Phospholipase activity in rat liver mitochondria studied by the use of endogenous substrates

PÅL BJØRNSTAD

Institute of Clinical Biochemistry, University of Oslo, Rikshospitalet, Oslo, Norway

ABSTRACT The hydrolysis of endogenous phosphatidyl ethanolamine and lecithin in rat liver mitochondria has been studied by using mitochondria from rats injected with ethanolamine-1,2-^{14}C or choline-1,2-^{14}C. A phospholipase A-like enzyme has been demonstrated, which catalyzes the hydrolysis of one fatty acid ester linkage in phosphatidyl ethanolamine and lecithin. Phosphatidyl ethanolamine is hydrolyzed in preference to lecithin and the main reaction products are free fatty acids and lysophosphatidyl ethanolamine. The further breakdown of lysophospholipids appears to be limited in mitochondria, which indicates that lysophospholipase activity is mainly located extramitochondrially. The enzymic system is greatly stimulated by calcium ions, and also slightly by magnesium ions, while EDTA inhibits it almost completely. These findings are discussed in relation to previous observations on the effect of calcium and of EDTA on the functions of mitochondria.

The possible function of the mitochondrial phospholipase for the formation of phospholipids with special fatty acids at the α- and β-position is discussed.

KEY WORDS rat · liver · mitochondria · enzyme · phospholipids · hydrolysis · calcium ions · inactivation

PHOSPHOLIPASES that catalyze the hydrolysis of the fatty acid ester linkages in phosphatidyl choline (lecithin) and phosphatidyl ethanolamine are known to be present in a number of animal tissues (1–5). Our knowledge, however, concerning the intracellular localization and substrate specificity of these enzymes is meager. Epstein and Shapiro (2) found that phospholipases of intestinal mucosa were bound to subcellular particles, and it has recently been claimed that phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) is present in rat liver mitochondria (6, 7).

In a recent report (8) we have demonstrated a calcium-activated enzymic system in rat liver microsomes which catalyzes the hydrolysis of the fatty acid ester linkages in endogenous lecithin and phosphatidyl ethanolamine. Phosphatidyl ethanolamine was hydrolyzed in preference to lecithin and the main reaction products were free fatty acids and glycerophosphoryl ethanolamine. Formation of small amounts of lysophosphatidyl ethanolamine was demonstrated and it was concluded that the hydrolysis was due to two enzymes, a phospholipase A and a lysophospholipase (lysolecithin acyl-hydrolase, EC 3.1.-1.5).

The present paper reports a corresponding study on the breakdown of endogenous lecithin and phosphatidyl ethanolamine in rat liver mitochondria.

A calcium-activated phospholipase in rat liver mitochondria which catalyzes preferentially the hydrolysis of one fatty acid ester linkage in phosphatidyl ethanolamine is demonstrated. Lysophosphatidyl ethanolamine is the main reaction product. The results are discussed in relation to the effect of aging and of calcium on mitochondrial function.

MATERIALS

Ethanolamine-1,2-^{14}C (specific activity 6.8 mc/mmole) and choline-1,2-^{14}C (specific activity 5.0 mc/mmole) were obtained from Philips-Duphar, Amsterdam, The Netherlands. Fatty acid methyl ester standards were obtained from The Hormel Institute, Austin, Minn.

Lecithin and phosphatidyl ethanolamine labeled in the base moiety, to be used in experiments in which the hydrolysis of added substrates was studied, were prepared in substrate amounts from livers of rats injected with labeled ethanolamine and labeled choline by the extraction procedure described by Folch, Lees, and Sloane Stanley (9). The extracted lipids were separated by thin-layer chromatography on Silica Gel G as previously

described (10). Lecithin and phosphatidyl ethanolamine were eluted from the silica gel with several portions of chloroform–methanol 1:9 and the solvent was evaporated under reduced pressure. The isolated phospholipds were solubilized in Tris buffer [tris(hydroxymethyl)-amino methane] (pH 8.5), 0.1 M, by ultrasonic vibration (20 kc/sec) at 0°C with a Branson sonifier (model S 75) for 30 sec at 6 amp. The same procedure was used for preparation of unlabeled phospholipids to be used in experiments in which the effect of added substrate on the breakdown of endogenous phospholipids in mitochondria was studied. All reagents used were of A.R. grade.

METHODS

Preparation of Labeled Rat Liver Mitochondria

Female rats of mixed strain, weighing 160–200 g, were used in all experiments. The labeled compounds were injected intraperitoneally (10 μc in 1 ml of 0.9% NaCl). One hour later, the animals were decapitated and the livers homogenized in ice-cold 0.25 M sucrose containing 2 mM EDTA (9 ml per g of fresh tissue) in a Potter-Elvehjem homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1000 $\times$ *g* for 10 min, and the mitochondria then sedimented at 12,000 $\times$ *g* for 10 min. The microsomes were sedimented at 100,000 $\times$ *g* for 45 min. The mitochondria were washed in sucrose–EDTA and then sedimented at 8000 $\times$ *g* for 10 min. All centrifugations were performed at 4°C. Both mitochondria and microsomes were finally suspended in 0.2 M Tris (pH 7.4)–1.15% KCl, 1:9.

