockford, Ill. or prepared from egg yolk phosphatidyl choline (Sigma Chemical Corp.) using phospholipase D (Calbiochem. Corp., Los Angeles, Calif.) (8).

Subcellular fractions were prepared from rat liver and bovine heart. Rat liver subcellular fractions were prepared from male Sprague-Dawley animals (150-200g) as described previously (9). Homogenates of bovine heart were prepared by modification of the procedure of Brierley (10). Hearts were obtained within several minutes after death of the animals and placed in ice. Each heart was trimmed of fat and connective tissue and ground in a precooled meat grinder. Fifty g of ground tissue were washed twice in 200 ml cold 0.25M sucrose containing 0.01M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) pH 7.6 (S-H buffer). The pH was maintained at 7.4 to 7.6 by addition of 4N KOH. The washed mince was suspended in S-H buffer to a final volume of 200 ml and one ml Nagarse (10 mg/ml S-H buffer) added. The suspension was stirred at 0 C for 20 minutes and then homogenized using a Dounce ball-type homogenizer. Time elapsed from death of the animal until preparation of the homogenate was less than one hour. The extent of cell breakage was monitored by microscopic examination of the homogenate. Subcellular fractions were prepared from the homogenate by differential centrifugation with g values computed from the bottom of the tube. The homogenate was centrifuged at 750 x g for 10 minutes and supernatant fluid A collected. The pellet was rehomogenized in one-half the original volume of S-H buffer and recentrifuged to obtain the nuclear fraction. The supernatant fluid was combined with supernatant A and centrifuged at 10,000 x g for 20 minutes. The fluffy pink layer was removed with supernatant fluid B. The firmly packed pellet was resuspended in S-H buffer

and centrifuged at 10,000 g for 20 minutes. The supernatant fluid and any fluffy layer was removed and combined with supernatant B. The firmly packed pellet was resuspended in S-H buffer and designated the mitochondrial fraction. The combined supernatant fluid containing the fluffy layer was centrifuged at 105,000 x g for 1 hour. The pellet was resuspended in S-H buffer and designated the microsomal fraction and the supernatant fluid the cytosol fraction.

CTP: PA cytidyl transferase was assayed in an incubation mixture containing 0.5 µmole ^{3}H-CTP (specific activity 0.5 Ci/mole), 250 µg phosphatidic acid, 100 µmoles Tris-HCl pH 7.2, 300 to 500 µg protein, and 2.5 µmoles $MgCl_2$ in a final volume of 0.25 ml. The $MgCl_2$ was added after the addition of the protein (11) and the incubation carried out at 37 C in a gyratory shaker for 10 minutes. The incorporation of ^{3}H-CTP into lipid was measured by solvent extraction (11) or by a modified filter disc assay (12) to be described elsewhere. The identification of the ^{3}H-labelled lipid as CDP-diglyceride was accomplished by descending chromatography on formaldehyde-treated paper (11). The labelled lipid was co-chromatographed with authentic CDP-didecanin (obtained from Dr. B.W. Agranoff) and greater than 95% of the radioactivity applied to the chromatogram migrated with the CDP-didecanin. The remaining counts were found at the origin. Radioactivity was determined in a Packard TriCarb liquid scintillation spectrometer using a scintillation medium of the following composition: 2,5-diphenyloxazole, 4.0 g; 1,4-bis [2-(5-phenyloxazoly)] benzene, 50 mg; and toluene, 1000 ml. The efficiency of counting was determined using external standardization procedures and the results computed as disintegrations per minute (DPM) using an Olivetti Programma 101 computer. Relative specific activity was computed as the ratio of the percent total DPM to percent total protein.

Purity of the individual subcellular fractions from both rat liver and bovine heart was evaluated by marker enzyme assays and electron microscopy. Marker enzyme assays for evaluation of the subcellular fractions were as described previously (9). Alkaline phosphatase, assayed by modification of

the procedure of Neumann and Van Breedendaal (13), was used as a microsomal marker for the bovine heart subfractions. Relative specific activity was calculated as the ratio of the percent total activity to percent total protein. Protein was determined by a modification (12) of the colorimetric micro-Folin procedure of Heidelberger and MacPherson (14).

