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Isolation of Rat Liver Mitochondrial Membrane Fractions and Localization of the Phospholipase A*

Moseley Waite

ABSTRACT: Mitochondria were isolated by sucrose density gradient centrifugation from livers of rats previously injected with [^{14}C]ethanolamine, which was incorporated into the membrane phosphatidylethanolamine. Marker enzymes showed that these preparations were contaminated by no more than 1% of the microsomes and about 2% of the lysosomes present in the homogenate. Mitochondrial membranes were isolated either by osmotic shock or by incubation with Ca^{2+}, followed by density gradient centrifugation. Osmotic shock appeared to cause 75–85% of the outer membrane to be stripped from the mitochondria with little inner membrane contamination. Up to 40% of the matrix enzymes were solubilized under these conditions. Incubation of mitochondria with Ca^{2+} apparently caused cross-contamination of the membrane fractions and solubilized 85–95% of the matrix enzymes. Mitochondrial phospholipase A catalyzed the hydrolysis of the outer membrane phosphatidylethanolamine mainly. Comparison of the specific activity of the phospholipase A in the outer and inner membrane with that of outer membrane marker enzyme levels raises the possibility that some phospholipase A might be associated with the inner membrane however. The specific activity (disintegrations per minute per micromole of phosphorus) of the phosphatidylethanolamine only changed in the inner membrane during incubation which suggests that not all of the inner membrane phospholipid was hydrolyzed at the same rate.

Recent work (Bjørnstad, 1966; Waite *et al.*, 1969a) demonstrated that rat liver mitochondria contain a phospholipase A which hydrolyzes both endogeneous and added phospholipids. It was shown that there is a relation between the hydrolysis of mitochondrial phospholipid and certain types of mitochondrial swelling (Waite and van Golde, 1968; Waite *et al.*, 1969b), substantiating the earlier proposal of Wojtczak and Lehninger (1961). Addition of either fatty acid or Ca^{2+} to mitochondria stimulated phospholipase A activity and swelling. EDTA, however, inhibited both swelling and phospholipase A activity. A recent paper by Nachbaur and Vignais (1968) provided evidence that the outer membrane fraction of rat liver mitochondria contained a phospholipase A capable of hydrolyzing added phosphatidylethanolamine. The phospholipase A reported by them appears to be the same as that studied by Bjørnstad (1966) and Waite *et al.* (1969a,b) and that described here. In the present extension of these studies mitochondrial membranes were disrupted and separated in order to determine the phospholipase A activity in each membrane fraction.

Several conflicting reports have appeared regarding the character of mitochondrial fractions based on marker enzyme studies. Allmann *et al.* (1968) used snake venom phospholipase A or high levels of oleic acid to isolate membrane fractions. In contrast, Sottocase *et al.* (1967a,b), Parsons *et al.* (1966, 1967), and Schnaitman and Greenawalt (1968) have used either mechanical means such as osmotic shock and sonication or detergents to remove the outer membranes from mitochondria. In the present studies experiments using both these approaches for the separation and characterization of membranes were compared. This comparison has led to the conclusion that the distribution of the marker enzymes studied is drastically changed under conditions leading to phospholipid hydrolysis.

Experimental Procedures

Male Wistar rats were injected with 20 μCi of [^{14}C]ethanolamine (specific activity 3.7 mCi/mmole) in 50% ethanol (Bjørn-

* From the Department of Biochemistry, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103. *Received February 13, 1969.* Supported by grants from the American Heart Association, sponsored in part by the North Carolina Heart Association, and by U. S. Public Health Service Grant AM-11799 from the National Institutes of Health.

stad, 1966). After 90 min they were killed, the livers removed and homogenized in 0.25 M sucrose with 1.0 mM EDTA, and the mitochondria isolated according to a modification (Waite *et al.*, 1969b) of the procedure described by Parsons *et al.* (1967) described earlier. M-I refers to the sediment obtained between 10,000 and 45,000*g* (min) and M-II refers to the mitochondrial preparation after sucrose density gradient centrifugation. The M-II after sucrose gradient centrifugation was diluted twofold with cold H_2O and centrifuged 250,000*g* (min) to remove the sucrose.

