Altered subcellular and submitochondrial localization of CTP:phosphatidate cytidylyltransferase in the Morris 7777 hepatoma

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Abstract The subcellular and submitochondrial localization of CTP:phosphatidate cytidylyltransferase is altered in the Morris 7777 hepatoma. Mitochondria in this poorly differentiated tumor are the principal sites of CDP-diacylglycerol synthesis, in contrast to normal rat liver where the endoplasmic reticulum is most active. This enzyme activity was increased 17-fold in the outer mitochondrial membrane, and a 22% increase was noted in the inner mitochondrial membrane of the 7777 hepatoma as compared with the corresponding fractions from normal rat liver. Increased mitochondrial CTP:phosphatidate cytidylyltransferase was present in six other Morris hepatomas, but it was not found in fetal rat liver mitochondria, suggesting that rapid growth alone is not responsible for the difference. Evidence is presented which indicates that mitochondrial lipid degradation is similar in normal liver and the 7777 hepatoma, in vitro. The increased activity of CTP:phosphatidate cytidylyltransferase is thought to be responsible in part for the moderately increased diphosphatidylglycerol content of 7777 hepatoma mitochondria.

Supplementary key words cardiolipin · CDP-diglyceride

Previous studies with the Morris 7777 hepatoma showed that mitochondria from this poorly differentiated tumor have a significantly increased content of phospholipid relative to that of mitochondria from normal rat liver (1). This has recently been independently confirmed by Reitz, Thompson, and Morris (2). These findings suggest disturbances in the regulation of mitochondrial membrane phospholipid metabolism in the 7777 hepatoma.

We have previously shown that diphosphatidylglycerol (cardiolipin) biosynthesis in normal rat liver occurs only in the inner mitochondrial membrane (3, 4). Mitochondria can also synthesize the diphosphatidylglycerol precursors, phosphatidic acid (5, 6), cytidine diphosphate diacylglycerol (CDP-diacylglycerol) (3) and phosphatidylglycerol (3, 7).

CTP:phosphatidate cytidylyltransferase has recently been found to have an aberrant subcellular localization in the 7777 hepatoma where it is located primarily in the mitochondrial fraction (8). In contrast, this enzyme is principally localized in the endoplasmic reticulum of normal liver (3, 8). This difference may be of importance in the regulation of de novo synthesis of diphosphatidylglycerol by tumor mitochondria since the availability of CDP-diacylglycerol is probably rate-determining in acidic phospholipid biosynthesis in liver (8).

The origin of the other mitochondrial phospholipids is currently felt to be extramitochondrial since the biosyntheses of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin have not been observed in normal liver mitochondria (9, 10). By studying the metabolism of phospholipids in hepatomas that have an altered mitochondrial phospholipid content, it may be possible to shed some light on the mechanisms that govern the renewal of mitochondrial membrane lipids in normal liver tissue.

This publication reports studies of the subcellular and submitochondrial localization and relative activity of some of the enzymes involved in de novo synthesis of acidic phospholipids in the 7777 hepatoma and normal liver. The results indicate a greatly increased activity of CTP:phosphatidate cytidylyltransferase in the outer membrane of 7777 hepatoma mitochondria; inner mitochondrial membrane activity is also increased in the 7777 hepatoma while the microsomal activity of this enzyme is greatly reduced. Results are presented which suggest that the increase in tumor mitochondrial phospholipid content (1, 2) is not due to a decreased rate of mitochondrial phospholipid catabolism.

