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Phospholipid Metabolism in Plant Mitochondria

SUBMITOCHONDRIAL SITES OF SYNTHESIS[1]

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ABSTRACT

Intact mitochondria from the endosperm of castor bean were isolated on linear sucrose gradients. These mitochondria were ruptured and the membranes separated on discontinuous sucrose gradients into outer membrane, intact inner membrane, and ruptured inner membrane fractions. Each membrane fraction was examined for its capacity to synthesize phosphatidylglycerol, CDP-diglyceride, phosphatidylcholine via methylation, and phosphatidic acid. The syntheses of phosphatidylglycerol, CDP-diglyceride, and phosphatidylcholine were localized exclusively in the inner mitochondrial membrane fractions while phosphatidic acid synthesis occurred in both the inner and outer mitochondrial membranes.

In order to understand membrane synthesis fully, it is important to define the sites within the cell involved in synthesis of the membrane components. It has been reported that the ER is responsible for the bulk of the phospholipid synthesis in cells. However, substantial evidence has accumulated regarding the ability of intact mitochondria to synthesize at least a portion of the total phospholipids constituting their membranes. Animal mitochondrial phospholipid synthesis has been studied extensively and is well reviewed (15, 20, 29, 41), but plant mitochondria have received comparatively less attention (18, 28). In spite of this, it has been suggested that plant mitochondria have the capacity to synthesize many of their membrane lipids.

Among the phospholipids reportedly synthesized by plant mitochondria are phosphatidic acid (4, 5, 11, 42), CDP-diglyceride (1, 10, 11, 38, 39), phosphatidylglycerol (10, 12, 26, 30), cardiolipin (10, 12), and phosphatidylethanolamine via the decarboxylation of phosphatidylserine (27). In addition, there is evidence that plant mitochondria have the capacity to synthesize phosphatidylcholine via the methylation of phosphatidylethanolamine (31) as well as the phosphorylcholine-glyceride transferase reaction (9). Finally, Sumida and Mudd (40) presented some evidence that mitochondria from the inflorescence of cauliflower could also synthesize phosphatidylinositol.

It should be emphasized that in the majority of the work mentioned above the mitochondria were isolated by differential centrifugation techniques. This method, as pointed out by Lord *et al.* (22), can lead to erroneous conclusions concerning compartmentation. The purest plant organelle preparations which have been used for phospholipid synthesis investigations are those derived from sucrose density gradient fractionation of castor bean endosperm. In these investigations, only the research of Moore (30, 31) and Vick and Beevers (42) have described any phospholipid synthesis by the mitochondrial fractions. Thus, it is imperative that the capacity of the castor bean mitochondrial fraction for phospholipid synthesis be fully defined. Such data are critical toward understanding the autonomy of mitochondria with respect to membrane biogenesis, an area of current concern (6-8, 21).

Another matter of interest is the precise intramitochondrial sites of the phospholipid syntheses which occur in mitochondria. Several attempts have been made to define these sites in mammalian, particularly rat liver, mitochondria, but only one such study to date exists for plant systems. With rat liver mitochondria, Hostetler and Van Den Bosch (16) showed that phosphatidylglycerol, CDP-diglyceride, and deoxy-CDP-diglyceride were synthesized primarily on the inner mitochondrial membrane but with some synthesis occurring on the outer membrane; cardiolipin was synthesized exclusively on the inner membrane. In addition, Sarzala *et al.* (35) showed that both the syntheses of phosphatidylcholine via the acylation of lysophosphatidylcholine and of phosphatidic acid via the acylation of 1-lysophosphatidate occurred primarily on the outer mitochondrial membranes. Kaiser and Bygrave (17) showed that ^{14}C-labeled choline was incorporated into phosphatidylcholine in the outer membranes of intact rat liver mitochondria. Finally, Zborowski and Wojtczak (43) and Shephard and Hubscher (36) both determined that phosphatidic acid was synthesized primarily on the outer membrane of rat liver mitochondria. Only Douce *et al.* (13) examined the inner and outer membranes of plant mitochondria for their capacities to synthesize a phospholipid. They showed that CDP-diglyceride was synthesized exclusively on the inner membrane of mitochondria isolated from etiolated mung bean hypocotyls.

The present investigation was designed to define more fully the phospholipid-synthesizing capacity of mitochondria isolated from castor bean endosperm and to examine the inner and outer membranes of these organelles for their individual capacities to synthesize those phospholipids.

MATERIALS AND METHODS

The salts were obtained from the J. T. Baker Chemical Co. and were of reagent grade. CDP-Dipalmitoylglyceride and phosphatidic acid (egg) were purchased from Serdary Research Laboratories (London, Ontario, Canada). Palmitoyl-CoA and all other organic compounds (except sucrose) were obtained from Sigma Chemical Co. Sucrose was purchased from Mallinckrodt Chemical Works.

The disodium salt of [U-^{14}C]glycerol-3-P (117.4 mCi/mmol), the tetra sodium salt of [5'-^{3}H]cytidine-5'-triP (21.8 Ci/mmol), and *S*-[methyl-^{14}C]adenosylmethionine (57.7 mCi/mmol) were purchased from New England Nuclear.

Seeds of castor bean (*Ricinus communis* L. var. Hale) were soaked overnight in running tap water, planted in moist Vermiculite, and germinated in the dark at 30 C for 5 to 6 days.

TISSUE HOMOGENIZATION AND ISOLATION OF MITOCHONDRIA

Castor bean endosperm halves were homogenized and the

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mitochondria were isolated on linear sucrose density gradients according to methods described elsewhere (23).

PREPARATION OF SUBMITOCHONDRIAL FRACTIONS

Two methods were employed to subfractionate mitochondria. In the first, intact mitochondria were homogenized and the submitochondrial fractions were isolated similarly to the method of Maisterrena *et al.* (25) as modified by Bowles *et al.* (3). This procedure initially results in crude inner and outer membrane fractions separated by differential centrifugation. These crude membrane fractions subsequently were purified on separate discontinuous sucrose gradients designated as the inner and outer membrane gradients. The first method was employed for the initial marker enzyme identification of the submitochondrial membrane fractions as well as for the submitochondrial localization of phosphatidylglycerol synthesis.

