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ASSOCIATION OF MONOAMINE OXIDASE WITH LIPID

A COMPARATIVE STUDY OF MITOCHONDRIA FROM NOVIKOFF HEPATOMA AND RAT LIVER*

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Abstract—Monoamine oxidase (MAO) from Novikoff hepatoma and liver of the rat host has been studied. MAO in the mitochondria from the fast growing hepatoma was determined to be predominantly of the A type by studies carried out with the irreversible inhibitors clorgyline and deprenyl. The enzyme from the host liver was similar to that reported for normal rat liver MAO. In order to investigate the role lipid may play in the genesis of different types of MAO, the hepatoma mitochondria were investigated as a model for type A MAO and compared to the host liver mitochondria which were shown to have both types of MAO activity. Phospholipid analysis of both hepatoma and host liver mitochondria revealed no significant difference in the proportion of outer membrane phospholipids. Delipidation with methyl ethyl ketone of both mitochondrial preparations resulted in the inactivation of type A MAO, which could be reactivated partially by mitochondrial lipids, but not by purified phosphatidylcholine. Low recovery of type B MAO after delipidation of the hepatoma mitochondria suggests that transformation of type A to type B MAO does not occur. Arrhenius plots of MAO activity revealed that type B MAO was sensitive to a phase transition of the outer mitochondrial membrane. The energy of activation of type B MAO as estimated by benzylamine deamination was reduced by 47 per cent at temperatures below 13°. Delipidation of mitochondria also resulted in a similar decrease in the energy of activation of type B MAO at all temperatures tested. Both types of MAO were shown to be labile to freeze/thawing as a result of delipidation. The results suggest that both types of MAO are integrally associated with the lipid environment of the outer mitochondrial membrane and that the association of lipid with the two types of MAO is different.

Monoamine oxidase (monoamine: O_2 oxidoreductase, EC 1.4.3.4) (MAO) is an enzyme, located primarily in the outer membrane of mitochondria [1], which catalyzes the oxidative deamination of a variety of biogenic amines. This enzyme may exist in multiple forms although the exact nature of these forms is a subject of controversy. Differing substrate specificity and sensitivity of MAO to irreversible inhibitors are some of the principal reasons for postulating that multiple forms of the enzyme may occur, although other criteria have been used [2]. From studies on the inactivation of MAO using the irreversible inhibitor clorgyline, MAO has been proposed to exist as a sensitive "A" type MAO and a less sensitive "B" type MAO in mitochondrial preparations from a number of tissues [3, 4]. In a large number of tissues, including rat liver, 5-hydroxytryptamine (5-HT) is deaminated by type A MAO, benzylamine is deaminated by type B MAO, and tyramine is deaminated by both types of MAO [4].

Rat hepatomas have not been well characterized with regard to the type or even the presence of MAO activity. Pedersen *et al.* [5] found that MAO activity measured with benzylamine was low or undetected in mitochondria from Morris hepatomas 9618A, 7800 and 3924A. MAO activity measured in mitochondria from the Novikoff hepatoma grown as solid, transplantable tumors and monolayer cell cultures was essentially undetectable when assayed with benzylamine and low with other substrates tested [6, 7].

In view of the limited range of substrates used for previous studies and the lack of information on the existence of multiple MAO types in neoplastic tissues, the present investigation represents a more detailed study of the properties of MAO in the solid, transplantable Novikoff hepatoma compared with the host liver enzyme. In addition, as a result of these experiments, the Novikoff hepatoma is used as a model for type A MAO to examine the possible role of lipids in modulating the multiple forms of MAO. The mitochondria from the hepatoma and host liver are compared with respect to their phospholipid composition and MAO activity after delipidation. Finally, Arrhenius plots of temperature studies were done in an attempt to detect changes in MAO activity due to lipid phase transition in the outer mitochondrial membrane.

MATERIALS AND METHODS

Materials. [Methylene-^{14}C]benzylamine hydrochloride was obtained from ICN Pharmaceuticals Inc., Irvine, CA, [^{3}H]tyramine hydrochloride from New England Nuclear, Boston, MA, and [^{3}H]-5-hydroxytryptamine creatine sulfate from Amersham-Searle, Arlington Heights, IL. Clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride, M & B 9302] was a generous gift from May & Baker Ltd, Dagenham, Essex, England. Deprenyl (phenylisopropylmethylpropinyl hydrochloride) was kindly provided by Dr. P. H. Kelly, Department of Pharmacology, Michigan State University, East Lansing, MI. Phospatidylcholine purified from egg yolk was obtained from Dr. L. Huang, Department of Bio-

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Preparation of mitochondria. Hepatomas were maintained in male Holtzman rats (Holtzman Co., Madison, WI) by serial transplantation of Novikoff hepatomas obtained from Dr. F. Snyder, Oak Ridge Associated Universities, Oak Ridge, TN. Hepatomas were removed from cervically dislocated rats on day 5 or 6 following transplantation, for isolation of mitochondria and continued transplantation of the tumor. Livers from the tumor-bearing rats were removed at the same time, and mitochondria from this tissue were isolated by a method described previously [8] in H medium [70 mM sucrose, 200 mM mannitol, 2 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), and 0.05% bovine serum albumin (BSA), adjusted to pH 7.4 with NaOH]. Mitochondria from the hepatoma were isolated as above except with the following modifications. The hepatoma was homogenized in H' medium (BSA added to 0.1% final concentration) with ten strokes of a Teflon pestle (motor driven) in a glass homogenizer. The homogenate was centrifuged at 660 *g* for 10 min; the pellet was rehomogenized with five strokes of the homogenizer and recentrifuged. The supernatant fractions from the first and second centrifugation were pooled and mitochondria were isolated by differential centrifugations as described previously [8]. When necessary, mitochondrial fractions were further purified by equilibrium sedimentation in a continuous sucrose gradient (1.0 to 2.5 M) [6].

