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Lysolecithin inhibition of mitochondrial metabolism

Considerable evidence has been obtained that lysolecithin brings about inhibition of mitochondrial respiration, uncouples oxidative phosphorylation, and induces swelling of mitochondria[1-4]. However, the site of the inhibitory effect of the lysolecithin on the electron transport system remained to be elucidated. In the present report, evidence based on examination of mitochondria and submitochondrial particles is presented, indicating that lysolecithin acts at more than one site of the respiratory chain with different sensitivity.

Preincubation of liver mitochondria with lysolecithin at 11 μg/mg of mitochondrial protein (L/M ratio = 0.01) caused a progressive inhibition of the State 3 respiration with glutamate as a substrate, paralleling closely with the marked decrease in absorbance at 520 mμ due to mitochondrial swelling (Fig. 1). Although the results obtained with α-ketoglutarate, pyruvate *plus* malate, and β-hydroxybutyrate were essentially similar to those found with glutamate, the State 3 respiration with succinate *plus* glutamate as substrate was only slightly impaired. On the other hand, the phosphorylation rates associated with glutamate or glutamate *plus* succinate diminished rapidly. In agreement with observations reported by WITTER AND COTTONE[3], the phosphorylation with succinate appeared to be more sensitive to the lysolecithin than did the oxidation.

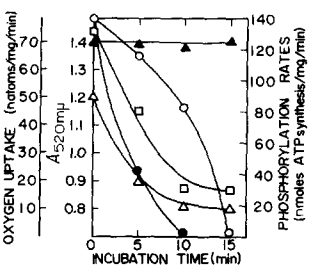

Fig. 1. Effect of incubation with lysolecithin on oxidative phosphorylation and swelling of liver mitochondria. Liver mitochondria (4.6 mg), which were prepared according to the method described for brain mitochondria by OZAWA *et al.*[5], were preincubated with lysolecithin (50 μg). At the end of the incubation, samples were removed. Oxygen consumption was measured polarographically at 22° at pH 7.4 in a medium containing 0.3 M mannitol, 0.01 M KCl, 0.01 M Tris–HCl buffer, 0.005 M potassium phosphate buffer, 0.2 mM EDTA and 4 mM $MgCl_2$. Glutamate and succinate were added at a concentration of 4 mM. The mitochondrial swelling was measured by following the decrease in absorbance at 520 mμ. The phosphorylation rate was calculated as ADP/O ratio × rate of oxygen uptake (nmoles ATP synthesis/mg protein per min). O—O, phosphorylation rate with glutamate *plus* succinate as substrate; ▲—▲, State 3 respiration with glutamate *plus* succinate; ●—●, phosphorylation rate with glutamate as substrate; △—△, State 3 respiration with glutamate; □—□, absorbance at 520 mμ.

The rate of glutamate oxidation with the lysolecithin-lysed mitochondria was greatly stimulated by an addition of NAD^+ into the assay system, suggesting a release of mitochondrial-bound pyridine nucleotide by the action of lysolecithin. As shown in Table I, about half of the bound pyridine nucleotides was released into

Abbreviations: L/M or L/S, ratio of the mg quantities of lysolecithin (L) to mitochondrial (M) or sonic particle (S) protein.

TABLE I

EFFECT OF LYSOLECITHIN ON THE MITOCHONDRIAL NAD^+ CONTENT

Mitochondria, 30 mg protein, were incubated in a medium (pH 7.4) containing 0.3 M mannitol, 0.01 M Tris–HCl buffer, 0.01 M KCl, 0.1 mM EDTA and 1.5 mg of lysolecithin in a final volume of 1.5 ml for 20 min at 22°. Bound pyridine nucleotides were assayed by the previously described method[6].

	*Water uptake** ($\Delta A_{520\,m\mu} \times 10^3$)	*Added mitochondria* (*mg*)	*Bound* NAD^+ (*nmoles*)	*Residue particles*** (*mg*)	NAD^+/*residue particles* (*nmoles/mg*)
Control	5	30	74.5	30.0	2.48
Lysolecithin-treated mitochondria	177	30	40.5	6.4	6.35

* Values are given in terms of the decrease in absorbance $\times 10^3$ at 520 mμ which occurs during the incubation.

** Residue particles obtained after centrifugation for 30 min at 100000 $\times$ *g*.

the supernatant fluid by centrifugation of the mitochondria which were treated at 22° for 20 min in the presence of lysolecithin (L/M = 0.05). However, the NAD^+ content per mg protein of the residue particles was about twice that of the original mitochondria, indicating that the loss of the oxidative activity with glutamate may be partly attributed to the disordered disposition of NAD^+ in the mitochondria.

In order to decide whether lysolecithin inhibits a component of the NADH

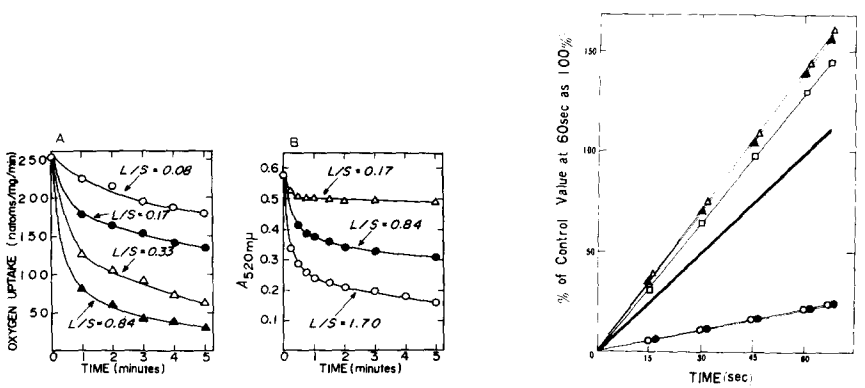

Fig. 2. Effect of lysolecithin on NADH oxidase activity. Oxygen consumption of sonic particles was measured polarographically at 22° in 70 mM potassium phosphate buffer (pH 7.4). Various levels of lysolecithin were added after the addition of NADH (A). B. Swelling of sonic particles.