For subsequent investigations this procedure was modified and the modified procedure is shown in Figure 1. Intact mitochondria from four standard sucrose gradients (22; approximately 10–14 mg of mitochondrial protein) were collected, diluted in half with water, and concentrated by centrifugation at 20,000*g* for 15 min in a Beckman model JA-20 rotor. The 20,000*g* supernatant (lacking any detectable fumarase activity) was discarded and the pellet of intact mitochondria was resuspended in 1 to 2 ml of 10 mM phosphate buffer (pH 7.4). The resuspended mitochondria were transferred to a Potter homogenizer and homogenized for 10 min (about 10 rotating strokes/min). The mitochondrial homogenate was then diluted with the 10 mM phosphate buffer (pH 7.4) to a protein concentration of approximately 0.1 mg/ml and the membranes were left to swell for 20 min. After swelling, the homogenate, consisting of outer membranes, intact inner membranes (mitoplasts), and vesicles of ruptured inner membranes, was centrifuged at 100,000*g* for 60 min in a Beckman type 50 or Ti-75 rotor. The 100,000*g* supernatant (containing considerable fumarase activity) was discarded and the pellet was resuspended in 5 ml of 8.6% (w/w) sucrose in 10 mM Tris-HCl (pH 7.4). This 5-ml resuspension of mitochondrial fragments was layered onto step gradients composed of sucrose (%, w/w) in 10 mM Tris-HCl (pH 7.4) as indicated in Figure 1. The final discontinuous gradients with their samples were centrifuged at 96,300*g* for 2 h in a Beckman model SW 27 rotor. Finally, the step gradients were fractionated on an ISCO model 640 density gradient fractionator and the fractions were assayed for the various marker and phospholipid-synthesizing enzyme activities.

ENZYME ASSAYS

Marker Enzymes. Enzymes used to identify the submitochondrial fractions were as follows: fumarase, according to Racker (34); succinate-Cyt *c* reductase, according to Douce *et al.* (14); antimycin A-sensitive and -insensitive NADH-Cyt *c* reductases, according to Douce *et al.* (14).

Phospholipid Synthesis. Phosphatidic acid and CDP-diglyceride syntheses were assayed according to the methods of Vick and Beevers (42) and Douce *et al.* (13), respectively. The syntheses of phosphatidylglycerol and phosphatidylcholine via methylation were assayed by the methods of Moore (30, 31).

PHOSPHOLIPID EXTRACTIONS AND IDENTIFICATIONS

Phospholipid enzyme reactions were terminated and extracted according to the method of Bligh and Byer (2). Phospholipid products were identified by thin layer co-chromatography with known standards on Silica Gel H. Solvent systems were chloroform-methanol-7 N NH_4OH (65:30:4) or chloroform-methanol-

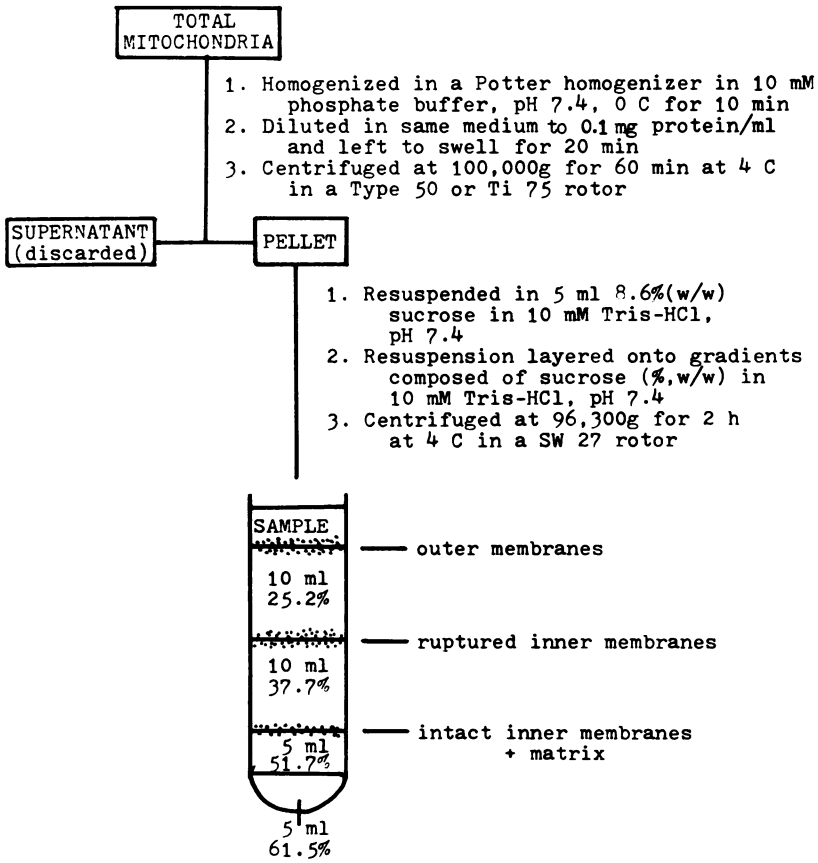

FIG. 1. Modified procedure for mitochondrial homogenization and separation of submitochondrial membrane fractions.

water (65:25:4, v/v). The radioactivity of the chloroform fractions was measured with a Beckman model LS-250 liquid scintillation counter in a scintillation cocktail consisting of 5 g PPO and 0.3 g dimethyl-POPOP/l toluene.

PROTEIN DETERMINATION

Protein determinations were performed according to the method of Lowry *et al.* (24).

ELECTRON MICROSCOPY

Pellets of intact mitochondria and inner mitochondrial membrane fractions were fixed in 1.0% (w/v) OsO_4 in 0.1 M phosphate buffer (pH 7.4) at 4 C. The samples were dehydrated in increasing concentrations of ethanol and embedded in Spurr's (37) medium. Thin sections were stained with 2% (w/v) uranyl acetate in 50% (v/v) ethanol and 2% (w/v) lead citrate. Outer membranes were fixed in vapors of OsO_4 and negatively stained with 1% (w/v) uranyl acetate. All specimens were viewed on an RCA EMU 3G electron microscope operated at 50 kv.