Assay of monoamine oxidase. MAO activity was routinely estimated by a modification of the radiochemical method of Callingham and Laverty [9]. Three substrates, benzylamine, tyramine and 5-HT, were used at final specific activities of 0.1, 1.0 and 2.0 μCi/μmole respectively. Substrate at a 1 mM final concentration was incubated with samples containing about 0.5 mg protein. Fifty μl mitochondrial samples diluted with water, and 50 μl substrate prepared in 0.2 M phosphate buffer, pH 7.8, were mixed and oxygenated on ice before being transferred to a water bath at 37°. The assay was terminated by transfer to ice and addition of 10 μl of 3 N HCl after an interval in which the assay is linear with time. Deaminated metabolites were extracted into 0.5 ml of ethyl acetate–benzene (1:1, v/v). After mixing and separation of phases by centrifugation, 0.4 ml of the organic phase was counted for radioactivity in 4 ml of 0.4% butyl-PBD in toluene (w/v) in a Beckman LS-230 liquid scintillation spectrometer.

MAO activity was also estimated by a polarographic method. Oxygen consumption was measured with a Clark oxygen electrode [10]. Samples containing 3–5 mg protein were assayed at 37° in a final volume of 1.5 ml of H medium (pH 7.4) containing 1 mM KCN. Substrates were added to a final concentration of 3 mM, and O_2 consumption was linear for at least 2 min.

The activity of MAO using benzylamine as substrate was also measured by the spectrophotometric determination of benzaldehyde formation at 250 nm [11]. This assay was performed at a final benzylamine concentration of 1.0 mM in 0.1 M phosphate buffer, pH 7.8, at room temperature. Mitochondria were solubilized in 2% Lubrol WX to reduce light scattering.

Phospholipid analysis. Total mitochondrial lipid was extracted by the method of Bligh and Dyer [12]. Phospholipids were separated by two dimensional thin-layer chromatography (t.l.c.) using silica gel H, first in a solvent of chloroform–methanol–NH_4OH (13:5:1), followed by a solvent containing chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1). The phospholipid fractions were visualized and eluted from the t.l.c. plates as described by Morton *et al.* [13]. Lipid phosphorus was determined as described by Ames and Dubin [14].

Preparation of lipid-depleted mitochondria. Water-lysed mitochondria from hepatoma and host liver were prepared by suspending mitochondria in distilled water with a Dounce homogenizer, and were then centrifuged at 15,000 *g* for 15 min. This procedure was repeated with the resulting pellet. Water-lysed mitochondria were extracted with methyl ethyl ketone (MEK) as described by others [15, 16]. Briefly, the mitochondria preparation was extracted with 8 vol. MEK. The organic phase was decanted, and the residue was resuspended in 0.1 M phosphate buffer (pH 7.2) containing 1 mM EDTA and centrifuged at 15,000 *g* for 15 min. The supernatant fraction (buffer extract) was decanted, and the pellet (delipidated mitochondria) was resuspended in the same buffer.

Addition of lipid to delipidated mitochondria. Mitochondrial lipids were pooled from several preparations of host liver mitochondria. Lipid dispersions were prepared by evaporating the lipids to dryness, adding phosphate buffer (0.1 M, pH 7.2, + 1 mM EDTA) to give a lipid concentration of 5.0 mg/ml, and sonicating for 10 min under nitrogen in a bath sonicator at approximately 15°. Reactivation of MAO activity was attempted by addition of 1.0 mg of delipidated mitochondrial protein to an aliquot of lipid dispersion, followed by adjustment of the resulting volume to 1 ml with the same phosphate buffer, and then incubation at 37° for 30 min. MAO activity was then determined as described previously for the radiochemical method, except that blanks containing either heat-inactivated or acid-inactivated delipidated mitochondria and the corresponding amounts of lipid were prepared for each lipid concentration used.

Protein was determined by the biuret method [17] with crystalline BSA as the standard. Glucose-6-phosphatase activity was determined as a marker of microsomal contamination [18].

RESULTS

Inhibition of host liver and hepatoma MAO by clorgyline and deprenyl. By definition, type A MAO is more sensitive to inhibition by the irreversible inhibitor clorgyline than is type B MAO [3]. Another irreversible inhibitor, deprenyl, selectively inactivates type B MAO at low concentrations [19]. Inhibition of MAO activity in hepatoma and host liver mitochondria by these inhibitors was studied, therefore, in an attempt to determine whether the type A and B enzymes are present in these tissues.

Figure 1 shows the inhibition of MAO in mitochondria from Novikoff hepatoma and host liver by both clorgyline and deprenyl. 5-HT deamination in mitochondria from both tissues was inhibited in a single sigmoid manner, with complete inhibition at concentrations of 10^{-6} M clorgyline and 10^{-4} M deprenyl. These results indicate that 5-HT is a substrate for type A