Fig. 3. Effect of preincubation with lysolecithin on the reduction of several electron acceptors, and NADH oxidase and cytochrome oxidase activities. The sonic particles (2.43 mg), which were prepared according to the method described by RILEY AND LEHNINGER[8], were incubated in the presence of 0.5 mg of lysolecithin for 20 min at 22°. NADH oxidase activity (●) was measured polarographically in the medium (pH 7.4, at 22°) containing 70 mM potassium phosphate buffer and 0.5 mM NADH. Cytochrome oxidase activity (▲) was measured polarographically by the system of WHARTON AND GRIFFITHS[9]. NADH–ferricyanide activity[10,11] (△) was determined in the presence of antimycin A. NADH–cytochrome *c* reductase[10] (□) and NADH–coenzyme Q_6 reductase[12] (○) activities were measured in the presence of sodium azide. Activity is expressed as percentage of the activity at 60 sec of sonic particles (—) similarly treated except for the presence of lysolecithin.

oxidase mediating the reaction between NADH and oxygen, the NADH oxidase activity of sonic particles or electron transport particles was measured after preincubation with lysolecithin. Fig. 2 shows that lysolecithin caused an inhibition of oxidation in the sonic particles proportional to L/S ratio. The inhibition was essentially the same with electron transport particles prepared by the method of CRANE, GLENN AND GREEN[7]. These inhibitions of the NADH oxidase activity paralleled closely with the marked decrease in absorbance at 520 mμ. The extent of inhibition by lysolecithin appears to be related to the extent to which the particles were swollen by lysolecithin. The high degree of instability of these submitochondrial particles suggests that some compound integral to the NADH oxidase chain is either displaced or destroyed specifically by lysolecithin. The preparations of sonic particles used in this study possess very little antimycin A, or amytal-insensitive NADH oxidase activity, while the NADH–cytochrome *c* reductase activity is insensitive to these inhibitors. Upon preincubating the sonic particles with lysolecithin, the NADH–ferricyanide reductase, NADH–cytochrome *c* reductase, and cytochrome oxidase were significantly stimulated (Fig. 3). On the other hand, a pronounced decrease in the NADH–coenzyme Q reductase activity was observed. The percentage inhibition was the same with the NADH oxidase. From these results, it is inferred that lysolecithin inhibits in the region between flavoprotein and coenzyme Q.

The above results indicate that the effect of lysolecithin has a striking similarity to that of snake venom phospholipase reported previously[10]. In fact, the preincubation of mitochondria or submitochondrial particles with heated snake venom gave identical effects on respiration, oxidative phosphorylation, and difference spectrum with those observed by lysolecithin[13,14]. Therefore, the role of phospholipid in electron transport suggested from the studies in which phospholipase A was used to evaluate the effect of phosphatides[15–19], should be reconsidered; that is, the action of phospholipase A on the mitochondria is probably due to lysolecithin liberated by the enzymic action rather than to the cleavage of phospholipid which is required for the structural integrity of the mitochondria.

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Some characteristics of cytochrome b_5 from outer membranes of rat liver mitochondria

Outer and inner membranes have been prepared from mitochondria from different sources. The outer membrane of liver mitochondria contains the rotenone insensitive NADH dehydrogenase system and bears some similarities to the endoplasmic reticulum[1,2]. In particular, a hemoprotein with the spectral characteristics of microsomal cytochrome b_5 has also been found in this outer membrane. The existence of a mitochondrial cytochrome b_5 was first proposed by RAW *et al.*[3]. In contrast to the microsomal cytochrome, which can be solubilized only through treatment of microsomes with lipolytic or proteolytic enzymes[4–6], the hemoprotein of outer membranes can be extracted by sonication of swollen and shrunken mitochondria[1]. It is not known whether this dissimilarity is due to differences in the structure of the two cytochromes, of the membranes to which they are attached, or both.

In the present communication, some properties of rat liver mitochondrial cytochrome b_5 are compared with those of the microsomal hemoprotein solubilized with lipase. The two cytochromes are similar in molecular weight, as judged by chromatography on Sephadex G-75, but differ in electrophoretic mobility in acrylamide gels and, particularly, in their solubility in ammonium sulfate solutions.

Cytochrome b_5 from rat liver microsomes was prepared as described for calf liver by STRITTMATTER[7], using lipase (Nutritional Biochem., Type 448) in the presence of trypsin inhibitor as the solubilizing agent. The purity was judged by the ratio of the absorbance at 413 and 280 mμ of the oxidized cytochrome. Preparations with ratios of 4 to 5 were used in different experiments. Mitochondria were obtained by differential centrifugation of the liver homogenate in 0.25 M sucrose. The "soluble" subfraction of the mitochondria containing cytochrome b_5 was prepared according to SOTTOCASA *et al.*[1] using the alternative two-layer gradient system. Other preparations of mitochondrial cytochrome b_5 were obtained by following the above procedure only to the step where mitochondria are swollen for 5 min in Tris–phosphate buffer. They were subsequently centrifuged at 35000 $\times$ g for 20 min. Surprisingly, this supernatant contains about the same amount of cytochrome b_5 as the extract obtained after contraction and sonication of the mitochondria. Generally, our yields were only about 30 % of those described[1]. Extracts obtained by these procedures were passed through