

## AN APPRAISAL OF THE USE OF MONOAMINE OXIDASE AS AN ENZYME MARKER FOR THE OUTER MEMBRANE OF RAT LIVER MITOCHONDRIA

JOHN W. GREENAWALT and CARL SCHNAITMAN. From the Department of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, and the Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22901

### INTRODUCTION

In a recent paper we reported that monoamine oxidase (MAO) is localized on the outer membrane of rat liver mitochondria. We concluded that this enzymic activity could be used as a biochemical marker to monitor the physical separation of the outer membrane (OM), from the inner membrane plus matrix fraction (IMM), and from components housed between the membranes (6). However, the use of MAO and rotenone-insensitive DPNH cytochrome *c* reductase (RIDCR) as markers for the outer membrane of rat liver mitochondria has been severely criticized by Green

et al. (2). In contrast to other workers, (1, 3-6) Green and co-workers assign enzymes of the citric acid cycle (except succinate dehydrogenase) and of fatty acid oxidation to sites on the outer membrane and MAO to the inner membrane. Green contends that, with regard to MAO localization, estimations of MAO are incorrect (*a*) due to errors in the spectrophotometric assay using benzylamine as substrate and (*b*) due to the high level of aldehyde dehydrogenase (ADH) in the mitochondria.

The present paper should help to clarify this controversy as it specifically relates to the use of MAO as a marker for the OM of rat liver mito-

chondria; the data indicate that the objections raised by Green et al. (2) are without validity. In addition, data are presented which indicate that the enzyme(s) oxidizing benzylamine, tyramine, and serotonin is also associated with the OM fraction of rat liver mitochondria, whereas the intramitochondrial site of kynuramine oxidation is less certain.

## METHODS

### Isolation of Mitochondria

Mitochondria were isolated from the livers of adult male albino rats (250-350 g, Sprague-Dawley strain) at 0°C in a medium containing D-mannitol (220 mM), sucrose (70 mM), HEPES (N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid) buffer (2 mM) and 0.5 mg/ml crystalline bovine serum albumin. The mitochondria were routinely washed twice by resuspension in one-half and then in one-fourth of the original volume of isolation medium followed by centrifugation at 7000 g for 15 min.

The final sediment was resuspended in isolation medium to give 100 mg of mitochondrial protein/ml.

### Digitonin Fractionation of Mitochondria

Digitonin treatment of the mitochondria and fractionation by differential centrifugation were performed as described (6); the modifications in the digitonin concentrations are indicated in the legends to the figures. In some experiments the mitochondria were fractionated into only the OM plus soluble fraction (9500 g supernatant) and the IMM fraction (9500 g sediment). In others the OM was separated from the 9500 g supernatant by sedimentation at 144,000 g for 1 hr after removal of a fluffy layer which was sedimented at 40,000 g for 10 min. Fractionation was monitored both with electron microscopy and by enzymic activities; freshly isolated mitochondria served as reference standards for these procedures.

### Biochemical Assays

Aldehyde dehydrogenase (ADH) was measured optimally in a medium containing Tris-HCl buffer, pH 8.5 (0.05 M), NAD (0.5 mM), benzaldehyde

TABLE I  
Distribution of Aldehyde Dehydrogenase Activity in Submitochondrial Fractions and its Effect on Monoamine Oxidase Activity

Fraction plus additions	Enzyme* activity		Distribution (% of total)		Inhibition (% of total)	
	ADH‡	MAO§	ADH	MAO	ADH	MAO
Mitochondria	155	136	100	100		
+ Chloral hydrate	31	133			80	2
+ Pargyline¶	—	0			—	100
IMM (9500 × g sediment)	135	44	87	32		
+ Chloral hydrate	21	44			84	0
+ Pargyline	—	0			—	100
OM + "soluble" (9500 × g supernatant)	26	102	16	75		
+ Pargyline	—	0			—	100
Recovered activity	161	146	103	107		

\* Arbitrary units.

‡ ADH = Aldehyde dehydrogenase activity; reaction medium contained Tris buffer, pH 8.5 (0.05 M), KCN (0.5 mM), NAD (0.5 mM), benzaldehyde (0.5 mM); NADH formation measured at 340 mμ, 37°C.

§ MAO = Monoamine oxidase activity; reaction medium contained phosphate buffer, pH 7.6 (0.05 M), benzylamine-HCl (2.5 × 10<sup>-3</sup> M); benzaldehyde formation measured at 250 mμ, 37°C.

|| Chloral hydrate and pargyline were added in final concentrations of 10<sup>-3</sup> M and 5 × 10<sup>-6</sup> M, respectively.