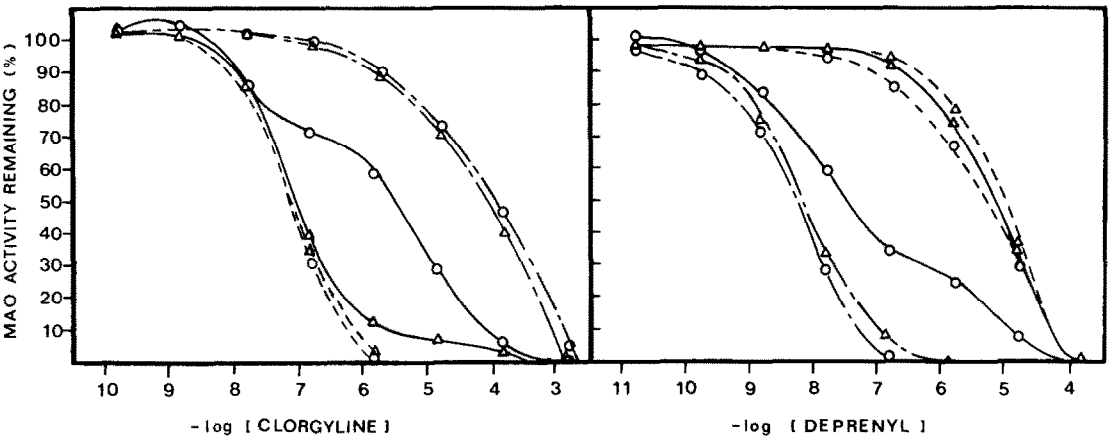

Fig. 1. Inhibition of MAO in Novikoff hepatoma and host liver mitochondria by clorgyline and deprenyl. Novikoff hepatoma mitochondria (△) and host liver mitochondria (○) were preincubated for 20 min at 37° with the indicated concentration of clorgyline or deprenyl. MAO activity was then determined by the radiochemical method with benzylamine (——— - ———), 5-HT (- - - -) and tyramine (———). Mitochondria protein content was approximately 60 μg. Each point is the mean of three determinations. Standard errors were ⩽7 per cent and not shown for clarity of the figure.

MAO in these tissues. Benzylamine deamination in both preparations was likewise inhibited completely at concentrations of 10^{-3} M clorgyline and 10^{-6} M deprenyl. Thus, benzylamine appears to be a substrate for type B MAO. The inhibition of tyramine deamination in both mitochondrial preparations by clorgyline resulted in a double sigmoid inhibition curve. The ratios of MAO types determined from this curve were 30 per cent type A to 70 per cent type B in the host liver mitochondria and 90 per cent type A to 10 per cent type B in the Novikoff hepatoma. Inhibition of tyramine deamination in host liver mitochondria by deprenyl also resulted in a double sigmoid curve that corresponds to 30 per cent type A and 70 per cent type B; however, the inhibition of tyramine deamination in the hepatoma mitochondria by deprenyl results in a single sigmoid curve with enzyme activity completely inhibited at 10^{-4} M deprenyl.

MAO activity in host liver and hepatoma mitochondria. The specific activities of MAO in mitochondria from Novikoff hepatoma and host liver measured, when possible, with radiochemical, polarographic and spectrophotometric assay are shown in Table 1. Substrates deaminated by either one or both types of MAO, when possible, were used to estimate MAO activity. The rate of benzylamine deamination in the hepatoma mitochondria was consistently much less than the rate of benzylamine deamination measured in mitochondria from host liver in all three assay systems. The rates of deamination of tyramine and 5-HT in the hepatoma mitochondria were determined to be about 10-fold higher than the rate of benzylamine deamination by the more sensitive radiochemical assay. These activities correspond to about 25 per cent of the rate of tyramine deamination and 45 per cent of the rate of 5-HT deamination in host liver mitochondria.

The substrate concentrations used for the determination of MAO activity were well above K_m concentrations for the enzyme both in host liver and in Novikoff hepatoma. The initial velocities of deamination with different substrate concentrations were measured in additional experiments and analyzed according to the Lineweaver and Burk double reciprocal method. These results gave estimated K_m values of 200 μM for benzylamine, 150 μM for tyramine, and 95 μM for 5-HT in the hepatoma mitochondria and about 100 μM for all substrates in the host liver mitochondria.

Phospholipid composition of host liver and hepatoma

Table 1. Monoamine oxidase activities of freshly isolated mitochondria from Novikoff hepatoma and host liver

Substrate	Tissue	Assay method: Radiochemical (nmoles/hr/mg)	Spectrophotometric (nmoles/hr/mg)	Polarographic (nmoles O_2/hr/mg)
Benzylamine	Host liver	617 ± 68*	570 ± 78	1038 ± 252
	Hepatoma	27.7 ± 6.5 (4.5%)†	30 ± 12 (5.3%)	132 ± 53 (12.7%)
Tyramine	Host liver	1135 ± 65	NA	1014 ± 132
	Hepatoma	282 ± 21 (24.8%)	NA	402 ± 78 (39.8%)
5-Hydroxytryptamine	Host liver	725 ± 33	NA	834 ± 120
	Hepatoma	325 ± 60 (44.8%)	NA	462 ± 160 (55.4%)

* Values are mean ± S.E. of four preparations of mitochondria.

† Activity of hepatoma mitochondria expressed as per cent of host liver mitochondria.

‡ NA = not applicable since these substrates do not yield a product with an absorbance maximum convenient for assay.

Table 2. Phospholipid composition of mitochondria from Novikoff hepatoma and host liver*

Tissue	% Total lipid phosphorus in t.l.c. fractions CL	PC	PE	PS + PI	SM	Unknown
Host liver	7.6 ± 0.6†	50.2 ± 1.1	36.8 ± 1.3	1.8 ± 1.0	1.8 ± 0.4	2.3 ± 1.0
Hepatoma	3.7 ± 0.4	52.1 ± 1.2	37.3 ± 1.0	3.0 ± 0.5	2.1 ± 0.7	1.0 ± 0.6
	Total phospholipid concentration (nmoles P/mg protein)‡					
Host liver	11.9 ± 0.9	79.9 ± 1.7	60.6 ± 2.0	4.3 ± 1.5	4.3 ± 0.6	3.6 ± 1.0
Hepatoma	10.5 ± 1.1	148.0 ± 3.3	106.0 ± 2.8	8.5 ± 1.5	6.0 ± 1.1	5.4 ± 0.8

*Abbreviations used: CL, cardiolipin; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS + PI, phosphatidyl serine + phosphatidyl inositol; and SM, sphingomyelin.

† Values are the mean ± S.E. of four preparations of mitochondrial lipids.