MATERIALS AND METHODS

Passage of tumors and preparation of subcellular fractions

The various Morris hepatomas were maintained in male rats of the Buffalo strain (Simonsen, Gilroy, CA.) and subcellular fractions were prepared as noted previously (1, 8). Inner and outer mitochondrial membranes were isolated from normal liver and the 7777 hepatoma by the method of Schnaitman, Erwin, and Greenwalt (11). Matrix proteins were released by brief sonication in distilled water as described by Hoppel and Tomec (12). Protein was determined by the method of Lowry et al. (13) and the fractions were suspended in 0.25 M sucrose–5 mM Tris-HCl (pH 7.4) and stored at −65°C until used. The purity of the respective fractions was assessed by means of the following marker enzymes: succinate dehydrogenase (14), rotenone-insensitive NADPH-cytochrome *c* reductase (15), and rotenone-insensitive NADH-cytochrome *c* reductase (15). The latter enzyme was used as a marker for the outer mitochondrial membrane since the activity of monoamine oxidase, another commonly used outer membrane marker, was found to be nearly absent in the 7777 hepatoma mitochondria.

Assays of phospholipid synthesis

For studies of the acylation of glycerol-3-phosphate, the incubation mixture contained 35 mM Tris-HCl (pH 8.3); 3.6 mg/ml bovine serum albumin; 0.15 mM dithiothreitol; 60 mM NaF; 150 μM *sn*-[U-^{14}C]glycerol-3-phosphate, sp act 16 mCi/mmol; 25 μM palmitoyl CoA; 25 μM oleoyl CoA and 1 mg/ml protein in a final volume of 200 μl. The mixture was incubated for 20 min at 37°C and formation of radioactive phospholipid was measured with the trichloroacetic acid–filter paper assay of Goldfine (16). Radioactivity measurements were made with 0.04% *p*-bis[2-(5-phenyloxazole)]benzene and 0.5% 2,5-diphenyloxazole in toluene–Triton X-100–water, 2:1:0.2 (by vol) as counting fluid, using a Searle/Nuclear Chicago Mark III liquid scintillation counter. Quench corrections were made with an external standard method. In some experiments a portion of the incubation mixture was also extracted by the method of Folch, Lees, and Sloane Stanley (17) and the radioactive products were examined by thin-layer chromatography with 0.25-mm silica gel G layers developed with chloroform–pyridine–formic acid 50:30:7 (by vol). The chromatograms were scanned with a Panax thin-layer scanner, (Panax Equipment Ltd., Redhill, Surrey, England). With microsomes from normal liver and 7777 hepatoma, only one peak was found that cochromatographed with reference phosphatidic acid (R_f 0.46) whereas mitochondria from both sources produced both phosphatidic acid and lysophosphatidic acid (R_f 0.12).

CDP-diacylglycerol synthesis from phosphatidic acid and [5-^{3}H]CTP was measured as previously described (8). The incubation mixture contained 50 mM Tris-HCl (pH 7.2); 1 mM phosphatidic acid (sonicated in buffer); 1 mg/ml protein; and 2 mM [5-^{3}H]CTP, sp act 10 mCi/mmol, in a final volume of 0.333 ml. 40 mM $MgCl_2$ was added last and the complete mixture was incubated for 20 min at 37°C. Radioactive lipid was measured with a filter disc assay (16, 18) and characterized as previously described (8, 18).

Phosphatidylglycerol synthesis from CDP-diacylglycerol and *sn*-[U-^{14}C]glycerol-3-phosphate was assayed as previously described (18). The incubation mixture contained 50 mM Tris-HCl (pH 7.4); 340 μM CDP-diacylglycerol; 40 μM *sn*-[U-^{14}C]glycerol-3-phosphate, sp act 16 mCi/mmol; and 3 mg protein/ml in a final volume of 0.250 ml. After a 60-min incubation at 37°C, duplicate 50-μl aliquots were analyzed by a filter disc method (16), and the radioactive product was identified as previously described (18).

Diphosphatidylglycerol synthesis from phosphatidyl[U-^{14}C]glycerol and CDP-diacylglycerol was measured by the method of Hostetler et al. (19). The incubation mixture contained 50 mM Tris-HCl (pH 8.0); 2.6 μM phosphatidyl[U-^{14}C]glycerol, sp act 130 mCi/mmol; 30 μM CDP-diacylglycerol; 2 mM EDTA; 3.2 mg/ml protein; and 20 mM $CoCl_2$ (added last) in a final volume of 125 μl. After incubation at 37°C for 60 min, the lipids were extracted (17) and analyzed by thin-layer chromatography as previously described (19).