¶ N-Benzyl-N-methyl-2-proponylamine-HCl.

Mitochondria were fractionated as previously described (6), by treatment with 0.1 mg digitonin/mg protein followed by differential centrifugation for 10 min.

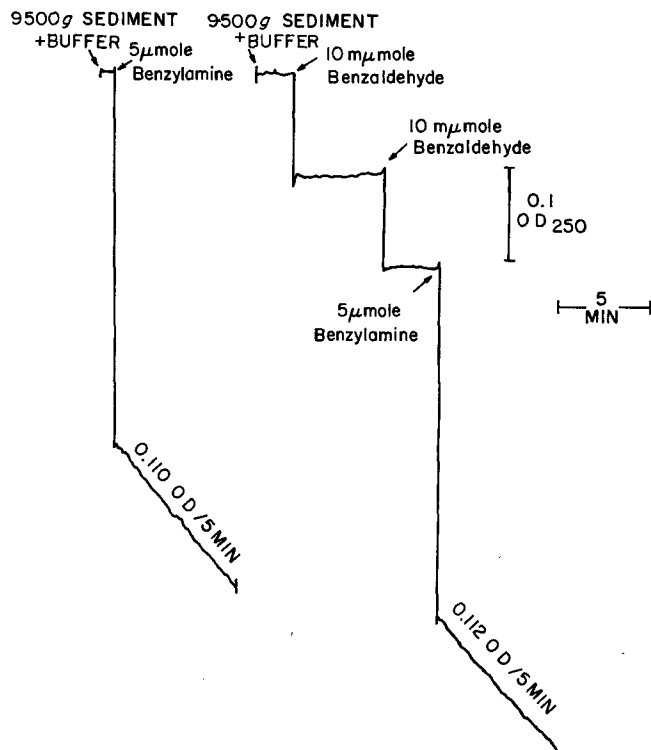


FIGURE 1 Effect of benzaldehyde on oxidation of benzylamine. Monoamine oxidase was assayed as previously described (6) except that a double-beam spectrophotometer was used. Each cuvette contained 0.5 mg protein and 0.2 mg Lubrol in 2.0 ml phosphate buffer, pH 7.6. The 9500 *g* sediment was obtained from mitochondria treated with 0.1 mg digitonin/mg mitochondrial protein, so that both MAO and ADH activities were in this fraction. Separate assays showed that 31% of the monoamine oxidase activity, and 87% of the aldehyde dehydrogenase activity of unfractionated mitochondria were present in the 9500 *g* sediment.

(0.5 mm); NADH formation was followed at 340  $m\mu$  at 37°C. Malate dehydrogenase (MDH) was measured by the method of Ochoa (7) as described previously. Monoamine oxidase (MAO) was measured by five different methods: (a) by the spectrophotometric method of Tabor et al. (8) using benzylamine as substrate; (b) by isotopic methods, using  $^{14}C$  substrates: (i) serotonin- $^{14}C$  or tyramine- $^{14}C$  by the method of Otsuki and Kobayashi (9) and (ii) tyramine- $^{14}C$  as assayed by McCaman et al. (10); (c) by following the change in absorbancy at 360  $m\mu$  using kynuramine as substrate as described by Weissbach et al. (11); and (d) by measuring oxygen uptake polarographically with a Clark oxygen electrode according to the method of Creasey (12) as modified by Tipton (13).

Protein was determined according to the procedure of Lowry et al. (14) or by the biuret method (15).

Tyramine-1- $^{14}C$  (*p*-hydroxyphenethylamine-1- $^{14}C$ ) and serotonin-2- $^{14}C$  (5-hydroxytryptamine-2- $^{14}C$ ) were obtained from New England Nuclear Corporation, Boston, Mass.

## RESULTS

At a pH 7.6 (the optimal pH for MAO assay) relatively little ADH activity was found in the various mitochondrial fractions; therefore, estima-

tions of ADH were made at pH 8.5. The distribution of ADH activity in the submitochondrial fractions was compared with that of MAO. Mitochondria were treated with digitonin as previously described (6), but only the IMM and the OM plus soluble (9500 *g* supernatant) fractions were studied. Treatment with this digitonin concentration provides a fraction containing both MAO and ADH activities (see Table I). In addition, the effect of inhibitors specific for ADH (chloral hydrate) and MAO (pargyline) were investigated. If ADH interfered with the estimation of MAO, the assay for MAO in the presence of chloral hydrate should reflect the inhibition of ADH and should provide a more accurate estimation of MAO activity.