For osmotic swelling and contraction the washed M-II was resuspended in 0.01 M Tris (pH 7.4), allowed to remain swollen for 5–20 min at 0°, and then contracted by the addition of a 1.4 M sucrose solution equal to one-half the initial volume of the M-II suspension. After 2–3 min 4.0 ml of the contracted M-II was sonicated for 10 sec with a Branson Sonifier at the lowest power setting using a microtip in a water-jacketed polyethylene tube (15 × 75 mm) maintained at 0° (Sottocasa *et al.*, 1967b). This treatment of mitochondria is referred to here as osmotic shock. Studies on hydrolysis of phospholipid were done in washed M-II suspended in 0.125 M glycylglycine (pH 8.2) containing 1.0 mM $CaCl_2$. The M-II then was incubated at 37° for the indicated periods of time, followed by the addition of sucrose to give a final concentration of 0.5 M and EDTA to a concentration of 2.0 mM. The preparation was then sonicated as before. Preparations treated by these procedures were centrifuged on a discontinuous sucrose density gradient for 2.5 hr in a Spinco SW25 rotor at 25,000 rpm. The gradient consisted of 8.0 ml of 1.4 M sucrose on the bottom, 6.0 ml of 0.7 M sucrose in the middle, and 7.0 ml of the M-II suspension on top (G. L. Sottocasa, personal communication). After centrifugation the I fraction was obtained from the bottom of the tube, the O fraction at the 1.4–0.7 M sucrose interface, and the soluble fraction from above the 0.7 M sucrose.

Determination of the hydrolysis of [^{14}C]phosphatidylethanolamine was done by extraction of the reaction products as described earlier (Waite and van Deenen, 1967) followed by chromatography on thin-layer chromatographic plates (silica gel G) in the chloroform–methanol–H_2O–NH_4OH system (65:35:3:4, v/v). The silica gel containing the radioactive compounds was scraped into scintillation vials containing the Omnifluor scintillation mixture in toluene and a thixotropic gel to suspend the silica gel.

The enzymes used as markers were assayed according to established procedures. Cytochrome oxidase (EC 1.9.3.1), rotenone-insensitive DPNH–cytochrome C reductase (EC 1.6.99.3), and TPNH–cytochrome C reductase (EC 1.6.99.1) were assayed spectrophotometrically according to Sottocasa *et al.* (1967b). Glucose 6-phosphatase (EC 3.1.3.9) and acid phosphatase (EC 3.1.3.2) were assayed as described by de Duve *et al.* (1955) and by Gianetto and deDuve (1955), respectively. Malate dehydrogenase (EC 1.1.1.37) was assayed by the procedure of Ochoa (1955) and α-ketoglutarate dehydrogenase (EC 1.2.4.2) by the procedure of Allmann *et al.* (1968). Monoamine oxidase (EC 1.4.3.4) was assayed using [^{14}C]tyramine as described by McCaman *et al.* (1965) to circumvent problems arising using a spectrophotometric assay due to the further oxidation of the aldehyde (Allmann *et al.*, 1968). This method was modified so that the final volume of the incubation mixture was 1.0 ml.

Quantitation of phospholipid phosphorus was done using the method of Parker and Peterson (1965) and protein determinations by the procedure of Lowry *et al.* (1951).

[1,2-^{14}C]Ethanolamine and [1-^{14}C]tyramine and Omnifluor, the fluor used for scintillation counting, were purchased from New England Nuclear Corp., Boston. All other compounds were obtained from Sigma, St. Louis.

