LOCALIZATION OF PHOSPHOLIPASE A_2 IN OUTER MEMBRANE OF MITOCHONDRIA.

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It has been possible to detect and measure phospholipase A activity in different subcellular organites : in mitochondria (Rossi et al., 1965; Scherphof and Van Deenen, 1965; Scherphof, Waite and Van Deenen, 1966; Waite and Van Deenen, 1967; Bjørnstad, 1966a;Vignais and Nachbaur, 1968 a), in microsomes (Bjørnstad,1966b; Waite and Van Deenen, 1967), in lysosomes (Mellors and Tappel, 1967; Blaschko et al., 1967; Vignais and Nachbaur, 1968a) when using either endogenous or exogenous phospholipids as substrates.

Each type of particles appears to exhibit a specific phospholipase activity which can be discriminated by the nature of the fatty acids released, by a different pH for optimal activity, by a reactivity towards ions such as calcium ions. Microsomes would contain a phospholipase A_1, lysosomes a phospholipase $A_1 + A_2$, insensitive to calcium ions and with an optimal activity at pH 4.5 and mitochondria a phospholipase A_2 activated by Ca^{++} and having a pH optimum between 8 and 8.5.

The presence of phospholipase activities in several distinct subcellular fractions renders the purification of these fractions of critical importance to ascertain the measured activity to a specific type of organites. It is well known that mitochondria isolated in 0.25 M sucrose by the classical differential centrifugation method (Hogeboom, 1965) are contaminated by lysosomes and microsomes. Whereas microsomal contamination may be lowered by repeated washings of the mitochondria suspension, by isotonic sucrose, lysosomal contaminants are more difficult to eliminate (Vignais and Nachbaur, 1968b).

Contamination by lysosomes becomes even more critical when the distribution of phospholipase in mitochondrial membranes is studied since lysosomal membranes behave as mitochondrial membranes on sucrose density gradients (Vignais and Nachbaur, 1968b) and since the specific activity of lysosomal phospholipase is considerably higher than that of mitochondria (Mellors and Tappel, 1967).

Results presented in this paper show that mitochondria contain a phospholipase A_2 bound to membranes and located in the outer membrane.

MATERIALS AND METHODS

Four to six days prior to sacrifice rats were injected intraperitoneously with 170 mg of Triton WR-1339 (Rohm and Haas, Philadelphia, U.S.A.). Rat liver mitochondria were isolated in 0.27 M sucrose by differential centrifugation and washed three times with 0.27 M sucrose adjusted to pH 7.4 with Tris buffer. The Triton filled lysosomes were eliminated by floatation on a two-layer sucrose density gradient (Vignais and Nachbaur, 1968b). Inner membrane plus matrix, outer membrane and soluble fractions were obtained by the method described by Parsons and Williams (1967) after disrupting the outer mitochondrial membrane by swelling of mitochondria in 20 mM phosphate buffer, pH 7.3. The degree of purification of the different fractions was monitored by enzyme markers : acid phosphatase for lysosomes, monoamine oxidase for the outer membrane, cytochrome oxidase for the inner membrane fractions (for details cf. Vignais and Nachbaur, 1968b).

Phospholipase activity was estimated either from the amount of lysoderivatives formed, after their isolation by thin layer chromatography and measurement of the phosphorus content by the method of Bartlett (1959) or from the amount of fatty acids released as evaluated by gas liquid chromatography.

RESULTS AND DISCUSSION

Table I shows the distribution of endogenous phospholipids in mitochondria before and after incubation, at 37° and at pH 8. Whereas cardiolipins were poorly hydrolyzed, about 38% of phosphatidylethanolamine disappeared. About 8% of the initial lecithin content underwent hydrolysis. These results are in good agreement with those obtained by Scherphof and Van Deenen (1965)

and by Bjørnstad (1966a) according to whom phosphatidylethanolamine is more readily attacked by mitochondrial phospholipase than lecithin.

TABLE I

Hydrolysis of endogenous mitochondrial phospholipids

	% of Total Phosphorus	
	Zero Time	1 hr
Cardiolipins	10.6	9.8
PE	38.4	23.6
PC + LysoPE	42.1	51.3
LysoPC	2.6	6.0

Mitochondria (5.2 mg of protein) were shaken for one hour at 37° in 0.02 M triethanolamine buffer, pH 8.0, containing 2.5 mM $CaCl_2$ in a final volume of 4 ml. The reaction was stopped by addition of 4 ml of chloroform/methanol (2/1) and the acidified medium extracted twice by 4 ml of chloroform/methanol. Phospholipids were separated by thin layer chromatography first using the chloroform-light petroleum-acetic acid (65:35:2, v/v) system and then the chloroform-methanol-water (65:35:4, v/v) system described by Waite and Van Deenen (1967). The spot of lysophosphatidylethanolamine (easily detected by spraying ninhydrin) overlapped the spot of lecithin.