RESULTS

IDENTIFICATION OF SUBMITOCHONDRIAL FRACTIONS

Marker Enzymes. Marker enzymes used to identify mitochondrial outer and inner membrane fractions were antimycin A-insensitive and -sensitive NADH-Cyt *c* reductases, respectively. Succinate-Cyt *c* reductase was used to confirm the identity of the inner membranes. Fumarase was employed to detect soluble matrix enzyme activity. The results of these marker enzyme assays on the mitochondria fractionated by the method of Bowles *et al.* (3) are shown in Figures 2 and 3. As expected, the amount of protein in the outer membrane gradient (Fig. 2) is considerably less than that of the inner membrane gradient (Fig. 3) and is near the lower limits of detection by the method of Lowry *et al.* (24). However, three bands of protein peaking in fractions 11, 28, and 51 of the outer membrane gradient are weakly discernible. Two

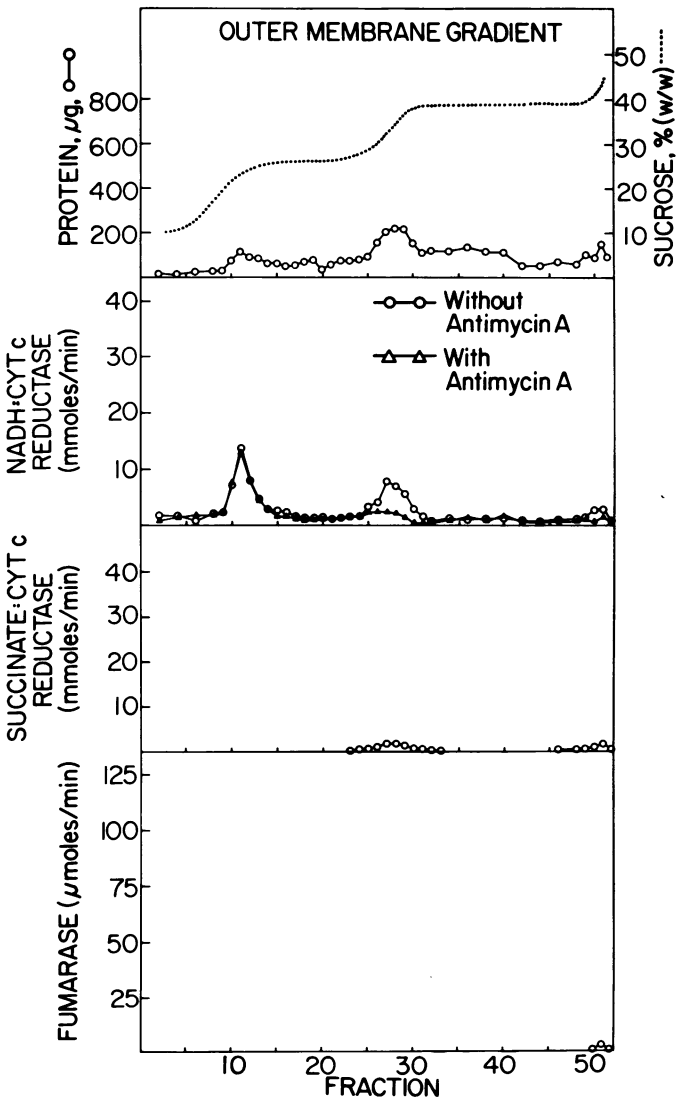

FIG. 2. Distribution of marker enzyme activities through outer membrane gradient. The outer membrane gradient derived from the submitochondrial fractionation method of Bowles *et al.* (3) was fractionated and each gradient fraction was assayed for protein content, antimycin A-sensitive and -insensitive NADH-Cyt *c* reductase, succinate-Cyt *c* reductase, and fumarase activities.

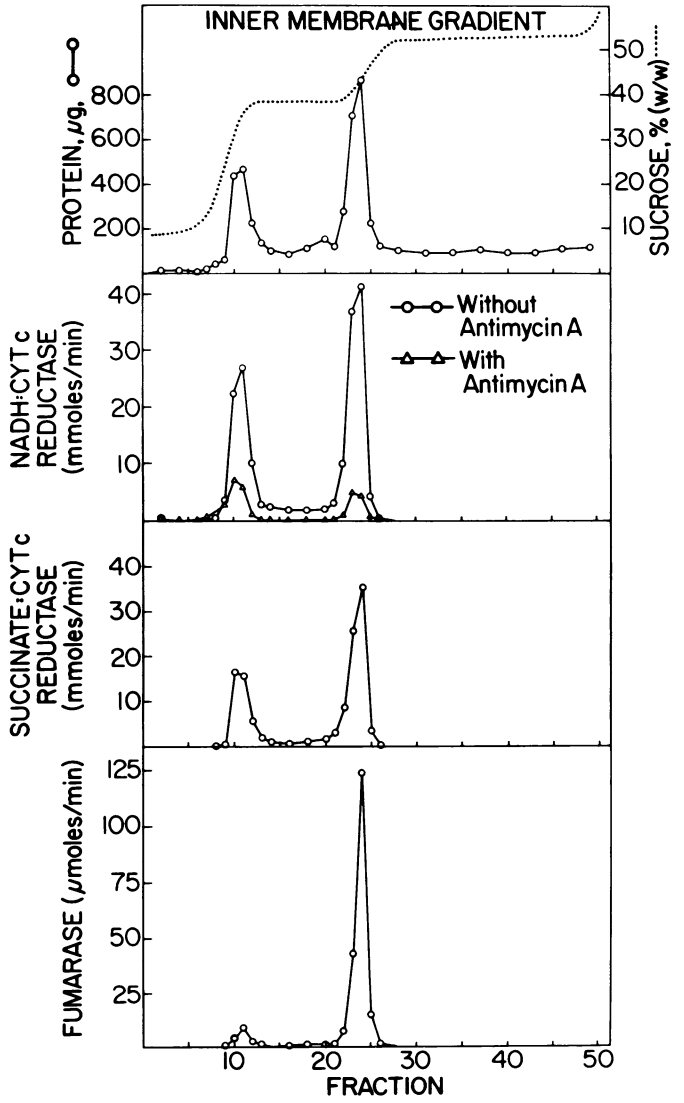

FIG. 3. Distribution of marked enzyme activities through inner membrane gradient. The inner membrane gradient derived from the submitochondrial fractionation method of Bowles *et al.* (3) was fractionated and each gradient fraction was assayed for protein content, antimycin A-sensitive and -insensitive NADH-Cyt *c* reductase, succinate-Cyt *c* reductase, and fumarase.