Results

Mitochondrial, lysosomal, and microsomal marker enzymes were compared in the rat liver homogenate and in mitochondria preparations at two stages of purification. Table I shows that mitochondrial preparations at the first stage (M-I) contain about 2–4% of the homogenate microsomal activity as measured by their TPNH–cytochrome C reductase and glucose 6-phosphatase content. This was decreased five- to sixfold by purification of the mitochondria using sucrose density gradient centrifugation to yield M-II. Acid phosphatase activity in the M-I was nearly 20% of that in the homogenate which indicated considerable contamination of M-I by lysosomes. This was reduced tenfold by the gradient centrifugation. 40% of the original cytochrome oxidase was present in the M-I and M-II preparations. Rotenone-insensitive DPNH–cytochrome C reductase, known to be microsomal as well as mitochondrial (Sottocasa *et al.*, 1965a,b) was not localized in the M-I fraction to the same extent as cytochrome oxidase. Also, its total activity decreased by a third on gradient centrifugation apparently due to the removal of some microsomal contamination. This decrease was considerably less than that found with microsomal marker enzymes which were lowered five- to sixfold. Twenty per cent of the malate dehydrogenase which has been found in the cytosol as well as the mitochondria (Christie and Judah, 1954) was in the M-I fraction. After gradient centrifugation there was an increase of the malate dehydrogenase specific activity even though the total activity decreased slightly.

M-II from the livers of rats previously injected with [^{14}C]-ethanolamine, which became incorporated in phosphatidylethanolamine, were subjected to osmotic shock and sonication, or were incubated with Ca^{2+} followed by sonication. Table IIa shows that most of the untreated M-II were intact based on the observation that 85–90% of the protein and lipid was in the I fraction which contained intact mitochondria as well as inner membranes and matrix enzymes. Osmotic shock and sonication solubilized one-third of the protein and caused an approximately equal distribution of the lipid content between I fraction and the O fraction. The lipid to protein ratio in the O fraction was 0.50–0.65 μmole/mg whereas the ratio of the I fraction was generally around 0.20. Incubation of the mitochondria for 45 min solubilized about two-thirds of the protein. About 25% of the protein and 70% of the lipid was in the O fraction which suggests that much of the outer membrane had been stripped from the mitochondria.

Five marker enzymes were assayed in these mitochondrial fractions (Table IIb). In the control 85–95% of the enzyme activities are found with the I fraction containing intact mitochondria. The outer membrane was detached from about 15% of the mitochondria, based on the distribution of the marker enzymes rotenone-insensitive DPNH–cytochrome C reductase and monoamine oxidase. Osmotic shock and sonication caused 75–85% of the outer membranes of this preparation to be freed from the mitochondria as shown by the amount of

TABLE I: Marker Enzyme Characterization of Mitochondria.[a]

	TPNH Cytochrome C Reductase		Glucose 6-Phosphatase		Acid Phosphatase		Cytochrome Oxidase		Rotenone-Insensitive DPNH–Cytochrome C Reductase		Malate Dehydrogenase	
	Sp Act.	Per cent	Sp Act.	Per cent	Sp Act.	Per cent	Sp Act.	Per cent	Sp Act.	Per cent	Sp Act.	Per cent
Homogenate	0.038	100	0.254	100	0.18	100	0.39	100	0.31	100	1.6	100
M-I	0.004	1.5	0.08	4.3	0.25	19	1.11	39	0.20	9.0	2.5	22
M-II	0.001	0.3	0.02	0.7	0.03	1.8	1.70	40	0.18	5.6	3.1	18

[a] Enzymes were assayed by the procedures listed in Experimental Procedures. The specific activities are expressed as micromoles of product formed per milligram of protein per minute. Per cent activity values are calculated as the total activity in the fraction divided by that total activity in the homogenate times 100. M-I and M-II refer to the mitochondria at two stages of isolation.

TABLE II: Separation of Outer and Inner Membranes by Osmotic Shock and by Incubation with $CaCl_2$.