Incubations, Extractions, and Separations of Labeled Phospholipids

Incubations were performed at 37°C in a shaking incubator and stopped by adding one-half volume of 20% trichloroacetic acid. After centrifugation, samples of the supernatant fractions were taken for determination of water-soluble radioactivity. The precipitates were extracted with chloroform–methanol 2:1 and the phases separated (9). The chloroform phase was evaporated to a small volume and the phospholipids were separated by thin-layer chromatography on Silica Gel G as previously described (8). After the lipids had been detected with iodine vapor, the zones corresponding to the phospholipid fractions were scraped off for determination of radioactivity or for measurement of lipid–phosphorus. Controls were run on the effect of trichloroacetic acid on mitochondrial phospholipids, and it was found that the phospholipid composition was the same with the procedure described as when mitochondria were solvent-extracted first.

The amount of labeled phospholipids hydrolyzed and the amount of labeled reaction products formed are given in per cent of radioactivity in phosphatidyl ethanolamine (animals injected with ethanolamine-1,2-^{14}C) or in lecithin (animals injected with choline-1,2-^{14}C) initially present. Incubations were performed in duplicate.

In experiments in which released fatty acids were to be analyzed, the incubations were stopped by adding one-half volume of 0.2 N H_2SO_4 and the fatty acids immediately extracted and titrated according to Dole (11). Prior to titration, the heptane phase was washed twice with 0.05% H_2SO_4 as proposed by Trout, Estes, and Friedberg (12). Quantitative determination of the individual fatty acids was performed by gas–liquid chromatography after the addition of 100 μg of pentadecanoic acid as internal standard. Pentadecanoic acid was chosen as internal standard since experiments had shown that this acid was not released during incubation. Prior to methylation, the fatty acids were separated from other lipids by thin-layer chromatography on Silica Gel G with a solvent system of petroleum ether (bp 60–80°C)–diethyl ether–concd. acetic acid 85:15:1. The R_f value for free fatty acids in this system was 0.20. The fatty acids were located by spraying the plates with 0.01% dichlorofluorescein in methanol, eluted from the gel with petroleum ether–diethyl ether 1:1, methylated with freshly distilled diazomethane prepared from *N*-nitrosomethyl urea, and reduced to a small volume prior to injection into the gas chromatograph. The analyses were obtained with a dual column gas chromatograph with differential flame ionization detector (Perkin-Elmer, Model 800). The stationary phase was 8% butanediol succinate polyester on 80–100 mesh Chromosorb W. The chromatograph was run isothermally at 190°C, and nitrogen, at a flow rate of 20 ml/min, was the carrier gas. The retention time for the fatty acid methyl esters was determined by comparison with standards, and the concentrations were calculated from the peak area of the internal standard. Peak areas were determined by triangulation. The validity of this procedure was checked with standard mixtures of methyl esters (The Hormel Institute, Austin, Minn.), and the values obtained were within 2% of the stated value.

Other Assay Methods. Protein and total phosphorus were determined as previously described (8). Glucose 6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed according to de Duve, Pressman, Gianetto, Wattiaux, and Appelmans (13), acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) according to Wattiaux and de Duve (14), and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) according to Skrede, Bremer, and Eldjarn (15). All measurements of radioactivity were per-

TABLE 1 DISTRIBUTION OF PHOSPHORUS IN RAT LIVER MITOCHONDRIAL PHOSPHOLIPIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Fraction Assayed	Phosphorus Content
	μmole/mg protein
Total	0.264
Lipid	0.135
Lecithin	0.054
"Cephalin"	0.040

"Cephalin," phosphatidyl serine + phosphatidyl ethanolamine.

TABLE 2 DISTRIBUTION OF RADIOACTIVITY IN LIVER MITOCHONDRIA FROM RATS INJECTED WITH ETHANOLAMINE-1,2-^{14}C OR CHOLINE-1,2-^{14}C

Compound Isolated	Precursor Injected: Ethanolamine-1,2-^{14}C	Precursor Injected: Choline-1,2-^{14}C
	%	
Phosphatidyl ethanolamine	84.1 ± 3.0	0
Phosphatidyl choline	13.6 ± 2.0	86.0 ± 3.2
Water-soluble	2.4 ± 0.7	14.0 ± 3.2

The mitochondrial suspension was precipitated with trichloroacetic acid, and the lipids were extracted and separated as described in Methods. The values are means ± SD of 15 mitochondrial preparations from 15 different animals, expressed as radioactivity in each fraction in per cent of total radioactivity recovered. Determinations were performed in duplicate.

formed in a liquid scintillation spectrometer (Tricarb, model 500 D) as previously described (8).