‡ Based on 157 nmoles lipid phosphorus/mg of host liver mitochondrial protein and 288 nmoles lipid phosphorus/mg of hepatoma mitochondrial protein.

mitochondria. Lipids were extracted from mitochondria and quantified as described in Materials and Methods. Table 2 lists the phospholipid compositions of freshly isolated mitochondria from host liver and Novikoff hepatoma. The phospholipid compositions of the two mitochondrial preparations were qualitatively the same. Quantitative differences in the hepatoma and host liver mitochondrial phospholipid composition were small when expressed as a percentage of total lipid phosphorus, with the exception of a lower percentage of cardiolipin in the hepatoma mitochondria. Table 2 also shows the phospholipid composition expressed as nmoles of lipid phosphorus per mg of protein. In this case, the host liver and hepatoma phospholipid compositions were different, due to the greater phospholipid to protein ratio measured in the hepatoma mitochondria. A ratio of 157 nmoles lipid phosphorus/mg of mitochondrial protein was determined for the host liver mitochondria and 288 nmoles lipid phosphorus/mg of mitochondrial protein was determined in the Novikoff hepatoma mitochondria.

The host liver mitochondria used for phospholipid analysis were isolated by differential centrifugation and repeated washing. Microsomal contamination, expressed as the percentage of total cellular glucose-6-phosphatase activity in the mitochondrial fraction, was always less than 5 per cent. Novikoff hepatoma mitochondria that contained 2 per cent of the total cellular glucose-6-phosphatase activity after isolation by differential centrifugation were purified further by sedimentation in a continuous sucrose gradient before the lipids were extracted for estimation of phospholipid composition.

Extraction of lipids by methyl ethyl ketone. Methyl ethyl ketone (MEK) extraction was performed in order to investigate the effect of lipid extraction on MAO activity in the host liver and hepatoma mitochondria. The extraction was carried out with pooled samples of frozen/thawed and then water-lysed mitochondria from several host liver and hepatoma mitochondrial preparations, since single preparations, particularly from the hepatoma, did not yield sufficient quantities of mito-

Table 3. Phospholipid compositions of water-lysed* and delipidated mitochondria from Novikoff hepatoma and host liver†

	Total lipid phosphorus (μmoles)	Lipid phosphorus (μmoles) in t.l.c. fractions CL	PC	Lyso PC	PE	Lyso PE	PS + PI	SM	Unknown
Host liver mitochondria	9.55	0.66	3.92	0.52	2.72	0.52	0.26	0.54	0.41
Delipidated host liver mitochondria‡	1.03	0.42	0.18	0.15	0.25	<0.01	0.02	0.02	<0.01
% Lipid phosphorus extracted	89.20	36.40	95.40	71.20	90.80	>98.00	92.30	96.30	>98.00
Novikoff hepatoma mitochondria	15.40	0.51	6.55	1.09	4.48	0.75	0.80	0.34	0.88
Delipidated hepatoma mitochondria§	0.97	0.37	0.39	<0.01	0.19	<0.01	<0.01	<0.01	0.02
% Lipid phosphorus extracted	93.70	27.50	96.00	>99.00	95.70	>98.70	>99.00	>97.00	97.70

* Mitochondria were frozen and then water-lysed.

† Abbreviations used:
CL, cardiolipin; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS + PI, phosphatidyl serine + phosphatidyl inositol; and SM, sphingomyelin.

‡ MEK extract contained 8.4 μmoles and buffer extract contained 0.27 μmole; 101 per cent recovery of lipid phosphorus.

§ MEK extract contained 13.6 μmoles and buffer extract contained 0.20 μmole; 94.6 per cent recovery of lipid phosphorus.

Table 4. Effect of lipid extraction with methyl ethyl ketone on MAO activity*

Source	Substrate	Specific activity before MEK extraction (nmoles/hr/mg)	Specific activity after MEK extraction† (nmoles/hr/mg)	% Recovery
Novikoff	Tyramine	323	29.5	8.0 (9.7 ± 2.2)‡
hepatoma	5-HT	311	21.0	5.9 (6.8 ± 1.7)
mitochondria	Benzylamine	51.7	26.5	44.9 (42.3 ± 9.2)
Host	Tyramine	1075	643	35.2 (29.6 ± 2.8)
liver	5-HT	888	67	4.4 (4.7 ± 1.3)
mitochondria	Benzylamine	644	489	77.0 (76 ± 5.0)

*MAO activity was assayed by the radiochemical method described in Materials and Methods.
† All detectable activity was found in the delipidated residue; no activity was detected in the buffer wash and activity could not be determined in the MEK extract.
‡ (Mean per cent recovery ± S.E.) for extractions on five different preparations.

chondria for this procedure. Table 3 shows the phospholipid compositions of frozen, water-lysed mitochondria from hepatoma and host liver before and after MEK extraction. Extraction by MEK resulted in an effective extraction of all mitochondrial phospholipids except cardiolipin. Phospholipids were extracted in a similar manner in mitochondria from both host liver and hepatoma, although MEK was slightly more efficient in extracting phospholipids from the hepatoma mitochondria (93.7 per cent lipid phosphorus extracted) than from host liver mitochondria (89.2 per cent lipid phosphorus extracted).

Effect of methyl ethyl ketone extraction on MAO activity. Extraction of mitochondria from both hepatoma and host liver with MEK resulted in a loss of MAO activity toward all three substrates. Table 4 shows the specific activities of MAO in a representative preparation of hepatoma and host liver mitochondria before and after MEK extraction and the total recovery of activity after extraction. The recovery of MAO activity after extraction differed among the substrates tested. In the delipidated mitochondria from Novikoff hepatoma, 5.9 per cent of the 5-HT deaminating activity, 8.0 per cent of the tyramine deaminating activity, and 44.9 per cent of the benzylamine deaminating activity remained of the total activity before extraction. After delipidation of the host liver mitochondria 4.4 per cent of 5-HT deaminating activity, 35.2 per cent of tyramine deaminating activity, and 77.0 per cent of benzylamine deaminating activity remained of the total activity before delipidation.