Treatment	Fraction	Protein (A)		Lipid-Extractable Phosphorus (B)		μmoles of Phospholipid (B)/mg of Protein (A)
		mg	Per cent	μmoles	Per cent	
A. Characterization of Isolated M-II Fractions by Protein and by Lipid Phosphorus[a]						
None	S	2.8	7.2	0.33	4.3	0.12
	O	1.3	3.4	0.82	10.6	0.63
	I	34.5	89.4	6.55	85.1	0.19
Osmotic shock	S	11.2	32.9	0.53	7.2	0.05
	O	5.5	16.2	3.06	41.4	0.56
	I	17.3	50.9	3.80	51.4	0.22
Incubation	S	25.4	65.3	0.78	9.5	0.03
	O	8.9	22.9	5.75	70.7	0.65
	I	4.6	11.8	1.60	19.8	0.35

		Enzyme									
		Malate Dehydrogenase		α-Ketoglutarate Dehydrogenase		Rotenone-Insensitive DPNH–cytochrome C Reductase		Monoamine Oxidase		Cytochrome Oxidase	
				Sp Act. (× 10^2)	Per cent	Sp Act. (× 10^2)	Per cent	Sp Act.	Per cent	Sp Act. (× 10^2)	Per cent
B. Characterization by Marker Enzymes[b]											
None	S	4.5	6.7	3.0	5	0.5	0.4		0		0
	O	1.6	1.1		0	54	14.9	68.3	16.6	8.7	4.2
	I	5.1	92.2	4.6	95	12	84.7	11.5	83.4	7.4	95.8
Osmotic shock	S	5.3	40.1	2.3	16	0.4	1.0	4.8	10.6	1.1	5.7
	O	2.6	9.4		0	66	86.3	70.7	75.8	6.6	16.9
	I	4.3	50.5	7.6	84	3.2	12.7	4.0	13.6	9.6	77.4
Incubation	S	5.4	84.3	12.9	97	1.6	10.8	2.3	10.7	1.3	18.7
	O	1.1	6.0		0	26	58.5	34.7	56.2	10.5	51.0
	I	3.4	9.7	1.8	3	27	30.7	39.6	33.0	12.0	30.3

TABLE II (*Continued*)

		[^{14}C]Monoacyl-glycerophos-phorylethanol-amine (cpm)	Phosphatidyl-ethanolamine (cpm)	% Hydrolysis	% of Total Monoacyl-glycerophos-phoryl-ethanolamine	Cpm of Monoacyl-glycero phosphoryl-ethanolamine mg of Protein
		C. Hydrolysis of Membrane [^{14}C] Phosphatidylethanolamine[c]				
None	S	46	1,812	2.5		
	O	189	8,428	2.2		
	I	1,040	68,343	1.5		
Osmotic shock	S	162	7,181	2.2		
	O	743	38,384	1.9		
	I	587	32,053	1.8		
Incubated	S	694	7,450	8.5	9.1	11
	O	4,913	43,328	10.4	64.1	814
	I	2,059	13,080	13.6	26.8	174

[a] Osmotic shocking of M-II was done by swelling and shrinking followed by sonication as described in Experimental Procedures. Incubation of M-II was run for 45 min at 37° followed by addition of sucrose and EDTA, and sonication. The control had the sucrose and EDTA added and was not sonicated. Separation of the fraction was described in Experimental Procedures. Percentage values were calculated as the amount protein or phospholipid recovered in the fraction divided by total protein or phospholipid recovered times 100. [b] Enzyme assays were done as outlined in Experimental Procedures on the fractions obtained in A part of the experiment. Specific activities are expressed as micromoles of product formed per milligram of protein per minute with the exception of MAO which is per 20-min incubation period. Percentage values were calculated in the same manner as protein and phospholipid percentages. [c] [^{14}C]Monoacylglycerophosphoryl and [^{14}C]Phosphatidylethanolamine were determined in the fractions obtained in the A part of the experiment using the thin-layer chromatography and scintillation counting as outlined in Experimental Procedures. Per cent hydrolysis was calculated as the [^{14}C]monoacylglycerophosphoryl counts per minute divided by the sum of [^{14}C]Phosphatidylethanolamine counts per minute times 100. Per cent [^{14}C]monoacylglycerophosphoryl of total monoacylglycerophosphoryl in each fraction was calculated as the counts per minute of [^{14}C]monoacylglycerophosphoryl in the fraction divided by the sum of the [^{14}C]monoacylglycerophosphorylethanolamine counts per minute in the three fractions times 100.