Phosphatidylcholine synthesis from *sn*-1,2-diacylglycerol and cytidine diphosphocholine[methyl-^{14}C] was done by the method of van Golde, Fleischer, and Fleischer (9). The incubation contained 50 mM Tris-HCl (pH 7.4); 1 mg/ml *sn*-1,2-diacylglycerol; 0.016% Tween 20; 89 mM CDP-choline[methyl-^{14}C], sp act 5.7 mCi/mmol; 4 mM glutathione; and 10 mM $MgCl_2$ in a final volume of 0.150 ml. The mixture was incubated at 37°C for 15 min and duplicate aliquots were analyzed as previously noted (16).

Studies with $^{32}P_i$-labeled mitochondria

To prepare $^{32}P_i$-labeled mitochondria, 0.25–1.0 mCi of $^{32}P_i$ was injected intravenously into the tail vein of a tumor-bearing male Buffalo rat. The animal was killed after 18 hr, the tumor and the host liver were removed, and gradient-purified mitochondria were prepared as noted above.

TABLE 1. Acidic phospholipid biosynthesis in mitochondria and microsomes from normal liver and the Morris 7777 hepatoma[a]

	Phosphatidic Acid		CDP-diacylglycerol		Phosphatidylglycerol		Diphosphatidylglycerol		% Contamination Microsomes[b]	% Contamination Mitochondria[b]
	pmol mg^{-1} min^{-1}	RSA[b]	pmol mg^{-1} min^{-1}	RSA[b]	pmol mg^{-1} min^{-1}	RSA[b]	pmol mg^{-1} min^{-1}	RSA[b]		
Normal liver mitochondria	503 ± 59 (3)[c]	100	82 ± 13 (3)	19	89 ± 4 (4)	100	3.8 ± 0.5 (4)	100	2.7	
Normal liver microsomes	238 ± 43 (3)	47	441 ± 7 (3)	100	20 ± 2 (4)	22	0.1 ± 0.08 (4)	3.2		5.7
7777 Hepatoma mitochondria	567 ± 85 (3)[c]	100	412 ± 83 (3)	100	144 ± 35 (4)	100	5.5 ± 1.2 (4)	100	8.2	
7777 Hepatoma microsomes	328 ± 61 (3)	58	166 ± 11 (3)	58	37 ± 4 (4)	26	0.1 ± .06 (4)	3.2		5.8

[a] Mean ± 1 SD. The numbers in parentheses represent the number of replicates.

[b] RSA, relative specific activity, where the most active fraction is set equal to 100. The mitochondrial contamination of microsomes is based on succinate dehydrogenase; microsomal contamination of mitochondria is based on rotenone-insensitive NADPH-cytochrome *c* reductase.

[c] Mitochondria produced both lysophosphatidic acid and phosphatidic acid in a ratio of 1:2. Microsomes produced only phosphatidic acid. The results represent the total lysophosphatidate and phosphatidate produced.

Endogenous phospholipase A activity was determined by incubating the $^{32}P_i$-labeled mitochondria from host liver and the 7777 hepatoma. The decline in ^{32}P content of phosphatidylcholine and diphosphatidylglycerol was determined as a function of incubation time. The incubation conditions are shown in the legend to Table 4. Total lipid extracts were prepared (17) and chromatographic analysis of the total lipids was done as noted in the legend. The spots representing phosphatidylcholine and diphosphatidylglycerol were identified by radioscanning and development with iodine vapors and were scraped into vials for liquid scintillation counting, as noted above.