RESULTS

Lipid-Phosphorus and Radioactivity in Labeled Mitochondria

Table 1 shows the amount of lecithin and "cephalin" phosphorus found in a typical mitochondrial preparation ("cephalin" includes phosphatidyl serine and phosphatidyl ethanolamine, since these phospholipids were not separated by the chromatographic procedure used). The values varied only slightly between different mitochondrial preparations and are in good agreement with results reported by others (16, 17). Lysophospholipids were not present in measurable amounts.

The labeling of mitochondrial phospholipids of rats injected with ethanolamine-1,2-^{14}C and choline-1,2-^{14}C is shown in Table 2. Acid hydrolysis (10) of the isolated phospholipids and determination of radioactivity in each of the degradation products showed that all the radioactivity was recovered in the choline or ethanolamine moiety. No radioactivity was found in serine isolated from phosphatidyl serine. In the ethanolamine-injected animals, about 10% of the radioactivity recovered was found in lecithin, a result in agreement with our previous findings (10), and a mean of 84% in phosphatidyl ethanolamine. In the choline-injected animals a mean of 86% was found in lecithin.

The standard deviations shown in Table 2 were mainly due to differences between animals, as the methods used showed a high degree of precision.

Hydrolysis of Mitochondrial Phospholipids

Table 3 shows the effect of calcium ions on the hydrolysis of endogenous, labeled phospholipids of rat liver mitochondria. In the absence of added calcium ions 7–8% of the radioactive phosphatidyl ethanolamine and lecithin was hydrolyzed after 1 hr at 37°C. Addition of calcium ions greatly stimulated the degradation of labeled phosphatidyl ethanolamine and to a lesser degree also that of lecithin. A mean of 55% of the radioactive phosphatidyl ethanolamine was hydrolyzed as compared to 16% of lecithin. About 75% of the labeled phospholipids hydrolyzed was recovered as the corresponding lyso compounds. Column chromatography (8) of the water-soluble radioactive reaction products formed showed it to be glycerophosphoryl ethanolamine or glycerophosphoryl choline, as appropriate.

In Table 4 the hydrolysis of mitochondrial and microsomal phospholipids is compared. In this experiment both lipid-phosphorus and radioactivity in the various phospholipids were determined prior to (zero time sample) and after incubation for 1 hr. The decrease in cephalin-phosphorus paralleled the decrease in radioactivity in

TABLE 3 EFFECT OF CALCIUM ON HYDROLYSIS OF RAT LIVER MITOCHONDRIAL PHOSPHOLIPIDS

Compound Isolated	Incubated without Ca^{2+}	Incubated with Ca^{2+}
	%	
Ethanolamine-labeled mitochondria		
Phosphatidyl ethanolamine hydrolyzed	8.1 ± 1.0	55.0 ± 3.0
Lysophosphatidyl ethanolamine formed	4.8 ± 0.5	44.5 ± 3.0
Water-soluble reaction products formed	3.8* ± 0.5	15.3* ± 1.3
Choline-labeled mitochondria		
Lecithin hydrolyzed	7.5 ± 1.0	16.4 ± 1.2
Lysolecithin formed	4.5 ± 0.5	11.5 ± 0.9
Water-soluble reaction products formed	3.0 ± 0.5	4.5 ± 0.6

The incubation system contained 5 mg of mitochondrial protein isolated from rats injected with ethanolamine-1,2-^{14}C or choline-1,2-^{14}C), Tris buffer (pH 8.5) 60 μmoles, in a final volume of 0.6 ml. The concentration of calcium ions was 10 mM. Incubation time 1 hr. The values are means ± SD of 10 mitochondrial preparations from 10 different animals, expressed as increase or decrease in radioactivity in each fraction in per cent of radioactivity initially present in phosphatidyl ethanolamine or phosphatidyl choline. Incubations were performed in duplicate.

* Includes a small amount of radioactivity from hydrolyzed labeled lecithin.

TABLE 4 HYDROLYSIS OF ENDOGENOUS, LABELED PHOSPHATIDYL ETHANOLAMINE IN RAT LIVER MITOCHONDRIA AND MICROSOMES

	Mitochondria		Microsomes	
	Zero Time	Incubated	Zero Time	Incubated
	*mμmoles/ mg protein**			
Lipid-phosphorus				
Phosphatidyl ethanolamine and serine	40.0	20.5	62.0	24.6
	38.3	18.9	64.3	27.2
Lysophosphatidyl ethanolamine and serine	0	20.3	0	1.8
	0	18.6	0	1.4
Lecithin	53.5	42.1	148.0	126.0
	51.6	43.8	151.2	123.6
	*cpm/mg protein**			
Radioactivity				
Phosphatidyl ethanolamine	2780	1250	7360	2990
	2650	1180	7250	2870
Lysophosphatidyl ethanolamine	0	1520	0	300
	0	1480	0	290
Water-soluble	105	410	180	4500
	110	430	190	4580

The incubation system contained 5 mg of microsomal or mitochondrial protein, Tris buffer (pH 8.5) 60 μmoles, and calcium chloride 10 mM, in a final volume of 0.6 ml. Incubation time 1 hr. Zero time samples were precipitated prior to incubation.
* The two values represent two identical, independent incubations.

phosphatidyl ethanolamine, which indicated that no selective hydrolysis of the newly synthesized labeled phospholipids took place. Table 4 further shows that the main reaction product in the breakdown of microsomal phosphatidyl ethanolamine is a water-soluble compound [previously (8) shown to be glycerophosphoryl ethanolamine], while the main reaction product in mitochondria is the corresponding lyso compound.