rotenone-insensitive DPNH–cytochrome C reductase and monamine oxidase in the O fraction. The two dehydrogenases were distributed between the S and I fractions under these conditions. More malate dehydrogenase was solubilized than α-ketoglutarate dehydrogenase generally. Solubilization of the matrix enzymes never exceeded 50%, although longer periods in the swollen state favor their release.

Incubation of the mitochondria with Ca^{2+} for 45 min solubilized almost all of the two dehydrogenases. The rotenone-insensitive DPNH–cytochrome C reductase and monoamine oxidase had nearly the same specific activity in the two membrane fractions, unlike their distribution after osmotic shock. Similarly, the cytochrome oxidase was almost equally distributed between O and I fractions. These findings indicate that the apparent localization of the marker enzymes is primarily dependent upon the method used for separation of the membranes rather than being the result of contamination by other subcellular fractions. Total recovery of enzyme activities in M-II fractions usually agreed within 10% although long incubations caused a decrease in cytochrome oxidase activity.

The amount of [^{14}C]phosphatidylethanolamine and [^{14}C]-monoacylglycerophosphorylethanolamine in fractions from M-II is presented in Table IIc. About 12% of the [^{14}C]phosphatidylethanolamine was hydrolyzed during the incubation, roughly two-thirds of the product [14C]monoacylglycerophosphorylethanolamine being in the O fraction. The I fraction had the highest percentage of the [^{14}C]phospholipid as [^{14}C]monoacylglycerophosphorylethanolamine even though the highest specific activity (counts per minute of monoacylglycerophosphorylethanolamine per milligram of protein) was in the O fraction. In a similar experiment it was found that a 10-min incubation period was sufficient to solubilize about 60% of the two dehydrogenases and to cause 40% of the cytochrome oxidase to be recovered in the O fraction. These conditions gave rise to about a 4–5% hydrolysis of the [^{14}C]phosphatidylethanolamine.

Even though the foregoing experiment suggests that the main site of [^{14}C]phosphatidylethanolamine hydrolysis was in the outer membrane, it was possible that these observations were due in part to cross-contamination of the membrane fractions during incubation. To test this possibility phospholipase A activity was determined on membranes separated by osmotic shock and sonication and isolated by gradient cen-

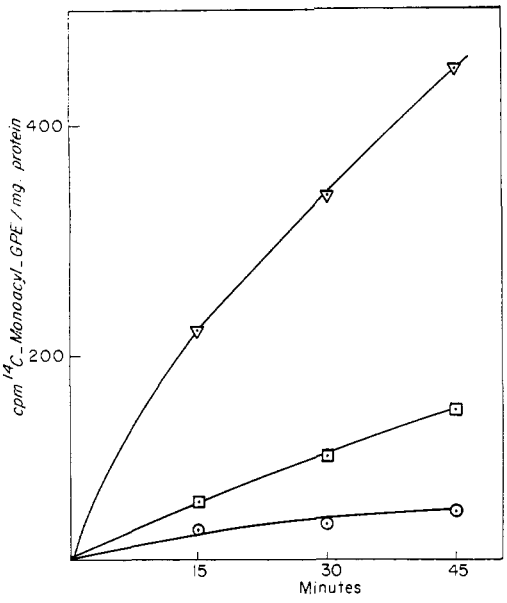

FIGURE 1: The S, O, and I fractions were separated by osmotic shock and sonication followed by sucrose density gradient centrifugation as described in Experimental Procedures. Samples of these three fractions suspended in 0.05 M glycylglycine buffer (pH 8.2) containing 2.0 mM $CaCl_2$ were incubated for the indicated times. Determination of [^{14}C]phosphatidylethanelamine hydrolysis is described in Experimental Procedures. The S fraction (○) had 2.82 mg of protein and 1630 cpm/incubation, the O fraction (▽) had 0.75 mg of protein and 3090 cpm, and the I fraction (□) had 7.85 mg of protein and 18,300 cpm. All values were corrected for zero-time controls.