The data presented in Table I show, first, that over 85% of the ADH was recovered in the IMM fraction, whereas 75% of the MAO was found in the OM + soluble fraction. Thus, the two enzymes are localized at different sites within the mitochondria, the MAO being associated with the OM and ADH with the IMM fraction. Secondly, chloral hydrate ( $10^{-3}$  M) inhibited ADH activity in the untreated mitochondrial fraction and in the

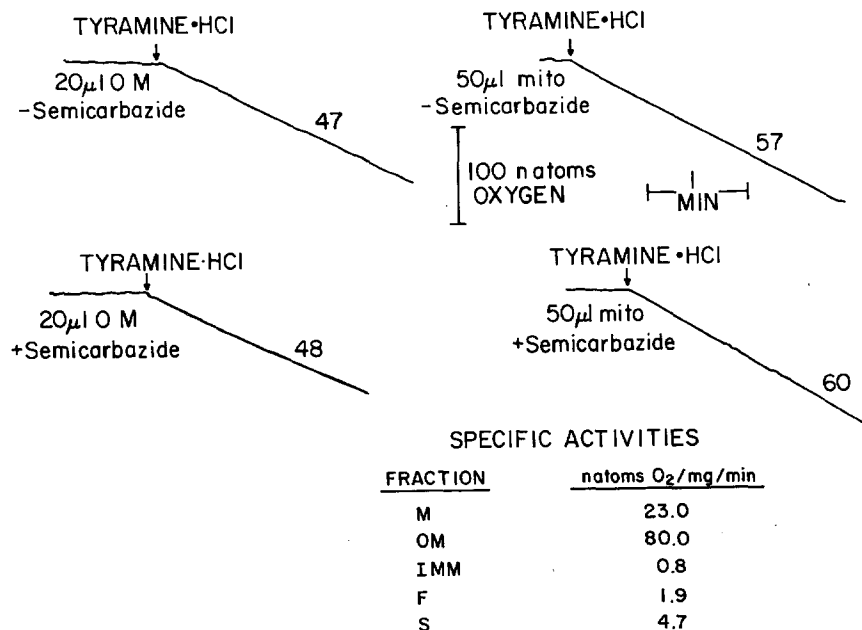


FIGURE 2 Effect of semicarbazide on monoamine oxidase activity. Measured polarographically using a Clark oxygen electrode. Assays were made according to the method of Creasey (12) as modified by Tipton (13). Assay system contained: 200  $\mu$ moles K-phosphate buffer, pH 7.0; 20  $\mu$ moles semicarbazide; 2  $\mu$ moles KCN; enzyme + water to give a total volume of 2.4 ml. Incubation was at room temperature; after equilibration, reaction was started by addition of 100  $\mu$ l of 0.2 M tyramine-HCl. Polarographic traces compare the rates of oxidation of tyramine-HCl by unfractionated mitochondria vs. the OM fraction in the presence and absence of semicarbazide. Table *insert* shows specific monoamine oxidase activity of various submitochondrial fractions: M = unfractionated mitochondria; OM = outer membrane (144,000 g sediment); IMM = inner membrane plus matrix (9500 g sediment); F = "fluffy" layer (40,000 g sediment) and S = "soluble" fraction (144,000 g supernatant).

IMM fraction about equally (80%). Also, the concentration of chloral hydrate used showed no effect on the MAO activity whatsoever, whereas pargyline ( $5 \times 10^{-6}$  M) completely inhibited MAO. These results suggest that the spectrophotometric assay for MAO was not influenced by the presence of ADH in the system. Also a simple reconstitution experiment was done in which IMM (containing ADH activity) was added to OM (MAO-containing fraction). No change in the rate of oxidation of benzylamine was detected.

Two experiments were used to check the possibility that other enzymes other than ADH might be interfering with the assay. Fig. 1 shows that the addition of benzaldehyde directly to the standard spectrophotometric assay of MAO caused no change in absorbancy, indicating that conversion of the aldehyde by the IMM fraction did not take place. Furthermore, it can be seen that benzylamine added to the reaction mixture already con-

taining added benzaldehyde was oxidized at the same rate as in the absence of added benzaldehyde.

