To the Graduate School:

I am submitting herewith a dissertation written by Stephen T. Sawyer entitled "Multiple Forms of Mitochondrial Monoamine Oxidase in the Novikoff Hepatoma and Rat Liver." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Leaf Huang

Leaf Huang, Major Professor

We have read this dissertation
and recommend its acceptance:

Jeffrey M Becker

Stuart W. Hawkinson

Jan C Chuda

Accepted for the Council:

L Evan Pugh

Vice Chancellor
Graduate Studies and Research

MULTIPLE FORMS OF MITOCHONDRIAL MONOAMINE OXIDASE IN THE NOVIKOFF HEPATOMA AND RAT LIVER

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Stephen T. Sawyer

March 1980

ACKNOWLEDGMENTS

I want to dedicate this dissertation to my wife Lynda who patiently and lovingly endured and shared the frustrations and setbacks that I faced during this phase of my life. I also wish to dedicate this dissertation to the memory of John W. Greenawalt, whose guidance and inspiration at the early stages of my studies will be valued for the rest of my life.

I want to especially acknowledge the guidance of Geoff Lyles, who most influenced the direction in which this research proceeded in the first phases, and Leaf Huang whose suggestions, criticisms and moral support made this dissertation possible. Special thanks go to the other members of my committee, Jeffrey Becker, Jorge Churchich and Stuart Hawkinson, for their suggestions and support. I also want to thank Jay Joshi for the valuable experience I received in his laboratory, Liz Childress for her proficient typing and all those associated with the Biochemistry Department who have contributed suggestions and support as well as friendship.

This research was funded by grants to John W. Greenawalt from NIH and the American Cancer Society.

ABSTRACT

Mitochondrial monoamine oxidase activity in rat liver and Novikoff hepatoma was studied in an attempt to determine the molecular nature of the two types of enzyme activity. Monoamine oxidase activity in the Novikoff hepatoma was determined to be predominantly of the A type by studies carried out with the irreversible inhibitors clorgyline and deprenyl. In order to investigate the role that lipid may play in the genesis of different forms of monoamine oxidase activity, the phospholipid composition of the hepatoma mitochondria and rat liver mitochondria (contains both A and B activities) were compared. No significant difference in the proportion of outer membrane phospholipids was revealed in this study. Studies utilizing delipidation and reconstitution of monoamine oxidase into defined lipid environments ruled out the sole role of lipid in the genesis of the multiple forms of monoamine oxidase activity.

The interaction of lipid with the two forms of monoamine oxidase was determined to be different. Arrhenius plots of monoamine oxidase activity revealed that only type B activity was sensitive to a bulk phase transition of the outer mitochondrial membrane. Delipidation with organic solvent, phospholipase and detergents resulted in selective inactivation of type A monoamine oxidase. Reconstitution of partially purified enzyme and addition of lipid to delipidated mitochondria demonstrated that type A activity could be reactivated partially by selected phospholipids. However, the maximum reactivation occurred

with different lipids with the two reactivation methods. The results suggested that a net negative charge of the lipid is required for lipid to initially interact with delipidated type A monoamine oxidase, but that the charge of the lipid is not important in activating the enzyme. Type B activity seems to be dependent of the fluidity of the lipid surrounding the enzyme but retains some activity after delipidation. The requirement of a specific lipid for either type of activity seems unlikely but could not be ruled out.

Treatment of the outer mitochondrial membranes from rat liver with digitonin in the absence of sonication resulted in the formation of two types of outer membrane fragments which were resolved by DEAE cellulose chromatography. The less anionic fraction was enriched in type B monoamine oxidase activity while the more anionic fraction was enriched in type A monoamine oxidase activity. Equilibrium sedimentation showed that both fractions were of similar density and contained most of the outer membrane phospholipid. Gel filtration showed that the molecular weight of the fragments exceeded 4×10^6.

Investigation of monoamine oxidase activity in coupled mitochondria showed that the activity was inhibited by rapid respiration. This study is not consistent with the hypothesis that electrons flow from monoamine oxidase to the electron transport chain.

A new purification method for pig liver monoamine oxidase is described. Immunological technique showed that monoamine oxidase from pig liver and beef kidney share identical antigenic sites.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

INTRODUCTION

General Characteristics of Monoamine Oxidase

The metabolic deamination of physiologically active amines by monoamine oxidase (E.C. 1.4.3.4.) is of current interest to many related scientific disciplines. Present interest in the physiological role of monoamine oxidase involves the oxidation of various biogenic amines which may serve a neurotransmitter role in the central nervous system. Discovery of multiple types of monoamine oxidase activity which differ with respect to properties such as substrate specificity and inhibitor sensitivity has heightened research activity in this field since the multiple types of activity differ with respect to the ability to metabolize neuroactive amines. The exact nature of the multiple forms has not been thoroughly characterized and is considered in detail in this dissertation.

The discovery of monoamine oxidase is credited to Hare (1) who showed the existence of an enzyme which metabolized tyramine via oxidative deamination. Early work from the 1930's to 1950's established the general enzymatic reaction:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

In this era, a wide variety of compounds were recognized to be substrates of this enzyme. Among these were noradrenaline, dopamine, octopamine, β-phenylethylamine, 5-HT, many aliphatic amines and some long chain (13 carbon) aliphatic diamines. The discovery that histamine

is not metabolized by monoamine oxidase (2) led to the separate classification of monoamine oxidase and diamine oxidase, which attacks histamine, instead of previous classification of amine oxidase for both enzymes. It has since been established that monoamine oxidase is a membrane bound enzyme having a flavin cofactor while the diamine oxidase is a soluble enzyme which requires either pyridoxal phosphate or copper as a cofactor.

Monoamine oxidase occurs in all vertebrate and some invertebrate animals. Unicellular organisms do not contain monoamine oxidase although a bacterial tyramine oxidase is known to exist. This enzyme is also a flavin oxidase but is not membrane bound.

Cellular monoamine oxidase activity is recovered primarily in the mitochondrial fraction after subcellular fractionation. Schnaitman, Erwin and Greenawalt (3) demonstrated that mitochondrial monoamine oxidase is exclusively located in the outer membrane of rat liver mitochondria. Other workers have shown this to be the case in all tissues examined. Microsomal monoamine oxidase may result from the shearing off of outer membranes of mitochondria during homogenization. Alternatively, the enzyme may be synthesized in the microsomes and transported to the mitochondria. Erwin and Simon (4) have provided some evidence for the latter possibility on the basis of increased rates of recovery of monoamine oxidase activity in the microsomal fraction after <u>in vivo</u> inhibition of all the enzyme activity. Purified monoamine oxidase from pig liver and bovine liver have been best characterized with respect to the cofactor requirement, amino acid composition and functional groups. Purification of the enzyme led to the demonstration that covalently

bound FAD is the probable cofactor. A flavin pentapeptide is released after digestion of bovine liver monoamine oxidase with trypsin and chymotrypsin. The following structure of the pentapeptide was determined (5):

ser-gly-gly-cys-tyr
|
FAD

A similar flavopeptide (aspartic acid substituted for tyrosine) has been isolated from pig liver (6). The flavin is attached to the cysteine residue by a thioether linkage to the 8-position of the flavin (5). Other purified monoamine oxidase preparations, including rat liver, have been shown to be covalently linked to FAD (7).

Copper and iron have both been implicated as required for monoamine oxidase activity. However, a number of investigators have purified a fully active, copper free monoamine oxidase from several sources. Evidence that iron is required for monoamine oxidase activity is based on direct measurement of iron in purified rat liver enzyme (8) and the decrease of enzyme activity in animals on an iron deficient diet.

Different detailed descriptions of the reaction mechanism of various monoamine oxidases have been reported. However, there is general agreement that the oxidation and reduction of the flavin cofactor at the active site takes place in an anaerobic phase and aerobic phase according to the following scheme:

$$\text{E}\cdot\text{FAD} + \text{RCH}_2\text{NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{(anaerobic)}} \text{E}\cdot\text{FADH}_2 + \text{RCHO} + \text{NH}_3$$

$$\text{E}\cdot\text{FADH}_2 + \text{O}_2 \rightarrow \text{E}\cdot\text{FAD} + \text{H}_2\text{O}_2 \text{ (aerobic)}$$

The kinetics of the overall reaction indicate that the reaction proceeds via a "ping-pong" catalytic mechanism (9).

Sulfhydryl groups, probably cysteine residues, have been implicated in the function of the active site. However, apparent species differences have confused this possibility. Bovine monoamine oxidase has 7 to 8 -SH groups per 10^5g of enzyme protein. Monoamine oxidase substrates protect two of these -SH groups from reaction with sulfhydryl reagents (10). However, rat liver monoamine oxidase contains only one reactive -SH group (11).

Kinetic analysis of bovine liver monoamine oxidase catalysis at different pH values have implicated both cysteine and histidine residues in the deamination of benzylamine (12). Six to nine histidine residues are present per 10^5g bovine liver monoamine oxidase, two of which are protected from photo-oxidation by substrate (13). The nature of the active site of monoamine oxidase is not clear. In addition to the implication of histidines and cysteine at the active site, many workers have suggested that both hydrophobic and hydrophilic regions are necessary for the binding of amines. A further consideration is the possible influence of lipid on the conformation and catalytic properties of the enzyme in the outer mitochondrial membrane.

Km values of around 0.3mM have been determined for oxygen utilization by monoamine oxidases from several sources. This value is similar to the concentration of oxygen in air saturated water at physiological temperatures, 0.217mM at 37°C (14). This implies that monoamine oxidase can function at only half of its maximal velocity in the cell. If the

rate of oxygen diffusion in the cell were rate limiting, monoamine oxidase activity could be affected by competition with other oxygen consuming reactions within the cell. Blaschko (15) proposed that monoamine oxidase used an unidentified electron acceptor other than oxygen in the cell. Greenawalt and Decker (16) showed for the first time that monoamine oxidase could reduce another electron acceptor, cytochrome c, other than oxygen. These workers proposed that electrons from the FAD component of monoamine oxidase in the outer membrane were shuttled to the respiratory chain of the inner membrane. Smith and Reid (17) showed that monoamine oxidase activity in coupled mitochondria was dependent on the state of respiration of the mitochondria. The possibility that monoamine oxidase is connected to the respiratory chain of the mitochondria is considered in this dissertation.

Multiple Forms of Monoamine Oxidase

The discovery of the irreversible monoamine oxidase inhibitors clorgyline and deprenyl has provided the greatest evidence for the existence of multiple forms of monoamine oxidase activity. Johnston (18) reported the inhibition of rat brain monoamine oxidase with different concentrations of clorgyline [N-methyl-N-propargyl-3-(2,4 dichlorophenoxy) propylamine]. Deamination of tyramine was inhibited in a biphasic manner with a plateau region which was interpreted as the existence of two types of enzymes designated A and B. Type A enzyme activity is defined as the more sensitive to inactivation while type B activity is less sensitive to inactivation by clorgyline. Hall, Logan and Parsons (19) repeated this experiment in rat liver and observed the same type of

inhibition of tyramine deamination with clorgyline and demonstrated that 5-HT is metabolized solely by the A type of enzyme and benzylamine solely by the B type enzyme in this tissue. Deprenyl (phenylisopropylmethylpropinylamine) is an irreversible inhibitor which inhibits monoamine oxidase in a similar manner as clorgyline except the relative sensitivities of type A and B monoamine oxidase toward this inhibitor are reversed (20).

The mode of action of these irreversible monoamine oxidase inhibitors has not been completely characterized. Paragyline, a similar inhibitor, binds in a stoichiometric and covalent fashion to the flavin of the enzyme (21). Radiolabeled pargyline has been useful in following monoamine oxidase under conditions where the enzyme is inactive such as SDS-polyacrylamide electrophoresis (22). Combination of clorgyline and deprenyl with radiolabeled pargyline has allowed the specific labelling of the two types of monoamine oxidase (23,24).

In vivo evidence of multiple forms of monoamine oxidase activity has also been demonstrated. Christmas, Coulson, Maxwell and Riddel (25) found differential effects of these drugs on the rat brain enzyme when tested in vitro and after in vivo administration of the drugs. Furthermore, selective effects were observed in the levels of biogenic amines corresponding to the selective inactivation of monoamine oxidase types. This finding has been repeated by others (26). Additionally, changes in the ratio of monoamine oxidase types due to both the aging process and hormonal status support an in vivo significance of the two types of enzyme activity. Lyles and Callingham (27) have shown that type A

monoamine oxidase increases in the rat heart during aging and after treatment with thyroid hormones. Others have shown the absence of type B monoamine oxidase activity in the prenatal rat brain and the increase of this activity relative to type A activity after birth (28).

Monoamine oxidase in the Novikoff hepatoma will be considered in this dissertation. Prior to this investigation, the nature of multiple forms of monoamine oxidase activity in rat hepatomas had not been investigated. Pedersen, Greenawalt and Chan (29) found that monoamine oxidase metabolizing benzylamine in a series of Morris hepatomas was inversely related to the growth rate of the hepatoma. White and Tewari (30,31) studied mitochondria from Novikoff hepatoma grown in tissue culture and rat hosts. In both cases the metabolism of benzylamine and tryptamine by monoamine oxidase was lower than in rat liver mitochondria. Martin et al. (32) and Cornbleet et al. (33) reported decreased specific activities of monoamine oxidase in homogenates of Morris hepatomas 21 and R3B and "normal" activity in 7794A compared to the metabolism of benzylamine in rat liver homogenates. Morris hepatoma 16 was determined to have a very high monoamine oxidase specific activity. All of these tumors were shown to have an increased number of smaller mitochondria than rat liver cells.

Murphy et al. (34) demonstrated that rat glioma cell line C_6 grown in tissue culture had only type A monoamine oxidase activity. These workers extended the hypothesis that neurons contained type A monoamine oxidase in the rat brain. Subsequently, Breakefield et al. (35) determined that all but one cell line of more than 20 rodent cell lines

examined contained greater than 95% of type A activity. The predominance of type A monoamine oxidase activity may be due to a factor associated with tissue culture.

Molecular Basis of Monoamine Oxidase Forms

The exact molecular nature of the multiple forms of monoamine oxidase activity is not known. Immunological studies of monoamine oxidase have been contradictory. Hartman, Yasunobu and Udenfriend (36) prepared antiserum against purified bovine liver monoamine oxidase. It was shown that this antiserum would also precipitate 80% of bovine brain enzyme (37). The remaining 20% of brain monoamine oxidase isolated by immunoabsorption differed in inhibitor and substrate specificity from bovine brain enzyme precipitated by antiserum against bovine liver enzyme (38). McCauley and Racker (39) also found two species of bovine brain monoamine oxidase using antiserum against the bovine liver enzyme. Youdim and Collins (40) reported two immunological distinct forms of rat brain and liver monoamine oxidase based on double immunodiffusion lines. Dennick and Mayer (41) prepared antiserum against purified rat liver monoamine oxidase which had only type B activity (no type A activity has ever been purified). This antiserum precipitated both type A and type B monoamine oxidase activity from rat liver mitochondria. However, the purified enzyme from which the antiserum was prepared may have contained inactive type A monoamine oxidase.

All attempts to separate and purify the two types of monoamine oxidase activity have failed due to the inactivation of the A type activity. Radiolabelling of the two types of monoamine oxidase enzymes has

allowed investigators to circumvent the necessity of stabilizing the activity. McCauley (22) demonstrated that only one band of [^{14}C]-pargyline label is resolved during SDS-polyacrylamide electrophoresis of rat liver outer mitochondrial membranes in which both types of monoamine oxidase had been labeled with the inhibitor. Edwards and Pak (24) also could not separate the labeled A and B type subunits by electrophoresis in SDS. Since only one subunit contains FAD (42) and can react with the labeled inhibitor, the non-flavin containing subunits of type A and type B monoamine oxidase may differ in molecular weight. These workers also determined by titration of the A and B active sites with [^{3}H]-pargyline that 3.3 type B monoamine oxidase active sites existed for each type A active site in rat liver mitochondrial outer membranes. A higher turnover number of the A type enzyme may account for the similar A and B type activity in the outer membrane.

Recently, the radiolabel inhibitor approach has provided evidence that the two forms of monoamine oxidase are different polypeptides. Cawthon and Breakefield (23) have used the technique of proteolytic digestion and peptide mapping of monoamine oxidase selectively labeled with [^{3}H]-pargyline to demonstrate that the resultant peptide maps of type A and type B enzymes are different. The different peptide maps obtained for the two forms of monoamine oxidase indicate differences in the location or sensitivity of sites of proteolytic cleavage by the protease which could be the result of different amino acid sequences.

Lipid has been proposed as the basis of the multiple forms of monoamine oxidase activity. Polyacrylamide electrophoresis of mitochondria

solubilized in Triton X-100 has resulted in up to five bands of monoamine oxidase activity. It has been shown that these bands differ in sensitivity to inhibitors and substrates and also have different amounts of phospholipid material (9,43,44). Treatment of solubilized monoamine oxidase preparations from rat liver and human brain with the chaotropic agent sodium perchlorate in order to remove phospholipid material, not only abolished the selective nature of the inhibition by clorgyline, but also resulted in a single band of activity after polyacrylamide electrophoresis, in contrast to the untreated material (45,46).