The hydrolysis of 8% of the mitochondrial phosphatidyl ethanolamine when mitochondria were incubated in the absence of calcium ions (Table 3) could be due to the presence of endogenous calcium, as it has been shown that homogenization and washing of mitochondria in sucrose–EDTA do not remove all the calcium from mitochondria, although they reduce it considerably (18). To test this possibility, we performed the experiments reported in Table 5, which shows that the hydrolysis of endogenous phosphatidyl ethanolamine in mitochondria prepared in the absence of EDTA was about twice as rapid as in mitochondria prepared in the presence of EDTA, when calcium ions were not added for the incubation. Addition of calcium ions induced a rapid and about equal hydrolysis of phosphatidyl ethanolamine in both preparations, while the addition of EDTA to the incubation system prevented the hydrolysis almost completely. The inhibition produced by EDTA could be reversed by adding calcium ions in excess. Thus, EDTA does not act by inhibiting the enzyme, but probably by complexing endogenous calcium, which indicates that calcium is an absolute requirement for enzymic activity.

TABLE 5 EFFECT OF EDTA ON HYDROLYSIS OF ENDOGENOUS PHOSPHATIDYL ETHANOLAMINE IN RAT LIVER MITOCHONDRIA

Incubation System	Phosphatidyl Ethanolamine Hydrolyzed	
	Preparation 1	Preparation 2
	%	
Buffer only	10	21
Buffer + 10^{-2} M Ca^{2+}	58	60
Buffer + 5×10^{-3} M EDTA	2	3
Buffer + 5×10^{-3} M EDTA + 10^{-2} M Ca^{2+}	53	56

Mitochondria were prepared in 0.25 M sucrose containing 2 mM EDTA (preparation 1) and in sucrose without EDTA (preparation 2). Incubations were performed in Tris buffer (pH 8.5) 10^{-2} M, for 1 hr. EDTA and calcium were added as indicated.

The results given in Tables 1 and 3 show that 20–25 mμmoles of phosphatidyl ethanolamine and 8–10 mμmoles of lecithin were hydrolyzed per mg of protein per hr. Since the amount of lecithin is higher than that of phosphatidyl ethanolamine, the endogenous phosphatidyl ethanolamine is more readily hydrolyzed than endogenous lecithin. A similar substrate specificity has been reported for the hydrolysis of added phospholipids by rat liver mitochondria (6), and for the enzymes that hydrolyze phospholipids in rat liver microsomes (8).

Heat Stability of the Enzyme(s)

The enzymic nature of the reactions studied was further established by heat treatment of the mitochondrial suspension. Table 6 shows that prior incubation of the

TABLE 6 STABILITY OF THE PHOSPHOLIPID-HYDROLYZING ENZYME PRIOR TO INCUBATION AT VARIOUS TEMPERATURES

Treatment	Phosphatidyl Ethanolamine Hydrolyzed
	%
10 min at 60°C	0
10 min at 50°C	37
10 min at 0°C	51

Mitochondria were suspended in Tris (pH 7.4)–1.15% KCl 1:9. The incubation system contained Tris buffer (pH 8.5) 60 μmoles, and calcium chloride 10 mM, in a final volume of 0.6 ml. Incubation time 1 hr. The amount hydrolyzed is given in per cent of the radioactivity initially present in phosphatidyl ethanolamine.

mitochondrial suspension at 60°C for 10 min at pH 7.4 inactivated the hydrolysis completely, while prior incubation at 50°C for 10 min inhibited the hydrolysis only 30%.

Cellular Localization of the Enzyme(s)

Since phospholipase A and lysophospholipase have been demonstrated in rat liver microsomes, it was important to examine carefully whether the enzyme(s) under study are truly localized in the mitochondrial compartment. The amount of other subcellular particles present in our mitochondrial preparations was determined by assaying the amount of marker enzymes present in the mitochondrial and microsomal fractions (glucose 6-phosphatase for microsomes, 3-hydroxybutyrate dehydrogenase for mitochondria, and acid phosphatase for lysosomes). The distribution of glucose 6-phosphatase (Table 7) showed that a maximum of 10% of the protein, 15% of the cephalin-phosphorus, and 20% of the radioactivity in phosphatidyl ethanolamine in the mitochondrial fraction could be due to the presence of microsomes.

Acid phosphatase activity was more equally distributed between the two fractions, which indicated that lysosomes were present to a great extent in both fractions.

