LECITHIN BIOSYNTHESIS IN LIVER MITOCHONDRIAL FRACTIONS

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SUMMARY: The pretreatment of rat liver mitochondrial fractions with phospholipase C preparations enhanced the incorporation of cytidine diphospho-[^{14}C]-choline into phospholipids several-fold. Similar pretreatment of the microsomal fraction produced a similar stimulation. When the extent of microsomal contamination in the mitochondria was determined, and increments of pretreated microsomes were added to the mitochondria, the incorporation values extrapolated to zero for zero microsomal contamination. It was concluded that lecithin biosynthesis from endogenous diglycerides in the mitochondrial fractions could be ascribed to contaminating microsomes.

INTRODUCTION

Although the original investigation of the subcellular distribution of CDP-choline: 1,2 diglyceride choline phosphotransferase [E.C.2.7.8.2.] demonstrated that the enzyme was essentially confined to the microsome fractions in rat liver (1,2), from time to time reports have appeared describing the biosynthesis of lecithin by mitochondrial fractions from mammalian liver (3-5) and from other tissues and species (6-9). Our previous experiments (10) indicated that the small amount of incorporation of CDP-[^{14}C]-choline into phospholipids of isolated mitochondria could be accounted for by the irreducible contamination of highly active microsomal components, either in the presence of absence of exogenous 1,2-diglyceride acceptors. However, a recent note from Scherphof's laboratory (11) suggests that brief pretreatment of the mitochondria with phospholipase C to generate endogenous diglycerides enhances the incorporation of CDP-[^{14}C]-choline into lecithin to an extent which can not be attributed to microsomal contamination. While confirming the observed stimulation by phospholipase C pretreatment, the experiments described here are not consistent with the

interpretation that the stimulated incorporation derives from mitochondria *per se*.

METHODS AND MATERIALS

Mitochondria and microsomes were isolated from adult rat liver by the procedures described previously (10), using a homogenization medium containing 0.25M sucrose, 0.0001M EDTA, pH 7.4, and a washing medium containing 0.25M sucrose, 0.00375M Tris, pH 7.4. The cell-fractions derived from 1.0g of fresh liver suspended in 1 ml of sucrose-Tris were treated with phospholipase C (0.5 units) in the presence of 5.0 μmoles $CaCl_2$ at 37° for the time stipulated (usually for 1 min). An excess of EGTA (15 μmoles, pH 7.4) was added, the fractions were sedimented at 10,000 x g* for 10 min, and the precipitates were washed twice with sucrose-Tris. Treated or untreated fractions in the amounts indicated were incubated for 30 min. at 37° with CDP-[1,2-^{14}C]-choline (0.2 μmoles; 0.028 μc) in Tris-buffered medium (10) composed of 0.17M sucrose, 0.01M cysteine, 0.02M $MgCl_2$, 0.0375M Tris, pH 7.4 in a final volume of 0.5 ml. The reaction was stopped, and the lipids were extracted by addition of 4 ml of $CHCl_3$:CH_3OH (1:1 v/v). The chloroform-methanol extract was removed from precipitated protein by centrifugation, rendered biphasic by addition of 2 ml of $CHCl_3$ + 0.7 ml of 0.9% aqueous NaCl, and the lower phase was washed three times with 0.75 ml of theoretical upper phase ($CHCl_3$:CH_3OH:0.9% NaCl-3:48:47 by vol) (10). Portions of the washed lower phase were evaporated to dryness in scintillation vials, and the radioactivity determined in a liquid scintillation spectrometer (toluene-POPOP-PPO system), with an efficiency of 85-90% for ^{14}C. NADPH-cytochrome C reductase (10) and glucose-6-phosphatase (13) were determined on both mitochondrial and microsomal fractions, before and after phospho-

*After the addition of Ca^{++}, despite subsequent removal with EGTA, the microsomes sedimented completely at low speed (12).

lipase treatment.** Phospholipase C preparations (*Clostridium welchii* enzyme, 2.0 units/mg and *Bacillus cereus* enzyme, 80 units/mg) were obtained from Sigma, and CDP-[1,2-^{14}C]-choline was obtained from New England Nuclear.

RESULTS AND DISCUSSION

The incorporation of CDP-[^{14}C]-choline into lipids of microsomes and mitochondria was enhanced several-fold by brief pretreatment of the fractions with phospholipase C (Fig. 1). Although the extent of the increase