The buffer wash of the delipidated mitochondria and organic solvent contained no detectable MAO activity. The buffer wash from the extraction of the hepatoma mitochondria (data for Table 4) contained 8 per cent of the total unextracted mitochondrial protein and the total protein recovered in the delipidated mitochondria plus buffer wash was 92.5 per cent of the unextracted material. The buffer wash from the extraction of the host liver mitochondria contained 22 per cent of the

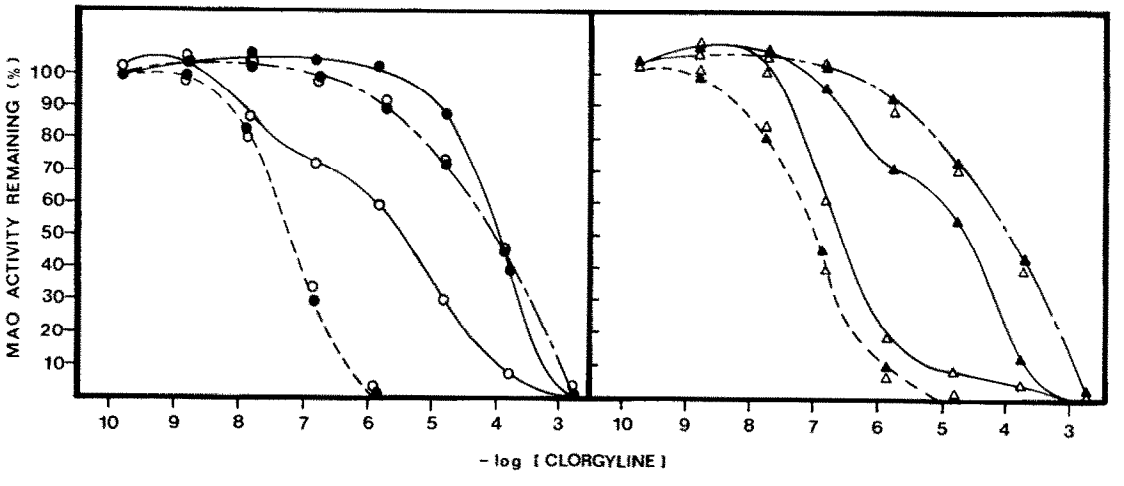

Fig. 2. Inhibition of MAO by clorgyline in Novikoff hepatoma and host liver mitochondria before and after extraction with methyl ethyl ketone. The left panel is the inhibition of MAO in host liver mitochondria (○) and delipidated host liver mitochondria (●); the right panel is the inhibition of MAO in Novikoff hepatoma mitochondria (△) and delipidated hepatoma mitochondria (▲). Substrates for MAO activity using the radiochemical method were benzylamine (—— · ——), 5-HT (· · · ·) and tyramine (——). Each point is the mean of three determinations. Standard errors were ≤5 per cent and not shown for clarity of the figure.

Table 5. MAO activity before and after freeze/thawing*

Tissue	Treatment	Substrate	Activity† (nmoles/hr/mg) Fresh	Freeze/thaw	% Recovery
Host liver mitochondria	Fresh	Tyr‡	1070	996	93.1
		5-HT	738	714	96.1
		BA	612	582	95.1
	Water-lysed	Tyr	800	766	95.7
		5-HT	515	508	98.6
		BA	532	505	94.9
	Delipidated	Tyr	284.1	89.6	31.5
		5-HT	35.0	12.6	36.0
		BA	296.0	69.2	23.4
Novikoff hepatoma mitochondria	Fresh	Tyr	296	278	93.9
		5-HT	298	276	92.6
		BA	43.0	41.0	95.3
	Water-lysed	Tyr	225	198	88.4
		5-HT	301	274	91.9
		BA	44.0	40.1	90.1
	Delipidated	Tyr	70.7	4.8	6.8
		5-HT	35.9	5.5	7.8
		BA	29.6	2.1	4.6

* Freshly isolated mitochondria were assayed and stored frozen for 2 weeks at −20°, at which time they were assayed. Water-lysed mitochondria were prepared from the frozen mitochondria, assayed, and stored frozen at −20° for 4 days, and then reassayed. Delipidated mitochondria were prepared from frozen mitochondria which were water-lysed shortly before MEK extraction. The delipidated residue was assayed, stored frozen overnight at −20°, and reassayed. Data are the mean of two preparations.

† MAO activity was assayed by the radiochemical method.

‡ Abbreviations used:
Tyr, tyramine; 5-HT, 5-hydroxytryptamine; and BA, benzylamine.

total unextracted protein and the total recovery of protein after delipidation was 80 per cent.

The relative proportions of type A and type B MAO are changed after lipid depletion. Figure 2 shows the inhibition of MAO by clorgyline in host liver and hepatoma mitochondria before and after delipidation. The substrate specificity of the two enzyme forms was not altered by the MEK extraction in either the host liver mitochondria or hepatoma mitochondria. However, the relative proportions of type A and type B MAO metabolizing tyramine in both host liver and hepatoma mitochondria were altered such that type B MAO predominated in the delipidated residue. The inhibition of tyramine deamination in host liver mitochondria and delipidated residue was studied; the double sigmoid inhibition curve, which indicated a ratio of 70:30 for type A:type B MAO activity before lipid extraction, became a single sigmoid curve after MEK extraction, indicating that only type B MAO activity remained. Delipidation of the hepatoma mitochondria altered the ratio of 90 per cent type A to 10 per cent type B MAO before MEK extraction to 30 per cent type A to 70 per cent type B MAO after extraction, as shown in Fig. 2.

Effect of freezing and thawing on hepatoma and host liver MAO. Table 5 shows the effects of freeze/thawing on the MAO activities of fresh mitochondria, water-lysed membranes, and delipidated residue from both Novikoff hepatoma and host liver. While fresh and water-lysed mitochondrial MAO from both host liver and Novikoff hepatoma were almost completely stable to storage by freezing at −20° and subsequent thawing for assay, the delipidation of host liver and hepatoma mitochondria rendered the residual MAO activity labile as a result of freeze/thawing. The recovery of MAO activity after freeze/thawing of the delipidated residue varied only slightly with the substrate used to determine activity. In the delipidated residue from host liver mitochondria, 31.5 per cent of tyramine, 36.0 per cent of 5-HT, and 23.4 per cent of benzylamine deaminating activity were recovered after freezing. In the delipidated residue from hepatoma mitochondria 6.8 per cent of tyramine, 7.8 per cent of 5-HT and 4.6 per cent of benzylamine deaminating activity were recovered after freezing.

