MITOCHONDRIAL AUTONOMY: INCORPORATION OF MONOSACCHARIDES INTO ENDOGENOUS GLYCOLIPID ACCEPTORS IN ISOLATED RAT LIVER MITOCHONDRIA.[1]

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SUMMARY

Isolated, sterile rat liver mitochondria homogenized in Triton X-100 incorporated glucose-^{14}C and mannose-^{14}C into endogenous lipid acceptors forming glycolipids. In addition, choline-^{14}C was incorporated into phosphatidyl choline in the system. The glycolipid incorporations were highly specific and showed an absolute requirement for Mg++. One of the products formed with each monosaccharide was identified as a diglyceride glycolipid. Fractionation of the mitochondria into inner and outer membrane fractions indicated that the choline, glucose and mannose systems were associated with the outer membranes of the mitochondria.

Of late, the mitochondrion has been described as being capable of synthesizing various lipid macromolecules independently of other subcellular structures. Isolated mitochondria synthesize long chain fatty acids (1), serine containing phospholipids (2), choline and ethanolamine containing phospholipids (3,4) and incorporate fatty acids into their outer and inner membranes (5). Recently, this laboratory reported on the incorporation of monosaccharides into glycoprotein by isolated mitochondria in vitro (6). Since rat liver mitochondria have been reported to contain 7.1% glycolipid (7) it seemed reasonable to determine whether or not isolated mitochondria could transfer monosaccharides onto endogenous lipid acceptors. The present communication indicates that mitochondria can independently transfer two

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sugars--mannose and glucose--onto lipid acceptors in isolated rat liver mitochondria. Also since questions have arisen (8) concerning the incorporation of choline into phospholipid in isolated mitochondria this communication documents the incorporation of choline into lecithin by isolated rat liver mitochondria.

MATERIALS AND METHODS

Mitochondria. Sterile mitochondria were prepared from male rat liver as previously described (6) with five washes by the method of Schneider and Hogeboom (9). Such mitochondria have been demonstrated to be free of microsomes (10), smooth internal membranes (11), and plasma membranes (12) when assayed for appropriate marker enzymes (13-16) and showed an increase in monoamine oxidase specific activity (17) with successive washes (6). Immediately after isolation of the mitochondria, they were homogenized for 30 strokes in a Ten Broeck homogenizer in 0.1% Triton X-100 at the temperature of melting ice. This extract was used for the assay of glycolipid transferase activity.

Assay for Bound Lipid Monosaccharide-^{14}C. After incubation of the indicated mixtures for 30 minutes, the extracted mitochondria were precipitated with 10% CCl_3COOH and washed 2 times with 10% CCl_3COOH. The resulting pellet was twice extracted with $HCCl_3$:CH_3OH (2:1 v,v) and the extracts were combined. The extracts were evaporated on glass fiber filters by repeated application and counted in a liquid scintillation counter or were evaporated at 37° for paper or thin layer chromatography.

Analytic methods. Protein was determined by the method of Lowry et al.(18); crystalline bovine serum albumin was used as a standard. Choline radioactivity was determined by paper electrophoresis in acetic acid/formic acid solution at pH 2 for 1 hour under constant voltage application after hydrolysis of the sample for 4 hours in 1 N HCl. Monosaccharide radioactivity was determined by paper chromatography in n-butanol: pyridine: 0.1 N HCl (5:3:2,

by vol.) for 44 hours or butanol: acetic acid: water (4:1:5, by vol.) for 24 hours after sample hydrolysis for 4 hours in 4 N HCl. Lipids in dried $HCCl_3$:H_3COH samples were chromatographed on silicic acid impregnated paper (Whatman SG81) developed with diisobutyl ketone: acetic acid: water (40:25:5, by vol.) (19) (DAW) or on thin layer chromatography developed with Solvent A, $HCCl_3$:CH_3OH:H_2O (65:25:4, by vol.) or Solvent B, $HCCl_3$:CH_3OH: H_3CCOOH: H_2O (25:15:4:2, by vol.) and alkaline hydrolysis products were developed on Whatman No. 1 paper developed with Solvent C, butanol: pyridine: water (6:4:3, by vol.). Radioactivity was determined by counting scraped samples or strips in a liquid scintillation counter or on a radioactivity strip scanner. Inner and outer mitochondrial membranes were separated by the digitonin method of Schnaitman (20) and were homogenized in 0.1% Triton X-100 after separation and before incubation.

RESULTS

The data in Table 1 indicate that Triton X-100 extracts of isolated rat liver mitochondria incorporate mannose and glucose from nucleotide diphosphate monosaccharide precursors into endogenous glycolipid. Since the same amount of approximately equal specific activity precursor was used in each assay the data indicate that the incorporation of mannose was much more efficient than the incorporation of glucose. However, it cannot be stated which transferase enzyme is present to the greatest extent since either enzyme or glycolipid acceptor could be limiting in the systems. In both systems, boiling of the extracted mitochondria for two minutes completely abolished incorporation of the sugars into lipid (Table 1).