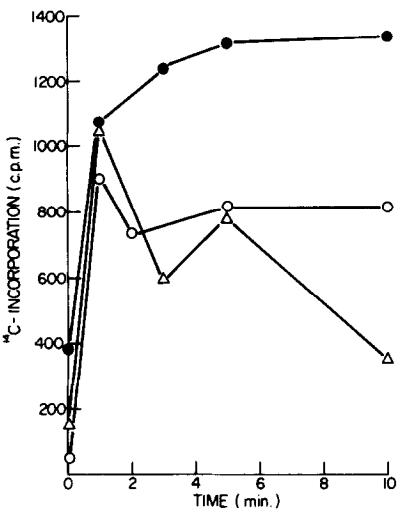

Figure 1. Effect of pretreatment of mitochondria and microsomes for varying times with phospholipase C preparations upon CDP-[^{14}C]-choline incorporation into lipids. The phospholipase treated fractions (mitochondria, 200 µl ≡ 200 mg. original liver; microsomes, 10 µl ≡ 10 mg. original liver) were incubatedwith CDP-[^{14}C]-choline for 30 min. at 37° in the Tris-buffered medium described in the text. ●——●, microsomes treated with C. welchii enzyme; Δ——Δ, mitochondria treated with C. welchii enzyme; o——o, mitochondria treated with B. cereus enzyme.

was variable (4-24-fold), in preparations from the same animal similar increases were noted for microsomes as for the mitochondria under similar conditions; the variability may be due to differences in endogenous

**Glucose-6-phosphatase activity was completely lost after phospholipase treatment, so the NADPH-cytochrome C reductase activity was used as microsomal marker for the treated fractions.

diglyceride levels throughout the liver fractions in different animals. The microsomes which contain both higher total phospholipids relative to protein, and higher phosphatidylcholine relative to total phospholipid showed greater susceptibility to phospholipase attack than the mitochondria. In the experiment depicted in Fig. 1 >50% of microsomal lipid P was depleted by 1 min. treatment with phospholipase C compared with <20% of mitochondrial lipid P hydrolysis under the same conditions, when related to total protein in the fractions. Since the maximal effect upon ^{14}C-incorporation was observed with 1 min. phospholipase C pretreatment and prolonged treatment sometimes depressed the incorporation this condition was standardized in subsequent experiments.

In order to examine the possible role of microsomal contaminants in the mitochondrial incorporation of CDP-[^{14}C]-choline a procedure similar to that used earlier (10) was followed. The procedure involves adding measured increments of microsomal membranes to a fixed quantity of the mitochondrial fraction, and plotting the incremental incorporation values. If pure mitochondria contain a significant ability to synthesize lecithin, the curve should extrapolate to a finite value at zero microsomal content. To establish the degree of microsomal contamination in the washed mitochondria, the marker enzymes NADPH-cytochrome C reductase and glucose-6-phosphatase were determined, yielding values that were in substantial agreement. In the phospholipase C treated, washed mitochondria the microsomal contaminations based on these criteria were the same as in untreated mitochondria (2-6%). As shown in Fig. 2 the addition of 5-20 µl of phospholipase-treated microsomes to 250 µl of phospholipase-treated mitochondria produced a linear increase in the CDP-[^{14}C]-choline incorporation; since the marker enzyme values showed that the mitochondrial fraction contained 3.85% microsomal membranes on a volume basis, the low point on the curve corresponding to 250 µl of mitochondria alone is plotted as containing 9.6 µl of microsomes. The curve clearly does not extrapolate to

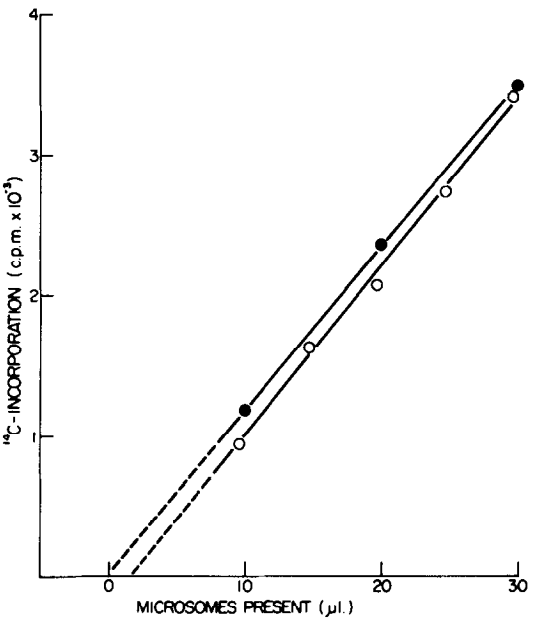

Figure 2. Effect of the addition of varying amounts of microsomes in the absence or presence of a fixed quantity of mitochondria (250 µl ≡ 250 mg. original liver) upon the CDP-[^{14}C]-choline incorporation. Both cell-fractions had been pretreated for 1 min. at 37° with C. welchii phospholipase C. See text for calculation of microsomes present based upon marker enzyme activities. ●———●, microsomes alone; o———o, mitochondria plus microsomes.

a significant finite value at 0 µl of microsomes. In fact, the curve is somewhat depressed below that obtained for microsomes, showing that mitochondria not only fail to support lecithin biosynthesis themselves, but also do not contribute to microsomal synthesis by co-operation.