In order to specify the localization of phospholipase A in mitochondria a fraction enriched in inner membranes plus matrix and a fraction enriched in outer membranes were prepared. In Table II are reported the activities of the marker enzymes. It has been already emphasized that the same distribution of monoamine oxidase was found when using either benzylamine or kynuramine as substrates. These results limit a possible underestimation of MAO due to aldehyde oxidase activity in inner membranes when using the benzylamine test.

Since the specific activity of acid phosphatase in lysosomal membrane is of the order of 4,000 nmoles/min./mg protein (Vignais and Nachbaur, 1968b) it is clear, from the very low acid phosphatase activity found in the outer mitochondrial membrane fraction, that the amount of lysosomal membrane present in that fraction is negligible. Data in Table III show the distribution of phospholi-

TABLE II

Enzymic activities in submitochondrial fractions

Fractions	Cytchrome Oxidase		Monoamine Oxidase		Acid Phosphatase	
	Spec.* Act.	Total Act.	Spec.** Act.	Total Act.	Spec.** Act.	Total Act.
Mitochondria	1.6	1,140	3.1	2,220	37	20,800
Inner Memb.	1.9	1,260	2.1	1,390	9	2,700
Outer Memb.	0.4	14	23.5	827	28	2,100
Soluble	0	0	0	0	4	4,150

* µmoles/min./mg protein ** nmoles/min./mg protein

Mitochondria were purified by centrifugation on a two-layer sucrose gradient as described in the preceding paper (Vignais and Nachbaur, 1968b). The submitochondrial fractions were obtained after swelling of mitochondria in 20 mM phosphate buffer and are the crude fractions collected at low speed (inner membrane) and high speed (outer membrane) centrifugation as described by Parsons and Williams (1967).

pase A among the submitochondrial fractions described in TableII. The fractions used, very evidently, were not extensively purified as shown by the distribution of some of the marker enzymes. However, when comparing results in Table II and III one can note a parallelism between the activities of monoamine oxidase and of phospholipase with the highest specific activity for both enzymes in outer mitochondrial membranes.

The outer mitochondrial membrane preparation exhibits a typical phospholipase A_2 activity as estimated by the nature of the fatty acids released (mostly unsaturated) and from the optimal activity found at pH 8. This is in contrast with the phospholipase activity of lysosomal membranes which is markedly stimulated at acid pH, is not stimulated by Ca^{++} not inhibited by EGTA, and which results in the release of saturated and unsaturated fatty acids in equal proportion.

TABLE III

Distribution pattern of Phospholipase A in submitochondrial fractions

Fractions	Fatty Acids released		
	Spec. Act.*	Total Act.	Unsaturated %
Mitochondria	23	18,147	67
Inner Memb. + Matrix	19	13,248	70
Outer Memb.	72	4,141	84
Soluble	6	1,405	99

* nmoles of fatty acids released/hr/mg protein.

Incubation conditions : 0.3 to 4 mg of protein were incubated with shaking at 37° for one hour in 0.02 M triethanolamine buffer, pH 8.0, 2 mM $CaCl_2$ and ultrasonicated egg phosphatidylethanolamine (0.4 mg) in a final volume of 2 ml. The fatty acids extracted by 4 ml x 2 of chloroform/methanol (2/1) were isolated by chromatography on a silicic acid column, methylated and analyzed by gas liquid chromatography.

TABLE IV

pH-dependent Phospholipase activities

Fractions	pH	Fatty Acids released	
		nmoles/hr/mg prot.	Unsaturated %
Inner Memb.	4.5	3	30
	8.1	25	79
Outer Memb.	4.5	26	60
	8.1	110	74
Lysosomal Memb.	4.5	266	48
	8.1	118	50

The membrane fractions were purified by centrifugation on a three-layer sucrose gradient as described by Parsons and Williams (1967)(cf. Vignais and Nachbaur, 1968b).

Incorporation of fatty acids into phospholipids of brain mitochondria has been recently reported (Webster and Alpern, 1964). In agreement with this finding preliminary results in this laboratory have shown that rat liver mitochondria devoid of lysosomes and of microsomes and supplemented with CoA and ATP are capable to incorporate (^{14}C) oleic acid in their endogenous phosphatidylethanolamine and phosphatidylcholine. Although the enzymatic mechanism involved in the incorporation and the distribution of the enzyme in mitochondrial membranes is not defined yet, it is tempting to assume that the reacylation of the lysophosphatides formed by the action of phospholipase A_2 is catalyzed by an acylCoA:phospholipid acyl-transferase located at the same place as phospholipase A_2, i.e. in the outer membrane of mitochondria.

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