ENZYME LOCALIZATION IN THE INNER AND OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA

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Several methods have been introduced to separate the mitochondria into inner and outer membrane fractions (Schniatman et al., 1967; Sottocasa et al., 1967; Parsons et al., 1967; Bachman et al., 1966). Recently, Green et al. (1968) have questioned the validity of these procedures based primarily on the assumption that rotenone-insensitive DPNH cytochrome c reductase (RIDCR), which had been assigned to the outer membrane after a careful and lengthy investigation by Sottocasa et al. (1967), was removed from the mitochondrial fraction by repeated washings in a similar fashion to glucose-6-phosphatase.

The present communication demonstrates that RIDCR activity cannot be removed from the mitochondrial fraction after repeated washings in a similar fashion to two microsomal enzymes, glucose-6-phosphatase and TPNH cytochrome c reductase and that after fractionation by digitonin according to the procedure of Schnaitman and Greenawalt (1968), RIDCR is removed from the inner membrane-matrix fraction in the same way as monoamine oxidase (MAO) and kynurenine hydroxylase, a strictly mitochondrial enzyme (Okamato et al., 1967). All three enzymes are recovered in the outer membrane fraction with a concomitant increase in specific activity. Electron microscopic observations of the inner membrane-matrix fraction, prepared by this method, clearly demonstrate the lack of a limiting outer membrane. This fraction also contains

the citric acid cycle enzymes and the enzymes of β-oxidation of fatty acids, as well as the intact respiratory chain.

EXPERIMENTAL SECTION

Adult male albino rats weighing 175-200 g were used. The animals were killed by decapitation, exsanguinated and the livers removed quickly and placed in ice-cold 0.25 M sucrose adjusted to pH 7.4 with Tris. The livers were minced and homogenized with two passes of a Teflon-glass homogenizer in 9 volumes of sucrose. The homogenate was centrifuged at 750 g for 10 min, the pellet discarded and the supernatant recentrifuged at 750 g for 10 min. The supernatant was carefully decanted and centrifuged at 8,700 g for 10 min. The fluffy layer was discarded and the pellet washed 4 times by resuspension in 1/4 the original volume of sucrose followed by centrifugation at 7,700 g for 10 min. Microsomes were prepared by centrifuging the first mitochondrial supernatant at 12,000 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 144,000 g for 1 hour to sediment the microsomes. Light microsomes were prepared by centrifuging the first mitochondrial supernatant at 30,000 g before centrifugation at 144,000 g for 1 hour.

RESULTS AND DISCUSSION

Table I indicates that repeated washings of a crude mitochondrial pellet reduces the activity of the two microsomal enzymes, glucose-6-phosphatase and TPNH-cytochrome c reductase, associated with the mitochondrial fraction to 3-4%, on a weight basis, of that in the microsomes, while increasing the specific activity of the mitochondrial enzyme, kynurenine hydroxylase, over 2-fold. In confirmation of the results of Okamato et al. (1967) this enzyme was only found in the mitochondrial fraction. RIDCR activity, however, was only slightly reduced by repeated washings leading to a value for microsomal contamination of 17-24%, an obvious discrepancy when compared to the contamination as calculated by the former two enzymes. Hence, significant RIDCR activity is associated with mitochondria prepared in such a way that the microsomal contamination (3-4%) is as

TABLE I

Effect of Repeated Washings on Enzymatic Activities of Rat Liver Mitochondria

	Glu.-6-Phosphatase			RIDCR			TPNH-Cytochrome c Reductase		Kynurenine Hydroxylase
	Sp. Act.	Ratio		Sp. Act.	Ratio		Sp. Act.	Ratio	Sp. Act.
Mitochondria		a	b		a	b		a	
No washes	21.8	25.3	20.5	127	26.7	19.7	3.96	8.3	1.39
1 wash	7.14	8.2	6.7	118	24.7	18.4	2.38	5.0	1.74
4 washes	3.47	4.0	3.1	114	23.7	17.7	1.72	3.6	2.77
Microsomes	86.8			480			47.9		0.51
Light microsomes	107			642					0

Glucose-6-phosphatase activity was assayed at 37° by the method of Hübscher and West (1965); DPNH and TPNH cytochrome c reductase by the method of Sottocasa et al. (1967); and kynurenine hydroxylase by the method of Okamato et al (1967).

a. Ratio of Mitochondrial/Microsomal activity x 100.
b. Ratio of Mitochondrial/Light Microsomal activity x 100.
Sp. Act. expressed as mμmoles/min/mg.