Formation of Free Fatty Acids

Table 8 shows that the release of free fatty acids was nearly that expected if the mitochondrial phospholipids which disappeared were converted to the corresponding lyso compounds. As part of the phospholipids hydrolyzed lost both of their fatty acids, the measured release of fatty acids is somewhat low.

Table 9 shows the results of a gas–liquid chromatographic determination of the free fatty acids present in mitochondria and those released during incubation in the presence of calcium ions. The total amount of fatty acids found was in good agreement with the result obtained by titration (Table 8). Of the fatty acids released, 43% was unsaturated.

Effect of Added Substrates

Table 10 shows the results of experiments with added substrates. When labeled mitochondria were incubated with added unlabeled substrates (phosphatidyl ethanolamine or lecithin) the same amount of endogenous phosphatidyl ethanolamine was hydrolyzed as when added substrates were omitted. On incubation of labeled phosphatidyl ethanolamine with unlabeled mitochondria, a mean of 24% of the labeled phospholipid was hydrolyzed. These results indicate that the enzyme(s) is bound to the mitochondrial membrane and hydrolyzes endogenous phospholipids in preference to added substrate. The finding that small amounts of added substrates were hydrolyzed may be due to liberation of the enzyme during incubation. In a recent communication (6) it was reported that added phospholipids were hydrolyzed by intact mitochondria in preference to endogenous phospholipids. Our results do not agree with this finding.

Properties of the Mitochondrial Enzyme That Hydrolyzes Phospholipids

Some properties of the mitochondrial enzyme that hydrolyzes endogenous phospholipids have been determined. In these studies, mitochondria from rats injected with labeled ethanolamine were used.

TABLE 7 DISTRIBUTION OF MARKER ENZYMES AND PHOSPHATIDYL ETHANOLAMINE-HYDROLYZING ACTIVITY IN MITOCHONDRIAL AND MICROSOMAL FRACTIONS ISOLATED FROM RAT LIVER

	Mitochondria		Microsomes	
	Total Activity	Specific Activity	Total Activity	Specific Activity
Glucose 6-phosphatase	603	22.9	5740	184.9
Acid phosphatase	1020	38.7	856	27.6
3-Hydroxybutyrate dehydrogenase	930	35.4	127	4.1
Phosphatidyl ethanolamine-hydrolyzing activity	11.3	0.43	33	1.1

The recovery of marker enzymes was somewhat low, as care was taken to obtain each fraction as pure as possible.

Total activity is expressed as mμmoles/min in subcellular particles isolated from 1 g of liver, and specific activity as mμmoles/min per mg of protein in the fractions.

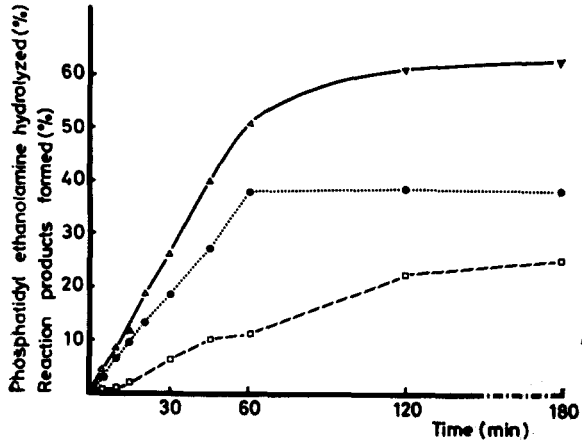

FIG. 1. Time–activity curves for the hydrolysis of phosphatidyl ethanolamine and for the formation of lysophosphatidyl ethanolamine and water-soluble reaction products. The incubation system was the same as given in the legend to Table 2. The amount hydrolyzed is given in per cent of labeled phosphatidyl ethanolamine initially present. The amount of reaction products formed is given as the increase in radioactivity in per cent of phosphatidyl ethanolamine radioactivity initially present (a small fraction of the increase in water-soluble radioactivity is due to the hydrolysis of labeled lecithin).
Δ—Δ—Δ Phosphatidyl ethanolamine hydrolyzed; ●...●...● lysophosphatidyl ethanolamine formed; □---□---□ water-soluble radioactivity formed.

TABLE 8 MEASUREMENT OF FREE FATTY ACIDS RELEASED DURING INCUBATION OF RAT LIVER MITOCHONDRIA

Substance Measured	Amount
	mμmoles/mg protein
Free fatty acids released	30
Phosphatidyl ethanolamine hydrolyzed	25
Lecithin hydrolyzed	13

The incubation system contained 10 mg of the mitochondrial protein, Tris buffer (50 μmoles) pH 8.5, and calcium chloride 10 mM, in a final volume of 0.6 ml. Incubation time 1 hr.