Effect of added lipid on MAO activity in delipidated mitochondria. The effects of mitochondrial phospholipids on MAO activities in both delipidated host liver mitochondria and hepatoma mitochondria are shown in Fig. 3. Mitochondria were extracted with MEK and incubated with lipid dispersions of total mitochondrial lipid extracts of host liver mitochondria for 30 min at 37° as described in Materials and Methods. Hepatoma mitochondrial lipids were not used; however, the lipid composition is similar to that of host liver mitochondria. The rate of deamination of 5-HT in both delipidated hepatoma and host liver mitochondria was increased after incubation with mitochondrial lipids. MAO activity with 5-HT in the delipidated hepatoma mitochondria, which corresponded to 3.5 per cent of the total MAO activity before delipidation, was maximally reactivated to 11.7 per cent of the total activity before delipidation. Mitochondrial lipid reactivated MAO activity with 5-HT in the delipidated host liver

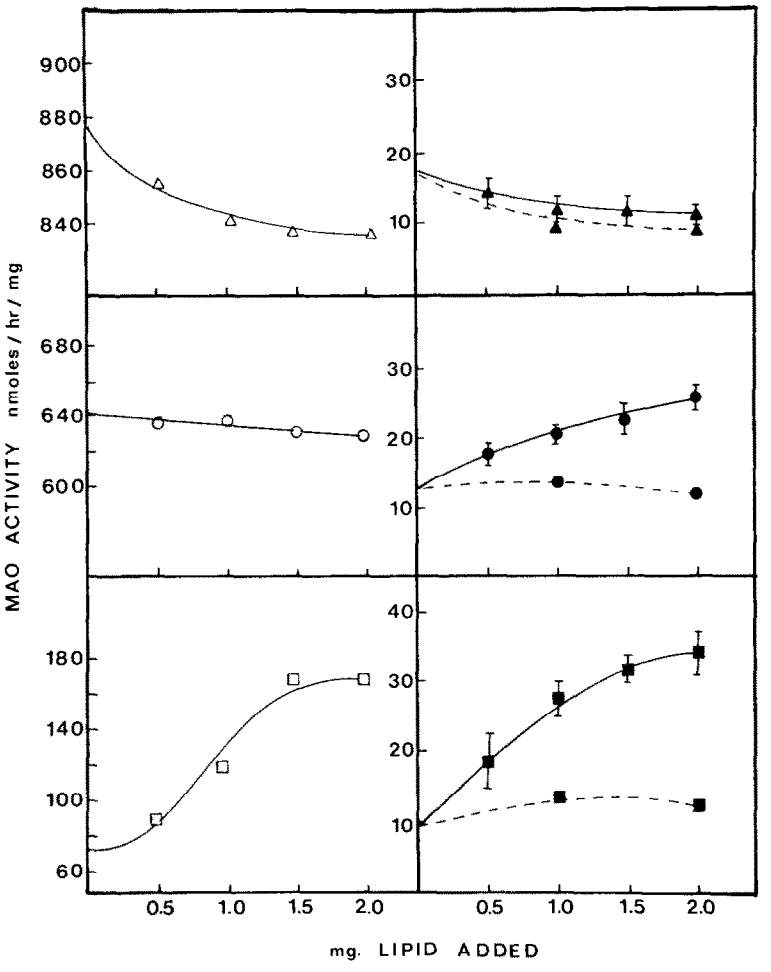

Fig. 3. Effect of added lipid on MAO activity in delipidated mitochondria from host liver and Novikoff hepatoma. The left panels show the effect of host liver mitochondrial lipids on MAO in the delipidated host liver mitochondria, as described in Materials and Methods. MAO activity was measured with benzylamine (△——△), tyramine (○——○) and 5-HT (□——□). The right panels show the effects on MAO activities in delipidated Novikoff hepatoma mitochondria of host liver mitochondrial lipids (——); and purified phosphatidyl choline from egg yolk (- - -). MAO activity was measured with benzylamine (▲), tyramine (●) and 5-HT (■) by the radiochemical method. Each point is the mean of three determinations. Standard errors for host liver data were smaller than the symbols and not shown. Error bars for the effect on MAO activity in the delipidated hepatoma mitochondria of host liver mitochondrial lipid represent the variation of two aliquots of hepatoma mitochondria which were extracted separately and added to lipid under identical conditions.

mitochondria from 4.5 to 10.7 per cent of the total activity before delipidation.

The MAO activity with tyramine increased 1.6-fold in the delipidated hepatoma mitochondria when incubated with mitochondrial lipid. However, no increase in the rate of tyramine deamination occurred in the delipidated host liver mitochondria. Incubation of delipidated mitochondria with mitochondrial lipids led to a decrease in the rate of benzylamine deamination in the delipidated hepatoma mitochondria of 30 per cent and in the delipidated host liver mitochondria of 5 per cent. The activity of MAO at zero lipid in Fig. 3 was determined for delipidated mitochondria which were incubated in buffer under the same conditions as the delipidated mitochondria plus lipid.

Figure 3 also shows the MAO activity of delipidated hepatoma mitochondria plus dispersions of increasing concentration of phosphatidylcholine purified from egg yolk. This phospholipid shows no ability to increase the activity of MAO toward any substrate tested in either the delipidated hepatoma or host liver mitochondria. The rate of benzylamine deamination in both delipidated mitochondrial preparations is reduced by egg phosphatidylcholine to the same extent as with mitochondrial lipids.

Dependence of MAO activity on temperature. MAO activities in Novikoff hepatoma mitochondria, host liver mitochondria, and delipidated host liver mitochondria were determined over a range of temperatures. A plot of the log of MAO activity versus the reciprocal of the absolute temperature gives an Arrhenius plot (Fig. 4) in which the slope is proportional to the activation energy of the reaction being assayed.