Determination of the protein content per mitochondrion

Gradient-purified mitochondria were prepared from normal liver and the 7777 hepatoma, protein content was determined by the method of Lowry et al. (13), and the protein concentration was adjusted to 0.5 mg/ml with 0.25 M sucrose. These suspensions of mitochondria were then mixed thoroughly with an equal volume of latex beads (481 ± 18 nm, Dow Diagnostics, Midland, Michigan) at an original concentration of 1.64×10^8 beads/μl. This mixture was placed on Formvar-coated grids, air dried, stained with 2% sodium phosphotungstate, and examined with a Zeiss EM-10 electron microscope. Twenty to forty electron micrographs were made of the respective mixtures and the ratio of beads to mitochondria was determined in each photograph. The amount of protein per mitochondrion was calculated from the initial concentration of protein, the number of beads/ml of suspension, and the observed ratio of beads to mitochondria.

Chemicals

Cytochrome *c*, glycerol-3-phosphate, NADH, and NADPH were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN; all radiochemicals were purchased from the New England Nuclear Corporation, Boston, MA. *sn*-1,2-Diacylglycerol was synthesized from egg lecithin by the action of phospholipase C as described by van Golde et al. (9). CDP-diacylglycerol and phosphatidyl[U-^{14}C]glycerol were synthesized, purified, and characterized as described previously (3, 19). CDP-choline and Tween 20 were purchased from the Sigma Chemical Company, St. Louis, MO. Other chemicals were of analytical reagent grade, obtained from usual commercial sources. Thin-layer chromatography solvents such as chloroform and methanol were redistilled before use. Silica gels G and H were obtained from EM Laboratories, Elmsford, NY.

RESULTS

The localization of the enzymes that catalyze the de novo synthesis of acidic phospholipids was determined in mitochondria and microsomes from normal Buffalo rat liver and in the Morris 7777 hepatoma; the results are shown in **Table 1.** In both normal liver and the 7777 hepatoma, phosphatidic acid synthesis was found in both mitochondria and microsomes when measured with radioactive glycerol-3-phosphate and a 1:1 mixture of palmitoyl-CoA and oleoyl-CoA. In both instances, mitochondria exhibited more activity than the microsomes. However, an analysis of the reaction products indicated that both preparations of mitochondria produced a mixture of phosphatidate

and lysophosphatidate in a ratio of 2:1 while microsomes produced only phosphatidate. The substantial activity of mitochondria in glycerol phosphate acylation cannot be explained by microsomal contamination. In addition, subcellular fractionation of the liver and hepatoma homogenates, as suggested by De Duve et al. (20) showed that the mitochondrial fractions contained the largest portion of the total activity for glycerol-3-phosphate acylation (not shown) in agreement with the results of Daae and Bremer (5).

As reported previously, CDP-diacylglycerol synthesis in the normal liver is primarily microsomal whereas in the Morris 7777 hepatoma the mitochondria are considerably more active than the microsomes (3, 8). As shown in Table 1, tumor mitochondria had a specific activity of 412 pmol mg^{-1} min^{-1} vs. 166 pmol mg^{-1} min^{-1} for tumor microsomes. This finding cannot be accounted for by microsomal contamination. The rate observed for 7777 hepatoma mitochondria should be regarded as a minimum because this fraction is contaminated with microsomes which have a much lower specific activity. In subcellular fractionations done as suggested by De Duve et al. (20), most of this enzyme activity was recovered in the microsomal fraction of normal liver, but in the case of the 7777 hepatoma most of the total activity sedimented with the mitochondrial fraction (not shown).

As shown in Table 1, phosphatidylglycerol synthesis was primarily mitochondrial in both the normal liver and the 7777 hepatoma, but significant activity was also found in both of the microsomal fractions in agreement with previous reports (3, 7). In these experiments, done at saturating concentrations of substrates, the observed maximal velocities of phosphatidylglycerol synthesis were higher in the tumor fractions; in the mitochondria 144 vs. 89 pmol mg^{-1} min^{-1}, and in the microsomes 37 vs. 20 pmol mg^{-1} min^{-1}, respectively.