Time–Activity Curve. Fig. 1 shows the time–activity curve for the hydrolysis of phosphatidyl ethanolamine and for the formation of lysophosphatidyl ethanolamine and water-soluble reaction products. The hydrolysis is nearly linear with time up to 1 hr, and at this time the main reaction product is lysophosphatidyl ethanolamine. When longer incubation times are used, a greater percentage of the reaction products is water-soluble.

pH. Fig. 2 shows the effect of pH on enzyme activity. The pH curve is nearly identical with that observed for the microsomal enzyme system (8). The enzyme is active over a wide pH range; optimum activity was found at pH 8–9, but considerable activity was observed even at pH 6.5. The activity decreased rapidly at pH above 9.0.

Concentration–Activity Curve for Calcium and Magnesium. Fig. 3 shows the concentration–activity curve for calcium and magnesium. The enzyme is activated by very low concentrations of calcium, and nearly optimum activity is obtained at a concentration of 3×10^{-3} M. In the experiments reported we have used a calcium concentration of 10×10^{-3} M. The enzyme is poorly activated by magnesium even at high concentrations.

TABLE 9 QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE FATTY ACID RELEASE FROM MITOCHONDRIA INCUBATED IN THE PRESENCE OF CALCIUM IONS

Fatty Acid	Zero Time	Incubated
	mμmoles/mg protein	
14:0	0.09	0.36
16:0	1.59	9.33
16:1	0.12	1.87
17:0	0	0.43
18:0	1.06	10.53
18:1	0.54	4.59
18:2	0.57	5.44
20:4	0	3.87
Total	3.97	36.42
Total unsaturated	1.23	15.77
Per cent unsaturated	31	43

The incubation system contained 50 mg of mitochondrial protein, Tris buffer (500 μmoles) pH 8.5, and calcium chloride 10 mM, in a final volume of 6.0 ml. Incubation time 1 hr. The zero time samples were extracted immediately.

TABLE 10 EFFECT OF ADDED SUBSTRATE ON THE HYDROLYSIS OF ENDOGENOUS PHOSPHATIDYL ETHANOLAMINE IN RAT LIVER MITOCHONDRIA

	Phosphatidyl Ethanolamine Hydrolyzed	
	Endogenous	Added
	%	
Labeled mitochondria	58 59	—
Labeled mitochondria + unlabeled substrate	57 59	—
Unlabeled mitochondria + labeled substrate	—	25 23

The incubation system contained 5 mg of mitochondrial protein, Tris buffer (60 μmoles) pH 8.5, and calcium chloride 10 mM, in a final volume of 0.6 ml. Where indicated, 100 mμmoles of labeled or unlabeled phosphatidyl ethanolamine was added to the incubation system. Incubation time 1 hr. The two values represent two identical, independent incubations.

DISCUSSION

The experiments reported have demonstrated that calcium activates a hydrolysis of phosphatidyl ethanolamine and lecithin in rat liver mitochondria. Phosphatidyl ethanolamine is hydrolyzed in preference to lecithin and endogenous phospholipids in preference to added phospholipids. The main reaction products are free fatty acids and the corresponding lyso compounds. The heat

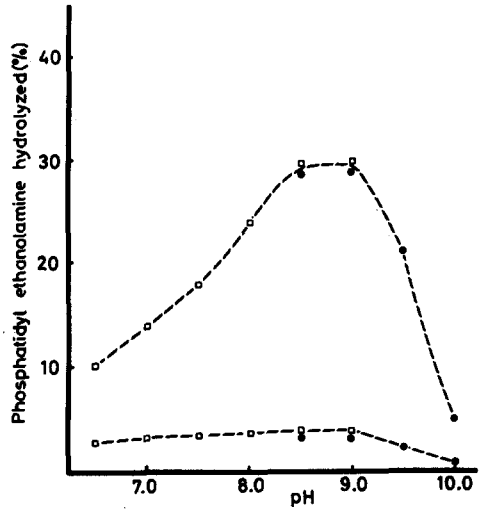

FIG. 2. Effect of pH on the hydrolysis of phosphatidyl ethanolamine. The incubation system was the same as given in the legend to Table 2, except that pH was varied as indicated. Incubation time 30 min. The lower curve represents incubations in the absence of calcium ions. The amount of phosphatidyl ethanolamine hydrolyzed is given as per cent of the amount initially present.

□, Tris buffer; ●, glycine buffer.

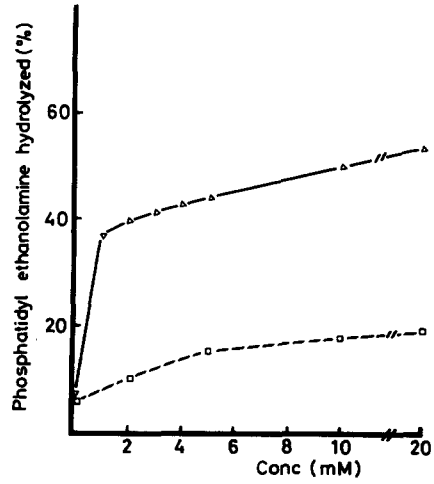

FIG. 3. Concentration–activity curves for calcium and magnesium. Incubation system was the same as in Table 2 except that calcium and magnesium were added as indicated. Incubation time 1 hr. Amount of phosphatidyl ethanolamine hydrolyzed was calculated as explained in the legend to Fig. 2.