TABLE III: Ratio of Membranous Marker Enzymes.[a]

Enzyme	Sp Act. O	Sp Act. I	Ratio (O/I)
Cytochrome oxidase (× 10)	5.6	11.2	0.50
Rotenone-insensitive-DPNH–cytochrome C reductase	58.7	5.1	11.5
Monoamine oxidase	67.3	4.9	13.7
Phospholipase A	464	149	3.1

[a] Enzyme specific activities were determined on membrane fractions separated by osmotic shock and sonication and are expressed in the same manner as those of Table II. PLA activity is expressed as counts per minute of [^{14}C]monoacylglycerophosphorylethanolamine formed per milligram of protein per 45-min incubation. The ratio of the phospholipase A specific activities at 15 min is 3.5 and at 30 min is 3.2. These data are the average of six experiments.

trifugation. These fractions were incubated for various time intervals to determine the hydrolysis of endogeneous [^{14}C]-phosphatidylethanolamine in each fraction. The results presented in Figure 1 demonstrate that the outer membrane fraction had the highest specific activity in these isolated fractions. Samples incubated 45 min underwent a 14% hydrolysis in the O fraction, 11% in the S fraction, and 8% in the I fraction.

If the phospholipase A were located only in the outer membrane obtained by osmotic shock, the ratio of its activities in the O and I fractions should be the same as those of the marker enzymes, rotenone-insensitive DPNH–cytochrome C reductase and monoamine oxidase. In six experiments the phospholipase A ratio was always lower than that of either of the two outer membrane enzymes but considerably higher than the ratio of cytochrome oxidase activities (Table III). These results do not support the possibility that the phospholipase A is located solely in the outer membrane.

Previously it was shown that not all the phosphatidylethanolamine in mitochondria was hydrolyzed at the same rate (Waite *et al.*, 1969b). It was suggested that this could be due to substrate specificity based on the fatty acids of the phosphatidylethanolamine or due to phosphatidylethanolamine hydrolysis occurring mainly in one membrane fraction, as shown here. Experiments were done to see if this preferential hydrolysis of the outer membrane could account for the earlier observations. After incubation of the membrane fractions and chromatography of the lipid extracts, [^{14}C]phosphatidylethanolamine and [^{14}C]monoacylglycerophosphorylethanolamine were eluted from the silica gel and the ^{14}C and phosphorus content were determined. Table IV shows that the specific activity of the isolated phosphatidylethanolamine and monoacylglycerophosphorylethanolamine in the O fraction were nearly equal after incubation. When the per cent hydrolysis was calculated from these data 17% of the radioactivity and 17% of the phosphorus was in monoacylglycerophosphorylethanolamine. The I fraction monoacylglycerophosphorylethanolamine specific activity (45,000) was considerably higher than that of the phosphatidylethanolamine both before (35,000) and after (31,000) incubation. This amounts to a 12% hydrolysis determined by radioactivity and a 9% hydrolysis based on phosphorus determinations. In these preparations the [^{14}C]phosphatidylethanolamine was about equally divided between the I and O fractions, whereas the distribution of the outer membrane marker enzymes indicated that only 25% of the outer membrane remained in the I fraction. These results would seem to eliminate the possibility that the [^{14}C]-phosphatidylethanolamine found in the I fraction was contaminated by the outer membrane.

Discussion

The results presented here demonstrate that M-II preparations used for these studies contained almost no lysosomal or microsomal contamination, as determined by marker enzymes. After sucrose density gradient centrifugation less than 1% of the original glucose 6-phosphatase and TPNH–cytochrome C reductase activity could be accounted for in the M-II. About 5% of the rotenone-insensitive DPNH–cytochrome C reductase was still associated with the M-II; 40% of the original cytochrome oxidase activity was recovered in the M-II which might be an index of the the recovery of mitochondria from from the original homogenate.