The slope of the Arrhenius plot in Fig. 4 for 5-HT deamination was linear over the range of temperatures that was assayed in the hepatoma and host liver mito-

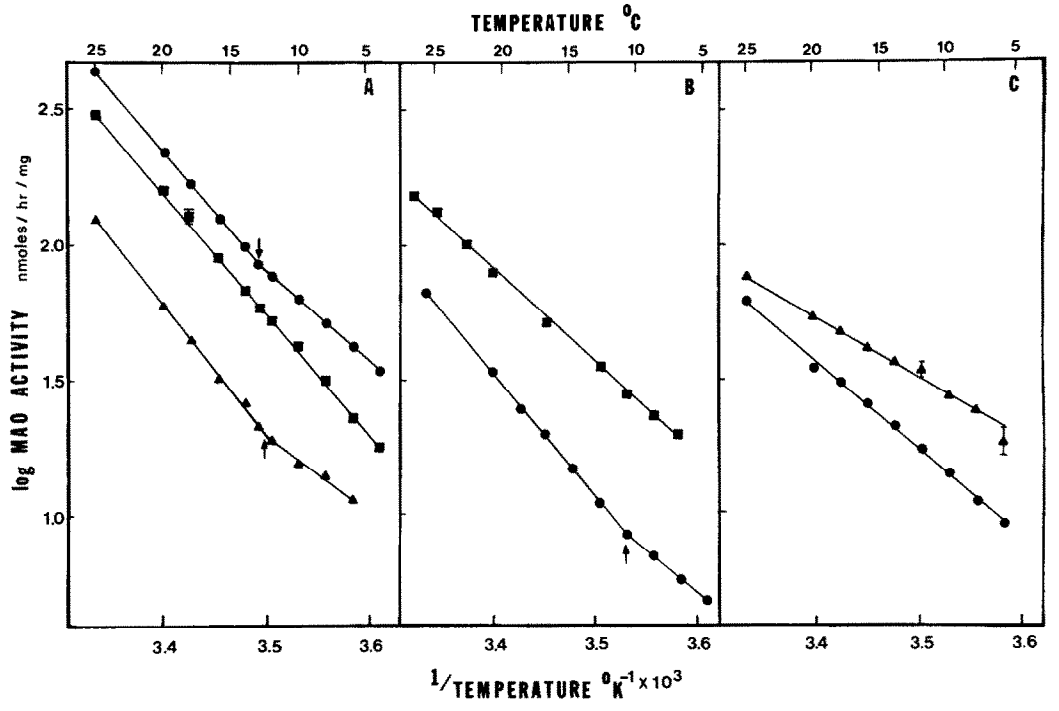

Fig. 4. Effects of temperature on MAO activities in host liver mitochondria, Novikoff hepatoma mitochondria, and delipidated host liver mitochondria. MAO activity was measured by the radiochemical method with benzylamine (▲——▲), 5-HT (■——■) and tyramine (●——●). Data for host liver mitochondria are shown in panel A, Novikoff hepatoma mitochondria in panel B, and delipidated host liver mitochondria in panel C. Each point represents the mean of four determinations. Standard errors were calculated for all points but are indicated only when they exceed the size of the symbol.

chondria; however, the Arrhenius plots for tyramine deamination had a break in the slope that resulted in a decreased slope below the temperature of inflection, which occurred at 13° in the host liver mitochondria and 10° in hepatoma mitochondria. The Arrhenius plot for benzylamine deamination in the host liver mitochondria had a lesser slope (below 12.5°) than with tyramine deamination. Arrhenius plots were not done with benzylamine deamination in the hepatoma mitochondria because of the difficulty of accurately assaying the very low MAO activity toward this substrate at low temperatures.

Arrhenius plots of delipidated host liver mitochondria are also shown in Fig. 4. No inflection points or breaks occurred in Arrhenius plots of tyramine and benzylamine deamination in host liver mitochondria extracted with MEK. Studies were not done with any substrates in delipidated hepatoma or with 5-HT in delipidated host liver mitochondria due to the difficulty of accurately assaying the low MAO activity remaining after MEK extraction.

The activation energies of MAO calculated from the Arrhenius plots in Fig. 4 are shown in Table 6. The decrease in slope that occurred at temperatures below the inflection point in the Arrhenius plot indicates a decrease in the energy of activation of MAO. In the host liver mitochondria, the energy of activation of MAO was 22.4 kcal for tyramine deamination above 13° and

Table 6. Energy of activation of monoamine oxidase at temperatures above and below lipid phase transition

Source of mitochondria	Substrate	E_a * (kcal)	Temperature of inflection	E_a † (kcal)
Host liver	Tyramine	22.4	13°	16.4
	5-HT	22.8	None	22.8
	Benzylamine	24.7	12.5°	13.0
Novikoff	Tyramine	22.8	10°	16.7
hepatoma	5-HT	17.5	None	17.5
Delipidated	Tyramine	16.6	None	16.6
host liver‡	Benzylamine	11.0	None	11.0

* E_{a_1} = energy of activation at temperature above inflection temperature.
† E_{a_2} = energy of activation at temperatures below inflection temperature.
‡ Delipidated mitochondria were prepared by MEK extraction.

16.4 kcal below 13°; the energy of activation of MAO estimated with benzylamine was 24.7 kcal above 12.5° and 13.0 kcal below 12.5°, while the energy of activation determined with 5-HT was 22.8 kcal over the entire temperature range. The energy of activation of MAO in the delipidated host liver mitochondria was 16.6 kcal for tyramine and 11.0 kcal for benzylamine over the entire temperature range. The energies of activation of MAO in the Novikoff hepatoma were found to be similar to the host liver MAO when tyramine was used as a substrate but lower when 5-HT was assayed.