Δ—Δ—Δ, calcium; □---□---□, magnesium.

inactivation experiments, the pH curve, the concentration–activity curve for calcium, and the finding that endogenous phospholipids are hydrolyzed in preference to added phospholipids clearly show that the reaction is enzymic and that it cannot be attributed to a base-catalyzed deacylation. The hydrolysis of phosphatidyl ethanolamine and lecithin observed must be due to a phospholipase A-like enzyme in mitochondria. However, phospholipase A is now believed to attack specifically the β-position in lecithin and phosphatidyl ethanolamine. It has been shown that the glycerophospholipids in rat liver contain predominantly unsaturated fatty acids at the β-position and saturated at the α-position (19). The composition of the fatty acids liberated by the mitochondrial phospholipase suggests that the fatty acids both in the α- and β-position are liberated. The calcium-stimulated hydrolysis observed might therefore be effected by one enzyme attacking both ester positions, or by two distinct phospholipases, each being responsible for the hydrolysis of one defined ester position.

We have previously shown (8) that rat liver microsomes contain phospholipid-hydrolyzing enzymes. The cellular localization of the enzyme in our mitochondrial fraction was therefore considered carefully. The distribution of marker enzymes (Table 7) showed that the mitochondrial fractions used in the present work were contaminated with about 10% of microsomal protein. The microsomes contain somewhat more phosphatidyl ethanolamine per mg of protein than mitochondria, and the specific radioactivity of the microsomal phosphatidyl ethanolamine is also somewhat higher (Table 4). However, at most 20% of the radioactivity in phosphatidyl ethanolamine in the mitochondrial preparations can be of microsomal origin. It is unlikely that this contamination by microsomal enzymes and substrates can account for the observed hydrolysis of phospholipids, as much more phospholipid is hydrolyzed in mitochondria than would be expected in view of the content of microsomal protein and substrate. This conclusion is based on the assumption that the enzymes that hydrolyze phosphatidyl ethanolamine in both fractions are saturated with substrate, an assumption which is supported by the observation that the reaction rate is nearly constant for 1 hr both in microsomes (8) and in mitochondria.

The possibility that the enzyme activity was derived from lysosomes is also unlikely. Both the total and specific activity of acid phosphatase are higher in the mitochondrial than in the microsomal fraction, which indicates that more lysosomes are present in the mitochondrial fraction. Despite this, the total and specific activity of the phosphatidyl ethanolamine-hydrolyzing enzyme are higher in microsomes than in mitochondria (Table 7). In an unpublished experiment the effect of lysosomes was tested by adding a lysosome-rich fraction (unlabeled) to an incubation of labeled mitochondria. No increased hydrolysis of the labeled phosphatidyl ethanolamine was observed.

The accumulation of lyso compounds in mitochondria indicates that lysophospholipase activity must be low or absent in mitochondria. In microsomes, however, both fatty acid ester linkages are hydrolyzed and only small amounts of lyso compounds accumulate. The lysophospholipase activity, therefore, is mainly located in the

extramitochondrial compartment of the liver cell. This conclusion is supported by results recently obtained by others (6, 7). The observed formation of glycerophosphoryl ethanolamine may possibly be due to the microsomal contamination of the mitochondrial fractions used, since it is not possible to know whether this enzyme is saturated with substrate formed by the microsomes themselves.

That calcium ions seem to be an absolute requirement for the activity of the mitochondrial phospholipase(s) is of interest in relation to the well-known effect of calcium on mitochondrial phosphorylating activity (20). Free fatty acids, one of the reaction products, are known to be deleterious to mitochondrial function (21, 22). In addition, the formation of the strongly lytic lysophospholipids within the mitochondrial membranes may cause a derangement of the mitochondrial structure, although it has been shown that addition of small amounts of lysophospholipids has no effect on mitochondrial P/O ratio (23). Lehninger and Remmert (21) have demonstrated that calcium ions stimulate an enzymic formation of a potent uncoupling factor (U-factor) in mitochondria. Most probably, this factor consists of free fatty acids. It seems likely that the reaction studied by these authors is the calcium-stimulated hydrolysis of endogenous phospholipids demonstrated in the present work. The finding that the composition of the fatty acids released from mitochondrial phospholipids is in good agreement with the fatty acid composition of U-factor (24) gives further support to this assumption.