Samples for electron microscopy were fixed in the cold for 1 hour using 1% osmium tetroxide. Samples were washed in sodium cacodylate buffer, dehydrated in progressive concentrations of ethyl alcohol, and propylene oxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a Hitachi 7S electron microscope.

RESULTS AND DISCUSSION

In the present study, rat liver and bovine heart homogenates have been fractionated by differential centrifugation into nuclear, mitochondrial, "intermediate" (rat liver only), microsomal, and cytosol fractions. Although the designation of the various fractions was arbitrary, their actual composition was determined by use of marker enzymes and electron microscopy. Figure 1 presents a direct comparison of the CTP: PA cytidyl transferase activity of rat liver with the distribution pattern of the marker enzymes. The results are representative of those obtained from three separate fractionations. The data show that the highest relative specific activity of the CTP: PA cytidyl transferase (5.3 times the specific activity of the homogenate) is associated with the mitochondrial fraction. Moreover, this fraction contained 92% of the total activity of the homogenate. The close relationship between the distribution pattern for CTP: PA cytidyl transferase and succinoxidase, a mitochondrial marker, is apparent.

The distribution of CTP: PA cytidyl transferase activity among subfractions of bovine heart homogenates is given in Table 1. The values represent the average of five separate fractionation experiments. As with rat liver subfractions, the highest relative specific activity for CTP: PA cytidyl transferase was observed in the mitochondrial fraction. The activity in this

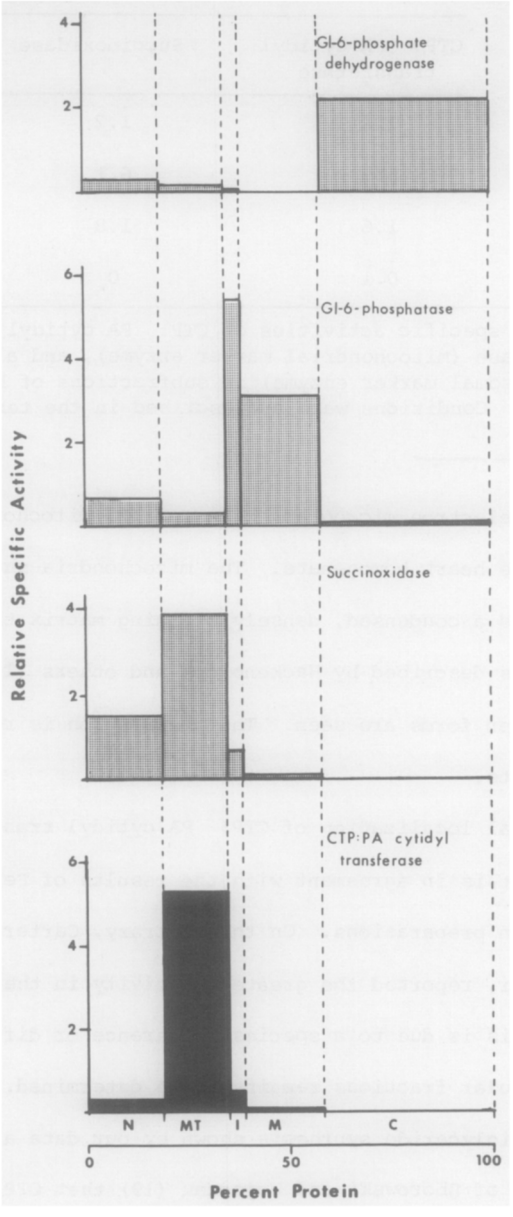

Figure 1. Distribution patterns for CTP: PA cytidyl transferase and marker enzymes among subfractions of rat liver homogenate (nuclear, N; mitochondrial, MT; "intermediate", I; microsomal, M; and cytosol, C). Conditions were as described in the text.