TABLE II

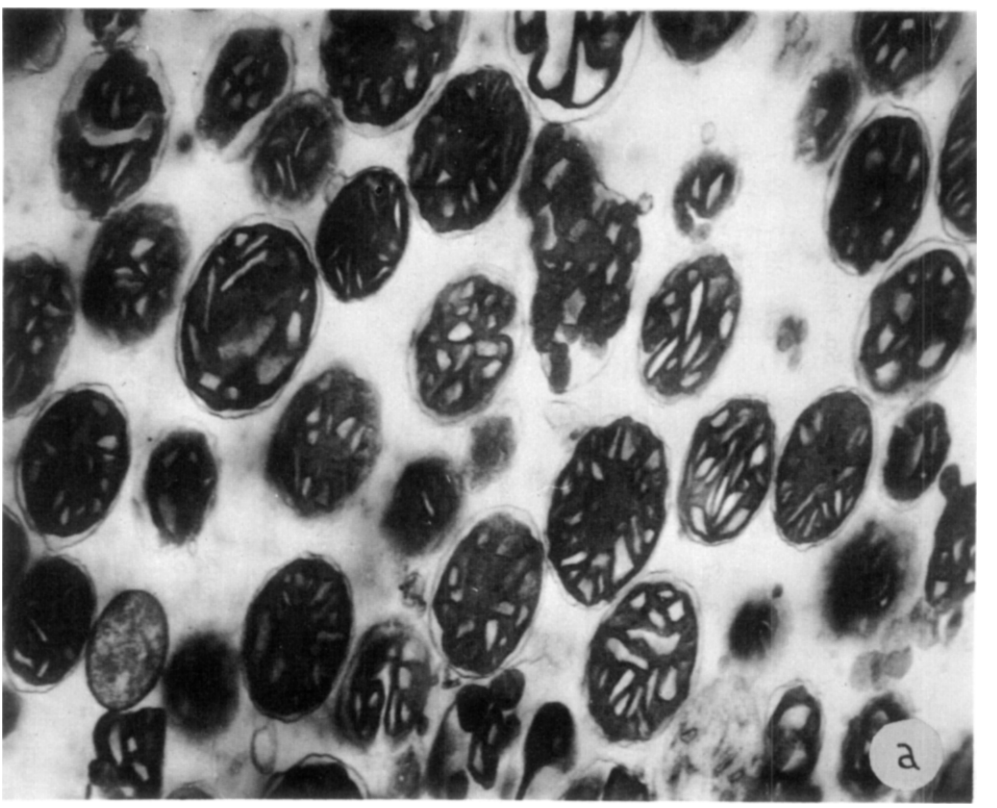
Distribution of Enzymatic Activities in Submitochondrial Fractions after Digitonin Treatment

	% Total Protein	RIDCR		Kynurenine Hydroxylase		MAO	
		Sp. Act.	% Total	Sp. Act.	% Total	Sp. Act.	% Total
Mitochondria	100	138		3.69		3.1	
Inner Membrane-Matrix	67	27.0	15.1	1.17	18	0.75	12
Outer Membrane	12	380	35.7	11.63	39	9.4	40
Soluble	20	59.0	49.6	1.94		4.7	
Light Microsomes		744		0		0.59	

Mitochondria were prepared in 0.220 M mannitol-0.070 M sucrose, pH 7.4.
MAO was assayed as described by Allmann et al. (1968).
Sp. Act. expressed as mμmoles/min/mg.

low as that of the mitochondria prepared by Green et al. (1968).

Mitochondria after four washings have normal morphology with an intact outer membrane and numerous cristae (Fig. 1a). Examination of many fields have confirmed the low level of microsomal contamination established by enzyme data. The biochemical integrity of these mitochondria has been determined in respiratory studies in which respiratory control of 7.5 and ADP/O ratios of 1.7 were obtained with succinate as substrate, assayed polarographically. It should be noted that these are considerably superior to those reported for