The mitochondrial phospholipase is active over a wide pH range and is activated even by the low concentrations of calcium present in mitochondria isolated in sucrose–EDTA. It is therefore reasonable to assume that the enzyme is active under the physiological conditions existing in the living cell. However, lysophospholipids have never been detected in fresh mitochondria. These compounds must therefore be removed as rapidly as they are formed. Since the further breakdown by lysophospholipase seems to be limited in mitochondria, this can only be caused either by reacylation to diacyl glycerophosphatides by the reaction described by Lands (25), or by transacylation reactions between two molecules of lysophospholipids as described by Erbland and Marinetti (26). According to these considerations, the glycerophosphatides in mitochondria will be in a dynamic state, the fatty acids being continuously exchanged. As regards the physiological significance of these reactions, the cycle may represent a mechanism by which phospholipids with special fatty acids at the α- and β-positions are formed, since it has been shown that the enzymes that catalyze the reacylations are position-specific (27, 28). Previous work gives support to the assumption that the renewal of fatty acid moiety in mitochondrial phospholipids is extremely rapid (29).

Since the reacylation reactions require activated fatty acids, either in the form of acyl-CoA or acyl carnitine, the theory outlined may at least in part explain the protective effect of ATP (30), succinate (30), acyl carnitine (31), and EDTA (32) on the functions of mitochondria. Substrate or ATP give energy necessary for fatty acid activation and reacylation, and acyl carnitines contain the fatty acids in an activated form. EDTA would act by removing calcium and thus inhibit phospholipase activity and accumulation of fatty acids and lysophospholipids.

The author expresses his thanks to Dr. Jon Bremer for many fruitful discussions and to Mrs. Brita Haga for her skillful technical assistance.

This work has been supported by grants from the Norwegian Council for Cardiovascular Diseases.

Manuscript received 21 February 1966; accepted 10 May 1966.

References

1. Magee, W. L., J. J. Gallai-Hatchard, H. Sanders, and R. H. S. Thompson. *Biochem. J.* **83:** 17, 1962.
2. Epstein, B., and B. Shapiro. *Biochem. J.* **71:** 615, 1959.
3. Gallai-Hatchard, J. J., W. L. Magee, R. H. S. Thompson, and G. R. Webster. *J. Neurochem.* **9:** 545, 1962.
4. Gallai-Hatchard, J. J., and R. H. S. Thompson. *Biochim. Biophys. Acta* **98:** 128, 1965.
5. Marples, E. A., and R. H. S. Thompson. *Biochem. J.* **74:** 123, 1960.
6. Scherphof, G. L., and L. L. M. Van Deenen. *Biochim. Biophys. Acta* **98:** 204, 1965.
7. Rossi, C. R., L. Sartorelli, L. Tato, L. Baretta, and N. Siliprandi. *Biochim. Biophys. Acta* **98:** 207, 1965.
8. Bjørnstad, P., *Biochim. Biophys. Acta* **116:** 500, 1966.
9. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
10. Bjørnstad, P., and J. Bremer. *J. Lipid Res.* **7:** 38, 1966.
11. Dole, V. P. *J. Clin. Invest.* **35:** 150, 1956.
12. Trout, D. L., E. H. Estes, and S. J. Friedberg. *J. Lipid Res.* **7:** 199, 1960.
13. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. *Biochem. J.* **60:** 604, 1955.
14. Wattiaux, R., and C. de Duve. *Biochem. J.* **63:** 606, 1956.
15. Skrede, S., J. Bremer, and L. Eldjarn. *Biochem. J.* **95:** 838, 1964.
16. Slotta, K. H. *J. Gerontol.* **18:** 326, 1963.
17. Collins, F. D., and V. L. Shotlander. *Biochem. J.* **79:** 321, 1961.
18. Cleland, K. W., and E. C. Slater. *Biochem. J.* **53:** 547, 1953.
19. Van Den Bosch, H., and L. L. M. Van Deenen. *Biochim. Biophys. Acta* **106:** 326, 1965.
20. Hunter, F. E., and L. Ford. *J. Biol. Chem.* **216:** 357, 1955.
21. Lehninger, A. L., and L. F. Remmert. *J. Biol. Chem.* **234:** 2459, 1959.
22. Pressman, B. C., and H. A. Lardy. *Biochim. Biophys. Acta* **21:** 458, 1956.

23. Aravindakshan, I., and B. M. Braganca. *Biochem. J.* **79:** 84, 1961.
24. Wojtczak, L., and A. L. Lehninger. *Biochim. Biophys. Acta* **51:** 442, 1961.
25. Lands, W. E. M. *J. Biol. Chem.* **235:** 2233, 1960.
26. Erbland, J. F., and G. V. Marinetti. *Federation Proc.* **21:** 295, 1962.
27. Lands, W. E. M., and I. Merkl. *J. Biol. Chem.* **238:** 898, 1963.
28. Merkl, I., and W. E. M. Lands. *J. Biol. Chem.* **238:** 905, 1963.
29. Pascaud, M. *Biochim. Biophys. Acta* **84:** 528, 1964.
30. Hansen, M., and A. L. Smith. *Biochim. Biophys. Acta* **81:** 214, 1964.
31. Vignais, P. V., and P. M. Vignais. *Symposium on the Regulation of Metabolic Processse in Mitochondria*. Bari, 1965. B.B.A. Library Volume 7, in press.
32. Myers, D. K., and E. C. Slater. *Biochem. J.* **67:** 558, 1957.