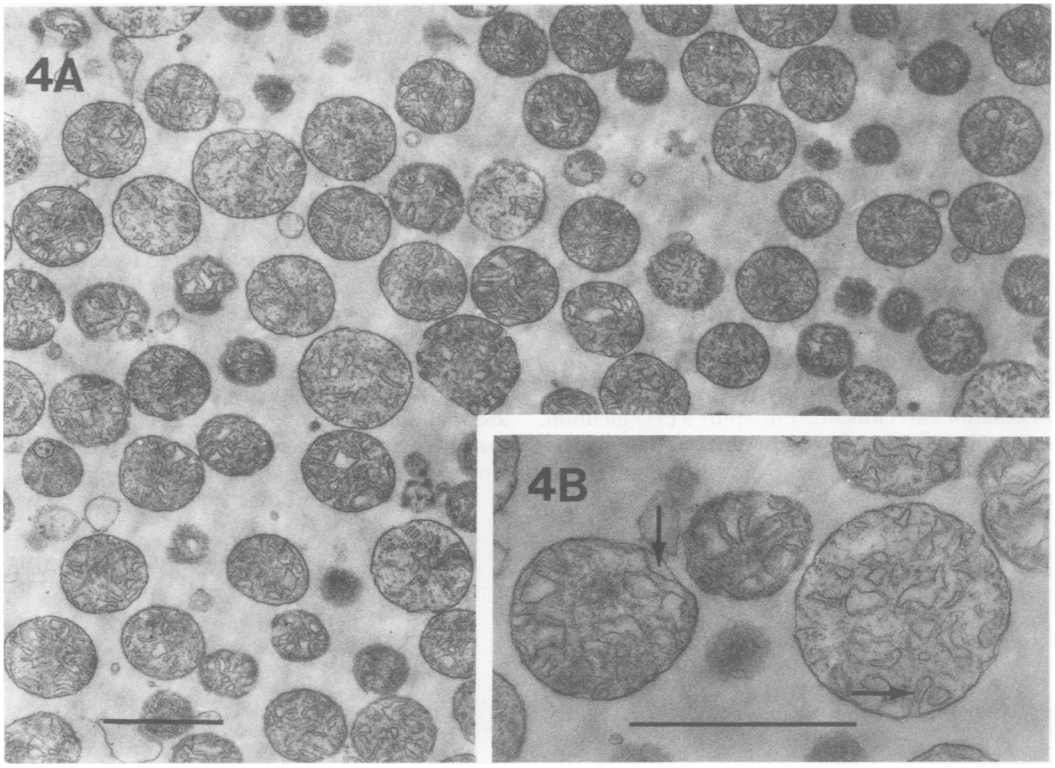

FIG. 4. Electron micrographs of intact mitochondria isolated from castor bean endosperm by the method of Lord *et al.* (22). A: low magnification of mitochondrial fraction indicating relative purity and homogeneity of the starting material; B: high magnification of mitochondrial fraction showing the presence of the characteristic double membrane and invagination of the inner membrane (arrows) indicating intactness of the mitochondria. Bars: 1.0 μm (A: × 16,225; B: × 30,250).

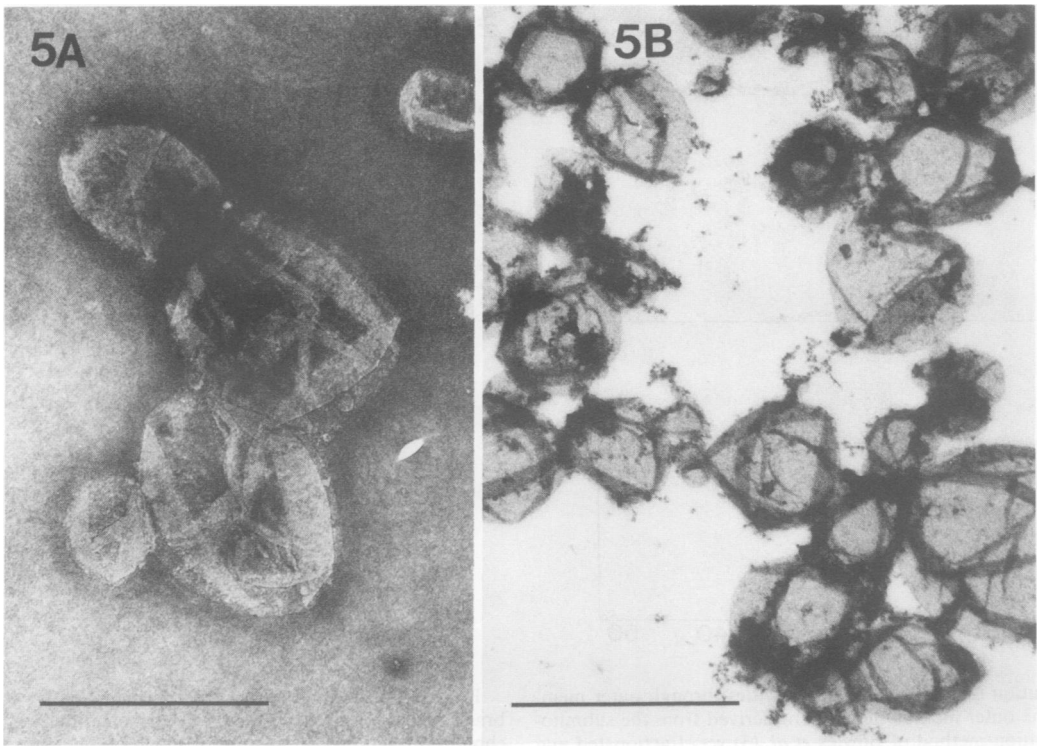

FIG. 5. Electron micrographs of outer membrane fraction occurring at 21.9% sucrose. A: Negatively stained membranes; B: unstained membranes. Note the characteristic "folded transparent membrane bag" appearance of these membranes. Bars: 1.0 μm (× 30,250).