Finally, Table 1 shows that the biosynthesis of diphosphatidylglycerol is exclusively mitochondrial in both normal liver and the 7777 hepatoma. It should be noted that these experiments were not done at saturating concentrations of phosphatidylglycerol due to limitations in the availability of the radioactive substrate. Although there appears to be a greater activity of the 7777 hepatoma in mitochondrial diphosphatidylglycerol synthesis, identical maximal velocities were calculated from Lineweaver-Burke plots obtained when the concentration of phosphatidylglycerol was varied (not shown). As can be seen by comparing the relative microsomal specific activities in diphosphatidylglycerol synthesis with the percent contamination of microsomes with mitochondria based on succinate dehydrogenase, diphosphatidylglycerol synthesis is the better mitochondrial marker in both normal liver and 7777 hepatoma.

The formation of phosphatidylcholine from *sn*-1, 2-diacylglycerol and CDP-choline[methyl-^{14}C] was measured by the method of van Golde et al. (9). In both the normal liver and the 7777 hepatoma, phosphatidylcholine synthesis was located in the microsomes where the specific activities were 4.5 and 2.0 nmol mg^{-1} min^{-1}, respectively. The specific activity of normal liver mitochondria was 0.003 nmol mg^{-1} min^{-1} vs. 0.12 nmol mg^{-1} min^{-1} for 7777 hepatoma mitochondria; this activity could be completely accounted for by contamination of these fractions with microsomes (shown in Table 1).

CDP-diacylglycerol synthesis is the only step in the de novo pathway for diphosphatidylglycerol formation that exhibits an aberrant subcellular localization in the 7777 hepatoma. To see if this is a feature of other rapidly-growing tissues, fetal rat liver and six other Morris hepatomas were studied and the results are shown in **Table 2.** Fetal liver mitochondria had a low activity in this reaction, 42 vs. 82 pmol mg^{-1} min^{-1} for normal liver mitochondria, while the activity of fetal liver microsomes was similar to that of normal liver microsomes, 429 vs. 441 pmol mg^{-1} min^{-1}. Increased mitochondrial CTP:phosphatidate cytidylyltransferase activity was found in all of the Morris hepatomas studied; this was most pronounced in tumors with rapid growth rates such as the 7777 or the 5123 tc (not to be confused with the minimal deviation 5123 or the 5123c), and in the hepatomas with intermediate growth rates such as the 7800 and the 7794a. The slowly growing hepatomas 7787 and 9633 had only slightly increased mitochondrial activities. Tumors with rapid growth rates (7777 and 5123 tc) had a decreased activity of CTP:phosphatidate cytidylyltransferase in the microsomes, while high or moderately reduced rates of CDP-diacylglycerol synthesis were found in microsomes from hepatomas with intermediate or slow growth rates. One tumor line, the 9618a minimal deviation hepatoma, exhibited an unusually high CTP:phosphatidate cytidylyltransferase activity in both mitochondria and microsomes.

To determine the submitochondrial distribution of CTP:phosphatidate cytidylyltransferase, mitochondria from normal liver and the 7777 hepatoma were treated with digitonin, and outer and inner membrane fractions were prepared according to the method of Schnaitman et al. (11) and the purity of the various fractions was assessed. Monoamine oxidase, which is frequently used as a marker for the outer mitochondrial membrane, was nearly absent in mitochondria from the 7777 hepatoma. Therefore,