In both systems the incorporations were linear with time up to 30 minutes, almost exactly linear with respect to added mitochondrial extract, and had optimum temperatures of 37° (Table 1). Each system was absolutely dependent on Mn^{++} and slightly dependent on the presence of the pH 7.6 $NaHPO_4$; EDTA completely abolished the activity with both sugars. Co^{++}

TABLE 1

Synthesis of Mannose and Glucose Containing Glycolipids by Isolated Mitochondria

	Component	mannose-^{14}C CPM/mg protein	glucose-^{14}C CPM/mg protein
I.	Complete System[1]	1303	410
	TX broken mito boiled 2 min.	7	7
II.	0 minutes incubation	4	3
	5 minutes incubation	210	70
	10 minutes incubation	416	146
	15 minutes incubation	640	211
	20 minutes incubation	830	280
	30 minutes incubation	1280	400
	45 minutes incubation	1302	410
	60 minutes incubation	1310	406
III.	100 µl TX broken mito	1540	460
	75 µl TX broken mito	1150	350
	50 µl TX broken mito	800	233
	25 µl TX broken mito	371	120
	5 µl TX broken mito	67	46
	0 µl TX broken mito	1	2
IV.	0°	185	40
	7°	248	31
	25°	652	141
	35°	1100	396
	37°	1287	409
	40°	938	321
	60°	90	101
	80°	86	10
V.	Complete	1365	405
	-$MnCl_2$	225	140
	-$NaHPO_4$	900	380
	-$MnCl_2$, - $NaHPO_4$	140	94
	+40 µl 0.2M EDTA	10	0
VI.	Complete-$MnCl_2$	225	140
	+Mn++[2]	1470	416
	+Mg++	224	124
	+Co++	823	393
	+Hg++	110	42
	+Fe+++	26	47
	+Cd++	480	16
	+Pb++	213	98
	+Ba++	144	141
VII.	Complete - TX broken mito.	0	0
	+ intact TX mito.	491	174
	+ intact mito in Tris	96	94

LEGEND TO TABLE 1

1. The complete system contained (as sterile solutions): 100 µl of the 0.1% Triton X-100 broken isolated rat liver mitochondria (between 2 and 6 mg protein), 30 µl of GDP-mannose (0.01 µC, 100 pMole), 10 µl of 0.1 M $MnCl_2$, 10 µl of 0.1 M sodium phosphate pH 7.6. It was incubated at 37° for 30 minutes and radioactivity determined as given in methods.
2. Ions tested by adding 10 µl of 0.1 M solution.

Abbreviations: TX = 0.1% Triton X-100. Mito = mitochondria. Tris = 0.1 M Tris buffer pH 7.6.

All volume adjustments were made with 0.1 M Tris buffer pH 7.6. All CPM have "0 time" and background CPM subtracted. The glucose -^{14}C system had 30 µl of UDP-glucose-^{14}C(0.01 µC, 100 p Mole) substituted for the GDP-mannose-^{14}C.

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and to a small extent Cd++ substituted for Mn++ in the mannose-^{14}C system, Mg++ and Pb++ did not affect the incorporation and Hg++, Fe+++ and Ba++ were inhibitory. Co++ was almost as efficient as Mn++ in the glucose-^{14}C incorporation, Mg++ and Ba++ had no effect while Hg++, Fe+++, Cd++ and Pb++ were inhibitory. Homogenization in 0.1% Triton X-100 was essential for optimal incorporation of UDP glucose-^{14}C and GDP-mannose-^{14}C into glycolipid, unbroken mitochondria in 0.1% Triton X-100 or in 0.1 M Tris pH 7.6 were ineffectual for incorporation.

The results presented in Table 2 indicate that the system was very specific for UDP-glucose and GDP-mannose; none of the other nucleotide diphosphate monosaccharides tested or glucosamine was incorporated. Although it is doubtful that this system would support much protein synthesis it is of interest that no leucine radioactivity was found with the system indicating no labelled protein was present in the chloroform extracts. In Table 3 are presented data indicating choline incorporation into lipid by broken Triton X-100 extracts of isolated rat liver mitochondria. The incorporation was absolutely dependent on Mg++ but only slightly dependent on an energy producing system (Table 3). It is of interest that in this system which supports some protein synthesis no leucine radioactivity was found in the extract. When an energy producing system was included with GDP mannose-C^{14} and UDP-glucose-C^{14} no increase in activity was found (cf Tables 1 and 3) indicating no dependence on energy for the incorporation of the monosaccharides.

When dried extracts were hydrolyzed and analyzed as given in Methods, the product of the UDP-glucose reaction was recovered as 91% glucose and 9% mannose, the product of the choline reaction as 100% choline and the product

TABLE 2

Specificity of Transfer of Sugars onto Endogenous Acceptors by Isolated Rat Liver Mitochondria. Complete System as Given in Table 1; Indicated Radioactive Compounds Substituted at the Same Amount.