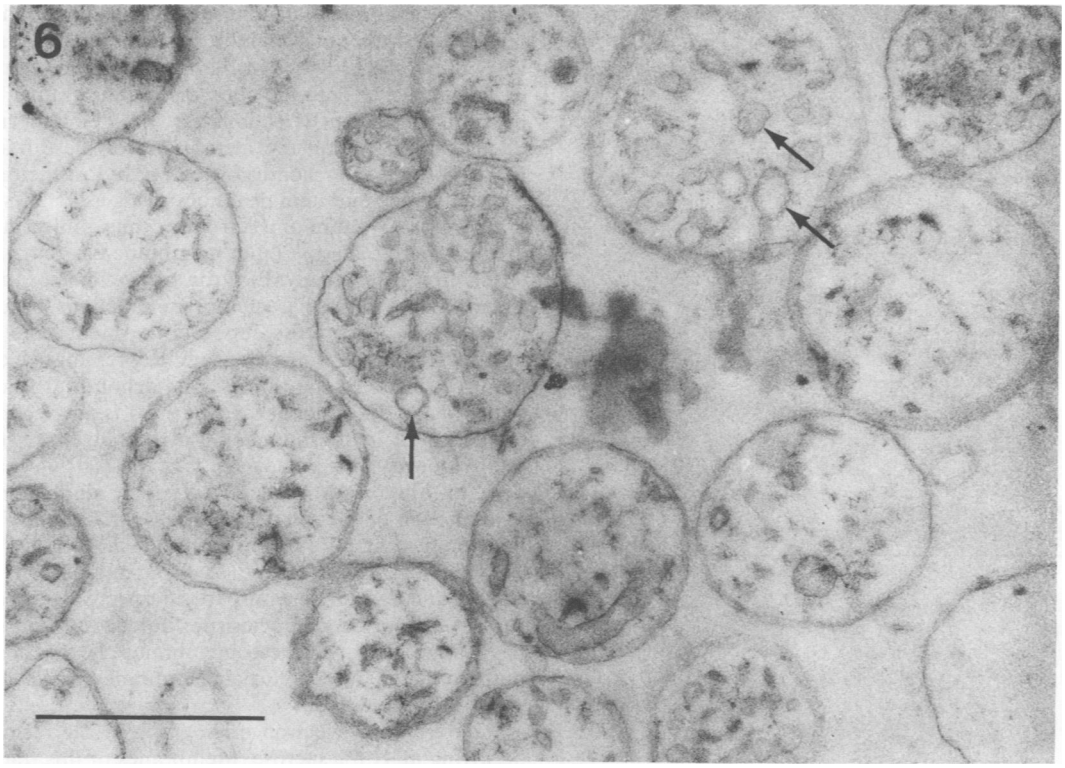

FIG. 6. Electron micrograph of membrane fraction occurring at 41.6% sucrose. These mitoplasts appear to be osmotically swollen intact inner membranes lacking outer mitochondrial membranes. In some instances (arrows) cristae appear to have broken off to form smaller inner membrane vesicles within the peripheral portion of the inner membrane. Bar: 1.0 μm (× 30,250).

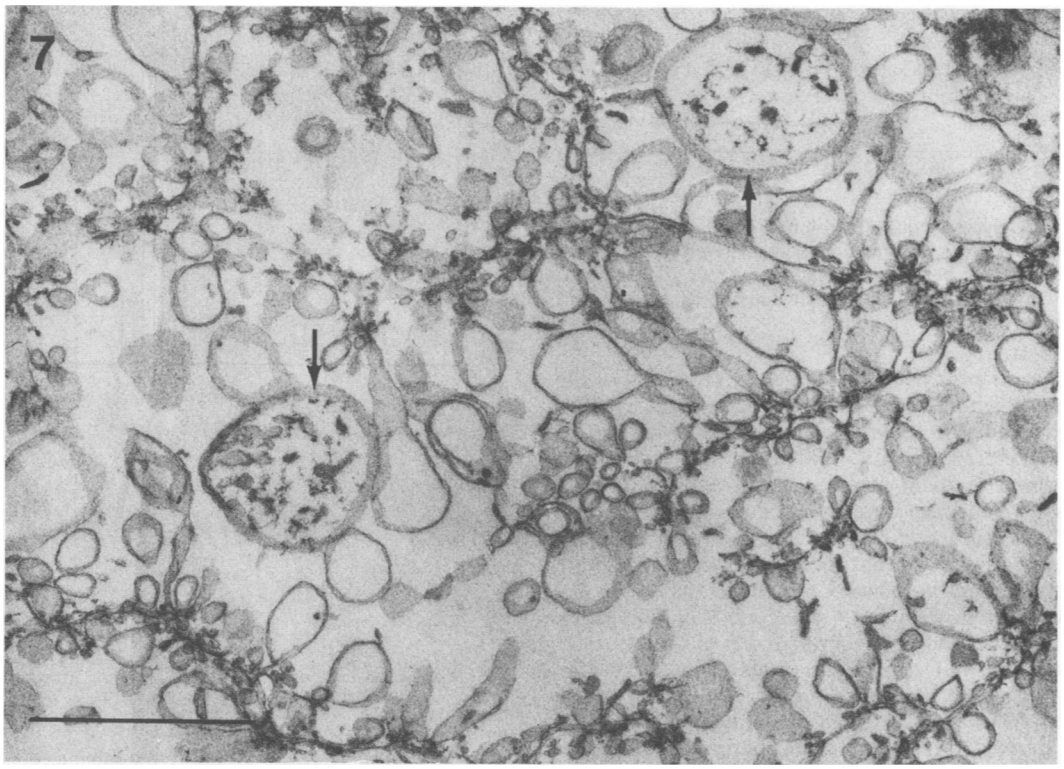

FIG. 7. Electron micrograph of membrane fraction occurring at 33.1% sucrose. This micrograph demonstrates the total disruption of conventional mitochondrial ultrastructure indicated by the presence of variously sized membrane vesicles. Arrows indicate the presence of minor contamination of this fraction by the intact inner membrane fraction. Bar: 1.0 μm (× 30,250).

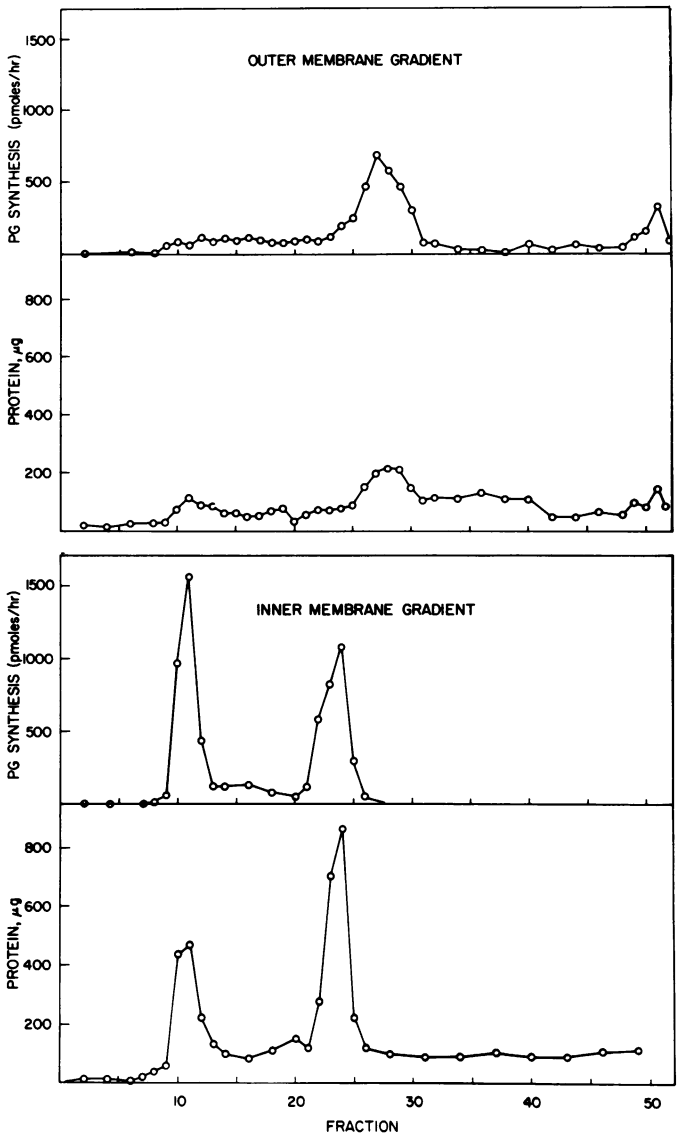

FIG. 8. Phosphatidylglycerol synthesis by submitochondrial membrane fractions. The outer and inner membrane gradients derived from the first fractionation procedure were both fractionated on sucrose gradients and each gradient was assayed for protein content and the location of and capacity for phosphatidylglycerol synthesis.