rotenone-insensitive NADH-cytochrome *c* reductase (found in both the microsomes and the outer mitochondrial membrane) was used to estimate the contamination of the various fractions with outer membranes. Rotenone-insensitive NADPH-cytochrome *c* reductase was used to assess contamination of the fractions with microsomes. As shown in **Table 3,** the specific activity of CTP:phosphatidate cytidylyltransferase was 1041 pmol mg^{-1} min^{-1} in the 7777 outer membrane vs. 59 pmol mg^{-1} min^{-1} in normal mitochondrial outer membranes after correction for contamination of normal outer membranes with microsomes.[1] Similarly, inner membrane CTP:phosphatidate cytidylyltransferase activity was higher in the 7777 after correction for contamination, 121 vs. 99 pmol mg^{-1} min^{-1} for normal liver, respectively. In the case of normal mitochondria, the interstitial soluble protein fraction was devoid of this activity when corrected for contamination with microsomal marker, but the interstitial soluble protein fraction from the 7777 hepatoma exhibited 100 pmol mg^{-1} min^{-1} of residual activity after correction for contamination by outer membrane, suggesting that some of the 7777 outer membrane enzyme is loosely bound and has been solubilized by treatment with digitonin. Corrected activities for the normal and 7777 hepatoma mitochondrial matrix fractions are 58 and 66 pmol mg^{-1} min^{-1}, respectively. It is difficult to determine whether the activity in these fractions represents a true matrix activity or whether some inner membrane-associated enzyme has been solubilized by the sonication that is necessary to release the matrix proteins.

[1] Sample calculation. The contribution of the microsomal contamination to the observed activity of the normal outer mitochondrial membrane was estimated by multiplying the microsomal contamination of the outer membrane (based on rotenone-insensitive NADPH–cytochrome *c* reductase) by the microsomal specific activity of CTP:phosphatidate cytidylyltransferase and subtracting this product from the observed outer membrane activity. E.g., $0.131 \times 709 = 92.8$, which represents the contribution of the contaminating microsomes. The observed specific activity of the outer membrane is 152. Therefore, $152 - 92.8 = 59.2$, which represents the residual activity of the normal outer mitochondrial membrane after correction for the contribution of the contaminating microsomes. Other corrections were made similarly, taking into account only the contamination due to the fraction that has the highest CTP:phosphatidate cytidylyltransferase activity.

TABLE 2. CDP-diacylglycerol synthesis by purified mitochondria and microsomes from normal liver, fetal liver, and hepatomas of varying growth rates[a]

Tissue	CTP:phosphatidate Cytidylyltransferase	
	Mitochondria	Microsomes
Normal liver	82 ± 13 (3)[b]	441 ± 7 (3)
Fetal liver	42	429
Hepatomas:		
7777	412 ± 83 (3)	166 ± 11 (3)
5123 tc	224	123
7800	321	590
7794a	309	675
7787	134	294
9633	110	305
9618a	533	846

[a] Mean ± 1 SD. The numbers in parentheses indicate the number of replicates; other values represent the average of two determinations.
[b] The results are given as pmol mg^{-1} min^{-1}.

Table 4 shows the results of experiments which measured the decrease of [^{32}P]diphosphatidylglycerol and [^{32}P]phosphatidylcholine from prelabeled mitochondria when incubated in vitro under conditions that are favorable for the endogenous mitochondrial phospholipase A. No significant differences were found in the rate of disappearance of [^{32}P]diphosphatidylglycerol from normal liver or 7777 hepatoma mitochondria. Regression analyses by the method of least squares showed nearly identical slopes (−0.24 vs. −0.23). Similar results were obtained for [^{32}P]-phosphatidylcholine (not shown).

The amount of protein per mitochondrion was determined for normal liver and 7777 hepatoma as

TABLE 3. Localization of CDP-diacylglycerol synthesis and marker enzymes in submitochondrial fractions and microsomes from normal liver and the 7777 hepatoma[a]