Allmann *et al.* (1968) have claimed that the rotenone-insensitive DPNH–cytochrome C reductase in mitochondrial preparations is due to microsomal contamination of mitochondrial preparations and that monoamine oxidase is localized in the inner membrane. Osmotic shock of M-II caused 75–85% of the rotenone-insensitive DPNH–cytochrome C

TABLE IV: Specific Activity of [^{14}C]Phosphatidylethanolamine in Membrane Fractions.

Time (min)	Fraction	Monoacylglycerophosphorylethanolamine			Phosphatidylethanolamine		
		Counts per minute	μmoles of P	Counts per minute/ μmole	Counts per minute	μmoles of P	Counts per minute/ μmole
0	O				9,500	0.222	43,000
	I				11,200	0.320	35,000
60	O	1,900	0.043	44,000	9,000	0.207	43,000
	I	1,100	0.024	45,000	8,000	0.254	31,000

[a] The phospholipid of O and I fractions at zero time and after 60-min incubation was extracted and chromatographed on thin-layer chromatography plates as described in Experimental Procedures. The silica gel containing monoacylglycerophosphorylethanolamine and phosphatidylethanolamine was scraped from the plates. The phospholipid was eluted from the silica gel with methanol. Phospholipid phosphorus, P, and radioactivity were determined on aliquots of this extract.

reductase and monoamine oxidase to be recovered in the O fraction, in agreement with the results of Schnaitman and Greenawalt (1968). Only about 15% of these enzymes was in the O fraction of the untreated control. These results do not support the suggestion of Allmann *et al.* because it would seem highly improbable that enzymes from contaminating microsomes and from the inner mitochondrial membrane would behave in a similar manner under the conditions used here.

Incubation of the mitochondria which led to hydrolysis of membrane phospholipid caused much of the two outer membrane enzymes to remain in the I fraction. The I fraction obtained by this procedure had a higher rotenone-insensitive DPNH–cytochrome C reductase and monoamine oxidase specific activity than that in the control or in the I fraction obtained by osmotic shock. The protein and lipid content of the incubated I fraction was the lowest of the three, however. This, plus the observation that about half of the cytochrome oxidase was recovered in the O fraction indicates that incubation led to degradation of the membrane structures.

The difference in the results obtained using the two approaches reported here points out the importance of the method used to separate and isolate membranes from mitochondria. Osmotic shock of mitochondria allows separation of the membrane fraction with little contamination of the outer by the inner membrane. Under conditions leading to hydrolysis of phospholipid, this separation is no longer achieved. It has been postulated by Green and Perdue (1966) that membranes are composed of repeating units which require phospholipid for their alignment, that is, detergents caused disaggregation of the membrane units which could only be restored to the vesicular form by removing the detergent and replacing the phospholipid. A similar phenomenon might account for the results obtained using methods which hydrolyze membrane phospholipid, since this gives rise to the detergent-like monoacyl phospholipids and fatty acids with the loss of diacyl phospholipids. The membranes under these conditions could dissociate into smaller complexes having centrifugation characteristics different from that of the membrane from which they were derived and thus account for the observed differences.