Pig liver mitochondria oxidase can be solubilized by the extraction of lipids from the mitochondria by methyl ethyl ketone only after the acidic phospholipids are extracted (47). Monoamine oxidase from this source is active in the presence of only two phospholipid molecules per enzyme, but has a high affinity for the binding of acidic phospholipids and will not bind neutral phospholipids (48,49). Treatment of rat liver mitochondria with methyl ethyl ketone, under conditions which result in selective extraction of all phospholipids except cardiolipin, results in inactivation of type A monoamine oxidase while type B activity is only reduced slightly (50). Evidence that no enzyme was solubilized by this treatment and the lack of greater than 100% recovery of type B activity led these workers to conclude that no transformation of the multiple types of monoamine oxidase activity occurred in this experiment.

There is some evidence that both types of monoamine oxidase activity in certain tissues are dependent on lipid for activity. Houslay and Tipton (45) showed that treatment of soluble enzyme from rat liver

with 0.7M sodium perchlorate for longer than 10 minutes resulted in complete inactivation of the enzyme. Similarly, treatment of the rat liver enzyme with phospholipase A for an extended period or dialysis against Triton X-100 resulted in complete inactivation (9). However, bovine kidney enzyme is resistant to phospholipase A treatment (51), and rat liver has been reported to be resistant to several phospholipases (52).

Detergent effects of monoamine oxidase activity in rat liver have also suggested the requirement of lipid for type A activity. Borges and D'Iorio (53) observed the instability of type A monoamine oxidase activity relative to type B activity in mitochondria solubilized in deoxycholate. These workers also showed that Lubrol and sonication produced minimal inactivation of either type of activity. A number of workers have demonstrated the inactivation of type A activity in Triton X-100 (54,55) by following both types of monoamine oxidase activity and [^{14}C]-pargyline binding activity during purification procedures. A conformation change in the enzyme upon solubilization has been suggested by these workers. Houslay and Tipton (56) proposed that a conformation change in monoamine oxidase upon solubilization in Triton X-100 explained an apparent alteration in the reaction mechanism of solubilized enzyme.

Detergents have also been responsible for the formation of large aggregates of monoamine oxidase. Shih and Eiduson (54) were able to isolate two high molecular weights ($> \times 10^6$) complexes of monoamine oxidase from rat brain after treatment with Triton X-100 and sonication. One complex was enriched in type A activity and the other was enriched in type B activity. These two complexes were resolved by gel filtration.

General Characteristics of Outer Mitochondrial Membranes

Mitochondria have two distinct membranes which differ in function, composition and morphology. In contrast to the highly specialized inner membrane which is involved in electron transport and highly regulated transport properties, the outer membrane is freely permeable to substances smaller than 5,000 daltons and has similarities to the membranes of the endoplasmic reticulum (57,58).

The morphology of outer membrane has been studied in detail by Parsons (59). He observed hollow cylinders projecting from the outer surface of rat liver mitochondria using negative staining electron microscopic techniques to look at thin sections of rat liver cells. These cylinders are not observed in isolated mitochondria or outer membranes. However, irregular surface patching of small particles was seen in both fixed and unfixed outer membrane vesicles.

An asymmetric distribution of intramembrane particles between the cytoplasmic and inner membrane leaflets of the outer membrane bilayer have been observed by free-fracture electron microscopy (60). The cytoplasmic half contained four times as many intramembrane particles as the inner membrane halves of the outer membrane. Polycationic ferritin was used to determine the distribution and density of anionic sites on the surfaces of rat liver mitochondrial outer membranes (61). This study showed that there were more randomly distributed anionic sites on the inner membrane surface of the outer membrane than the cytoplasmic surface except for an area around contact sites between the outer and inner membrane which did not bind the polycationic ferritin. An asymmetric structure of the outer membrane is implied by these studies.

The protein to phospholipid ratio and density of the outer membrane is much less than the inner mitochondrial membrane. The lipid composition is known for both membranes (62). Rat liver outer membranes are composed of 50% phosphatidylcholine, 30% phosphatidylethanolamine, 15% phosphatidylinositol plus phosphatidylserine and less than 5% of spingomylein, cardiolipin and other phospholipids. In contrast, the inner membrane is composed of 20% cardiolipin, only 2% phosphatidylinositol plus phosphatidylserine, and the remainder is equal molar quantities of phosphatidylcholine and phosphatidylethanolamine. All cholesterol is in the outer membrane and occurs in a ratio of 1 mole per 9 moles of phospholipid. The fatty acid composition of both membranes of rat liver mitochondria are known (63). Outer membrane lipids are 56% saturated fatty acid and 44% unsaturated fatty acids. Inner membrane lipids are 42% saturated (mostly 16.0 and 18.0) and 58% unsaturated.

Enzymes located in the outer membrane are listed in Table 1. This list was taken from the review by Chau (58) and is not complete. These enzymes are not necessarily exclusively located in the outer membrane. Electrophoresis of rat liver outer membrane in SDS-polyacrylamide gels results in five major bands (64) and seven minor bands. One of the major bands is a glycoprotein (M.W. 93,000). It is interesting that monoamine oxidase from pig liver has been reported to be a glycoprotein (47) but this has not been shown for the rat liver enzyme.

Fluidity of the outer membrane has been studied by the molecular lateral mobility of intramembrane particles, visualized by freeze-fracture

TABLE 1

Enzymes found in the outer mitochondrial membrane[1]

Monoamine oxidase
Kynuremine-3-hydroxylase
Cytochrome b_5
NADH-cytochrome b_5 reductase
ATP-dependent fatty-acyl-CoA synthetase
Hexokinase
Fatty acid elongating system
Glycerol phosphate transferase
Lysophosphatidate-acyl transferase
Choline phosphotransferase
Phosphatidate phosphatase
Phospholipase A_2
Bicarbonate-dependent ATPase

[1]From Chau (58)

electron microscopy, during phase transitions induced by cooling. Hackenbrock, Hochli and Chau (65) have shown that thermotropic lipid-protein separations occur in both membranes of rat liver mitochondria. These workers have concluded that both membranes are fluid and that proteins (intramembrane particles) can migrate to domains of more fluid lipid as phase transition occurs due to freezing of less fluid lipids occur. Higher temperature of phase transition in the outer membrane compared to the inner membrane agrees with the higher degree of saturation of the outer membrane lipids compared to the saturation of the inner membrane lipids.

Chau (58) found that an inflection in Arrhenius plots of monoamine oxidase metabolizing tryptamine corresponded to the temperature (13°) of a phase transition of the outer membrane. Other workers have previously demonstrated constant slopes in Arrhenius plots of monoamine oxidase activity in rat liver mitochondria in the range from 35° to 10° (66,67,68). Baker and Hemsworth (52) reported that inflection in Arrhenius plots of rat liver monoamine oxidase activity occurred at 26°. The Arrhenius behavior of monoamine oxidase types is also considered in this dissertation.

Chau (58) has purified antibody directed against rat liver monoamine oxidase and prepared ferritin labeled antibody. This antibody reacts with sites on both sides of the outer membrane in equal amounts as visualized by thin section electron microscopy. Russel, Davey and Mayer (69) have proposed that type A monoamine oxidase is located on the inner membrane side of human liver and brain outer mitochondrial membranes while type B monoamine oxidase is located on the outer surface of the membrane. This conclusion was based on the accessibility

of both types of activity to antiserum against the purified enzyme. The asymmetric distribution of the two types of enzymes in the outer membrane may give rise to the multiple forms of monoamine oxidase. Alternatively, the different location of the two types of enzyme activity on different sides of the outer membrane may explain why they interact with different lipid environments and behave differently when exposed to detergents or when a phase transition occurs.

CHAPTER II

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 and B 9302] was a generous gift from May and 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. Phosphatidylcholine and phosphatidylethanolamine purified from egg yolk were obtained from Dr. L. Huang, Department of Biochemistry, University of Tennessee, Knoxville, TN. Other lipids, phospholipase A_2, digitonin and cholic acid were obtained from Sigma Chemical Company, St. Louis, MO. Other reagents were obtained from Fisher Scientific Company.

Preparation of Mitochondria

Hepatomas were maintained in male Holtzman rats (Holtzman Company, Madison, WI) by serial transplantation of Novikoff hepatoms obtained from Dr. F. Snyder, Oak Ridge Associated Universities, Oak Ridge, TN. Hepatomas were removed from cervically dislocated rats on the fifth or sixth day 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 previously described method (70) in H' medium [70mM sucrose, 20mM mannitol, 2mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), 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 10 strokes of a Teflon pestle (motor driven) in a glass homogenizer. The homogenate was centrifuged at 660 x g for 10 minutes; the pellet was rehomogenized with five strokes of the homogenizer and recentrifuged. The supernatants from the first and second centrifugation were pooled and mitochondria were isolated by differential centrifugations as described previously (70). When necessary, mitochondrial fractions were further purified by equilibrium sedimentation in a continuous sucrose gradient (1.0M to 2.5M) (30).

Assay of Monoamine Oxidase

Monoamine oxidase activity was routinely estimated by a modification of the radiochemical method of Callingham and Laverty (71). Three substrates, benzylamine, tyramine and 5-HT, were used at final specific activities of 0.1, 1.0 and 2.0μCi/μmole, respectively. Substrate at 1mM final concentration was incubated with samples containing about 0.5mg protein. Fifty μl mitochondrial samples diluted with water and 50μl substrate prepared in 0.2M 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 10μl of

3N HCl after an interval in which the assay is linear with time. Deaminated metabolites were extracted into 0.5ml of ethyl acetate: benzene (1:1 v/v). After mixing and separation of phases by centrifugation, 0.4ml of the organic phase was counted for radioactivity in 4ml of 0.4% butyl-PBD in toluene (w/v) in a Beckman LS-230 liquid scintillation spectrometer.

Monoamine oxidase activity was also estimated by a polarographic method. Oxygen consumption was measured with a Clark oxygen electrode (72). Samples containing 3 to 5mg protein were assayed at 37° in final volume of 1.5ml of H' medium (pH 7.4) containing 1mM KCN. Substrates were added to a final concentration of 3mM, and O_2 consumption was linear for at least two minutes.

The activity of monoamine oxidase using benzylamine as substrate was also measured by the spectrophotometric determination of benzylaldehyde formation at 250nm (73). This assay was performed at 1.0mM final benzylamine concentration in 0.1M 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 (74). Phospholipids were separated by two dimensional thin layer chromatography (tlc) using Silica Gel H, first in a solvent of chloroform, methanol and NH_4OH (13:5:1), followed by a solvent containing chloroform, acetone, methanol, acetic acid and water (6:8:2:2:1). The phospholipid fractions were visualized and eluted from the tlc as described by Morton et al. (75). Lipid phosphorous was determined as described by Ames and Dubin (76).

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 then centrifuged at 15,000 x g for 15 minutes. This procedure was repeated with the resulting pellet. Water lysed mitochondria were extracted with methyl ethyl ketone (MEK) as described by others (47,48,49). Briefly, the mitochondria preparation was extracted with eight volumes of MEK. The organic phase was decanted, and the residue was resuspended in 0.1M phosphate buffer, pH 7.2, containing 1mM EDTA and centrifuged at 15,000 x g for 15 minutes. The supernatant (buffer extract) was decanted and the pellet (delipidated mitochondria) was resuspended in the same buffer.

Addition of Lipid to Methyl Ethyl Ketone Extracted Mitochondria

Lipid dispersions were prepared by evaporating the lipid to dryness, adding phosphate buffer (0.1M, pH 7.2, with 1mM EDTA) to give a lipid concentration of 5.0mg/ml, and sonicating for 10 minutes under N_2 in a bath sonicator at temperatures below 15°. An aliquot of lipid dispersion was added to 1.0mg of delipidated mitochondrial protein, and the volume was adjusted to 1.0ml with the same buffer at ice bath temperatures. After appropriate incubation, monoamine oxidase activity was determined by the radiochemical assay as described except that blanks containing enzyme inactivated by heat or acid plus lipid were assayed under identical conditions as the samples.

Reconstitution of Monoamine Oxidase into Lipid Vesicles

Outer mitochondrial membranes were prepared as described by Decker and Greenawalt (77). Fifty mg of outer membrane protein were suspended in 10ml of a 1.0% cholate solution made in phosphate buffer (50mM, pH 7.2) containing 1.0mM EDTA and 0.1mM dithiothreotol (DTT). This mixture was sonicated in a bath sonicator at ice temperature for 15 minutes, after which centrifugation at 150,000 x g was carried out for 90 minutes. Monoamine oxidase in both the supernatant and pellet were assayed. The supernatant was concentrated to a small volume and was applied to a continuous glycerol gradient (5% to 40%) containing 1.0% cholate. Centrifugation was carried out at 100,000 x g for 24 hours. Fractions containing monoamine oxidase activity were pooled and concentrated. This material was diluted to achieve a protein concentration of 1.0mg/ml. One half of one mg of dry lipid was resuspended in the enzyme solution by stirring for 30 minutes. Reconstitution was carried out by removing the cholate by gel filtration (78) on Sephadex G-50, coarse grade. This column was eluted with the same phosphate buffer used previously. Turbid fractions at the void volume were pooled and assayed for monoamine oxidase activity.

Treatment of Outer Mitochondrial Membranes with Digitonin

Digitonin suspensions were prepared immediately before use since this detergent is insoluble in water. Five to 10% solutions of digitonin in phosphate buffer (0.1M, pH 7.0) were prepared by adding solid digitonin to buffer heated to just below the temperature of boiling. This gave a clear suspension in which the detergent did not precipiate for

about two days. Other solutions were prepared from this stock. Digitonin was quantitated by the colorimetric determination of the digitonin carbohydrate using anthrone reaction (51).

Outer mitochondrial membranes isolated by the French press method were treated with varying concentrations of digitonin. DEAE cellulose chromatography of digitonin treated outer membranes was carried out in 20mM phosphate buffer, pH 8.0. Digitonin was present in the buffer used to wash the sample and in the 0 to 0.5M KCl gradient elution, but the column was not equilibrated with digitonin before the sample was applied. Sedimentation of digitonin treated outer membranes in a continuous sucrose gradient was done in the presence of 0.2% digitonin and 100mM phosphate buffer, pH 7.0. This condition was found to be optimal for maintaining the digitonin in suspension.

Assay of Monoamine Oxidase Activity in Coupled Mitochondria

Mitochondrial respiration was measured with a Clark oxygen electrode. One ml of a solution of 1.5 fold concentrated H' medium was added to the chamber and was diluted to 1.5ml by the addition of mitochondria, substrates, drugs and water. Additions of the following substances to the indicated final concentration were common: ADP, 0.5mM; Na succinate, 5.0mM; oligomycin in ethanol, 1.0mM; and KCN, 0.5mM.

Benzylaldehyde production in coupled mitochondria was determined by measuring the absorbance at 250nm in an Aminco (DW-2) split beam spectrophotometer. The assay mixture was described as above and the sample and reference cuvets contained identical additions except that

1.0mM benzylamide was present in the sample cuvet. An assay volume of 1.5ml was used, and less than 100µg of mitochondrial protein was added to reduce the turbidity.

Purification of Pig Liver Monoamine Oxidase

Pig liver outer mitochondrial membranes were isolated by the method of Decker and Greenawalt (77). These membranes were suspended in 100mM phosphate buffer, pH 7.2, at a protein concentration of 10mg/ml and extracted with eight volumes of methyl ethyl ketone (MEK). Solvent was discarded and the residue was resuspended in the same buffer. After stirring for five minutes, centrifugation was carried out at 30,000 x g for 20 minutes. The pellet was resuspended in the same buffer containing 10% cholate and allowed to stand overnight on ice before it was centrifuged at 150,000 x g for two hours. The pellet was discarded and the supernatant was stored on ice for further purification.

Gel filtration with Sephadex G-200 was carried out twice on this preparation. First, the activity was eluted with 100mM phosphate buffer, pH 7.2, and then the fractions containing activity were pooled and applied to another column which was equilibrated and eluted with the same buffer containing 2% cholate. Fractions containing activity were pooled and ammonium sulfate was added until the solution was 20% saturated. This solution was centrifuged at 30,000 x g for 15 minutes. Ammonium sulfate was added to the supernatant until the solution was 50% saturated. Centrifugation under the same conditions resulted in most of the monoamine oxidase activity in the pellet.

Immunodiffusion of Monoamine Oxidase

Agarose immunodiffusion plates (Hyland) were used to study the immunological nature of monoamine oxidase. Ten µl of IgG antibody against beef kidney monoamine oxidase, obtained from Dr. J.W. Greenawalt, was placed in the center well. Fifty µl of pig liver and beef kidney enzyme, both preparations containing about 1mg protein/ml, were placed in alternating wells. This plate was allowed to develop at 4° for one week. No further development occurred after this time.

Other Procedures

Protein was determined by the biuret (79) and Lowry methods (80). When the Lowry method was used for samples containing sucrose and/or detergents, these compounds were added to the standard containing crystalline BSA. Glucose-6-phosphatase activity was determined as a marker of microsomal contamination (81).

CHAPTER III

MITOCHONDRIAL MONOAMINE OXIDASE IN NOVIKOFF HEPATOMA AND RAT LIVER

Observations on Novikoff Hepatoma Mitochondria

This study on monoamine oxidase activity in the Novikoff hepatoma was initiated to examine the nature of multiple forms of monoamine oxidase in an undifferentiated, fast growing hepatoma. Although the growth rate, ultrastructure and some biochemical properties of the Novikoff hepatoma have been studied (82,83), it had not been characterized with regard to the substrate and inhibitor specificity of monoamine oxidase.

