ENZYMATIC SOLUBILIZATION OF MICROSOMAL NADH-CYTOCHROME $\underline{b}_5$ REDUCTASE BY LYSOSOMES

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Purification of microsomal NADH-cytochrome $\underline{b}_5$ reductase was first reported by Strittmatter and Velick (1957), who solubilized the enzyme from calf liver microsomes by digestion with the venom of a snake (Naja naja). However, the nature of the component of the snake venom, which was responsible for the release of the reductase from microsomal particles, has remained to be elucidated.

It is the purpose of the present communication to show that the lysosomal fraction of rat liver has an ability to solubilize microsomal NADH-cytochrome $\underline{b}_5$ reductase when incubated with microsomes under conditions very similar to those employed by the previous workers for digestion of microsomes with the snake venom (Strittmatter and Velick, 1957; Strittmatter, 1967). The principal role of snake venom in the solubilization of the reductase is doubted.

Methods. Microsomes and other subcellular fractions were prepared from the livers of adult male Sprague-Dawley rats. The liver was homogenized with 9 volumes of ice-cold 0.25 M sucrose solution containing 1 mM EDTA (pH 7.5), and the homogenate was centrifuged at 900 x g for 10 minutes to remove nuclei and cell debris. The supernatant fluid was then successively

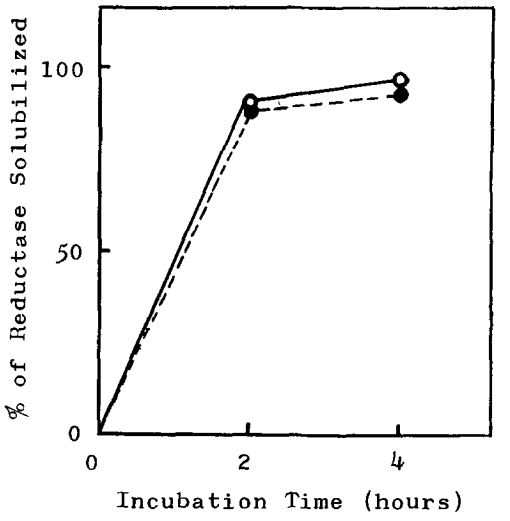
centrifuged at 5,000 x g for 10 minutes, at 8,000 x g for 10 minutes, at 12,000 x g for 30 minutes, and finally at 105,000 x g for 60 minutes, to precipitate mitochondrial (Mt), lysosomal (Ls), heavy microsomal (HMs), and microsomal (Ms) fractions, respectively. Each fraction was washed once by resuspending in fresh 0.25 M sucrose solution, and spinning down again.

Strittmatter and Velick (1957) used a microsomal preparation precipitated at an acidic pH (5.35) from the post-mitochondrial supernatant of calf liver homogenate. To repeat their experiments, acid-precipitated microsomal fraction of rat liver was prepared according to their procedures. The preparation thus obtained will be called "acid-precipitated microsomes".

The activity of NADH-cytochrome $\underline{b}_5$ reductase was assayed by measuring the reduction of purified cytochrome $\underline{b}_5$ by NADH. The reaction mixture contained 0.2 μmoles of NADH, 0.02 μmoles of cytochrome $\underline{b}_5$, and enzyme, in 2 ml of 0.1 M phosphate buffer (pH 7.5). The reduction of the cytochrome was measured by following the increase in the absorption at 423 mμ at 25°. The difference in the extinction coefficient at 423 mμ between reduced and oxidized cytochrome $\underline{b}_5$ was assumed to be 100 cm^{-1} mM^{-1} (Strittmatter and Velick, 1956). The purified cytochrome $\underline{b}_5$ used in the assay was prepared from rat liver by the procedure of Omura *et al.* (1967) with some modifications.

The extent of solubilization of the reductase was measured after centrifuging the digested suspension of microsomes at 105,000 x g for 90 minutes. The percentage of solubilization is expressed as the ratio of the enzyme activity in the supernatant to that of the whole digested suspension.

Results and Discussions. When a suspension of "acid-precipitated microsomes" was incubated with *Naja naja* venom (Calbio-



(Fig. 1) The suspension of "acid-precipitated microsomes" in 0.05 M Tris-acetate buffer (pH 6.5) containing 1 mM EDTA was incubated at 37° with (o—o) or without (●---●) Naja naja venom. The final concentrations of microsomes and venom in the incubation mixture were 30 mg protein/ml and 0.01 %, respectively.

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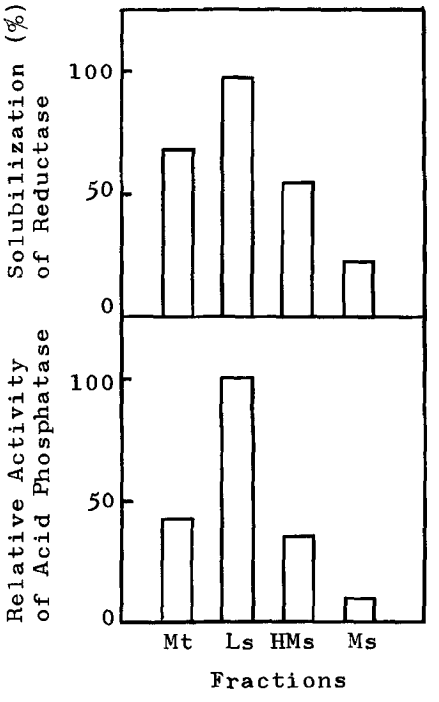
chem Co.) under the conditions specified by the previous workers (Strittmatter and Velick, 1957; Strittmatter, 1967), the reductase was effectively solubilized in agreement with their reports (Fig. 1). Unexpectedly, however, the omission of the snake venom from the incubation mixture did not appreciably affect the release of the reductase (Fig. 1). This finding was confirmed repeatedly. Although the extent of solubilization of the reductase varied somewhat from one microsomal preparation to another, the amount solubilized was always the same in the presence and absence of venom. Due to the weak buffering capacity of 0.05 M Tris-acetate buffer at pH 6.5, the pH of the microsomal suspension was lowered considerably (by 0.5 - 0.8 pH-unit) during the incubation. The "acid-precipitated microsomes" of rat liver thus contains an