α-Ketoglutarate dehydrogenase had a distribution in the three fractions similar to that of malate dehydrogenase, a matrix enzyme. The O fraction separated either by osmotic shock or by incubation had no detectable α-ketoglutarate activity. This distribution did not change upon incubation of the swollen mitochondria with the substrates required for mitochondrial contraction, ATP, $MgCl_2$, and albumin (Allmann, *et al.*, 1968). α-Ketoglutarate dehydrogenase appeared to be somewhat more difficult to solubilize by osmotic shock than the malate dehydrogenase and perhaps this reflects the higher affinity of this matrix enzyme for the inner membrane, a possibility pointed out by Schnaitman and Greenawalt (1968). Allmann *et al.* (1968) found that the α-ketoglutarate dehydrogenase was released from the inner membrane fraction by phospholipase A or oleic acid treatment. Since they were able to sediment two-thirds of the enzyme by high-speed centrifugation, they believed this to be associated with the outer membrane. They demonstrated using high-speed centrifugation that α-ketoglutarate dehydrogenase had characteristics different from a purified preparation (Sanadi *et al.*, 1952, 1956). It is possible that this was a reflection of a difference in structure of the enzyme complex (perhaps complexed with noncatalytic proteins, as suggested by Allmann *et al.*, 1968) due to the methods of isolation. Complexing with noncatalytic protein might account for the observation that it is solubilized more slowly from the inner membrane matrix fraction than the malate dehydrogenase. Beattie *et al.* (1967), Schnaitman and Greenawalt (1968) and Parsons *et al.* (1967) have located this enzyme in the inner membrane–matrix and suggested that the appearance of this enzyme with the outer membrane was due to contamination of the outer membrane by certain matrix proteins.

Assay of phospholipase A activity using either intact mitochondria or the membrane fractions separated by osmotic shock and sonication indicates that the majority of the activity is in the outer membrane. Nachbaur and Vignais (1968) obtained similar results from the hydrolysis of added egg yolk phosphatidylethanolamine. The finding that the ratio of phospholipase A specific activities was lower than the ratios of monoamine oxidase and rotenone-insensitive DPNH–cytochrome C reductase suggests the possibility that the phospho-

lipase A might have a dual localization, that is, in both the outer and inner membranes. Marker enzyme specific activity ratios calculated from the data of Nachbaur and Vignais are 0.21 for cytochrome oxidase, 11.2 for monoamine oxidase, and 3.8 for phospholipase A. These ratios are similar to those reported here and indicate that our preparations were comparable to those used in the study of Nachbaur and Vignais. It was not possible using endogenous phosphatidylethanolamine as substrate to vary the ratio of substrate to enzyme which presents the possibility that the substrate might be limiting and that the values might not be a true measure of the level of the phospholipase A. This doesn't seem to be the case however, since the values reported here agree so well with those of Nachbaur and Vignais who used high levels of added substrate.

Another indication that the phospholipase A activity acts on the inner membrane was the finding that incubation of mitochondria causes disruption of the inner membrane. This was shown by the increased amount of cytochrome oxidase recovered in the O fraction and by the solubilization of matrix enzymes. The action of the phospholipase A could be either direct, by hydrolysis of the inner membrane phospholipid or, indirect by absorption of the hydrolysis products (monoacylglycerophosphorylethanolamine and fatty acid) to the inner membrane. Further studies will be required to settle this point.

Examination of the specific activity (disintegrations per minute per micromole of phosphorus) of the [^{14}C]phosphatidylethanolamine and [^{14}C]monoacylglycerophosphorylethanolamine before and after incubation indicated that little, if any outer membrane phosphatidylethanolamine was preferentially hydrolyzed. On the other, the specific activity of the inner membrane phosphatidyl ethanol changed during incubation. One possibility would be the existence of a population of phosphatidylethanolamine in the inner membrane that was hydrolyzed more rapidly than the total. Similar results were found previously using intact mitochondria (Waite *et al.*, 1969b). However, the earlier observations can be explained only in part from the difference in the rate of the hydrolysis between the two membrane fractions. Another possibility is that the change in specific activity could be due to differences in the molecular species (variations in the fatty acid moieties) or to differences in the localization within the inner membrane, such as being located in the sections facing out toward the outer membrane or in parts folded into matrix (cristael *vs.* inner boundary of the outer membrane, according to Penniston *et al.*, 1968). The latter possibility seems to be more reasonable since the first requires the postulation that certain molecular species of phosphatidylethanolamine which were not hydrolyzed by phospholipase A are present in the inner but not the outer membrane.

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