Novikoff hepatoma mitochondria have been studied to some extent (30,31). However, these workers used equilibrium sedimentation techniques for subcellular fractionation. This method was initially used to isolate hepatoma mitochondria and resulted in severely damaged mitochondria. A differential centrifugation technique for subcellular fractionation developed for rat liver was then modified for use with the Novikoff hepatoma tissue such that intact, functional mitochondria could be isolated. Modifications were necessary to efficiently disrupt the hepatoma cell membrane and spin down the lighter, smaller mitochondria.

Substantial damage was still evident of the hepatoma mitochondria during isolation as indicated by electron micrographs (not shown). This damage was also apparent from the acceptor control ratio measured in hepatoma mitochondrial preparations. Acceptor control ratios of

hepatoma mitochondria ranged from 1.0 to 3.5 in preparations spanning a period of two years. In the same period, liver mitochondria from the rat host were determined to have acceptor control ratios ranging from 3.8 to 12.0. It is well established that the acceptor control ratio indicates the integrity of mitochondrial preparations.

Mitochondrial fractions from the hepatoma and host liver were further purified by equilibrium sedimentation in a continuous sucrose gradient when necessary. Figure 1 is a tracing of the sedimentation behavior of host liver and hepatoma mitochondria in a continuous sucrose gradient. Hepatoma mitochondria are less dense than the host liver mitochondria. Furthermore, the outer membrane of the hepatoma mitochondria was also of less density than the outer membrane of the host liver mitochondria. The basis of this conclusion was the observation that when crude outer membranes from both mitochondria preparations were centrifuged, 72% of the monoamine oxidase activity in the hepatoma outer membrane was not pelleted by the same centrifugation which pelleted 80% of the monoamine oxidase activity in rat liver outer membranes.

Inhibition of Host Liver and Hepatoma Monoamine Oxidase by Clorgyline and Deprenyl

By definition, type A monoamine oxidase activity is more sensitive to inhibition by the irreversible inhibitor clorgyline than type B activity (18). Another irreversible inhibitor, deprenyl, selectively inactivates type B activity at low concentrations (20). Inhibition of monoamine oxidase activity in hepatoma and host liver mitochondria by these inhibitors was therefore studied, in an attempt to determine whether the type A and B enzymes are present in these tissues.

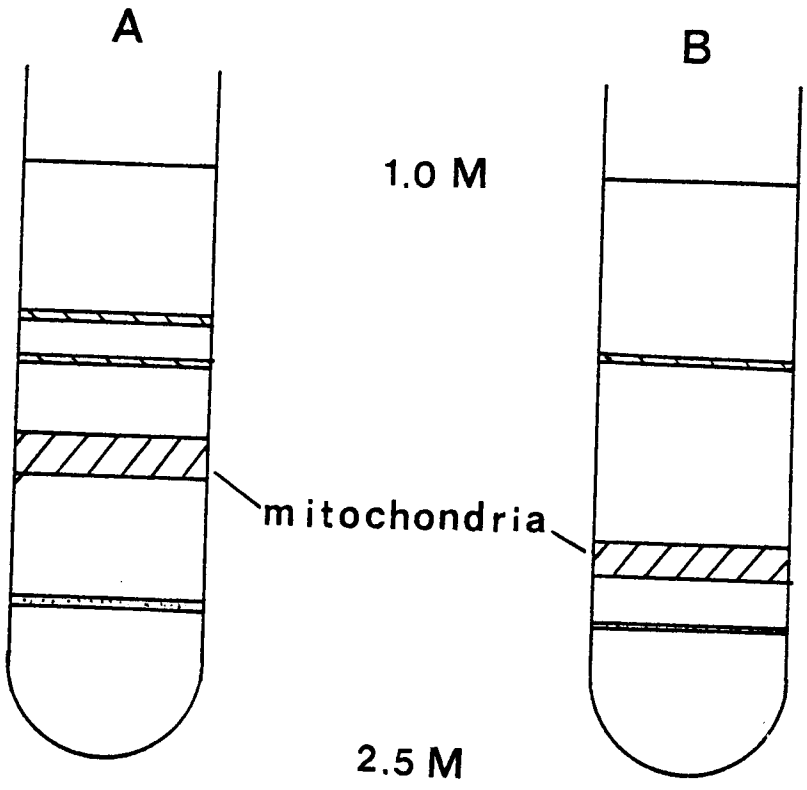

Figure 1. Equilibrium sedimentation of Novikoff hepatoma and host liver mitochondria. One hundred mg hepatoma mitochondrial protein (A) and host liver mitochondrial protein (B) were applied to the continuous sucrose gradient, 1.0 to 2.5 M. Centrifugation was carried out at 100,000 x g for 24 hours in a SW 27 rotor.

Figure 2 shows the inhibition of monoamine oxidase activity in mitochondria from Novikoff hepatoma and host liver by both clorgyline and deprenyl. Five-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 monoamine oxidase activity in these tissues. Benzylamine deamination in both preparations was likewise completely inhibited at concentrations of 10^{-3}M clorgyline and 10^{-6}M deprenyl. Thus, benzylamine appears to be a substrate for type B monoamine oxidase. The inhibition of tyramine deamination in both mitochondrial preparations by clorgyline resulted in a double sigmoid inhibition curve. The ratio of enzyme types determined from this curve were 30% type A - 70% type B in the host liver mitochondria and 90% type A - 10% 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% type A and 70% 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.

Monoamine Oxidase Activity in Host Liver and Hepatoma Mitochondria

The specific activities in mitochondria from Novikoff hepatoma and host liver measured when possible with radiochemical, polarographic and spectrophotometric assay are shown in Table 2. Substrates deaminated by either one or both types of monoamine oxidase when possible were used to estimate monoamine oxidase activity. The

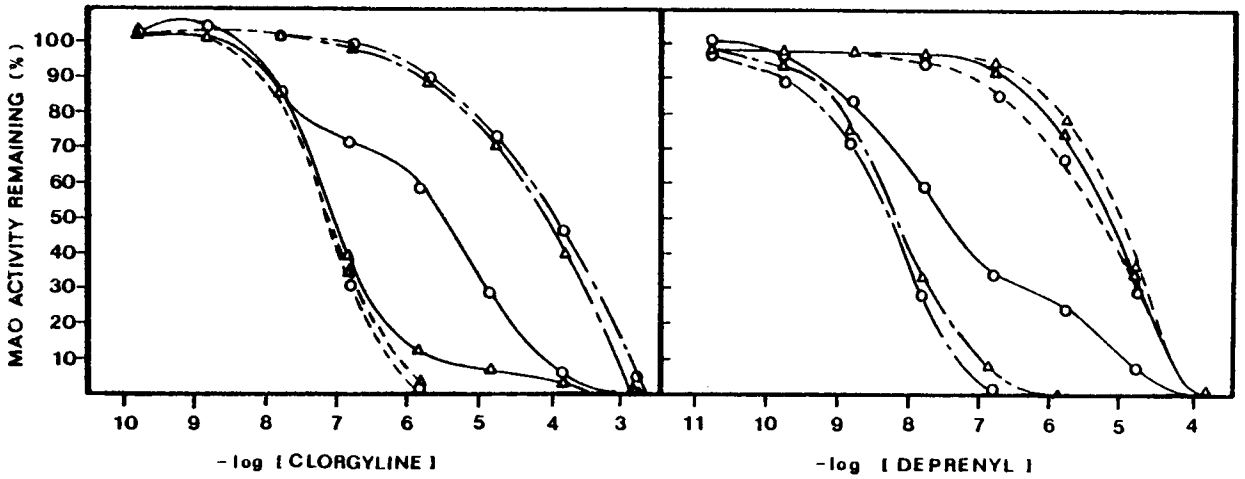

Figure 2. Inhibition of monoamine oxidase in Novikoff hepatoma and host liver mitochondria by clorgyline and deprenyl. Novikoff hepatoma (Δ) and host liver mitochondria (O) were preincubated for 20 minutes at 37° with the indicated concentration of clorgyline or deprenyl. Activity was then determined with benzylamine (—— - ——), 5-HT (----) and tyramine (——). Each point is the mean of three determinations. Standard errors were $\leq$7% and not shown for clarity of the figure.

TABLE 2

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

Substrate	Tissue	Radiochemical nmole/hr/mg	Spectrophotometric nmole/hr/mg	Polarographic nmole O_2/hr/mg
Benzylamine	Host Liver	617 ± 68*	570 ± 78	1,038 ± 252
	Hepatoma	27.7 ± 6.5 (4.5%)†	30 ± 12 (5.3%)	132 ± 53 (12.7%)
Tyramine	Host Liver	1,135 ± 65	N.A.	1,014 ± 132
	Hepatoma	282 ± 21 (24.8%)	N.A.	402 ± 78 (39.8%)
5-Hydroxytryptamine	Host Liver	725 ± 33	N.A.	834 ± 120
	Hepatoma	325 ± 60 (44.8%)	N.A.	462 ± 160 (55.4%)

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

†Activity of hepatoma mitochondria expressed as % of host liver mitochondria

N.A. = Not applicable since these substrates do not yield a product with an absorbance maximum convenient for assay

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% of the rate of tyramine deamination and 45% of the rate of 5-HT deamination in host liver mitochondria.

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

Discussion

Previous studies of monoamine oxidase activity in rat hepatoma have been performed with a limited range of substrates and without inhibitor differentiation of monoamine oxidase types. The present study of monoamine oxidase activity in the Novikoff hepatoma has utilized the sensitivity toward inhibition of activity by clorgyline and deprenyl in order to determine the relative proportions and substrate specificity of monoamine oxidase types in mitochondria from the Novikoff

hepatoma and liver of the rat host. While the substrate specificities of enzyme types in the hepatoma and host liver were similar, the proportions of enzyme types were greatly different. A plateau in the clorgyline inhibition curve for hepatoma monoamine oxidase deaminating tyramine at 10% activity indicated a ratio of 90% type A - 10% type B monoamine oxidase; however, inhibition of hepatoma monoamine oxidase with deprenyl did not detect any type B activity metabolizing tyramine. Discrepancies in the results obtained with these two inhibitors have been observed previously (84). There was no difference in the proportion of enzyme types determined in the host liver mitochondria by clorgyline and deprenyl, and the ratio of 30% type A - 70% type B monoamine oxidase compares favorably with that reported in the literature (19).

The substrate specificity of monoamine oxidase types in the hepatoma defined by the inhibitor study was identical to that in the rat liver. This allows the relative proportion of the two forms of monoamine oxidase activity to be determined from the specific activity of 5-HT deamination (A type) and benzylamine deamination (B type). Low rates of benzylamine deamination in the Novikoff hepatoma mitochondria reflects the almost absence of the type B activity in the tumor tissue. This result is in agreement with the finding of Tewari and coworkers (30).

The recent report that two different peptides correspond to the different types of monoamine oxidase activity (23) implies that genetic regulation of the enzyme proteins may account for the higher ratio of type A activity in the hepatoma mitochondria. Novikoff hepatoma may have been derived from the nonparenchymal cells of the liver which have

the same embryological origin as the spleen, which has a ratio of 95% type A to 5% type B monoamine oxidase activity (85).

A rapid rate of growth of a tissue or cell may influence the proportion of monoamine oxidase types. Pedersen et al. (29) observed that monoamine oxidase activity measured with benzylamine was inversely related to the growth rate of a series of Morris hepatomas. This activity was also observed to decrease by 67% in regenerating rat liver relative to control rat liver activity (85). Hawkins and Breakefield (35) have reported that greater than 95% of the total monoamine oxidase activity in all but one cell line in a large number of continuous rodent cell lines grown in tissue culture was type A activity.

Mitochondria from the Novikoff hepatoma and host liver will be compared in latter chapters in an effort to establish the molecular nature of the multiple types of monoamine oxidase. Most previous work on this problem has implicated an effect of lipid in the generation of the two forms of monoamine oxidase. Therefore, these two mitochondrial preparations were used as a model system to search for differences in lipid composition which might be responsible for the different ratios of monoamine oxidase types. Although genetic control of the synthesis of the two types of monoamine oxidase protein may be the cause of the increased ratio of type A activity, the possibility that an inactive B type enzyme, due to a lipid alteration, is synthesized cannot be ruled out.

CHAPTER IV

ASSOCIATION OF MONOAMINE OXIDASE WITH LIPID

Phospholipid Composition of Novikoff Hepatoma and Host Liver Mitochondria

Mitochondria from the Novikoff hepatoma and host liver were compared with regards to their phospholipid composition to examine the possible role of phospholipids in modulating the multiple forms of monoamine oxidase activity. Table 3 lists the phospholipid composition of freshly isolated mitochondria from host liver and Novikoff hepatoma. The phospholipid composition of the two mitochondrial preparations was qualitatively the same. Quantitative differences in the hepatoma and host liver mitochondrial phospholipid composition were small when expressed as a percentage of total lipid phosphorous, with the exception of a lower percentage of cardiolipin in the hepatoma mitochondria. Table 3 also shows the phospholipid composition expressed as nmoles of lipid phosphorus per mg 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 157nmole lipid phosphorus per mg of mitochondrial protein was determined for the host liver mitochondria and 288nmole lipid phosphorus per 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

TABLE 3

Phospholipid composition of mitochondria from Novikoff hepatoma and host liver

Tissue	CL*	PC	PE	PS + PI	SM	Unknown
	% Total Lipid Phosphorus in TLC Fractions					
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 (nmole 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

*CL, cardiolipin, PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS + PI, phosphatidylserine + phosphatidylinositol; 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 228 nmoles lipid phosphorus/mg of hepatoma mitochondrial protein

glucose-6-phosphatase activity after isolation by differential centrifugation were further purified 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 of monoamine oxidase 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 mitochondria for this procedure. Table 4 shows the phospholipid composition 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% lipid phosphorus extracted) than host liver mitochondria (89.2% lipid phosphorus extracted).

Effect of Methyl Ethyl Ketone Extraction on Monoamine Oxidase Activity

Extraction of mitochondria from both hepatoma and host liver with MEK resulted in a loss of monoamine oxidase activity toward all three substrates. Table 5 shows the specific activities of monoamine oxidase

TABLE 4

Phospholipid composition of water lysed† and delipidated mitochondria from Novikoff hepatoma and host liver

	Total Lipid Phosphorus (µmoles)	Lipid Phosphorus (µmoles) in TLC 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	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

*Same as Table 2

‡MEK extract contained 8.4 µmoles and buffer extract contained 0.27 µmoles; 101% recovery of lipid phosphorus

§MEK extract contained 13.6 µmoles and buffer extract contained 0.2 µmoles; 94.6% recovery of lipid phosphorus

TABLE 5

Effect of lipid extraction with methyl ethyl ketone on monoamine oxidase activity*

Source	Substrate	Specific Activity Before MEK Extraction (nmol/hr/mg)	Specific Activity After MEK Extraction† (nmol/hr/mg)	% Recovery
Novikoff Hepatoma Mitochondria	Tyramine	323	29.5	8.0 (9.7 ± 2.2)§
	5-HT	311	21.0	4.9 (6.8 ± 1.7)
	Benzylamine	51.7	26.5	44.9 (42.3 ± 9.2)
Host Liver Mitochondria	Tyramine	1075	643	35.2 (29.6 ± 2.8)
	5-HT	888	67	4.4 (4.7 ± 1.3)
	Benzylamine	644	849	77.0 (76 ± 5.0)

*Activity was assayed by the radiochemical method described in Materials and Methods

†All detectable activity is found in the delipidated residue; no activity is detected in the buffer wash and activity cannot be determined in the MEK extract

§(mean % recovery ± S.E.) for extractions on five different preparations

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 activity after extraction differed among the substrates tested. In the delipidated mitochondria from Novikoff hepatoma 5.9% of the 5-HT deaminating activity, 8.0% of the tyramine deaminating activity, and 44.9% of the benzylamine deaminating activity remained of the total activity before extraction. After delipidation of the host liver mitochondria 4.4% of 5-HT deaminating activity, 35.2% of tyramine deaminating activity, and 77.0% of benzylamine deaminating activity remained of the total activity before delipidation.

The buffer wash of the delipidated mitochondria and organic solvent contained no detectable monoamine oxidase activity. The buffer wash from the extraction of the hepatoma mitochondira contained from 5 to 10% of the total unextracted mitochondrial protein and the total protein recovered in the delipidated mitochondria plus buffer wash was always greater than 90% of the unextracted material. The buffer wash from the extraction of the host liver mitochondria contained from 10 to 25% of the total unextracted protein and the total recovery of protein after delipidation was usually greater than 80%.