An additional approach to this question was to add varying amounts of microsomal membranes to the mitochondria prior to the treatment with phospholipase C. The mitochondria were then washed to remove phospholipase, Ca^{++} and excess microsomes, and portions of the treated mitochondria were taken for measurement of NADPH-cytochrome C reductase and CDP-[^{14}C]-choline incorporation. In this way one could be assured that both mitochondria and microsomes were subjected to identical treatment, and that the marker enzyme value truly reflected the microsomal content remaining in the treated sample. When the lecithin synthesis was plotted against the microsomal marker enzyme content

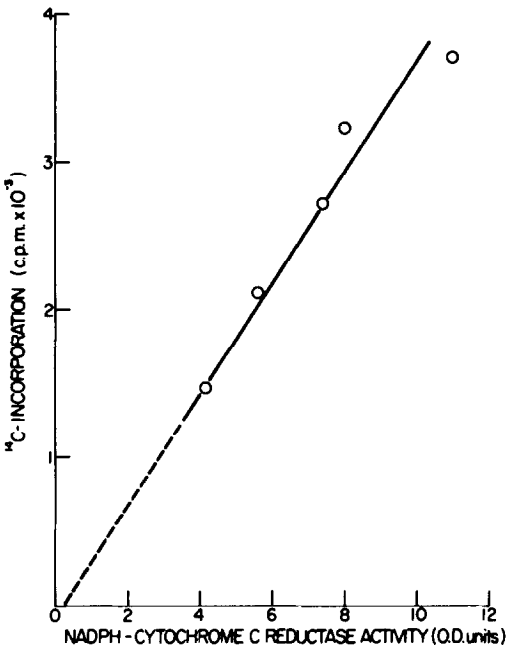

Figure 3. Effect of mixing varying amounts (5-20 µl) of microsomes with a fixed quantity (200 µl) of mitochondria prior to pretreatment with phospholipase C upon the CDP-[^{14}C]-choline incorporation. The amounts of microsomal membranes remaining in the phospholipase-treated, washed mitochondria are expressed in terms of the NADPH-cytochrome C reductase activity.

the curve was linear and again extrapolated essentially to zero at zero microsomal content (Fig. 3).

A variety of procedures was tried to demonstrate the presence of latency of mitochondrial CDP-[^{14}C]-choline incorporation since the above experiments, although negative, do not rule out the possibility that an intramitochondrial site for biosynthesis of lecithin is inaccessible either to the CDP-[^{14}C]-choline and added diglyceride, or to the action of added phospholipase C. Treatment of the mitochondria with ultrasound, by repeated freezing or thawing, or with a number of detergents either before or after phospholipase C digestion failed to activate the CDP-[^{14}C]-choline incorporation above that observed with phospholipase C pretreatment alone (Table 1). It was of interest that the lecithin biosynthesis from endogenous diglycerides was not increased by concentrations of oleate which have been shown to stimulate CDP-[^{14}C]-choline

TABLE I Incorporation of CDP-[^{14}C]-choline in Phospholipase-treated Mitochondria and Microsomes

Expt.	Treatment	Incorporation into Lipids (c.p.m.) Mitochondria	Microsomes
1	None	400	-
	*PlipaseC + Ca^{++}	1,532	-
	**Frozen-thawed	530	-
	Frozen-thawed + Ca^{++}	340	-
	Frozen-thawed then PlipaseC + Ca^{++}	1,471	-
	†Ultrasonicated then PlipaseC + Ca^{++}	1,412	-
	Deoxycholate (.8mM) then PlipaseC + Ca^{++}	1,048	-
2	PlipaseC + Ca^{++}	2,092	19,120
	PlipaseC + Ca^{++} then frozen-thawed	2,030	16,200
	PlipaseC + Ca^{++} then ultrasonicated	2,186	20,280
	PlipaseC + Ca^{++} then deoxycholate	1,920	8,330
	PlipaseC + Ca^{++} then NH_4 oleate (0.8 mM)	1,508	6,474

Mitochondria from 200 mg of liver or microsomes from 25 mg of liver were incubated with CDP-[^{14}C]-choline for 30 min. at 37° in the Tris-buffered medium. The following pretreatments of the cell-fractions were performed:

* - Phospholipase C + Ca^{++} = 0.5 units enzyme/g. of liver equivalent + 5mM $CaCl_2$ for 1 min. at 37°.

** - Frozen and thawed five times.

† - Ultrasonicated by immersion in a sonic disruption bath (Heat Systems Co.) for 5 min.

incorporation many-fold in the presence of added diglycerides (14).

Although the present findings cannot exclude a latent site for lecithin biosynthesis inside a mitochondrial permeability barrier, they provide no evidence for incorporation of significant amounts of exogenous CDP-[^{14}C]-choline into mitochondrial lecithin, in the presence or absence of endogenous diglycerides, that may not be accounted for by microsomal contaminants.

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