DISCUSSION

In this paper we have shown that type A MAO activity predominates in the mitochondria of the Novikoff hepatoma. Previous studies, which had reported low MAO activity in fast growing hepatoma [5–7], were done primarily using benzylamine as a substrate and without differentiation of MAO types by inhibitors. In this report, inhibitors were used to measure the proportion and substrate specificity of MAO types in mitochondria from the Novikoff hepatoma and liver of the rat host. While the substrate specificities of MAO types in the hepatoma and host liver were similar, the proportions of MAO types were greatly different. A plateau in the clorgyline inhibition curve for hepatoma MAO deaminating tyramine at 10 per cent activity indicated a ratio of 90 per cent type A to 10 per cent type B MAO; however, inhibition of hepatoma MAO with deprenyl did not detect any type B MAO metabolizing tyramine. Discrepancies in the results obtained with these two inhibitors have been observed previously [20]. There was no difference in the proportions of MAO types determined in the host liver mitochondria by clorgyline and deprenyl, and the ratio of 30 per cent type A to 70 per cent type B MAO compares favorably with that reported in the literature [4].

The majority of experiments reported here were directed toward the role that lipids may have in the generation of multiple forms of MAO activity. Although the nature of multiple forms of MAO activity is not known, there is evidence accumulating that the multiplicity of MAO activity is due to different lipid environments of a single polypeptide [21–23]. Using the hepatoma mitochondria as a model for type A MAO, we compared the two mitochondrial preparations in an attempt to correlate the difference in the proportion of MAO types with some difference in the mitochondrial membranes.

Phospholipid analysis of hepatoma and host liver mitochondria revealed that the major difference in phospholipid compositions was a lesser percentage of the inner membrane phospholipid, cardiolipin, in the hepatoma mitochondria. Since mitochondrial MAO activity is located exclusively in the outer membrane [1], the difference in the proportion of MAO types found in these two mitochondrial preparations cannot be attributed to any gross difference in the lipid composition. However, the possibility cannot be ruled out that differences in the composition of lipids in the immediate vicinity of the enzyme may give rise to a different proportion of the two types of MAO activity.

Although the phospholipid to protein ratio of the hepatoma mitochondria is greater than that of the host liver mitochondria, the lack of transformation of MAO types upon delipidation implies that the differences in the two types of MAO are more complex than the amount of protein in the mitochondrial membranes. MAO activity recovered after MEK extraction was primarily of the B type in both the delipidated hepatoma and host liver mitochondria, consistent with the observations of others [16, 22, 23]. The recoveries of total activity with all substrates tested were less than 100 per cent in both delipidated preparations, indicating an inactivation of MAO activity by this treatment. In contrast to other experiments designed to test for transformation of type A MAO to type B MAO in rat liver mitochondria upon delipidation [16], the hepatoma mitochondria is a better model to test this hypothesis. A low level of type B MAO in the hepatoma mitochondria reduces the difficulty of accessing possible transformation of type A to type B MAO while accounting for the potential loss of enzyme into the organic phase and inactivation of type B MAO by the extraction procedure.

Freeze/thawing inactivates the MAO activity in delipidated mitochondria. We have shown here that, while the MAO activities of fresh and water-lysed mitochondria were stable to freeze/thawing, this treatment greatly reduced the MAO activity with all substrates tested in the delipidated hepatoma and host liver mitochondria. The result suggests that lipid surrounding the enzyme can protect it from inactivation by freeze/thawing. Inactivation of MAO activity to the same degree with all substrates tested implies that both MAO types are associated with the lipid environment in a similar manner.

The delipidation of hepatoma mitochondria results in less recovery of type B MAO activity and more lability of total MAO activity toward freeze/thawing than in the host liver mitochondria. Type B MAO in the hepatoma mitochondria may be more dependent upon the lipid environment for activity than the enzyme in host liver mitochondria. Alternatively, the high level of cardiolipin remaining in the host liver mitochondria after MEK extraction may result in the greater recovery of type B MAO activity and more protection of MAO activity against inactivation by freeze/thawing.

The addition of mitochondrial lipids to both the delipidated host liver mitochondria and hepatoma mitochondria resulted in a partial reactivation of type A MAO. The reactivation of type A MAO activity by the mitochondrial lipids, but not phosphatidylcholine, indicates that some specificity of lipid is required for reactivation. However, it is not known if this is due to the lack of binding of phosphatidylcholine to the delipidated mitochondria or due to the lack of a direct interaction between phosphatidylcholine and the enzyme. In contrast, inhibition of type B MAO by either mitochondrial lipids or phosphatidylcholine was observed in both the delipidated mitochondrial preparations. This further indicates the differential sensitivities of the two types of enzyme to their lipid environment.

Activity of membrane enzymes is often studied as a function of temperature to probe the lipid environment surrounding the enzyme. Breaks and inflections in Arrhenius plots of membrane enzymes are usually attributed to changes in the fluidity of the membrane as lipid phase transitions occur [24]. Arrhenius plots of benzylamine and tyramine deamination, type B MAO activity,

in host liver mitochondria show a change of slope at about 13°. This temperature corresponds closely with the temperature of a phase transition in the outer membrane of rat liver mitochondria, as demonstrated by freeze fracture electron microscopy techniques [25]. Furthermore, the absence of a change in the slope of Arrhenius plots of MAO in delipidated host liver mitochondria also suggests that type B MAO in the intact mitochondria is affected by a lipid phase transition. Arrhenius plots of type A MAO activity in both host liver and hepatoma mitochondria have a constant slope over the range of temperatures tested. Type A MAO could be associated with lipid which does not undergo a phase transition at these temperatures. Arrhenius behavior of hepatoma MAO was similar to host liver MAO except that the change in slope of type B MAO activity was shifted to 10°.

In summary, the MAO types in this study differ in their association with lipid. Type A MAO is dependent upon lipid for activity and insensitive to bulk lipid phase transition, while type B MAO is less dependent upon lipid for activity and sensitive to bulk lipid phase transition of the outer mitochondrial membrane. From these experiments, there is little doubt that MAO in rat liver mitochondria is an integral membrane protein, intimately associated with lipid, as opposed to the previous classification of MAO as a peripheral membrane protein [26].

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