The relative proportions of type A and type B monoamine oxidase are changed after lipid depletion. Figure 3 shows the inhibition of monoamine oxidase 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

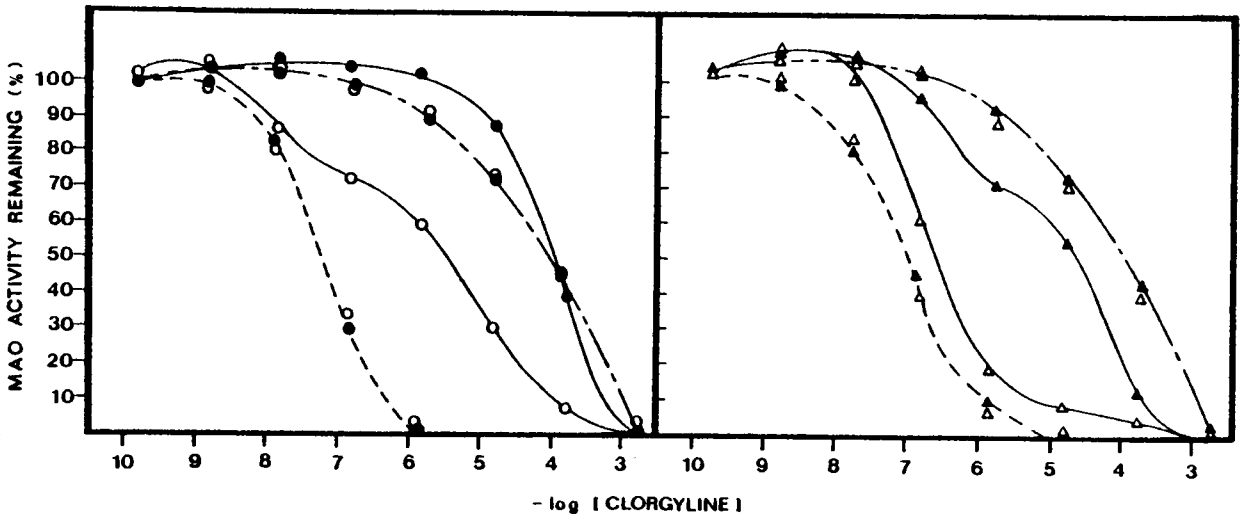

Figure 3. Inhibition of monoamine oxidase activity by clorgyline in Novikoff hepatoma and host liver mitochondria before and after extraction with methyl ethyl ketone. The left panel is the inhibition of monoamine oxidase in host liver mitochondria (0) and delipidated host liver mitochondria (●); the right panel is the inhibition of monoamine oxidase in Novikoff hepatoma mitochondria (△) and delipidated hepatoma mitochondria (▲). Substrates for monoamine oxidase activity using the radiochemical method were benzylamine (—— - ——), 5-HT (----) and tyramine (——). Each point is the mean of three determinations. Standard errors were 5% and not shown for clarity of the figure.

proportions of type A and type B enzyme metabolizing tyramine in both host liver and hepatoma mitochondria were altered such that type B activity 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 activity before lipid extraction, became a single sigmoid curve after MEK extraction, indicating that only type B monoamine oxidase activity remained. Delipidation of the hepatoma mitochondria altered the ratio of 90% type A - 10% type B activity before MEK extraction to 30% type A - 70% type B monoamine oxidase activity after extraction as shown in Figure 3.

Effect of Freezing and Thawing on Hepatoma and Host Liver Monoamine Oxidase Activity

Table 6 shows the effect of freeze/thawing on the monoamine oxidase activity of fresh mitochondria, water lysed membranes, and the delipidated residue from both Novikoff hepatoma and host liver. While fresh and water lysed mitochondrial monoamine oxidase activity from both host liver and Novikoff hepatoma was 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 monoamine oxidase activity labile as a result of freeze/thawing. The recovery of monoamine oxidase activity after freeze/thawing of the delipidated residue varied only slightly with the substrate used to determine activity.

TABLE 6

Monoamine oxidase activity before and after freeze/thawing

Tissue	Treatment	Substrate	Activity Fresh (nmole/hr/mg)*	Activity Freeze/Thaw (nmole/hr/mg)*	% 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 for two 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 four days, 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 is the mean of two preparations

*Activity was assayed by the radiochemical method

Tyr, tyramine; 5-HT, 5-hydroxytryptamine; BA, benzylamine

Monoamine Oxidase Activity as a Function of Temperature

Monoamine oxidase activity in Novikoff hepatoma mitochondria, host liver mitochondria, rat liver outer mitochondrial membranes, outer membranes solubilized in Triton X-100, and host liver mitochondria delipidated by MEK extraction was studied over a range of temperatures. A plot of the log of monoamine oxidase activity versus the reciprocal of the absolute temperature gives an Arrhenius plot in which the slope is proportional to the activation energy of the reaction (Figure 4).

Rates of 5-HT deamination at different temperatures gave an Arrhenius plot with a constant slope over the entire range of temperatures. However, the Arrhenius plots for tyramine and benzylamine deamination in the host liver mitochondria deflect to a decreased slope at temperatures below 13°. Similar deflections of Arrhenius plots of benzylamine deamination in outer mitochondrial membranes and tyramine deamination at 10° in the hepatoma mitochondria are shown in Figures 5 and 4. Arrhenius plots of benzylamine deamination were not done in the hepatoma mitochondria because of the difficulty of accurately assaying low levels of monoamine oxidase at low temperatures.

Arrhenius plots of monoamine oxidase activity in delipidated host liver mitochondria are also shown in Figure 4. No inflection points or breaks occurred in Arrhenius plots of tyramine and benzylamine deamination in this preparation. Arrhenius plots of monoamine oxidase activity in outer mitochondrial membranes solubilized in Triton X-100 are shown in Figure 6. No inflections or breaks occurred in these plots.

Figure 4. Effect of temperature on monoamine oxidase activity in host liver mitochondria, Novikoff hepatoma mitochondria and delipidated host liver mitochondria. Monoamine oxidase activity was measured by the radiochemical method with benzylamine, ▲——▲; 5-HT, ■——■; and tyramine, ●——●. Data for host liver mitochondria is 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.

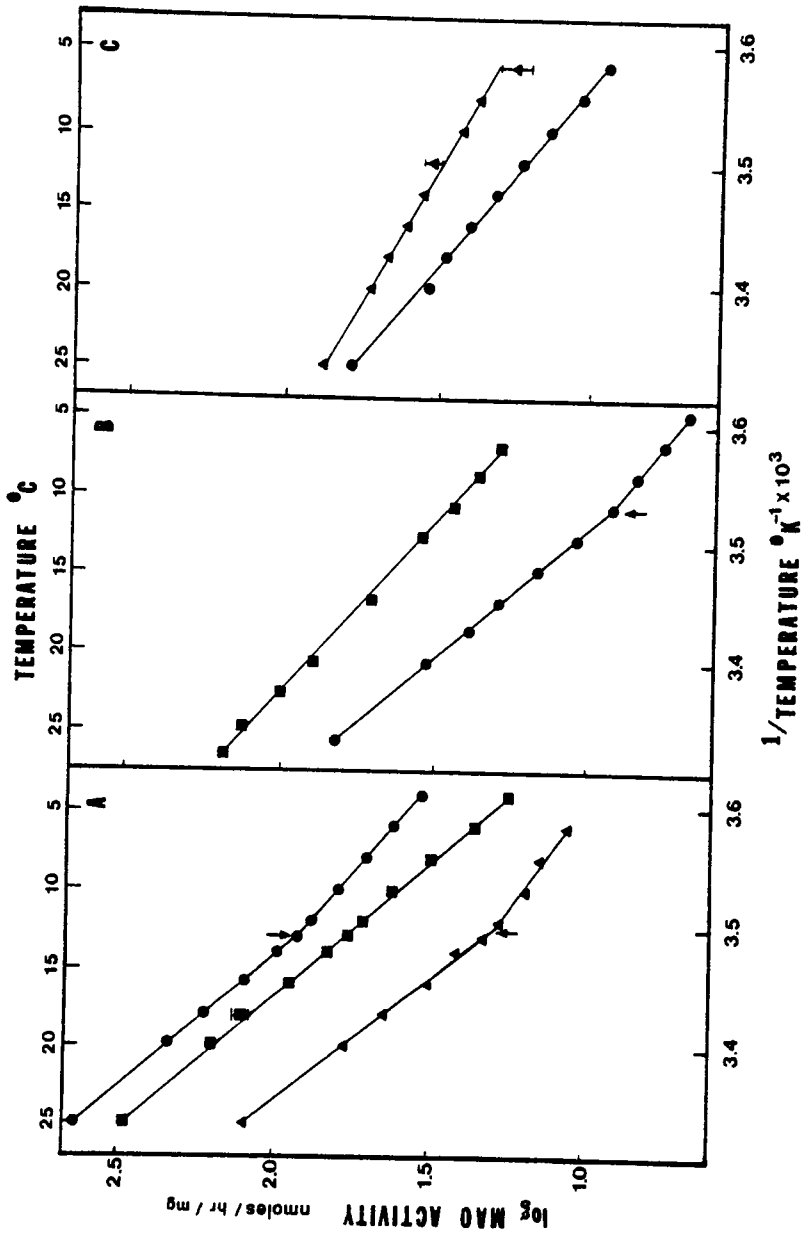
TEMPERATURE °C
log MAO ACTIVITY nmoles / hr / mg
1/TEMPERATURE °K⁻¹ x 10³
A
B
C

Figure 4

Figure 5. Arrhenius plot of monoamine oxidase activity in outer mitochondrial membranes. Monoamine oxidase activity was determined by the radiochemical method using benzylamine (▲) and 5-HT (●). Each point is the mean of four determinations. Standard error does not exceed the size of the symbols. Insert, Km of monoamine oxidase for benzylamine, linear point.

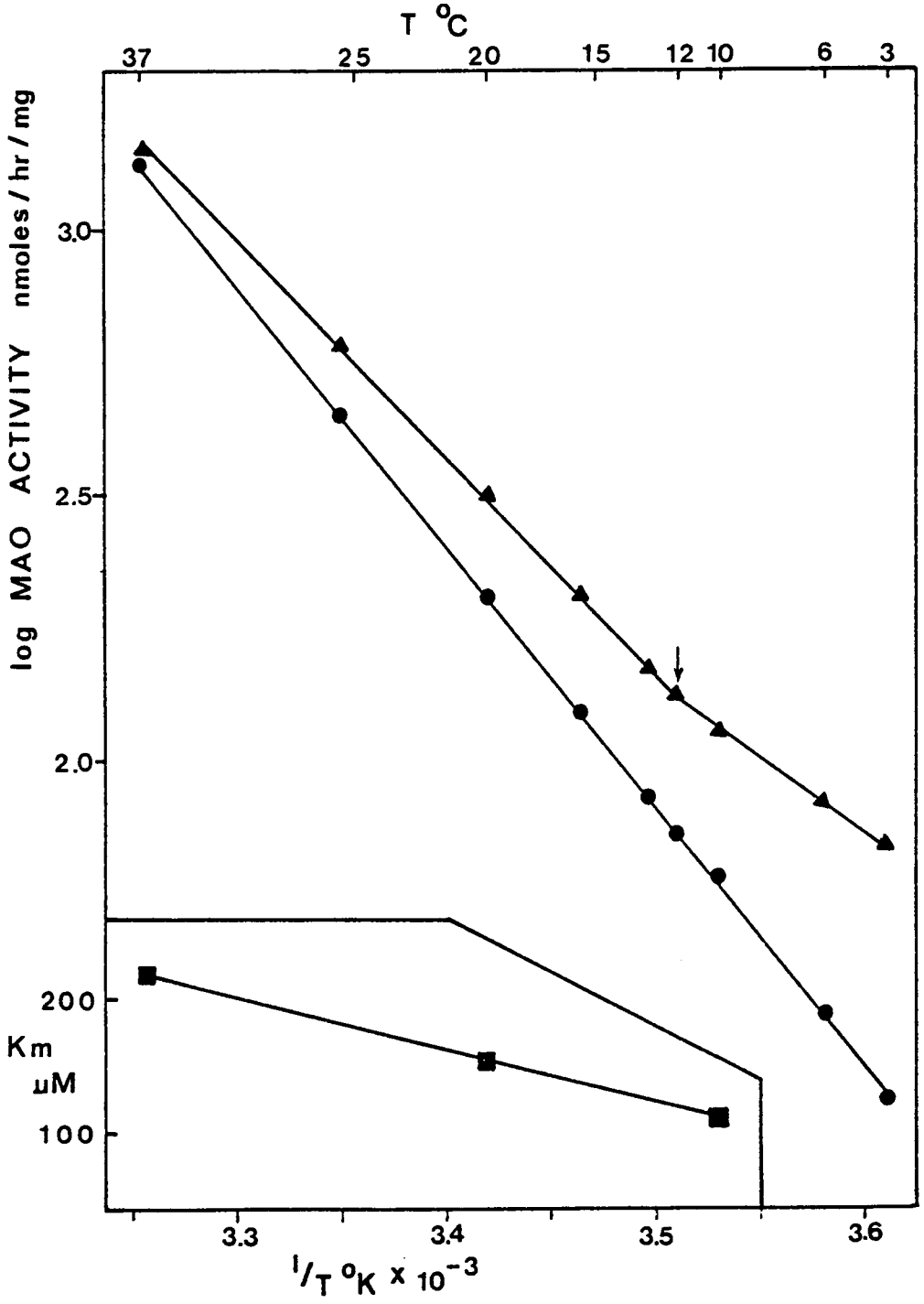
T °C
37
25
20
15
12
10
6
3
log MAO ACTIVITY nmoles / hr / mg
3.0
2.5
2.0
200
Km
µM
100
3.3
3.4
3.5
3.6
1/T °K x 10⁻³

Figure 5

Figure 6. Arrhenius plot of monoamine oxidase activity in outer mitochondrial membranes solubilized in Triton X-100. Monoamine oxidase activity was determined by the radiochemical method using benzylamine (▲) and 5-HT (●). Each point is the mean of three determinations.

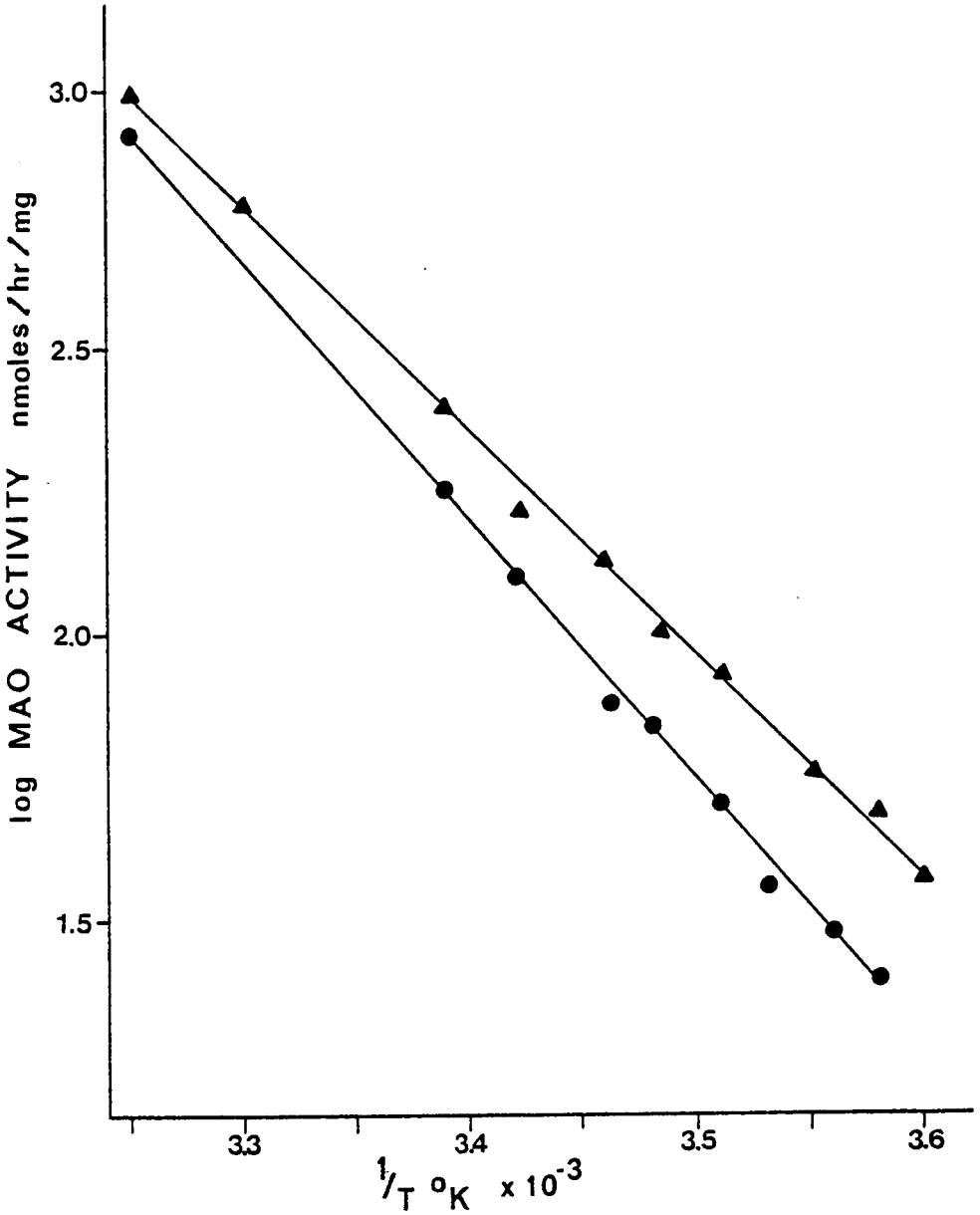

Figure 6

Activation energies of monoamine oxidase determined from the Arrhenius plots in Figures 4, 5 and 6 are listed in Table 7. Decreased slope of the Arrhenius plots of benzylamine deamination and tyramine deamination in the unsolubilized state indicate a reduction in the energy of activation of monoamine oxidase. The energy of activation of benzylamine deamination in host liver mitochondria to 13 kcal below 12° compared to an energy of activation is 25 kcal at temperatures higher than 12°. In the delipidated host liver mitochondria an activation energy of 11 kcal was calculated over the entire range of temperatures tested. However, in outer membranes solubilized in Triton X-100, the energy of activation was 18 kcal over the entire range of temperatures tested.