plainly evident protein peaks occur at fractions 11 and 24 of the inner membrane gradient. The presence of antimycin A-insensitive NADH-Cyt *c* reductase activity coinciding with the protein peak in fraction 11 (average density = 1.09 g/cm^3, 21.9% sucrose) of the outer membrane gradient provides evidence that this membrane fraction is derived from outer mitochondrial membranes. The absence of any succinate-Cyt *c* reductase and fumarase activities in this peak indicates that these outer membranes are free from inner membranes and matrix proteins.

The sensitivity of the NADH-Cyt *c* reductase to inhibition by antimycin A in the two remaining protein peaks of the outer membrane gradient as well as in both protein peaks (average density = 1.14 g/cm^3, 33.1% sucrose and 1.19 g/cm^3, 41.2% sucrose) of the inner membrane gradient indicates that these fractions all are derived from inner mitochondrial membranes. The coincidence of succinate-Cyt *c* reductase activity with the antimycin A-sensitive assay confirms this conclusion. In addition, the relative sensitivity of the NADH-Cyt *c* assay (72.1% inhibition in the 33.1% sucrose fraction and 80.2% inhibition in the 41.2% sucrose fraction) reveals that these inner membrane fractions are largely free from outer membranes. Finally, the absence of significant fumarase activities in the membrane fractions occurring at 33.1% sucrose in both the outer membrane gradient and the inner membrane gradient (fractions 28 and 11 of the respective gradients) indicates that the membranes of these fractions resulted from ruptured mitochondria which have lost their soluble fumarase activity. The protein peaks occurring as 41.2% sucrose in these gradients still retain this enzyme, thus indicating that in this membrane fraction the inner membrane has remained intact.

Electron Microscopy. Electron microscopic examination of the three submitochondrial membrane fractions occurring at 21.9, 33.1, and 41.2% sucrose supports the membrane identifications derived from marker enzyme data. Representative electron micrographs of each of the three submitochondrial fractions are presented in Figures 5, 6, and 7. Figure 4 is an electron micrograph of the initial intact mitochondria prior to homogenization. The relative purity and homogeneity of the starting material are evident in Figure 4A. The characteristic double membrane and invagination of the inner membrane (arrows) in Figure 4B show that the starting mitochondria are intact.

Figure 5, A and B, are electron micrographs of negatively stained and unstained membranes, respectively, occurring at 21.9% sucrose. Note the characteristic "folded transparent membrane bag" appearance of these membranes, a diagnostic feature of purified outer mitochondrial membranes prefixed in OsO_4 and negatively stained (33).

Figure 6 is an electron micrograph of the membrane fraction occurring at 41.6% sucrose. Membranes of this fraction appear to be osmotically swollen inner membranes lacking the outer mito-

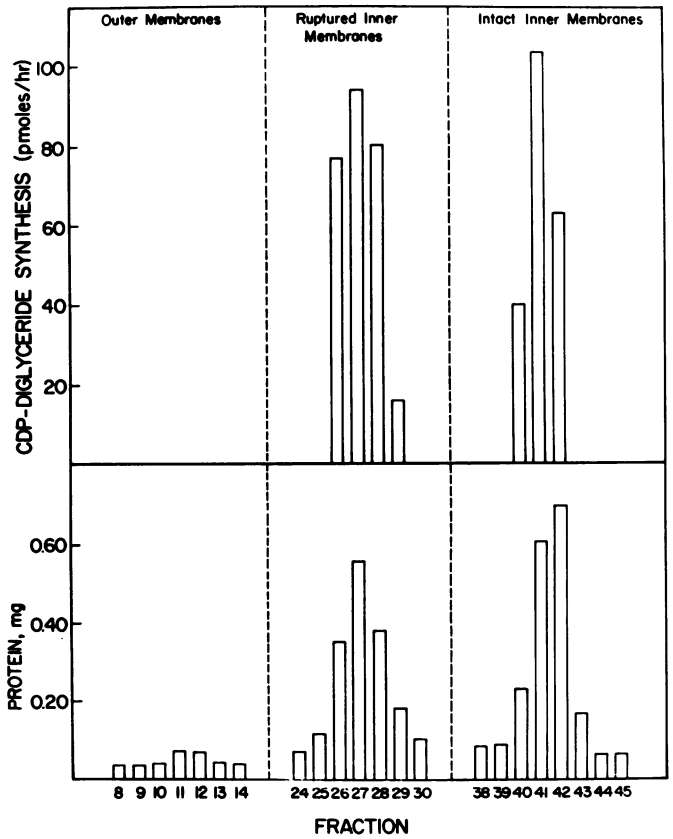

FIG. 9. CDP-Diglyceride synthesis by submitochondrial membrane fractions. A gradient obtained by the modified mitochondrial homogenization procedure in Figure 1 was fractionated and each gradient fraction was assayed for protein content and capacity to synthesize CDP-diglyceride.