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fraction was 6.9 times the specific activity of the homogenate and the distribution parallels that of the mitochondrial marker succinoxidase.

Subfraction	Relative Specific Activity		
	CTP: PA cytidyl transferase	Succinoxidase	Alkaline Phosphatase
Nuclear	1.1	1.2	1.0
Mitochondrial	6.9	6.1	1.1
Microsomal	1.6	1.8	10.2
Cytosol	0.1	0	0.3

Table 1. Relative specific activities of CTP: PA cytidyl transferase, succinoxidase (mitochondrial marker enzyme), and alkaline phosphatase (microsomal marker enzyme) in subfractions of bovine heart homogenates. Conditions were as described in the text.

Figure 2 is an electron micrograph of a typical mitochondrial fraction prepared from a bovine heart homogenate. The mitochondria show a distinct outer membrane and most have a condensed, densely staining matrix that is typical of active mitochondria as described by Hackenbrock and others (15,16,17). Very few orthodox or swollen forms are seen. The preparation is relatively free of other cellular elements.

The mitochondrial localization of CTP: PA cytidyl transferase in rat liver and bovine heart is in agreement with the results of Petzold and Agranoff (11) using chick brain preparations. On the contrary, Carter and Kennedy (18) using guinea pig liver, reported the greatest activity in the microsomal fraction. Whether this is due to a species difference or differences in preparation of the subcellular fractions remains to be determined. The mitochondrial localization of CDP-diglyceride synthesis shown by our data also is compatible with the observations of Zborowski and Wojtczak (19) that CTP stimulated incorporation of glycerol-3-phosphate into glycerophosphatides by isolated rat liver mitochondria.

The role of mitochondria in glycerophosphatide biosynthesis has been investigated in many laboratories (19,20,21,22,23). Although the final stage in the synthesis of many glycerophosphatides is known to be carried out by isolated liver microsomal fractions (23), conflicting evidence on the ability of