Km's for benzylamine and tyramine deamination in outer mitochondrial membranes were determined at 37°, 20° and 10° by the Lineweaver and Burk method. Figure 5 shows that the Km for benzylamine deamination decreases with lower temperature.

Inhibition of monoamine oxidase activity in outer membranes was done at different temperatures as shown in Figure 7. The temperature of preincubation with clorgyline and assay of tyramine deamination were the same. The plateau in the inhibition curve occurred at higher activity as the temperature was lowered. This indicates that the ratio of type B to type A monoamine oxidase increased as the temperature of preincubation with inhibitor and assay were reduced. Complete inhibition of activity occurred at 10^{-3}M inhibitor concentration when the experiment was carried out at 37°, while complete inhibition at lower temperatures required higher concentrations of inhibitor.

TABLE 7

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

Source of Enzyme	Substrate	Ea_1* (kcal.)	Temperature of Inflection	Ea_2† (kcal.)
Host Liver Mitochondria	Tyramine	22.4	13°	16.4
	5-HT	22.8	None	22.8
	Benzylamine	24.7	12.5°	13.0
Novikoff Hepatoma Mitochondria	Tyramine	22.8	10°	16.7
	5-HT	17.5	None	17.5
Delipidated‡ Host Liver Mitochondria	Tyramine	16.5	None	16.6
	Benzylamine	11.0	None	11.0
Rat Liver Outer Mitochondria	5-HT	22.4	None	22.4
	Benzylamine	18.7	12°	12.2
Triton X-100 Soluble Outer Membranes	5-HT	20.5	None	20.5
	Benzylamine	17.8	None	17.8

*Ea_1 = energy of activation at temperature above inflection temperature

†Ea_2 = energy of activation at temperature below inflection temperature

‡Delipidated mitochondria prepared by MEK extraction

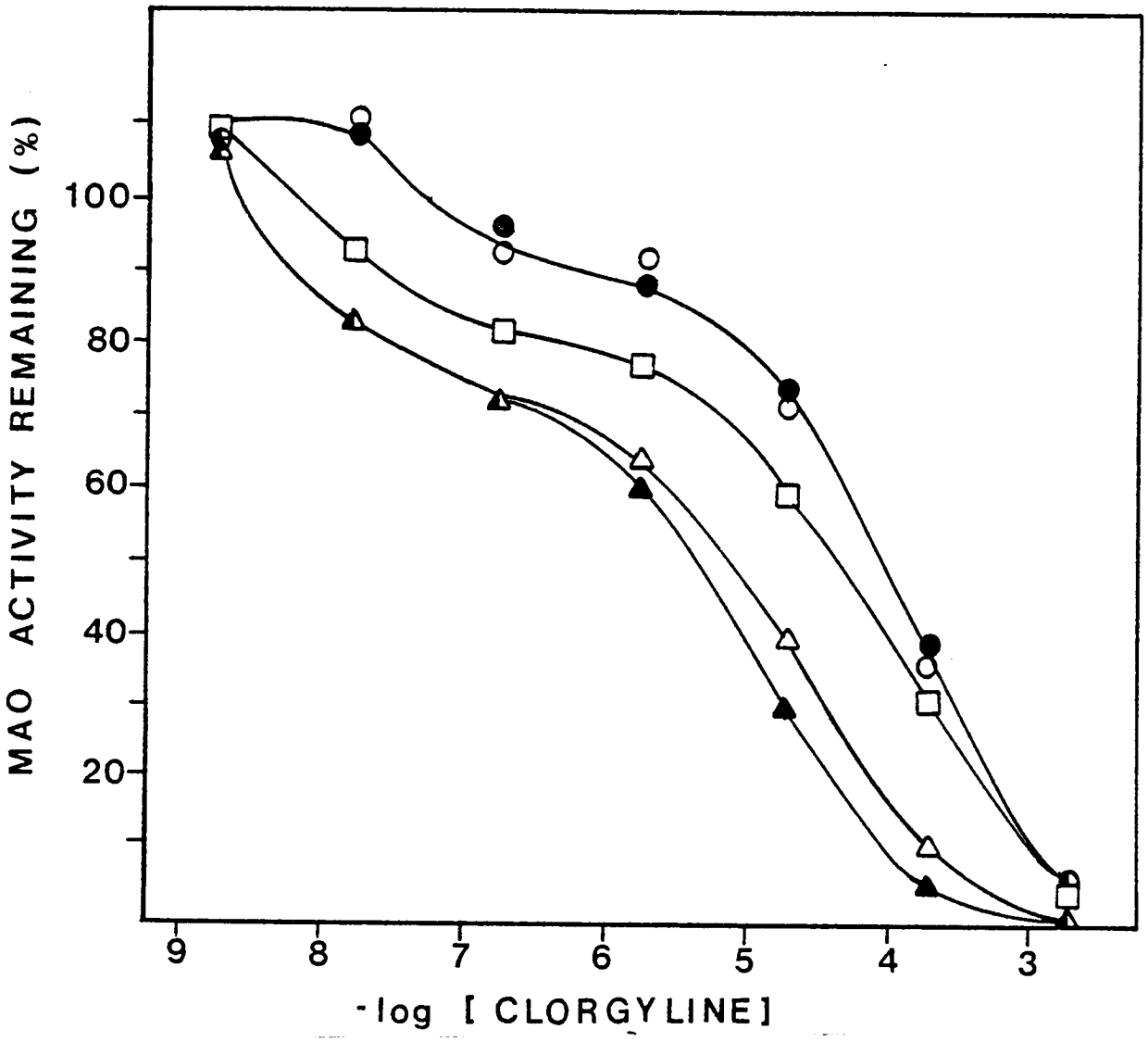

Figure 7. Inhibition of monoamine oxidase activity by clorgyline in rat liver outer mitochondrial membranes at different temperatures. Clorgyline was preincubated with the membranes at 37° (▲) and 25° (△) for 15 minutes and at 15° (□), 10° (●) and 8° (○) for 45 minutes. Monoamine oxidase activity was determined at the same temperature with tyramine.

Discussion

The only significant difference in the phospholipid composition of the hepatoma and host liver mitochondria was a reduction of the inner membrane lipid, cardiolipin, in the hepatoma mitochondria. There was no significant difference in the relative proportion of the phospholipids known to make up the outer membrane of rat liver mitochondria. Since mitochondrial monoamine oxidase is located exclusively on the outer membrane (30), the difference in the ratio of monoamine oxidase types in these mitochondrial preparations cannot be attributed to any gross difference in the phospholipid composition. However, the possibility that the phospholipid in the immediate vicinity of the enzyme in these two mitochondria is different cannot be ruled out.

A larger phospholipid to protein ratio in the hepatoma mitochondria relative to host liver mitochondria is consistent with the observation that the hepatoma mitochondria are of lower density than host liver mitochondria. The phospholipid to protein ratio of cardiolipin (located in the inner membrane) of the hepatoma mitochondria was the same as the host liver mitochondria. However, the same ratio for phosphatidylserine plus phosphatidylinositol (located in outer membrane) for the hepatoma mitochondria was twice that of the host liver. This implies that the increased phospholipid to protein ratio in the hepatoma is due to the altered composition of the outer membrane and not the inner membrane.

Although the phospholipid to protein ratio of the hepatoma mitochondria is greater than that of the host liver mitochondria, the lack

of transformation of monoamine oxidase types upon delipidation implies that the differences in the two types of enzyme activity are more complex than the amount of protein in the mitochondrial membranes. Monoamine oxidase 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 (49,55,85). The recoveries of total activity with all substrates tested were less than 100% in both delipidated preparations, indicating an inactivation of monoamine oxidase activity by this treatment. Since the proposal by Tipton and coworkers that the multiple forms of monoamine oxidase can be interconverted by changing the lipid environment surround one type of enzyme polypeptide (85), experiments have failed to substantiate this hypothesis but have not conclusively ruled out the possibility that transformation of type A to type B can occur (49). This study with the hepatoma mitochondria, enriched in type A activity, conclusively shows that type A monoamine oxidase is not transformed to type B activity after MEK extraction.

Freeze/thawing inactivates the monoamine oxidase activity in delipidated mitochondria. It is shown here that, while monoamine oxidase activity in fresh and water lysed mitochondria was stable to freeze/thawing, this treatment greatly reduced the 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 activity to the same degree with all substrates tested implies that both types are associated with the lipid environment.

The delipidation of hepatoma mitochondria results in less recovery of type B monoamine oxidase activity and more lability of total monoamine oxidase activity toward freeze/thawing than in the host liver mitochondria. Type B activity 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 activity and more protection of both types of activity against inactivation by freeze/thawing.

Activity of membrane bound enzymes is often studied as a function of temperature to probe the lipid environment surrounding the enzyme. Breaks and inflections of membrane enzyme Arrhenius plots are usually attributed to changes in the fluidity of the membrane as lipid phase transitions occur (87), although other factors are sometimes responsible for unusual Arrhenius behavior. Silvius et al. (88) have shown that large changes in apparent Km's of some membrane enzymes with temperatures were responsible for inflections and downward bending Arrhenius plots even though the energy of activation was constant.

Arrhenius plots of benzylamine and tyramine deamination, type B monoamine oxidase, 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 mitochondrial membrane, as demonstrated by freeze fracture electron microscopy techniques (65). Furthermore, the absence of a change in slope of Arrhenius plots of monoamine oxidase activity in delipidated mitochondria and detergent solubilized outer membranes also suggests that type B monoamine oxidase

in the intact outer mitochondrial membrane is affected by a lipid phase transition. Km values for type B monoamine oxidase substrates do not change enough to account for the observed Arrhenius behavior.

Arrhenius plots of type A monoamine oxidase activity in all preparations tested had a constant slope over the range of temperatures tested. This is taken as evidence of the different interaction of the two types of monoamine oxidase with the lipid environment. A constant slope of an Arrhenius plot of activity of a membrane enzyme in the range of temperatures in which the membrane undergoes a lipid phase transition is usually interpreted as indicating that the enzyme is a peripheral membrane protein or has tightly bound lipid which does not undergo a phase transition (87). The latter possibility is most likely the case for type A monoamine oxidase because this enzyme is dependent on lipid for activity.

In the vast majority of membrane enzymes for which Arrhenius inflection behavior has been observed, the energy of activation increased below the temperature of the inflection. Type B monoamine oxidase is an exception. Energy of activation decreased below the temperature of phase transition. Delipidation of host liver mitochondria with MEK resulted in a similar reduction of the energy of activation over the entire range of temperatures tested. Perhaps this observation reflects a shift in the lipid environment of type B monoamine oxidase upon phase transition such that the enzyme is less restricted by lipid and more closely resembles the delipidated enzyme (enzyme in mitochondria depleted of lipid except for cardiolipin). Hackenbrock et al. (65) have shown

that intramembrane particles migrate from freezing domains of lipid to fluid domains during the phase transition of the outer membrane of rat liver mitochondria. It is tempting to speculate that monoamine oxidase can migrate to fluid regions of the outer membrane as the phase transition occurs such that the energy of activation of type B monoamine oxidase is reduced due to the new lipid environment.

Inhibition of monoamine oxidase by clorgyline was studied at different temperatures above and below the temperature of the phase transition to investigate possible alterations in the specificity of monoamine oxidase types toward this inhibitor. Higher ratios of type B activity determined at lower temperatures reflect the relative specific activities of the two types of activity in response to lower temperature. Type A activity decreased more than type B activity as the assay temperature was reduced. Corresponding inhibition with clorgyline and assay of monoamine oxidase activity at these lower temperatures confirms that more type B enzyme is active relative to type A enzyme. It is doubtful that the phase transition has any effect on the interaction of clorgyline with either type of monoamine oxidase.

In summary, this study presents evidence that both types of monoamine oxidase are associated with lipid, but the association is different for each. Type A monoamine oxidase is dependent upon lipid for activity, yet is insensitive to a bulk lipid phase transition in the outer mitochondrial membrane. Type B monoamine oxidase is less dependent on lipid for activity, but is sensitive to the phase transition and requires lipid for stability in the cold. Similarity of the phospholipid composition of the hepatoma and host liver mitochondria and

the lack of transformation of monoamine oxidase types upon delipidation do not support the hypothesis that the multiple forms of monoamine oxidase are strictly due to effect of lipid. Recent evidence that the two types of monoamine oxidase activity are due to different proteins (23) and may be located on different sides of the outer membrane (69) may explain the different relationship of monoamine oxidase types with lipid. In this regard, asymmetric distribution of membrane phospholipids between the two faces of several biological membranes has been demonstrated. The significance of lipid interaction with monoamine oxidase types is not known and will be considered in latter chapters.

CHAPTER V

EFFECT OF LIPID ADDITION ON MONOAMINE OXIDASE ACTIVITY

Effect of Lipid Addition on Monoamine Oxidase Activity in Methyl Ethyl Ketone Extracted Mitochondria

As described in the previous chapter, extraction of mitochondria with MEK results in inactivation of monoamine oxidase activity. This strongly suggests that the activity is dependent on the lipid environment. These experiments were carried out to examine the sensitivity of monoamine oxidase activity to lipid added back to the extracted mitochondria. Delipidated Novikoff hepatoma mitochondria were also studied along with delipidated host liver and control rat liver mitochondria.

Figure 8 illustrates the effect of mitochondrial lipids on monoamine oxidase activity in both delipidated host liver and hepatoma mitochondria. Mitochondria were extracted with MEK and incubated with lipid dispersions of total mitochondrial lipid extracts for 30 minutes at 37° as described in Methods. Hepatoma mitochondrial lipids were not used, because their lipid composition is similar to that of host liver mitochondria. Rates of 5-HT deamination in both delipidated mitochondrial preparations were increased after incubation with mitochondrial lipids. Monoamine oxidase activity measured with 5-HT in the delipidated hepatoma mitochondria, which corresponded to 3.5% of total activity before extraction, was maximally reactivated to 11.7% of the total original activity. This treatment increased the monoamine oxidase

Figure 8. Effect of added lipid on monoamine oxidase activity in delipidated mitochondria from host liver and Novikoff hepatoma. The left panel is the effect of mitochondrial lipid on activity in the delipidated host liver mitochondria as described in the Methods section. Monoamine oxidase activity was measured with benzylamine (△——△), tyramine (○——○) and 5-HT (□——□). The right panel is the effect of host liver mitochondria lipids (——) and purified phosphatidylcholine from egg yolk (----) on monoamine oxidase activity in Novikoff hepatoma mitochondria. 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 of monoamine oxidase activity in the delipidated hepatoma mitochondria by host liver mitochondria lipid represent the variation of two aliquots of hepatoma mitochondria which were separately extracted and added to lipid under identical conditions.

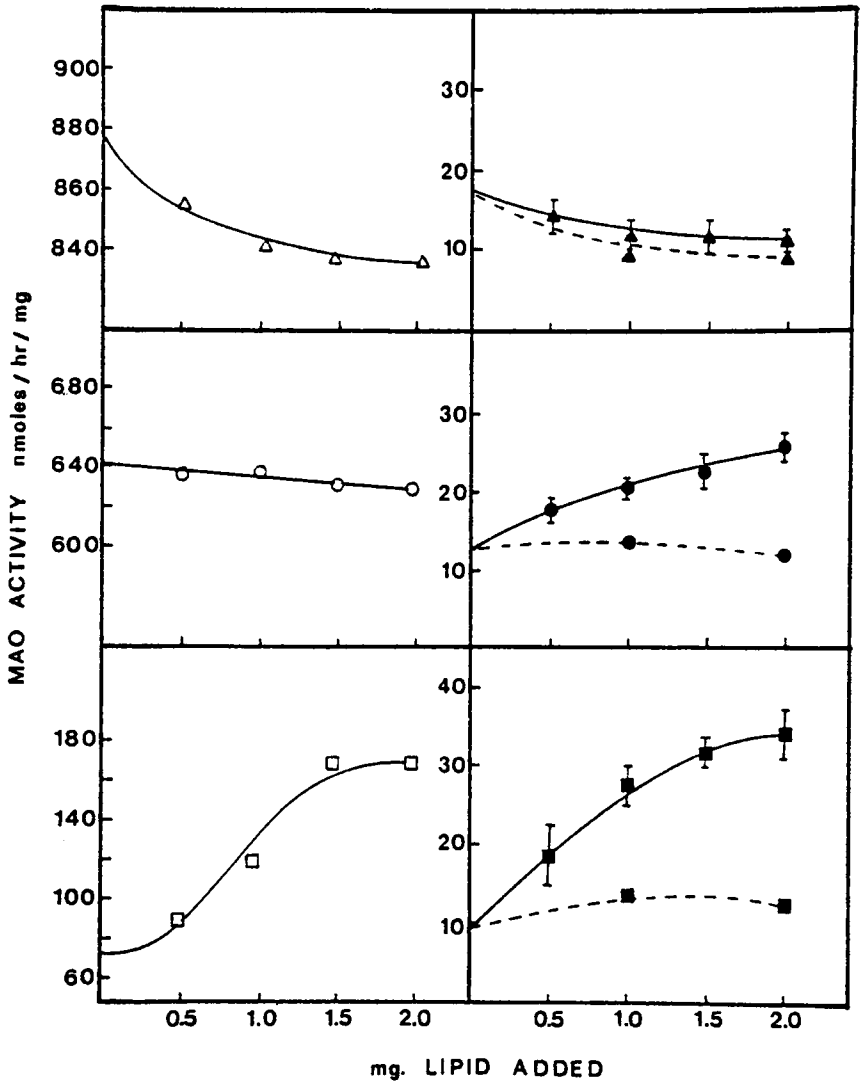
MAO ACTIVITY nmoles / hr / mg
900
880
860
840
680
640
600
180
140
100
60
30
20
10
30
20
10
40
30
20
10
0.5
1.0
1.5
2.0
0.5
1.0
1.5
2.0
mg. LIPID ADDED

Figure 8

activity measured with 5-HT in the delipidated host liver mitochondria from 4.5 to 10.7% of the total activity before extraction.