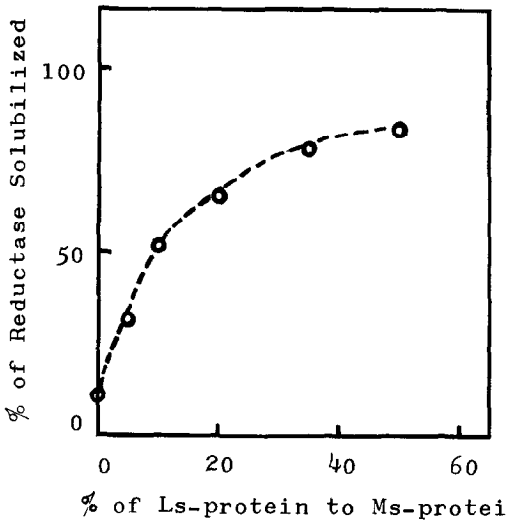


(Fig. 2) (a) Microsomes (8 mg/ml) were suspended in 0.1 M Tris-maleate buffer of pH 5.7, and incubated at 37° for 2 hours with various subcellular fractions (2.5mg/ml) as indicated in the Figure. (b) Acid phosphatase activity of the subcellular fractions was assayed according to the method of Gianetto and de Duve (1955).

endogenous solubilizing factor which is active in an acidic medium.

Since "acid-precipitated microsomes" contained a large amount of non-microsomal contaminants, the microsomal fraction obtained by the fractional centrifugation of a sucrose homogenate of liver, as described in Methods, was then used; practically no solubilization of the reductase was observed from the microsomes, even in the presence of the snake venom. The endogenous solubilizing factor which was present in "acid-precipitated microsomes" had been removed from the microsomal fraction during the course of fractional centrifugation of the sucrose homogenate of rat liver.

The various subcellular fractions of rat liver were then incubated with microsomes to determine which fraction was most active in the solubilization of the reductase. As shown in Fig. 2-a, the lysosomal fraction was most active. Indeed, the relative



(Fig. 3) Microsomes (8 mg/ml) were incubated in 0.1 M Tris-maleate buffer (pH 5.7) with increasing concentrations of lysosomal fraction at 30° for 2 hours, and the extent of solubilization of the reductase was determined as in Methods.

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effectiveness of these subcellular fractions closely paralleled the activity of acid phosphatase (Fig. 2-b) which is a typical lysosomal enzyme (de Duve, 1959). Since the pH-region at around 5.5 was found to be optimal for solubilization of the reductase when the pH of the incubation mixture was controlled during the incubation, Tris-maleate buffer of pH 5.7 was used in these experiments. When the incubation medium was more acidic than pH 5.5, considerable inactivation of the reductase occurred. This acidic pH optimum for solubilization also suggested the participation of a lysosomal enzyme in the release of the reductase from microsomal particles. The percentage of solubilization rose with increased amount of the lysosomal fraction added, and it was possible to solubilize most of NADH-cytochrome $\underline{b}_5$ reductase from microsomes if enough lysosomal fraction was used (Fig. 3).

The above findings strongly suggest the principal role of a

lysosomal enzyme or enzymes in the release of the reductase from microsomal membranes. The addition of Naja naja venom, at least in the case of rat liver microsomes, has no significance in the solubilization of the reductase in an acidic medium. The "acid-precipitated microsomes" seem to be contaminated by a sufficient amount of lysosomes to permit the solubilization of the reductase at a mildly acidic pH.

The activity of the reductase was considerably stimulated upon the solubilization from microsomes. Hence, the solubilization percentage calculated here is always somewhat higher than the actual extent of solubilization. This activation phenomenon will be described in detail in a later publication.

(Table I) Solubilization of microsomal components by digestion with lysosomal fraction.

component	solubilization (%)
NADH-cytochrome b_5 reductase	70.5
NADPH-cytochrome c reductase	22.0
cytochrome b_5	11.6
protein	18.7
phospholipid	0

Microsomes (8 mg/ml) were incubated with lysosomal fraction (2 mg/ml) at 30° for 2 hours in 0.1 M Tris-maleate buffer of pH 5.7.

An interesting feature of this solubilization by lysosomes is its specificity towards microsomal enzymes. While NADH-cytochrome b_5 reductase was readily made soluble by digestion with lysosomal fraction, the release of NADPH-cytochrome c reductase and cytochrome b_5 from microsomes occurred to a much lesser extent (Table I). The latter two microsomal enzymes were most readily solubilized by the treatment of microsomes with trypsin (Phillips and Langdon, 1962; Omura et al. 1967), or with a crude

pancreatic extract (Williams and Kamin, 1962; Omura and Sato, 1963). Although we have not yet characterized the active solubilizing enzyme in the lysosomal fraction, the enzyme should have some sort of specificity towards the microsomal membrane-components which are involved in the binding of these microsomal enzymes to the membrane structure.

We have also purified the reductase solubilized from rat liver microsomes by digestion with the lysosomal fraction. The purified enzyme has properties which are almost identical with those reported previously for the "snake venom-solubilized" enzyme. The details of the solubilization experiments and the properties of the purified "lysosome-solubilized" enzyme will be published elsewhere.

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