The traces in Fig. 2 were obtained using tyramine-HCl as substrate for MAO and oxidation was measured by following oxygen utilization polarographically. The data compare the rates obtained with the intact mitochondria vs. the OM fraction both in the presence and absence of semicarbazide. If, during the reaction, the aldehyde formed by the MAO activity were further oxidized, then the addition of the semicarbazide as a trapping agent for the aldehyde should produce a reduction in the rate observed. As can be seen, the rates were essentially the same in the presence and absence of semicarbazide. The table *insert* shows that the OM fraction exhibited a 10-fold greater MAO specific activity than did the IMM fraction and a 3- to 4-fold greater specific activity than the mitochondria.

The distributions of total protein and MAO in

the 9500 g pellet (IMM) and the 9500 g supernatant (OM plus soluble fraction) were measured, the latter by spectrophotometric assay, using benzylamine as a substrate, and by the tyramine-<sup>14</sup>C assay which Green et al. (2) used to determine MAO localization. Malate dehydrogenase (MDH) distribution in these fractions was also determined as an indicator for the matrix proteins. The results of this experiment are shown in Table II. The recoveries of total protein and of each enzyme assayed were nearly 100%; MDH was found primarily in the IMM fraction (9500 g sediment) as reported previously (6). The results of the two methods were in excellent agreement for the dis-

tribution of MAO; about 90% of the total MAO was recovered in the OM plus soluble fraction. These data indicate that the spectrophotometric assay for MAO is valid for the mitochondrial and submitochondrial fractions and that reported differences in the distribution of MAO are not due to error in the spectrophotometric assay.

The question of whether or not more than a single MAO activity are present in mitochondria has been considered for some time by several laboratories. We therefore looked at the distribution of the MAO activity in our submitochondrial fractions using different substrates and different methods of assay. Table III summarizes the data

TABLE II  
*Comparison of the Distribution and Recovery of Monoamine Oxidase Activity using Benzylamine vs. Tyramine-<sup>14</sup>C as Substrate*

Fraction	% of total enzymic activity or protein*			
	Monoamine oxidase		Malate dehydrogenase	Protein
	Benzylamine	Tyramine- <sup>14</sup> C		
IMM (9500 g sediment)	5.9	7.8	91	70
OM + "soluble" (9500 g supernatant)	87.4	89.7	10	28
% Recovery	93.2	97.5	101	98

\* The total protein or enzymic activity in the initial mitochondrial preparation assayed before fractionation equals 100%.

TABLE III  
*Distribution of Monoamine Oxidase Activity using Various Substrates and Assay Methods*

Fraction	Protein % of total	Benzylamine*		Serotonin†		Tyramine‡		Kynuramine		O <sub>2</sub> uptake§	
		Sp act	% re-covered activity	Sp act	% re-covered activity	Sp act	% re-covered activity	Sp act¶	% re-covered activity	Sp act**	% re-covered activity‡‡
Unfractionated mito	100	20	—	11.5	—	10.8	—	16.0	—	23.0	—
OM	3	383	48.3	94.3	45.0	91.3	48.8	125.8	32.8	80.0	59.5
IMM	76	3	9.6	0.79	9.6	0.31	4.4	6.7	46.4	0.8	16.0
Fluffy layer	5	21	4.4	8.1	6.8	5.47	5.1	12.0	5.4	1.9	2.4
Supernatant	18	48	37.7	12.7	38.6	12.3	41.7	9.4	15.3	4.7	22.1

\* Method of Tabor et al. (8).

† Method of Otsuki and Kobayashi (9).

§ Method of Creasey (12), and modified by Tipton (13).

|| mμmoles/min per mg protein.

¶ Arbitrary units measured as change in absorbancy at 360 mμ/min per mg protein by method of Weissbach et al. (11).

\*\* natoms O<sub>2</sub>/min per mg protein.

‡‡ % of total activity recovered.

obtained when serotonin, tyramine, kynuramine, or benzylamine were used as substrates for MAO. As indicated in Table III, four different methods of assay were used. With all of the substrates, the specific activity of MAO was significantly greater in the OM fraction than in the IMM fraction. The increase in the activity of IMM compared to unfractionated mitochondria ranged from about 3.5-fold (tyramine oxidation measured by oxygen uptake polarographically) to 19-fold (spectrophotometrically with benzylamine as substrate). In all cases the specific MAO activity of the IMM was very low, and, with the exception of kynuramine, contained only about 10–16% of the recovered activity. With each substrate, except kynuramine, about 50% of the recovered activity was found in the OM fraction, regardless of the assay method. In addition, about 20–40% was recovered in the 144,000 g supernatant from which the OM was sedimented. Thus, the activities found in these two fractions (OM and 144,000 g supernatant) accounted for over 80% of the total recovered activities using any of the substrates except kynuramine. In the case of this latter substrate, about 46% of the total activity appeared in the IMM fraction. It should be noted that in these experiments quantitative recovery of the mitochondrial MAO activity was obtained only in the case of benzylamine oxidation. Recoveries with the other substrates ranged from 50 to 70% whereas the mitochondrial protein was quantitatively recovered.