Incubation of delipidated mitochondria with mitochondrial lipids led to a decrease in the rate of benzylamine deamination in the delipidated hepatoma mitochondria of 30% and a 5.0% decrease in the delipidated host liver mitochondria. Rate of tyramine deamination was increased 1.6 fold in the delipidated hepatoma after incubation with mitochondrial lipids. However, no change in the rate of tyramine deamination occurred in the delipidated host liver mitochondria after incubation with mitochondrial lipids.

Monoamine oxidase activity in the delipidated hepatoma mitochondria plus increasing concentrations of phosphatidylcholine is also shown in Figure 8. This phospholipid had no ability to increase the activity of monoamine oxidase toward any substrate tested under the conditions of this experiment. However, the rate of benzylamine deamination was decreased by phosphatidylcholine to the same extent that the activity was reduced after incubation with mitochondrial lipids.

Attempted reactivation of monoamine oxidase in the delipidated mitochondria with mitochondrial lipid was carried out at low temperature because of the possible susceptibility of the delipidated enzyme to thermal inactivation (52,89). As shown in Figure 9, the magnitude of the increase of 5-HT deamination in response to added lipid was the same when lipid was added to the delipidated mitochondria at 4°C for 24 hours as compared to the condition used previously (37° for

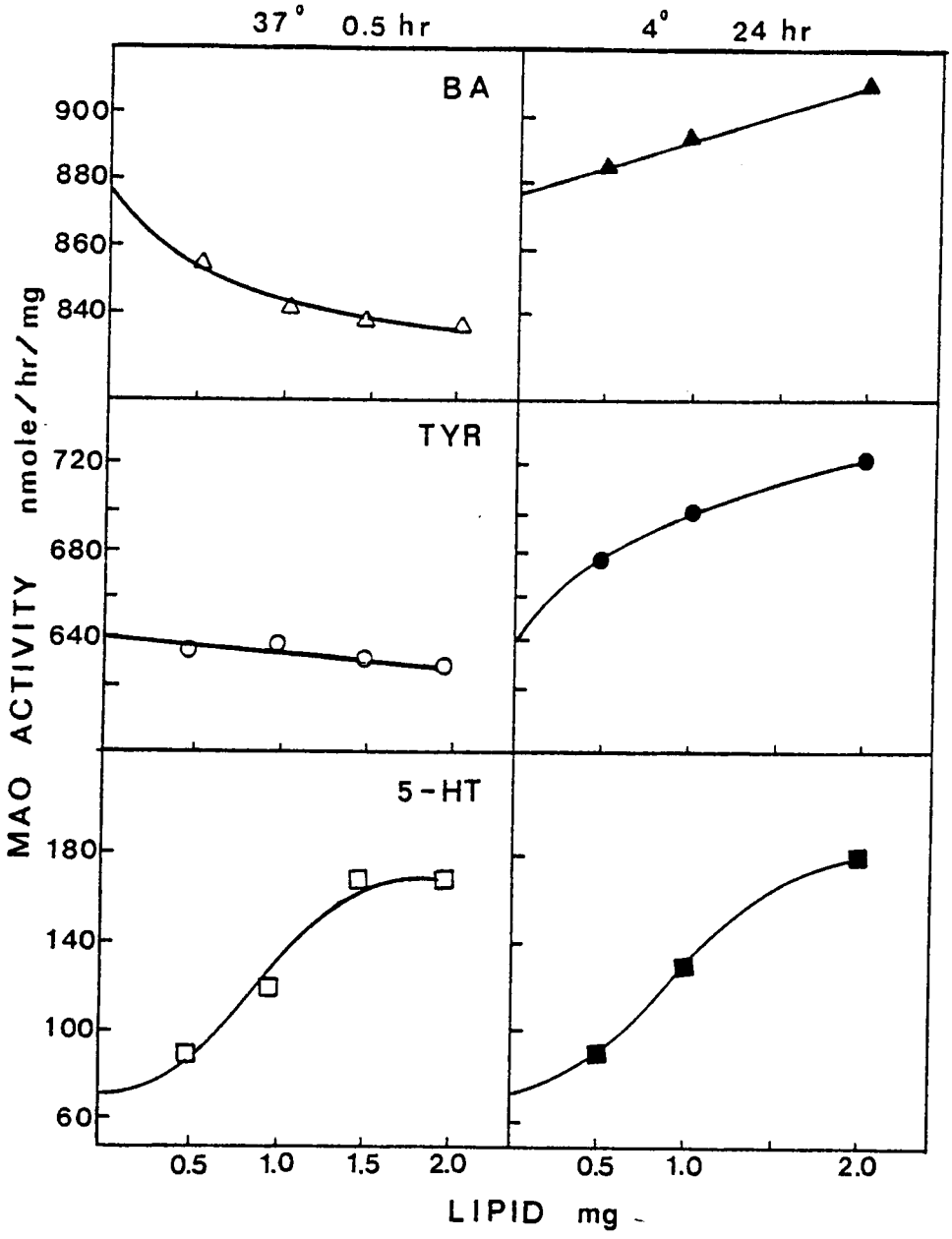

Figure 9. Effect of added lipid on monoamine oxidase activity in delipidated host liver mitochondria at 37° and 4°. Mitochondrial lipids were added to MEK extracted mitochondria at 37° for 30 minutes and at 4° for 24 hours; activity was then determined by the radiochemical method.

30 minutes). However, both the rates of tyramine and benzylamine deamination increased in response to added lipid. All additional experiments were carried out at low temperature since this condition gave increased reactivation.

Purified phospholipids and mixtures of phospholipids were incubated with delipidated hepatoma and host liver mitochondria, and the effect of monoamine oxidase activity was analyzed. Figures 10 and 11 show representative experiments in which phospholipids were added to delipidated host liver and hepatoma mitochondria, respectively. Additional experiments are summarized in Table 8. In all experiments done over a three year period, cardiolipin was always able to reactivate monoamine oxidase activity toward 5-HT to a greater extent than other phospholipids and mixtures of phospholipids tested. This phospholipid restored 5-HT deamination to a maximum of 10.8% of unextracted activity in the delipidated host liver mitochondria and to a maximum of 17.8% of the unextracted activity in the delipidated hepatoma mitochondria. Phosphatidylcholine did not greatly affect the rate of 5-HT deamination in the delipidated host liver mitochondria; however, this phospholipid was variable in its effect on 5-HT deamination in the delipidated hepatoma mitochondria. The result with phosphatidylserine in both delipidated mitochondrial preparations was intermediate between phosphatidylcholine and cardiolipin.

The rates of benzylamine and tyramine deamination were also measured after phospholipids were added to delipidated mitochondria. Addition of most phospholipids to delipidated host liver mitochondria

Figure 10. Effect of added phospholipid on monoamine oxidase activity in delipidated host liver mitochondria. Cardiolipin (▲), phosphatidylserine (●) and phosphatidylcholine (■) were added to the delipidated mitochondria at 4° for 12 hours. Monoamine oxidase activity was then determined by the radiochemical method. Each point is the mean of three determinations.

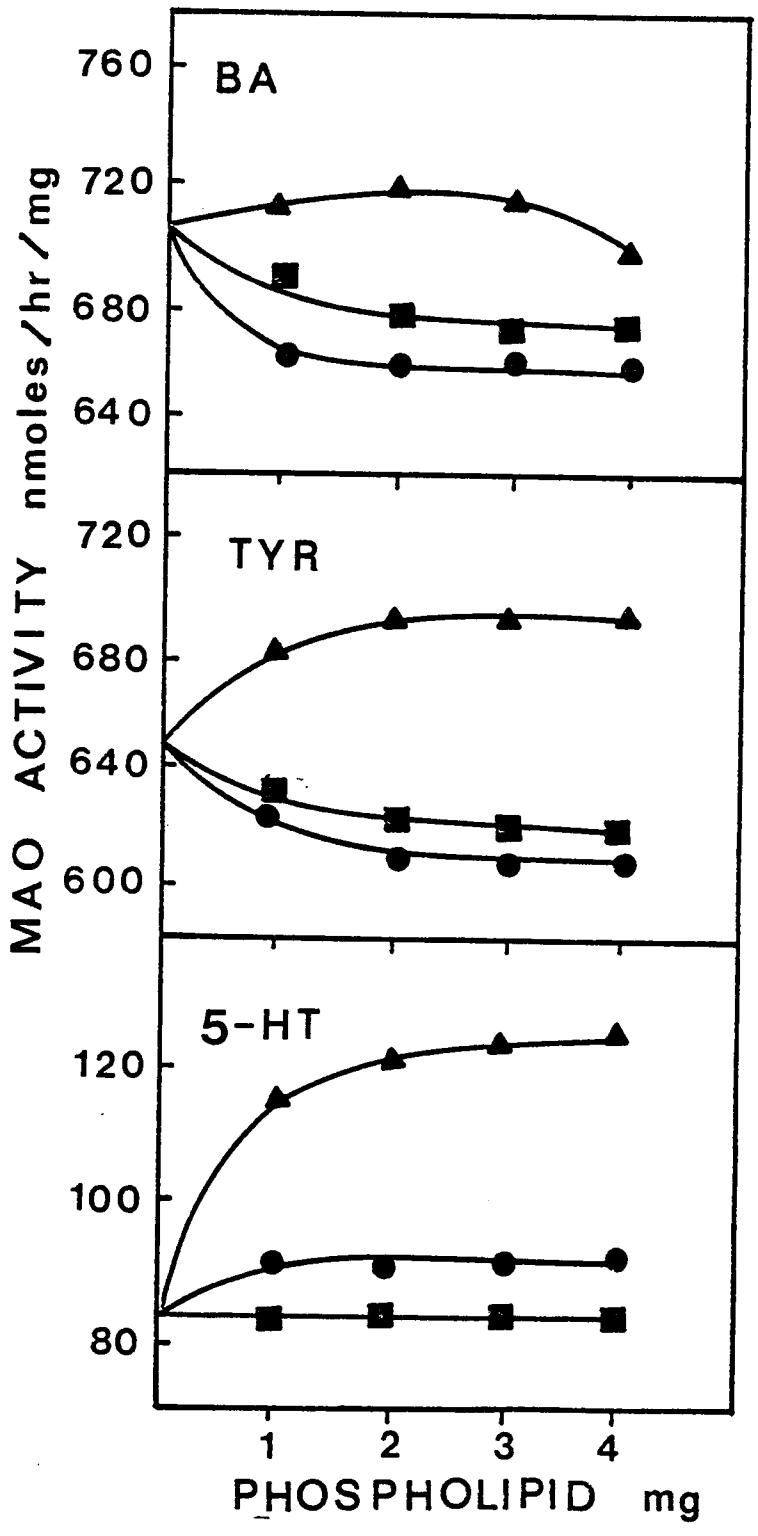
760
720
680
640
BA
720
680
640
600
TYR
120
100
80
5-HT
MAO ACTIVITY nmoles/hr/mg
1
2
3
4
PHOSPHOLIPID mg

Figure 10

Figure 11. Effect of added lipid on monoamine oxidase activity in delipidated hepatoma mitochondria. Cardiolipin (○,●), phosphatidylcholine (△,▲), phosphatidylcholine plus phosphatidylethanolamine (1:1) (□,■) and phosphatidylcholine plus cholesterol (4:1) (⬡,⬢) were added to the delipidated hepatoma mitochondria at 4° for 12 hours. Activity was then determined by the radiochemical method with benzylamine (closed symbols) and 5-HT (open symbols).

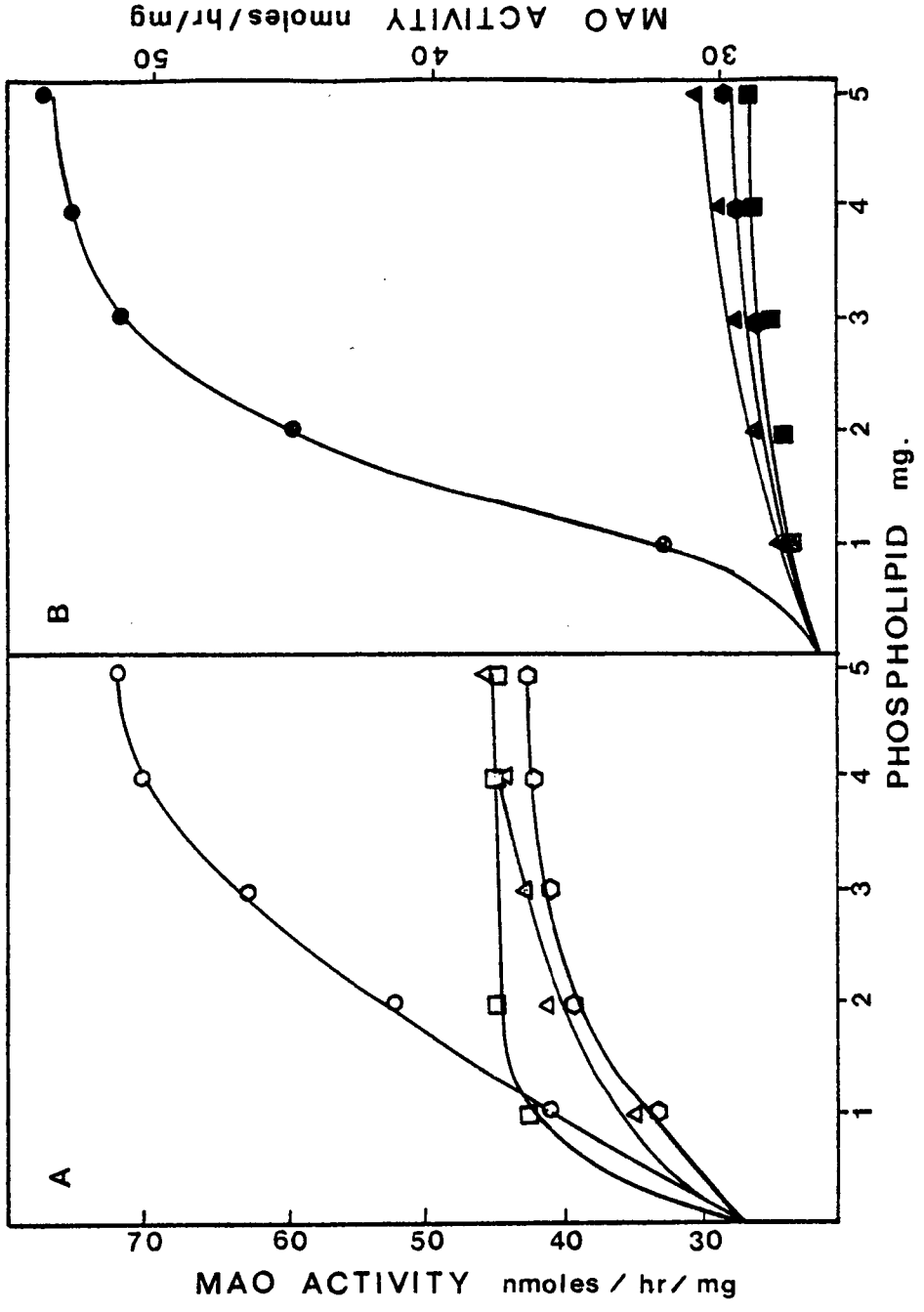
MAO ACTIVITY nmoles / hr / mg
70
60
50
40
30
A
B
PHOSPHOLIPID mg.
1
2
3
4
5
30
40
50
MAO ACTIVITY nmoles/hr/mg

Figure 11

TABLE 8

Effect of lipid addition on monoamine oxidase activity in delipidated mitochondria