C^{14}-Compound Substrate	CPM/mg protein
UDP-glucose	420
GDP-mannose	1360
UDP-galactose	52
GDP-fucose	12
UDP-xylose	11
UDP-N-acetyl-galactosamine	2
UDP-arabinose	0
leucine	0
glucosamine	72

TABLE 3

Incorporation of Choline-C^{14} into Lipid by Isolated Rat Liver Mitochondria

Component	CPM/mg protein	Component	CPM/mg Protein
Complete System[1]	707	Complete System	704
Boiled TX broken mito.	110	- ATP	602
0 min. incubation	7	- PEP	612
15 min. incubation	420	- PK	610
30 min. incubation	760	- ATP, PEP, PK	590
45 min. incubation	778	Complete System-Choline-C^{14}	0
60 min. incubation	719	+ leucine-C^{14}	0
Complete System	717	+ GDP-mannose-C^{14}+Mn++	1206
-$MgCl_2$	30	+ UDP-glucose-C^{14} + Mn++	350

[1]The complete system contained as sterile solutions: 20 µl of choline-C^{14} (1 µC, 1 µMole), 10 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.6) 50 mM tris (hydroxymethyl) amino methane (pH 7.6), 5 mM phosphoenol pyruvate (PEP), 2 µg of pyruvic kinase (PK), 2 mM adenosine triphosphate (ATP), 2 mM EDTA, 22.5 mg/ml of a complete amino acid mixture minus leucine, 100 µl of the 0.1% Triton X-100 broken isolated rat liver mitochondria and 0.154 M KCl to a final volume of 0.2 ml. Abbreviations as given in Table 1. Incubations were for 30 minutes at 37° and radioactivity was determined as given in methods.

of the GDP-mannose reaction as 89% mannose and 11% fucose. Chromatography of the reaction products in the DAW system gave 3 radioactive spots for the products formed from GDP-mannose (35% CPM, R_f 0.20, 31% CPM, R_f 0.31, 34% CPM, R_f 0.60), 3 radioactive spots for the products formed from UDP-glucose (31% CPM, R_f 0.18, 31% CPM, R_f 0.285, 38% CPM, R_f 0.64) and 2 radioactive spots (95% CPM, R_f: 0.55, 5% CPM R_f: 0.42) for the products formed from choline. Since the glucose-C^{14} containing glycolipid of R_f 0.64 had the same mobility in this system as monoglucosyl diglyceride (21) after elution it

was subjected to alkaline hydrolysis in 0.1 N KOH at 70° for 1.5 hours, adjusted to pH 2.5 and extracted with ether. The water soluble radioactive product was chromatographed in Solvent C and had R_f 0.33, the reported R_f of monoglucosyl glycerol (21). The glycolipid of R_f 0.60 of the GDP-mannose-C^{14} system was eluted and run in Solvent A in which it had an R_f of 0.65, that indicated for dimannosyl diglyceride (22). Acid hydrolysis (2 N HCl, 2 hours, 105°) yielded mannose-^{14}C and glycerol (identified in Solvents A and C). The R_f of 0.55 in the DAW system for the choline system is the same as that reported for lecithin (23); this lipid had an R_f of 0.33 in Solvent B, that reported for phosphatidyl choline (24). The other lipid products have not as yet been identified.

Fractionation of the mitochondria into inner and outer membranes by digitonin and extraction and homogenization with 0.1% Triton X-100 resulted in the following CPM/mg protein for the membranes substituted in the complete systems of Tables 1 and 3 respectively: UDP-glucose-C^{14}, 102 (inner) and 1580 (outer); GDP-mannose-C^{14}, 128 (inner) and 2020 (outer); and choline-C^{14} 14 (inner) and 1777 (outer).

DISCUSSION

Isolated rat liver mitochondria incorporate monosaccharides-C^{14} from nucleotide diphosphate monosaccharide-C^{14} into glycolipid. It is not known what the function of the glycolipids synthesized in the mitochondria is or whether or not they function as intermediates (25) in the synthesis of glycoproteins by mitochondria (6) or are part of the membrane structure of the mitochondria. The results do demonstrate that the mitochondrion can independently synthesize some glycolipids in agreement with the growing volume of literature demonstrating mitochondrial autonomy.

The results on the Mg^{++} dependence of the choline incorporation are in agreement with those of Kaiser (3), but the energy dependence for the incorporation reported (3) was not found in the present results presumably because in the present study the mitochondrial material was solubilized with

the non-ionic detergent before incubation. The choline incorporation at a higher specific activity in the outer membranes than the inner confirm the observations of Kaiser and Bygrave (26). The fact that the glycolipid transfers also occur at much higher specific activity in the outer membranes as opposed to the inner membranes leads one to speculate that synthesis of lipid and glycolipid macromolecules occurs at the outer membrane while synthesis of protein (27) and glycoprotein (6) macromolecules occur at the inner membrane level.

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