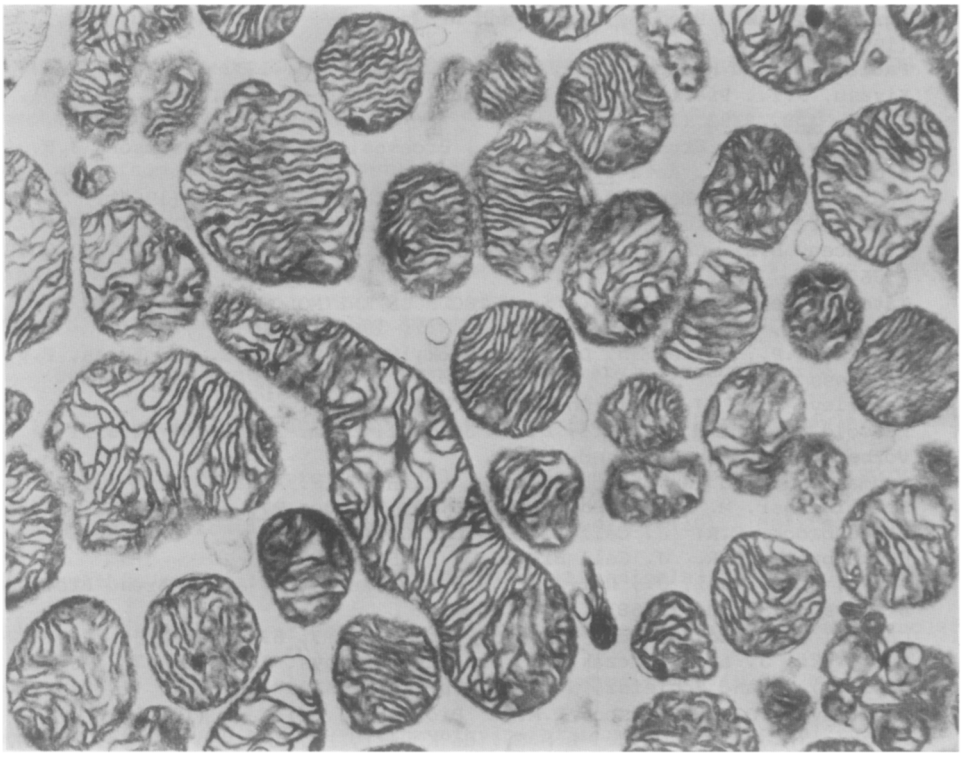

Figure 2. Electron micrograph of a freshly isolated mitochondrial fraction obtained from a bovine heart homogenate. The preparation was fixed with osmium tetroxide and stained with uranyl acetate and lead citrate. x 20,000

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isolated mitochondria to synthesize glycerophosphatides has been reported (20,24). The role of CTP: PA cytidyl transferase in mitochondrial glycerophosphatide biogenesis as well as its role in glycerophosphatide synthesis in other subcellular fractions is currently under investigation.

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REFERENCES

1. Agranoff, B.W., Bradley, R.M., and Brady, R.O. J. Biol. Chem. 233, 1720 (1958).
2. Paulus, H. and Kennedy, E.P. J. Biol. Chem. 235, 1303 (1960).
3. Kiyasu, J.Y., Pieringer, R.A., Paulus, H., and Kennedy, E.P. J. Biol. Chem. 238, 2293 (1963).
4. Stanacev, N.Z., Stuhne-Sekalec, L., Brookes, K.B., and Davidson, J.B. Biochim. Biophys. Acta 176, 653 (1969).
5. Kanfer, J.N. and Kennedy, E.P. J. Biol. Chem. 239, 1720 (1964).
6. Chang, Y.Y. and Kennedy, E.P. J. Lipid Res. 8, 447 (1967).
7. Stanacev, N.Z., Chang, Y.Y. and Kennedy, E.P. J. Biol. Chem. 242, 3018 (1967).
8. Kates, M. and Sastry, P.S., in METHODS OF ENZYMOLOGY, Editor J.M. Lowenstein, Vol 14, Academic Press, N.Y. 1969, p. 197.
9. Martin, A.P., Halterman, D.R., Vorbeck, M.L., Kuo, M.C. and Lucas, F.V. In press (1970).
10. Brierley, G.P. J. Biol. Chem. 242, 1115 (1967).
11. Petzold, G.L. and Agranoff, B.W. J. Biol. Chem. 242, 1187 (1967).
12. Vorbeck, M.L. Unpublished work.
13. Neumann, H. and Van Breedendaal, M. Clin. Chim. Acta 17, 183 (1967).
14. Heidelberger, M. and MacPherson, C.F.C. Science 97, 405; 98, 63 (1943).
15. Hackenbrock, C.R. J. Cell Biol. 30, 269 (1966).
16. Hackenbrock, C.R. J. Cell Biol. 37, 345 (1968).
17. Green, D.E., Asai, J., Harris, R.A., and Penniston, J.T. Arch. Biochem. Biophys. 125, 684 (1968).
18. Carter, J.R. and Kennedy, E.P. J. Lipid Res. 7, 678 (1966).
19. Zborowski, J. and Wojtczak, L. Biochim. Biophys. Acta 187, 73 (1969).
20. Stoffel, W. and Schiefer, H.G. Z. Physiol. Chem. 349, 1017 (1968).
21. McMurray, W.C. and Dawson, R.M.C. Biochem. J. 112, 91 (1969).
22. Sarzala, M.G., Van Golde, L.M.G., de Kruyff, B., and Van Deenen, L.L.M. Biochim. Biophy. Acta 202, 106 (1970).
23. Jungalwala, F.B. and Dawson, R.M.C. Eur. J. Biochem. 12, 399 (1970).
24. Fang, M. and Marinetti, G.V. Biochim. Biophys. Acta 202, 91 (1970).