Lipid*	% Change in Specific Activity†	Average Range % Total Activity Before	After	% Change in Total§ Activity
Delipidated Host Liver Mitochondria				
	5-HT Deamination			
PC	2.0 ± 3.2 (3)	4.6	4.9	0.3 ± 0.9 increase
PS	66.7 ± 29.8 (3)	4.6	7.7	3.2 ± 1.1 increase
CL	103.0 ± 24.0 (3)	4.6	8.9	4.7 ± 1.2 increase
ASO	100	4.5	8.9	4.4 increase
	Benzylamine Deamination			
PC	- 4.7 - 2.1 (3)	63.7	61.1	2.6 ± 1.1 decrease
PS	- 16.7 - 7.1 (3)	73.6	54.9	18.7 ± 6.7 decrease
CL	- 12.5 - 9.6 (3)	65.4	74.5	9.1 ± 6.3 increase
ASO	10.0	72.0	79.4	7.4 - increase
Delipidated Novikoff Hepatoma Mitochondria				
	5-HT Deamination			
PC	5.7 ± 23.2 (3)	3.5	4.6	1.1 ± 2.7 increase
PS	42.4	2.3	3.3	1.0 increase
CL	177.1 ± 36.0 (2)	4.0	11.9	7.9 ± 4.3 increase
PS:PC (3:7)	26.7	2.6	3.3	0.7 increase
PS:PC (3:2)	- 3.4	2.6	2.5	0.1 decrease
CL:PC (3:7)	- 14.7	2.6	2.2	0.4 decrease
CL:PC (3:2)	24.1	2.6	3.2	0.6 increase
PE:PC (1:1)	91.3	5.7	10.9	5.2 increase
	Benzylamine			
PC	20.1 - 8.4 (3)	48.6	38.8	9.8 ± 5.0 decrease
PS	47.6 -	42.7	63.0	20.3 increase
CL	112.5 - 8.5 (2)	43.8	93.0	49.2 increase
PS:PC (3:7)	- 39.1	58.1	35.4	22.7 decrease
PS:PC (3:2)	0	58.1	58.1	
CL:PC (3:7)	11.4	58.1	64.7	6.6 increase
CL:PC (3:2)	14.6	58.1	66.6	8.5 increase
PE:PC (1:1)	20.8	44.9	54.2	9.3 increase

*Abbreviations used: PC, phosphatidylcholine; PS, phosphatidylserine; CL, cardiolipin; ASO, asolectin. Molar ratio of lipid in mixture in parentheses

†Mean ± standard deviation (number of experiments) negative sign denotes decreased activity

§Mean ± standard deviation

resulted in a slight decrease in the rate of benzylamine deamination (10% or less). However, the addition of cardiolipin to delipidated host liver mitochondria either had no effect on the rate of benzylamine deamination or resulted in a slight increase in the activity. Monoamine oxidase metabolizing benzylamine in the delipidated Novikoff hepatoma was restored for approximately 40% to greater than 90% of the unextracted activity by the addition of cardiolipin. Phosphatidylserine was able to restore this activity to 63% of the unextracted activity while phosphatidylcholine had no effect. Changes in the rate of tyramine deamination in both delipidated mitochondrial preparations due to added lipid were similar to the changes in the rate of benzylamine deamination.

Mixtures of cardiolipin and phosphatidylcholine were only as effective as phosphatidylcholine alone in restoring monoamine oxidase activity toward any substrate tested. Likewise, mixtures of phosphatidylethanolamine and phosphatidylcholine had the same effect. Increasing concentrations of cholesterol in a cholesterol and phosphatidylcholine mixture were added to delipidated rat liver mitochondria and monoamine oxidase activity was estimated as shown in Figure 12. The rate of benzylamine deamination was reduced by 20% by the addition of phosphatidylcholine and was additionally reduced as a result of greater than 30% cholesterol in the lipid incubated with the delipidated mitochondria. Monoamine oxidase metabolizing 5-HT in the delipidated rat liver mitochondria was not influenced by the addition of cholesterol.

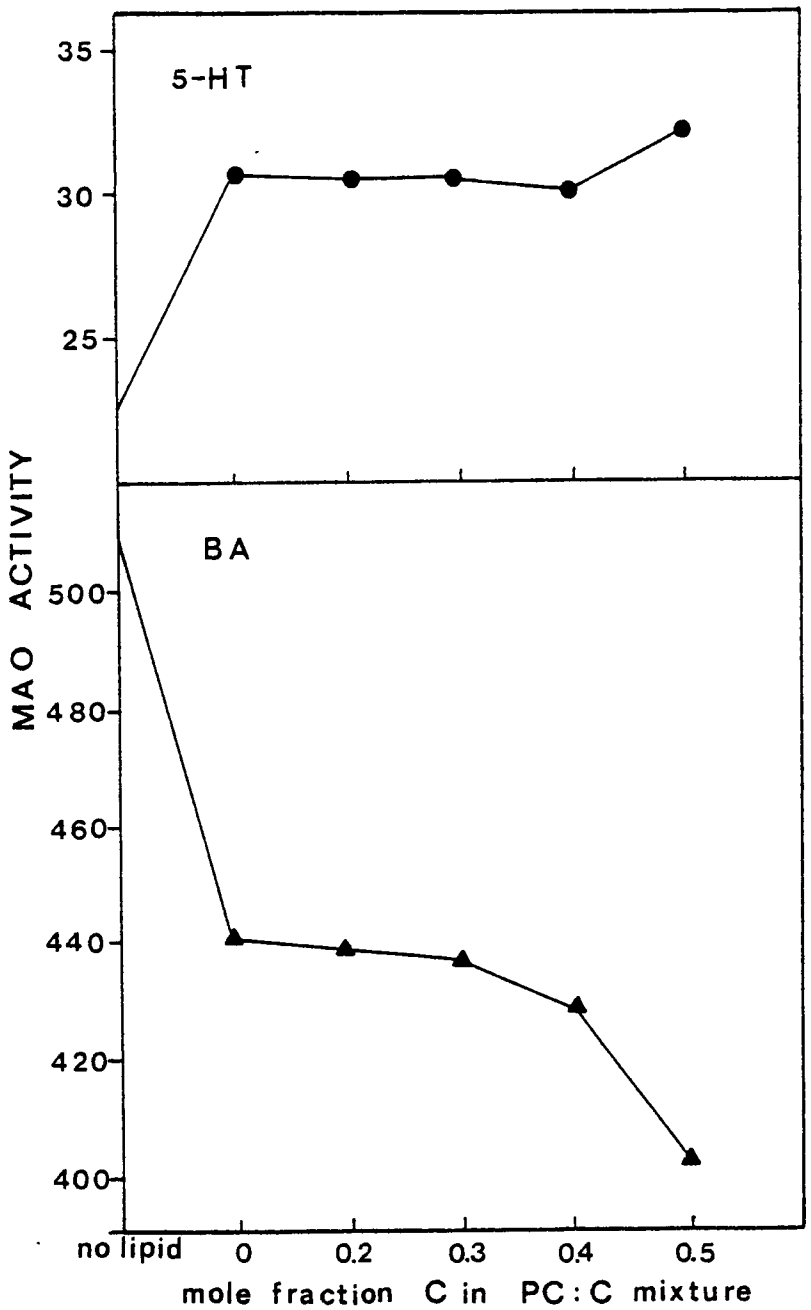

Figure 12. Effect of cholesterol on monoamine oxidase activity in delipidated rat liver mitochondria. Mixtures of cholesterol and phosphatidylcholine were added to delipidated mitochondria at 4° for 12 hours, after which monoamine oxidase activity was determined by the radiochemical method with 5-HT (●) and benzylamine (▲).

Binding of exogeneous phospholipid to the delipidated mitochondria was investigated by separating the free lipid from the mitochondrial residue by rapid sedimentation into a sucrose gradient as illustrated in Figure 13. Quantitation of the phospholipid which cosedimented with the monoamine oxidase activity was determined by the H^3-phosphatidylcholine tracer added to the lipid dispersions. Figure 13 shows that this label and total lipid phosphorus cosediment exactly. In the experiment shown here, 66.3% of phosphatidylserine, 63.5% of cardiolipin and 51.9% of phosphatidylcholine incubated with the delipidated mitochondria associated strongly enough with this material to enter the gradient. Lipid dispersions in the absence of delipidated mitochondria did not enter the gradient in experiments not shown.

Reconstitution of Partially Purified Monoamine Oxidase

Monoamine oxidase was partially purified from rat liver outer mitochondrial membranes solubilized in cholate by sedimentation in continuous glycerol gradients containing cholate. Recovery of monoamine oxidase activity was 3.1% of the total activity toward 5-HT and 19.0% of the total activity toward benzylamine in the outer membrane (corrected for the unsolubilized activity which accounted for greater than 70% of the total outer membrane monoamine oxidase activity). This procedure removed greater than 90% of the soluble lipid phosphorus from the pooled fractions containing monoamine oxidase activity determined in a parallel experiment done under the same conditions. Lipid was dissolved in the cholate containing enzyme solution and lipid vesicles were obtained after the removal of cholate by gel filtration as described in Methods.

Figure 13. Sedimentation of delipidated rat liver mitochondria and added phospholipid. Two mg of phospholipid dispersion was added to 1.0 mg of MEK extracted mitochondria at 4° for 12 hours. This mixture was applied to a 5 to 40% continuous sucrose gradient and centrifuged for one hour at 150,000 x g in a SW 50.1 rotor. Tracer quantities of tritium labeled phosphatidylcholine were included in the phospholipid dispersions as a marker of added lipid (●). Monoamine oxidase activity was determined by the spectrophotometric method (▲). Total lipid phosphorus was determined as previously described in Methods (0).

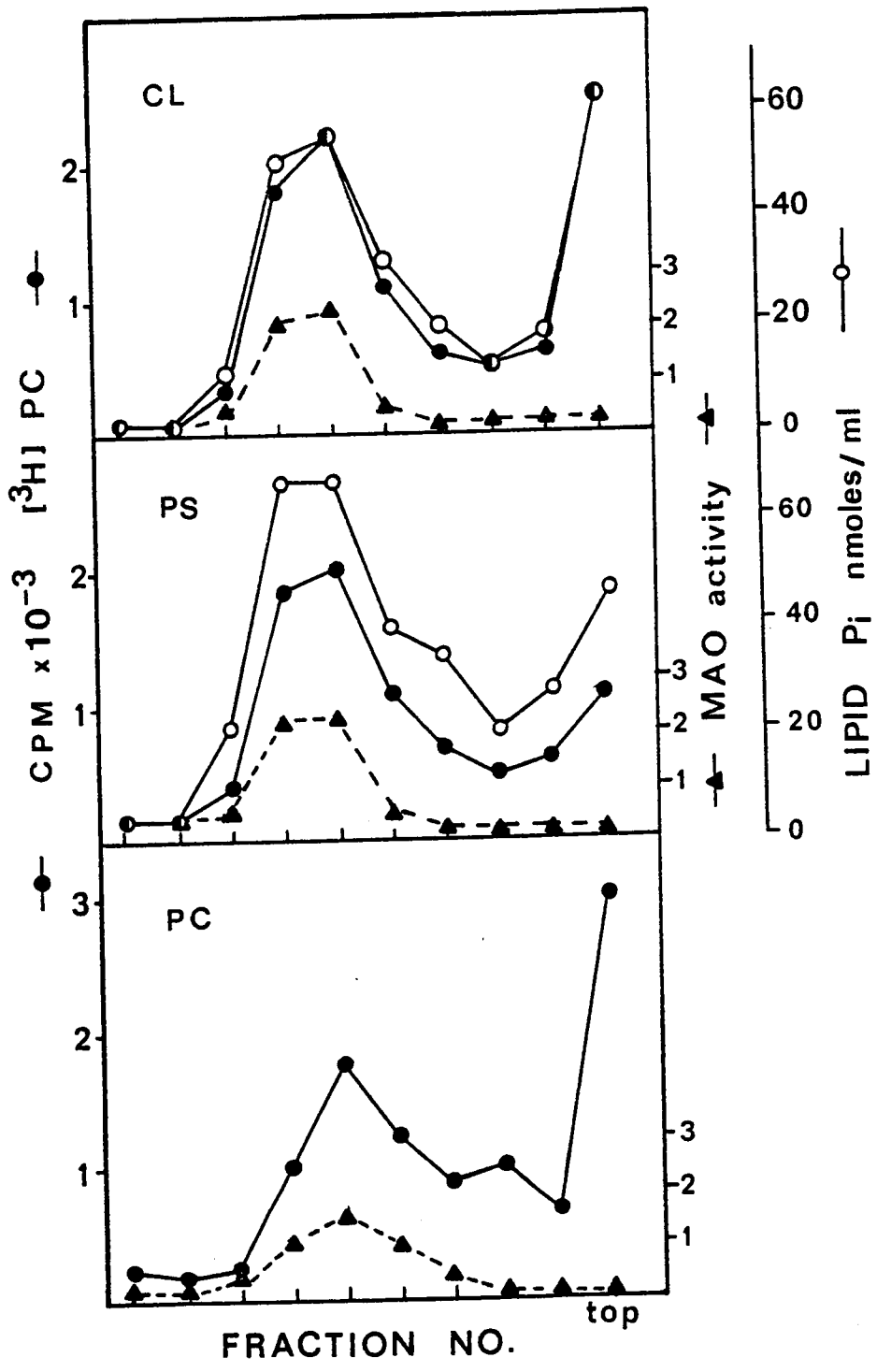
CL
PS
PC
CPM x10⁻³ [³H] PC
MAO activity
LIPID Pi nmoles/ml
FRACTION NO.
top

Figure 13

Table 9 shows the increase of monoamine oxidase activity upon incubation of the partially purified enzyme into lipid vesicles of purified phospholipid. Monoamine oxidase activity toward 5-HT increased from 3.1% of total activity to 16.4% of total activity in phosphatidylserine liposomes. Outer membrane lipid from rat liver mitochondria was capable of restoring activity toward 5-HT to 9.2% of the total activity, phosphatidylcholine to 13.2% and cardiolipin restored this activity to 8.3% of the total activity. Monoamine oxidase activity toward benzylamine increased from 19.0% of total activity to 52.0% of the total activity when partially purified enzyme preparation was reconstituted into liposomes of outer membrane lipid. Reconstitution of this preparation in other lipids resulted in similar increases in the rate of benzylamine deamination.

Discussion

Reactivation of type A monoamine oxidase activity in MEK extracted mitochondria varied as a result of added lipid in a consistent pattern: Greatest reactivation by cardiolipin, a comparable reactivation by mitochondrial lipids, lesser reactivation by phosphatidylserine and almost no reactivation by phosphatidylcholine. These lipids differ in both net charge and the degree of saturation of the fatty acids. Cardiolipin from beef heart has a net charge of -2, and linolenic acid makes up greater than 70% of the fatty acid. Phosphatidylserine from beef brain has a net charge of -1, and the fatty acid composition consists of equimolar quantities of stearic and oleic acid. Phosphatidylcholine from egg yolk has no net charge,

TABLE 9

Monoamine oxidase activity after reconstitution into lipid vesicles

Lipid	Monoamone Oxidase Activity (nmoles/hr/mg) 5-HT	Benzylamine	(% of Total) 5-HT	Benzylamine
None	66	785	3.1	19.3
OML	196	2,107	9.2	51.8
PC	282	2,045	13.2	50.3
PS	351	2,129	16.4	52.5
CL	178	2,274	8.3	55.9

and the fatty acid composition is a mixture of palmitic acid (32%), stearic acid (16%), oleic acid (33%) and linolenic acid (13%). Total mitochondrial lipid has a net negative charge, and the fatty acids are more than 50% unsaturated. Binding of phospholipid to the delipidated mitochondria is not a factor in the response of monoamine oxidase activity to added lipid since equal proportions of the three phospholipids cosediment with the delipidated mitochondria. However, the interaction of lipid with monoamine oxidase in the MEK extracted mitochondria may depend on the charge and/or the fatty acids of the lipid.

Olivecrona and Oreland (50) have shown that acidic phospholipids, cardiolipin and phosphatidylserine, will bind to purified pig liver monoamine oxidase forming soluble complexes. Phosphatidylcholine could not bind to the pig liver enzyme. This study also suggests that the interaction of lipid dispersions with type A monoamine oxidase in the delipidated mitochondria is dependent on a net negative charge of the lipid. MEK extracted mitochondria are not the best system to study the interaction of lipid with monoamine oxidase since this preparation is rich in cardiolipin. High levels of type B monoamine oxidase activity recovered after extraction of rat liver mitochondria with MEK may be the result of the inefficient extraction of cardiolipin. The observation that type B activity in the hepatoma mitochondria (poor in cardiolipin relative to rat liver mitochondria) is greatly inactivated by MEK treatment and reactivated by the addition of cardiolipin supports this idea. It is not understood why type A monoamine oxidase activity is reactivated by exogenous cardiolipin but seemingly insensitive to the residual cardiolipin unless the two types of monoamine oxidase have a different environment in the MEK extracted mitochondria.

Monoamine oxidase type B activity in the delipidated rat liver mitochondria was slightly decreased as a result of the addition of lipids other than cardiolipin and azolectin (crude soy bean phospholipids). This result was taken to indicate the dependence of type B activity on the fluidity of the lipid environment since both cardiolipin and azolectin are known to have large amounts of unsaturated fatty acids while the other lipids tested have much more saturated fatty acids. The effect is small due to the presence of the cardiolipin in the delipidated mitochondria. Cholesterol in dispersions of phosphatidylcholine was added to the delipidated rat liver mitochondria to increase the rigidity of the lipid (90). Additional inhibition of type B monoamine oxidase due to cholesterol further implicates the influence of the fluidity of the lipid surrounding this enzyme on its activity.