#### DISCUSSION

The data presented here show rather conclusively that MAO measured spectrophotometrically using benzylamine as substrate constitutes a reliable marker for the OM of rat liver mitochondria. Whether or not this activity should more properly be designated "benzylamine oxidase" has not been answered, since the existence of a multiplicity of enzymes which oxidize various monoamines has not been determined unequivocally. It seems certain, however, that the oxidation of tyramine, benzylamine, and serotonin is catalyzed by an enzyme or enzymes associated primarily with the OM of rat liver mitochondria. The localization of "kynuramine oxidase" cannot be ascertained from the present data, but it seems to be different from that of the other activities. It should be pointed out that the isolation and detailed characterization of highly purified enzymes with different sub-

strate specificities are required to show with certainty that multiple monoamine oxidases do function in rat liver mitochondria.

The present study upholds previous reports (1, 6) that treatment with digitonin under controlled conditions does provide a means for systematically subfractionating rat liver mitochondria. Thus, it has been possible to isolate mitochondrial subfractions with distinct biochemical properties which essentially correspond to the physical compartments of freshly isolated mitochondria as determined by ultrastructural studies of thin sections.

The excellent technical assistance of Mrs. Paula Bonitz is gratefully acknowledged. Line drawings for the figures were made by Mr. Ronald Garrett. The authors also thank Dr. Pedersen for many helpful discussions concerning this work and they appreciate the critical reading of this manuscript by Dr. A. L. Lehninger.

This study has been supported in part by Research Grant Number GM 12125 from The National Institute of General Medical Sciences.

Received for publication 25 November 1969, and in revised form 22 January 1970.

#### REFERENCES

1. SCHNAITMAN, C., V. ERWIN, and J. W. GREENAWALT. 1967. *J. Cell Biol.* **32**:719.
2. GREEN, D. E., D. W. ALLMANN, R. A. HARRIS, and W. C. TAN. 1968. *Biochem. Biophys. Res. Commun.* **31**:368.
3. PARSONS, D. F., G. R. WILLIAMS, W. THOMPSON, D. WILSON, and B. CHANCE. 1967. In *Mitochondrial Structure and Compartmentation*. E. Quagliariello, S. Papa, F. C. Slater, and S. M. Tager, editors. Adriatica Editrice, Bari, Italy. 29.
4. SOTTOCASA, G. L., B. KUYLENSTIerna, L. ERNSTER, and A. BERGSTRAND. 1967. *J. Cell Biol.* **32**:415.
5. BEATTIE, D. S. 1968. *Biochem. Biophys. Res. Commun.* **30**:57.
6. SCHNAITMAN, C., and J. W. GREENAWALT. 1968. *J. Cell Biol.* **38**:158.
7. OCHOA, S. 1955. In *Methods in Enzymology*. S. Colowick and N. Kaplan, editors. Academic Press Inc., New York **1**:735.
8. TABOR, C. W., H. TABOR, and S. M. ROSENTHAL. 1954. *J. Biol. Chem.* **208**:644.
9. OTSUKI, S., and Y. KOBAYASHI. 1964. *Biochem. Pharmacol.* **13**:955.
10. McCAMAN, R. E., M. W. McCAMAN, J. M.

- HUNT, and M. S. SMITH. 1965. *J. Neurochem.* **12**:15.
11. WEISSBACH, H., T. E. SMITH, J. W. DALY, B. WITKOP, and S. UDENFRIEND. 1960. *J. Biol. Chem.* **235**:1160.
12. CREASEY, N. H. 1956. *Biochem. J.* **64**:178.
13. TIPTON, K. F. 1967. *Biochim. Biophys. Acta.* **135**:910.
14. LOWRY, O., N. ROSEBROUGH, A. FARR, and R. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
15. GORNALL, A. G., C. T. BARDOWALL, and M. M. DAVID. 1963. *J. Biol. Chem.* **177**:574.