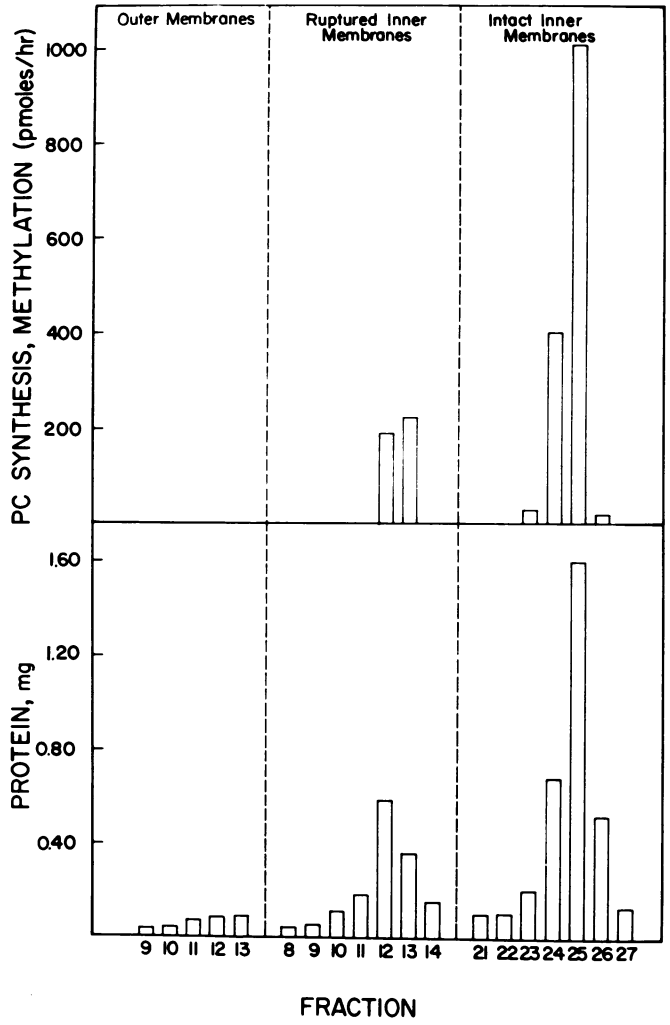

FIG. 10. Phosphatidylcholine synthesis by submitochondrial membrane fractions. A gradient obtained by the modified mitochondrial homogenization procedure in Figure 1 was fractionated and each gradient fraction was assayed for protein content and capacity to synthesize phosphatidylcholine via the methylation of phosphatidylethanolamine.

chondrial membranes. In some cases (arrows) cristae appear to have broken off to form smaller inner membrane vesicles within the peripheral portion of the inner membrane.

Finally, an electron micrograph of the submitochondrial membrane fraction occurring at 33.1% sucrose (Fig. 7) demonstrates the total disruption of conventional mitochondrial ultrastructure as seen by the presence of variously sized membrane vesicles. There is only minor contamination of this fraction by the intact inner mitochondrial membranes (arrows) which was predicted by the slight peak of fumarase activity in fraction 11 of Figure 3.

These electron micrographs, in conjunction with the marker enzyme data, provide evidence that three submitochondrial membrane fractions result from the homogenization and purification procedures discussed under "Materials and Methods." The identities of these three membrane fractions occurring at 21.9% sucrose (δ = 1.09 g/cm^3, 33.1% sucrose (δ = 1.14 g/cm^3), and 41.6% sucrose (δ = 1.19 g/cm^3) are, respectively, outer membranes, ruptured inner membranes, and intact inner membranes.

PHOSPHOLIPID SYNTHESIS

Phosphatidylglycerol. Each of the submitochondrial fractions obtained from the first purification procedure was assayed for its capacity to synthesize phosphatidylglycerol. The results of these assays on the separate inner and outer membrane gradients are shown in Figure 8. These data clearly indicate that no significant synthesis of phosphatidylglycerol occurs in the outer membrane fractions. Considerable phosphatidylglycerol-synthesizing activity does occur in both the ruptured and intact inner mitochondrial membrane fractions of both gradients.

The remainder of the phospholipid assays were performed with fractions obtained from the second, improved procedure and only those gradient fractions that contained the membrane peaks were tested.

CDP-Diglyceride. CDP-Diglyceride was found to be synthesized exclusively in the inner membrane fractions with no measurable activity occurring in the outer membrane (Fig. 9).

Phosphatidylcholine. Phosphatidylcholine synthesis via the methylation reaction also occurred only in the inner membrane fractions and not the outer membrane (Fig. 10).

Phosphatidic Acid. The synthesis of phosphatidic acid (Fig. 11) was the only phospholipid-synthesizing activity found to occur in the outer membrane fraction and this activity coincided precisely with the protein peak. This phospholipid was synthesized by both inner membrane fractions as well. The total activities in the outer and inner membranes are quite similar despite the large disparity of protein in these fractions. Another interesting phenomenon with this enzyme is the obvious skewedness of the activity peak to the low density side of the inner membrane protein peaks, with apparently a double peak of activity in both inner membrane fractions.

Recovery of Phospholipid Synthetic Activities. Appropriate assays were performed at several steps of the submitochondrial membrane preparation to measure the recovery of total protein

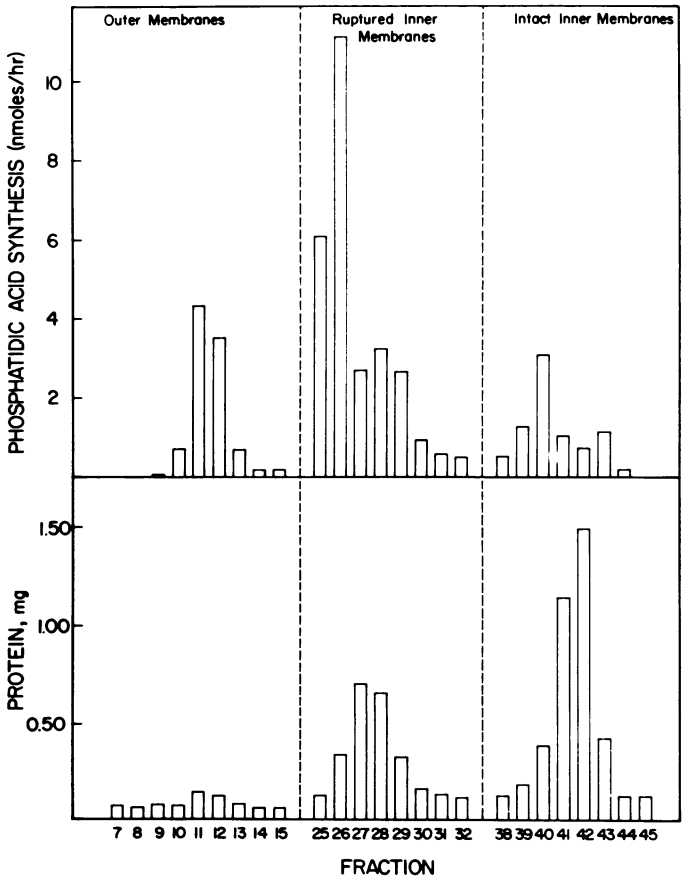

FIG. 11. Phosphatidic acid synthesis by submitochondrial membrane fractions. A gradient obtained by the modified mitochondrial homogenization procedure in Figure 1 was fractionated and each gradient fraction was assayed for its capacity to synthesize phosphatidic acid.

TABLE 1. Protein, total activity and specific activity of inner and outer mitochondrial membrane phospholipid syntheses.