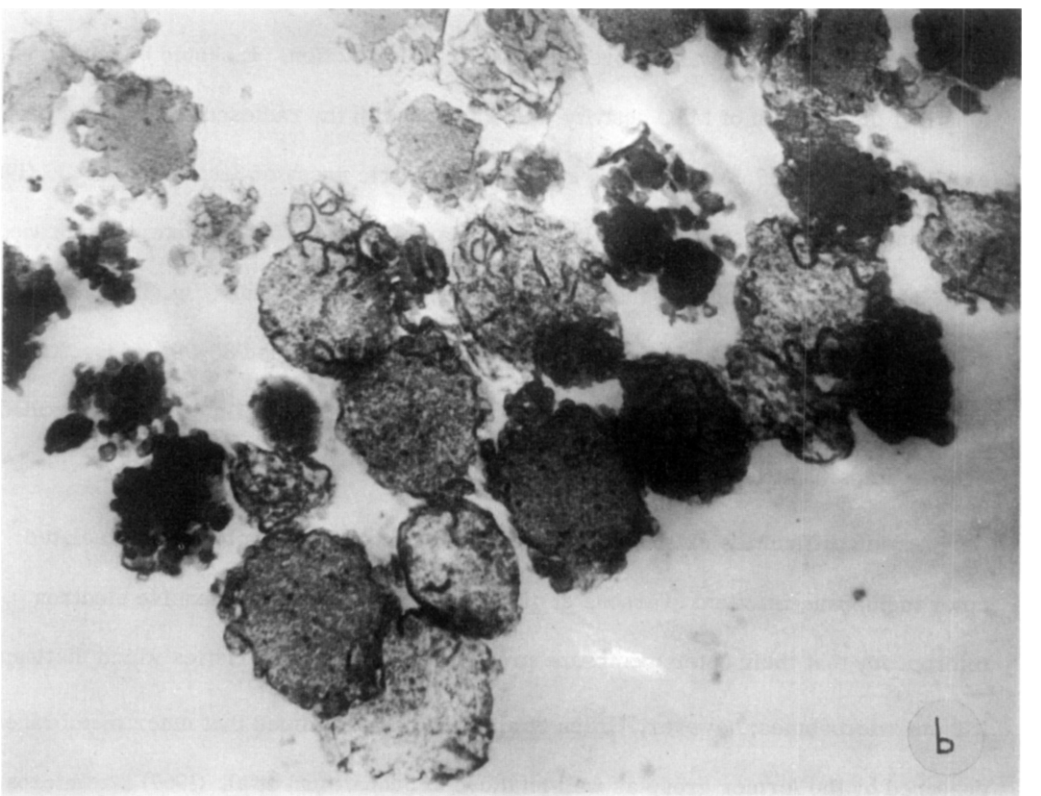


Figure 1. Electron micrographs of sections of aglutaraldehyde-OsO_4 fixed and urahlyl acetate-lead citrate stained suspension of (a) rat liver mitochondria washed four times, (b) inner membrane prepared by digitonin method. (27,400 X).

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beef liver mitochondria by Allmann et al. (1968). In addition, isocitric dehydrogenase activity could not be measured without prior sonication of the mitochondrial suspension.

After fractionation of rat liver mitochondria by the digitonin method of Schnaitman and Greenawalt (1968), 67% of the total mitochondrial protein was recovered in the 9,500 g pellet (inner membrane-matrix), which retained only 12-18% of the total kynurenine hydroxy-

lase, MAO, and RIDCR activity with a concomitant decrease in specific activity (Table II). The 144,000 g pellet (outer membrane) contained 40% of the total activity of these three enzymes with an approximate 3-fold increase in specific activity. The remaining activity of these three enzymes was recovered in the soluble fraction. It should be noted that an identical distribution of MAO activity was obtained with the radioactive tyramine procedure of Allmann et al. (1968) as with the spectrophotometric assay of Schnaitman et al. (1967), thus confirming the assignment of MAO as a marker enzyme for the outer membrane.

Electron micrographs of the inner membrane-matrix fraction (Fig. 1b) indicate the absence of an outer limiting membrane. This fraction contained 80-90% of isocitric dehydrogenase activity as well as 95% of the total mitochondrial ability for the β-oxidation of fatty acids (Beattie, 1968a).

Certain difficulties exist in attempting to discuss the morphology of an isolated outer membrane fraction. Parsons et al. (1967) concluded after extensive electron microscopy that their outer membrane preparation had characteristics which distinguish it from microsomes; however, Green et al. (1968) have claimed that outer membranes prepared by the former group as well as those of Schnaitman et al. (1967) are microsomes contaminating their mitochondrial preparations. If this were indeed true, then the microsomal contamination of the mitochondria would amount to 20-30%, a figure nearly 10-fold greater than that determined by enzyme data. In the present study, after digitonin fractionation of mitochondria with 3% microsomal contamination, two non-microsomal enzymes, (MAO and kynurenine hydroxylase) were removed from the inner membrane (determined morphologically as well as biochemically) and recovered in the outer membrane and soluble fractions. It should be emphasized that the MAO assay employed in this study satisfies the objections of Green et al. (1968) to previous methods (Schnaitman et al., 1967) and that kynurenine hydroxylase is an enzyme found only in the mitochondria (Okamato et al., 1967). Furthermore, additional evidence has been obtained that the outer membrane

fraction obtained by the method of Schnaitman and Greenawalt (1968) is not identical to the microsomes. The in vivo rate of incorporation of (^{14}C) leucine into the proteins of the outer membrane as well as its turnover show no relationship to that of the microsomes (Beattie, 1968b).

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