Membrane Fraction	CTP:phosphatidate Cytidylyltransferase pmol mg^{-1} min^{-1}				Rotenone-insensitive NADH Cytochrome *c* Reductase nmol mg^{-1} min^{-1}				Rotenone-insensitive NADPH-Cytochrome *c* Reductase nmol mg^{-1} min^{-1}			
	Normal Liver	RSA[b]	7777 Hepatoma	RSA[b]	Normal Liver	RSA[b]	7777 Hepatoma	RSA[b]	Normal Liver	RSA[b]	7777 Hepatoma	RSA[b]
Outer membrane	152	21.4	1041	100	1445	100	341	100	8.1	13.1	8.9	36.3
Interstitial sol. protein	29	4.1	194	18.6	22	1.5	31	9.0	5.0	8.1	3.7	15.1
Inner membrane	139	19.6	396	38.0	198	13.7	90	26.4	2.3	3.7	3.5	14.2
Matrix	65	9.1	118	11.3	29	2.0	20	5.9	1.2	1.9	3.0	12.2
Microsomes	709	100	171		700		158		62.0	100	24.5	100

[a] The enzyme activities represent the average of three separate preparations determined in duplicate.
[b] R.S.A., relative specific activity with the most active fraction set to 100.

TABLE 4. Hydrolysis of ^{32}P-labeled diphosphatidylglycerol in mitochondria from host liver or the 7777 hepatoma, in vitro

Incubation Time, min	% Control [^{32}P]Diphosphatidylglycerol Remaining	
	Host Liver	7777 Hepatoma
30	76 ± 7	90 ± 10
60	81 ± 26	79 ± 10
90	66 ± 12	71 ± 9
120	54 ± 18	74 ± 2
180	55 ± 14	56 ± 3

The values are the mean ± 1 SD of three replicates. Incubation conditions were as follows. The mixture containing 50 mM Tris-HCl (pH 8.4); 100 mM KCl; 3.5 mg/ml mitochondria prelabeled with $^{32}P_i$ and 5 mM $CaCl_2$ in a final volume of 0.200 ml. The samples were incubated at 37°C as noted and the lipids were extracted (17) and chromatographed on 0.25-mm layers of silica gel H prepared with 0.1 M $NaHCO_3$ and activated at 110°C for 30 min. The plates were developed with chloroform–methanol–1 M ammonium hydroxide 80:36:2 (by vol). The radioactive diphosphatidylglycerol was identified and assayed as noted in Methods.

described in Methods. Gradient-purified mitochondria from normal Buffalo rat liver were found to contain $8.2 \pm 2.0 \times 10^{-8}\mu g$ of protein per mitochondrion vs. $8.4 \pm 2.3 \times 10^{-8}\ \mu g$ per mitochondrion for the 7777 hepatoma. The difference is not statistically significant.

DISCUSSION

In this report CDP-diacylglycerol synthesis has been shown to be most active in mitochondria in the 7777 hepatoma, whereas in normal liver this process is principally microsomal. However, the subcellular localization of the biosynthesis of phosphatidate, phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylcholine in the 7777 hepatoma was not different from that of normal rat liver. The increase in the specific activity of CTP:phosphatidate cytidylyltransferase was greatest in the outer mitochondrial membrane of the 7777 hepatoma although the inner mitochondrial membrane activity was also increased substantially. A concomitant decrease in 7777 hepatoma microsomal CTP:phosphatidate cytidylyltransferase was observed. It is not known if CTP:phosphatidate cytidylyltransferase in mitochondria is the same enzyme as that in microsomes since these membrane-bound enzymes have not yet been characterized. Furthermore, it is not clear if these findings represent an increase in the total amount of enzyme present or whether the increased activity is due to alterations in the catalytic properties of the enzyme.

Fetal liver is a tissue which is often used as a control for the effects of rapid growth. Fetal liver mitochondria do not exhibit the increased CTP:phosphatidate cytidylyltransferase activity found in the 7777 hepatoma, and it is possible that the substantial increase in the activity of this enzyme in the mitochondrial outer and inner membranes in the 7777 hepatoma is a result of malignant transformation. All of the other Morris hepatomas studied showed increases in the mitochondrial activity of this enzyme, suggesting that increased mitochondrial CTP:phosphatidate cytidylyltransferase activity is a common occurrence in hepatomas and not just an isolated feature of the 7777 hepatoma.