SUBMITOCHONDRIAL FRACTION	PHOSPHOLIPID ASSAY											
	PHOSPHATIDYLGLYCEROL			CDP-DIGLYCERIDE			PHOSPHATIDYLCHOLINE			PHOSPHATIDIC ACID		
	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY
	mg	nmoles/ h	nmoles/ h /mg	mg	nmoles/ h	nmoles/ h /mg	mg	nmoles/ h	nmoles/ h /mg	mg	nmoles/ h	nmoles/ h /mg
INTACT MITOCHONDRIA	12.1	4421.0	364.8	10.8	0.84	0.077	12.0	264.8	22.1	14.4	867.1	60.4
	RECOVERY			RECOVERY			RECOVERY			RECOVERY		
		%			%			%			%	
INITIAL MITOCHONDRIAL VALUES	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
MITOCHONDRIAL HOMOGENATE SUPERNATANT	11.0	0.0	0.0	3.9	0.0	0.0	3.0	0.0	0.0	3.0	0.0	0.0
MITOCHONDRIAL HOMOGENATE PELLET	43.7	62.4	142.7	65.4	785.9	1225.7	53.7	23.7	44.2	60.1	74.5	123.8
OUTER MEMBRANE	2.8	0.1	5.1	2.6	2.2	82.7	0.9	0.0	0.0	1.5	1.1	71.4
RUPTURED INNER MEMBRANE	7.7	10.8	141.0	4.7	227.4	4794.0	2.8	0.7	26.3	5.8	3.3	56.4
INTACT INNER MEMBRANE	25.8	29.3	113.4	21.1	1561.2	7394.2	24.0	4.4	18.5	29.2	1.6	7.2
TOTAL RECOVERY	36.3	40.2		28.4	1790.8		27.7	5.1		36.5	6.0	

and total and specific activities of each of the phospholipid-synthesizing enzymes. Only 27 to 36% of the total mitochondrial protein was recovered in the various purifications, but the patterns of protein recovery were consistent (Table I).

Recovery of the total activity for phosphatidylglycerol synthesis generally paralleled the protein recovery, the specific activity increasing slightly. This enzyme appeared to be relatively stable throughout the purification procedure. The increase in specific activity suggests an increased substrate permeability and/or some degree of purification of the enzyme by this procedure.

The total and specific activities for CDP-diglyceride synthesis showed 1,500 and 7,400-fold increases, respectively, over those of the intact mitochondria. These data were not unexpected in light of the fact that intact mitochondria have been reported to be relatively impermeable to all but the adenine-containing nucleotides (19). Coincubation of the homogenate pellet with the mitochondrial homogenate supernatant had no effect on the activity.

The enzymes synthesizing phosphatidic acid and phosphatidylcholine via methylation of phosphatidylethanolamine appear somewhat sensitive to handling as evidenced by the decreased specific activities. The mitochondrial homogenate supernatant had no effect on either enzyme in the resuspended mitochondrial homogenate pellet.

DISCUSSION

Until the present investigation there have been only two reports unequivocally demonstrating phospholipid biosynthesis by mitochondria isolated from castor bean endosperm tissue. Moore (30) showed that mitochondria from the castor bean system could synthesize phosphatidylglycerol and later (31) reported for the first time in any system that these mitochondria also had the capacity to synthesize phosphatidylcholine via the methylation of phosphatidylethanolamine. Vick and Beevers (42) detected minor phosphatidic acid synthesis in these organelles, but attributed this activity to contamination by ER, concluding that the ER most likely was the exclusive site of synthesis of this phospholipid. By far the majority of the works involving phospholipid metabolism in castor bean endosperm report that mitochondria from this tissue are deficient in other phospholipid-synthesizing activities. Castor bean mitochondria were reported not to synthesize phosphatidylcholine via phosphorylcholine-glyceride transferase (22, 23, 31), phosphatidylinositol via CDP-diglyceride-inositol transferase, phosphatidylserine via the exchange reaction (phosphatidylethanolamine-L-serine transferase), or diglyceride via phosphatidic acid phosphatase (32). Instead, these synthetic activities were confined to the ER. The present report clearly confirms the capacity of these mitochondria to synthesize phosphatidylglycerol and phosphatidylcholine by methylation of phosphatidylethanolamine. In addition, evidence for CDP-diglyceride synthesis in the mitochondria as well as an unequivocable demonstration of phosphatidic acid synthesis in that organelle have been presented. This is not an exhaustive search for such enzymes and others, such as the enzyme involved in cardiolipin synthesis, probably do exist in these mitochondria.

The results presented here indicate that the syntheses of CDP-diglyceride, phosphatidylcholine via methylation and phosphatidylglycerol are exclusively associated with the inner mitochondrial membrane. The synthesis of phosphatidic acid was found to occur equally well in both the inner and outer mitochondrial membranes. The few similar studies with mammalian systems, described in more detail in the introductory section, are confined to rat liver tissues and compare variously with the castor bean system. Hostetler and Van Den Bosch (16) showed that the synthesis of phosphatidylglycerol and CDP-diglyceride occurred primarily on the inner mitochondrial membrane with minor syntheses occurring on the outer membrane. Cardiolipin was synthesized on the inner membrane exclusively (16). Phosphatidic acid synthesis was localized primarily on the outer membrane of mitochondria from rat liver (35, 36, 43). The single related work with plants indicated that CDP-diglyceride was synthesized only on the inner membrane of mitochondria from mung bean hypocotyls (13). In light of the quite different systems investigated and our relatively incomplete understanding of the compartmentation of phospholipid synthesis and utilization, it is premature to speculate on the apparent differences between the castor bean and other systems.

The double peaks of phosphatidic acid synthetic activity in both inner membrane fractions (Fig. 11), with major peaks of activity skewed to the less dense portions of these protein peaks, are interesting. Latency, or poor substrate permeability, may explain these results since the activity of the intact inner membranes is considerably less than that of the ruptured inner membranes, despite a greater amount of the membrane protein being present in the former fraction. The light peak of activity in the intact membrane fraction might be due to the presence of some partially ruptured membranes. The degree of breakage or number of right-side-out *versus* wrong-side-out membranes may be involved in skewed activity in the ruptured inner membrane fraction. ER from castor bean tissue, which has a density similar to that of the ruptured inner membrane fraction, does not contribute to this activity since similar skewed activity peaks would be expected for the other phospholipids assayed which are synthesized by both the ER and the mitochondria of this tissue (30, 31). Also, one would not expect this organelle to contaminate the more dense inner membrane fraction. One other possible explanation for this skewed activity is that subfractions of the inner membrane exist, one or more of which contains this enzyme. Further investigations are underway to examine these possibilities.

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