In principle, the increased 7777 hepatoma mitochondrial diphosphatidylglycerol content (1) could also reflect a decreased rate of phospholipid degradation. Mitochondria that had been prelabeled with $^{32}P_i$ were isolated from the host liver and a 7777 hepatoma from a tumor-bearing rat. No difference could be found in the in vitro rate of hydrolysis of [^{32}P]diphosphatidylglycerol by the two mitochondrial preparations (Table 4). Similar results were obtained for mitochondrial [^{32}P]phosphatidylcholine breakdown (not shown). These findings suggest that the increased content of diphosphatidylglycerol in tumor mitochondria is not due to a decreased rate of phospholipid breakdown.

The increase in the phospholipid content per mg membrane protein of the 7777 hepatoma (1, 2, 8) could be artifactual, due to a decrease in the protein content of the mitochondrion. This does not appear to be the case, since the protein contents of the normal liver mitochondria and of the 7777 hepatoma mitochondria were nearly identical, 8.2×10^{-8} vs. $8.4 \times 10^{-8}\ \mu g$ of protein per mitochondrion, respectively. These data are in general agreement with the results of White and Tewari (21) and Myers and Bosmann (22) who found no differences in the protein content of normal liver mitochondria and mitochondria from the Novikoff hepatoma and the Reuber H-35 hepatoma. However, the mitochondrial protein content in this study is much lower than that calculated by the other authors. This may be due to procedural differences since our calculations were made from the observed ratio of latex beads to mitochondria determined by negative-staining electron microscopy instead of the light microscopic counting procedure employed in the other studies (21, 22).

Our findings are probably sufficient to explain the moderate increase in the 7777 mitochondrial content of diphosphatidylglycerol which was previously observed (1). However, the most substantial increases in phospholipid content were due to phosphatidylcholine and phosphatidylethanolamine (1, 2). 7777 hepatoma mitochondria, like those of normal liver, are devoid of CDP-choline:diacylglycerol choline-

phosphotransferase, the enzyme that catalyzes the final step of phosphatidylcholine synthesis (this paper, 9). The mechanism of the renewal of mitochondrial phosphatidylcholine and phosphatidylethanolamine is still poorly understood. Most authorities agree that no de novo synthesis of these important membrane phospholipids takes place in liver mitochondria (9). The suggestion has been made that phosphatidylcholine-exchange proteins may supply mitochondria with phosphatidylcholine (23), but these proteins generally do not catalyze a net transfer of phospholipid. However, it should be noted that net transfer of phosphatidylinositol has recently been demonstrated between monomolecular films containing phosphatidylinositol and liposomes containing phosphatidylcholine and phosphatidic acid (24). Further studies will be necessary to elucidate the mechanisms that result in the increased content of neutral phosphoglycerides in 7777 hepatoma mitochondria.

In conclusion, these studies indicate that the subcellular localization of CTP:phosphatidate cytidylyltransferase, a membrane-bound enzyme, is altered in the 7777 hepatoma. The enzyme activity is greatly increased in the outer mitochondrial membrane (17-fold), slightly increased in the inner mitochondrial membrane (22%) and decreased in the microsomes (60%). Since mitochondria from fetal rat liver do not exhibit this increased activity, it seems possible that the changes are a result of malignant transformation rather than rapid growth as such. The increased activity of CTP:phosphatidate cytidylyltransferase in 7777 hepatoma mitochondria could lead to an increased content of diphosphatidylglycerol, since the availablity of CDP-diacylglycerol is rate-limiting in this reaction. The rate of hydrolysis of [^{32}P]diphosphatidylglycerol by 7777 hepatoma mitochondria was the same as that of normal liver mitochondria, thus the increased 7777 mitochondrial CTP:phosphatidate cytidylyltransferase activity may be the cause of the increased diphosphatidylglycerol content previously observed (1).

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