Reconstitution of purified and lipid free monoamine oxidase into artificial vesicles of known lipid composition is the approach to answer the question of what, if any, lipid is required for monoamine oxidase activity. This study never progressed beyond the reconstitution of a partially purified enzyme into liposomes. All the lipids tested were capable of reactivating type A monoamine oxidase to within a range of 8 to 16% of the initial outer membrane activity. The conclusion that a specific lipid head group is required for monoamine oxidase type A activity seems unlikely but cannot be excluded because the preparation is not completely delipidated. If a specific lipid is tightly bound to this enzyme after centrifugation in the cholate-glycerol gradient, additional lipid is required for activity.

It is interesting that the more saturated lipids, phosphatidylserine and phosphatidylcholine, reactivate type A monoamine oxidase activity to a greater extent than cardiolipin. This observation suggests that the fatty acid chains of the lipid interact with the enzyme directly or an enzyme plus boundary lipid complex such that type A monoamine oxidase activity is increased by saturated fatty acids. Alternatively, lipid having saturated fatty acids may be able to displace tightly bound cholate from the enzyme with greater efficiency than lipid containing more saturated fatty acids.

Type B monoamine oxidase activity was increased by reconstitution into vesicles irrespective of the lipid used. It will be shown in a later chapter that the majority of this increase in type B activity upon reconstitution is due to the removal of cholate which interferes with the radiochemical assay of benzylamine deamination and therefore can inhibit the activity. The absence of differential effects of the lipids used for reconstitution on type B activity may be due to the presence of residual cholate. On the other hand, the activity of this enzyme may be totally indescriminate in its requirement for a hydrophobic environment. A change in the reaction mechanism of type B monoamine oxidase upon solubilization has been reported (56). The possibility that the enzyme in the soluble state has little resemblence to the enzyme in the outer membrane should always be considered.

It is not known precisely why different types of lipid give maximum reactivation of type A monoamine oxidase activity in the two

experiments. The reactivation of delipidated monoamine oxidase with lipid depends on the ability of the lipid to bind to the enzyme and then to interact with the enzyme such that activity is restored. In this study and as shown by others (50), the binding of lipid with delipidated monoamine oxidase requires a negatively charged phospholipid. This phenomena has been shown for other mitochondrial proteins (91) and the Na^+ and K^+ ATPase (92). Presence of cholate may facilitate the binding of uncharged lipid to the enzyme such that monoamine oxidase activity can be reactivated by phosphatidylcholine. Therefore, the reconstitution experiment is probably the best measure of how well different types of lipid can restore monoamine oxidase activity. Results of the two reactivation experiments imply that the binding of lipid other than cardiolipin limit the reactivation of type A monoamine oxidase when lipid dispersions are added to delipidated mitochondria. However, when the binding was no longer limiting, phosphatidylserine reactivated type A monoamine oxidase best.

It is apparent that the type A activity is more dependent on the type of lipid present than the type B activity. This probably reflects the selective association of type A monoamine oxidase with a particular lipid in the outer membrane. It is tempting to speculate that type A monoamine oxidase might associate with phospholipids which are similar in structure to the beef brain phosphatidylserine used in this reconstitution experiment. These lipids may be a minor component of the outer membrane since outer membrane lipids did not result in the maximum reactivation of type A activity.

In conclusion, these results are compatible with the hypothesis that the multiple forms of monoamine oxidase are two different polypeptides which have different interactions with the lipids of the outer membrane. However, the hypothesis that lipid alone is responsible for the generation of the multiple forms of monoamine oxidase is inconsistent with these results since the two types of activity could not be interconverted by manipulation of the lipid environment.

CHAPTER VI

SOLUBILIZATION OF RAT LIVER MITOCHONDRIAL OUTER MEMBRANES IN DETERGENTS: EFFECT ON MONOAMINE OXIDASE ACTIVITY

Solubilization of Mitochondrial Outer Membranes

Purification of monoamine oxidase requires harsh treatment to liberate the enzyme from the mitochondrion. Organic solvents and sonication have been successfully used to solubilize monoamine oxidase activity in mitochondria from some tissues. However, monoamine oxidase from rat liver has been purified only in the presence of detergents, most commonly Triton X-100. This study is a limited survey of the ability of common detergents to solubilize monoamine oxidase activity from outer mitochondrial membranes. Differential effects on multiple types of monoamine oxidase activity by detergents were also investigated.

Table 10 shows the effectiveness of detergents in solubilizing total protein and both types of monoamine oxidase activity of outer mitochondrial membranes. Solubilized material was defined as that which did not pellet during centrifugation at 150,000 x g for 90 minutes. Digitonin and Triton X-100 solubilized greater than 75% of total membrane protein. Cholate and deoxycholate solubilized approximately 60% of membrane protein, while Lubrol WX and Tween 80 were able to solubilize less than 40% of the outer membrane protein. Centrifugation of outer membrane in the absence of detergents under identical conditions resulted in less than 5% of the membrane protein in the supernatant.

TABLE 10

Effectiveness of detergents in solubilizing monoamine oxidase activity from outer membranes of rat liver mitochondria

Detergent*	Concentration	% Protein in Supernatant	% Monoamine Oxidase Activity in Supernatant		% Recovery	
			Type A (5-HT)	Type B (benzylamine)	Type A	Type B
Cholate	1.0%	59.8	8.3	29.0	85.3	95.0
Deoxycholate	1.0%	54.9	9.7	32.1	71.6	95.7
Digitonin	1.0%	84.3	63.0	81.8	67.0	86.8
Digitonin	2.0%	74.5	54.6	76.2	n.d.	n.d.§
Lubrol WX†	2.0%	30.7	10.9	19.7	74.1	88.3
Triton X-100†	1.5%	76.5	32.2	56.7	44.2	64.8
Tween 80	2.0%	37.2	4.7	10.2	92.0	98.6

*10 mg of rat liver mitochondrial outer membrane protein were added to 10 ml of 20 mM phosphate buffer, pH 7.2, containing detergent. The mixture was stirred on ice for one hour and then centrifuged at 150,000 x g for 90 minutes.

†100 mM phosphate buffer, pH 7.2

§Not determined

Digitonin solubilized 63% of monoamine oxidase activity assayed with 5-HT and 82% of the activity measured with benzylamine, while only 4 to 5% of the activity measured with either substrate remained in the unsolubilized material. Triton X-100 solubilized 32% of the activity measured with 5-HT and 57% of the activity measured with benzylamine; however, the use of this detergent resulted in inactivation of the activity measured with 5-HT to 44% of the outer membrane activity and inactivation of activity measured with benzylamine to 65% of the outer membrane activity. Cholate, deoxycholate and Tween 80 were able to solubilize less than 30% of the monoamine oxidase activity of the outer membrane measured with benzylamine and less than 10% of the activity measured with 5-HT. The recovery of monoamine oxidase activity metabolizing 5-HT was 85% after treatment with cholate, 72% after treatment with deoxycholate, 92% after treatment with Tween 80 and 74% after treatment with Lubrol WX. Treatment of outer membranes with Lubrol WX resulted in an 88% recovery of monoamine oxidase activity metabolizing benzylamine.

Possible effects of high detergent concentration on the radiochemical assay were examined since it had become apparent that hydrophobic substances could alter the partitioning of the unreacted amine into the organic phase which gave falsely high activity measurements. Table 11 shows the comparison of benzylamine deamination in the presence of unusually high concentrations of detergents measured by the radiochemical and spectrophotometric assays. The spectrophotometric assay is not affected by high detergent concentration since the

TABLE 11

Effect of high detergent concentration on monoamine oxidase activity in outer mitochondrial membranes

Detergent	Concentration[†]	*Rate of Benzylamine Deamination	
		Spectrophotometric	Radiochemical
Cholate	10%	58	26
Deoxycholate	5%	102	160
Digitonin	5%	85	86
Lubrol WX	5%	42	4
Triton X-100	3%	65	121

*Expressed as the percentage of monoamine oxidase activity in the absence of detergents. Outer membranes were incubated with the assay mixture for five minutes on ice to standardize the two procedures

[†]Concentration of detergent in the assay mixture

absorbance of benzaldehyde at 250 nm was the same in the presence of detergents as in water. The activity determined by the radiochemical assay in the presence of greater than 2.0% cholate and 1.0% Lubrol WX are falsely low due to the precipitation of these detergents upon the addition of acid. Activity measured in the presence of greater than 1.0% Triton X-100 or deoxycholate were falsely high. This is probably due to a detergent induced change in the amine partition coefficient in the organic phase of the assay. Digitonin was the only detergent which did not interfere with the radiochemical assay. Therefore, this detergent was selected for further study.

Properties of Outer Membranes Treated with Digitonin

Purification of monoamine oxidase from outer mitochondrial membranes treated with digitonin was attempted by chromatography on DEAE cellulose. An elution profile of this column is shown in Figure 14. Monoamine oxidase activity and protein are eluted in two major fractions. Fraction I is the pooled fractions which did not adhere to the DEAE cellulose, and Fraction II is the pooled fractions eluted shortly after a gradient of increasing KCl concentration is applied to the DEAE cellulose column. Table 12 shows the distribution of monoamine oxidase activity, protein and lipid phosphorus between the two fractions. The ratio of monoamine oxidase activity metabolizing 5-HT to the activity metabolizing benzylamine was 0.14 in Fraction I and 0.93 in Fraction II compared to the ratio of 0.83 measured in mitochondria and outer membranes. Monoamine oxidase activity recovered in Fraction I was 67% of the activity metabolizing benzylamine and 15% of the activity metabolizing 5-HT in

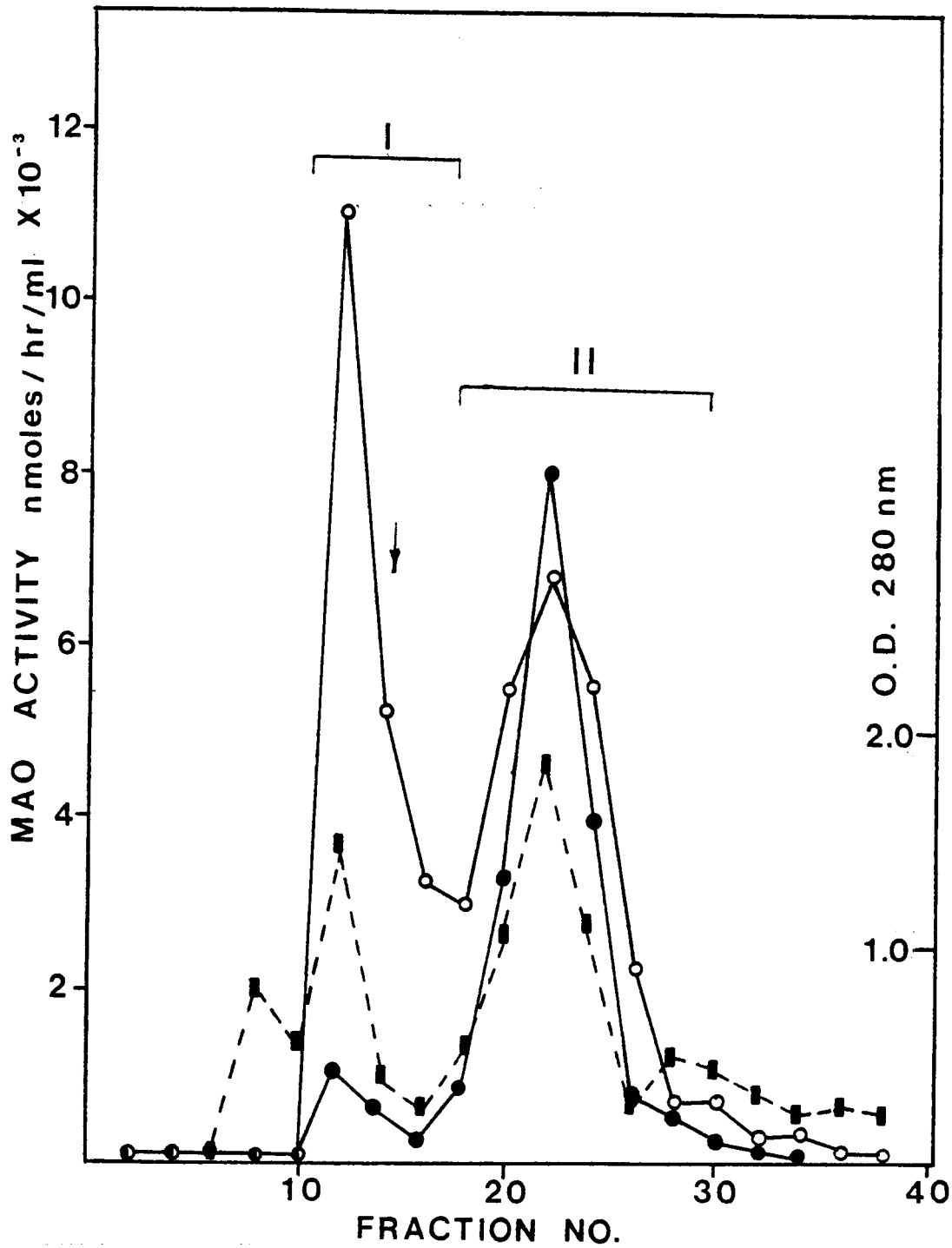

Figure 14. DEAE cellulose chromatography of outer mitochondrial membranes treated with digitonin. Arrow indicates the start of 0 to 0.5 M KCl gradient. Monoamine oxidase activity was determined by the radiochemical method with benzylamine (O) and 5-HT (●). Optical density at 280 nm is also shown (--▮-).

TABLE 12

Fractionation of outer mitochondrial membranes solubilized in digitonin by DEAE cellulose chromatography

Sample	Ratio Monoamine Oxidase 5-HT/Ba*	% Protein	% Monoamine Oxidase Activity 5-HT	TYR	BA	% Lipid Pi
Outer membranes	0.83	100.	100.	100.	100.	100
Solubilized outer† membranes	0.64	83.3	61.7	63.8	80.5	91
DEAE Fraction I	0.14	26.3	9.1	29.7	54.1	62
DEAE Fraction II	0.93	52.1	41.6	35.1	36.6	27

*Specific activity of monoamine oxidase determined with 5-HT divided by the activity determined with benzylamine.

†Outer mitochondrial membrane was solubilized in 2.0% digitonin

the solubilized outer membranes. Fraction II contained 45% of the monoamine oxidase activity metabolizing benzylamine and 67% of the activity metabolizing 5-HT in the solubilized outer membranes. Sixty-two per cent of the outer membrane lipid phosphorus and 26% of outer membrane protein were recovered in Fraction I. Fraction II contained 27% of the lipid phosphorus and 52% of the protein from the outer mitochondrial membrane.

DEAE cellulose chromatography of outer membrane treated with digitonin in buffer containing cholate resulted in two protein peaks eluted. However, both fractions adhered to the DEAE cellulose so that most of the digitonin was washed from the column before the protein fractions were eluted by increasing the KCl concentration. The first fraction eluted contained 0.07 mg digitonin per mg protein and the second fraction contained 0.47 mg digitonin per mg protein compared to a ratio of 20 mg digitonin per mg protein in the solubilized outer membranes. Only 8% of monoamine oxidase metabolizing benzylamine and 2% of the activity metabolizing 5-HT in the solubilized outer membranes were recovered in both fractions.

In additional experiments not shown, sonication of outer membranes in the presence of 2.0% digitonin for longer than two to five minutes resulted in only one peak of monoamine oxidase activity eluted from a DEAE cellulose when chromatography of this material was done under the same conditions as the experiment shown in Figure 14. This material was eluted from the DEAE cellulose at a similar ionic strength that Fraction II was eluted. Monoamine oxidase activity recovered in this fraction was 10% of the activity metabolizing 5-HT and 54% of the

activity metabolizing benzylamine in the outer membrane. This fraction contained 20% of the outer membrane protein and 21% of the outer membrane lipid phosphorus. Chromatography of this material on Sepharose 6-B resulted in only one peak of monoamine oxidase activity and protein eluted shortly after the void volume.

Equilibrium sedimentation of outer membranes solubilized in digitonin without sonication in a continuous sucrose gradient containing 0.2% digitonin resulted in only one band of protein and monoamine oxidase as shown in Figure 15. Monoamine oxidase activity metabolizing 5-HT and benzylamine cosediment in the gradient. Greater than 90% of monoamine oxidase activity metabolizing either 5-HT or benzylamine in the solubilized outer membranes was recovered after centrifugation. Fifty-nine per cent of the lipid phosphorus of the solubilized outer membrane was recovered in the pooled fractions containing monoamine oxidase activity. Chromatography of this material on Sepharose 6-B resulted in all material being eluted in the void volume.

Outer membranes and outer membranes treated with digitonin were treated with phospholipase A_2. Monoamine oxidase activity determined after this treatment is shown in Table 13. Activity measured with 5-HT and benzylamine was not affected when outer membranes were incubated with phospholipase A_2. When solubilized outer membranes were incubated in buffer without phospholipase A_2, monoamine oxidase activity metabolizing 5-HT was reduced to approximately 50% of control activity at ice bath temperature. Monoamine oxidase activity metabolizing benzylamine was not reduced by more than 6% of the control activity after this incubation in the absence of phospholipase A_2. Addition of

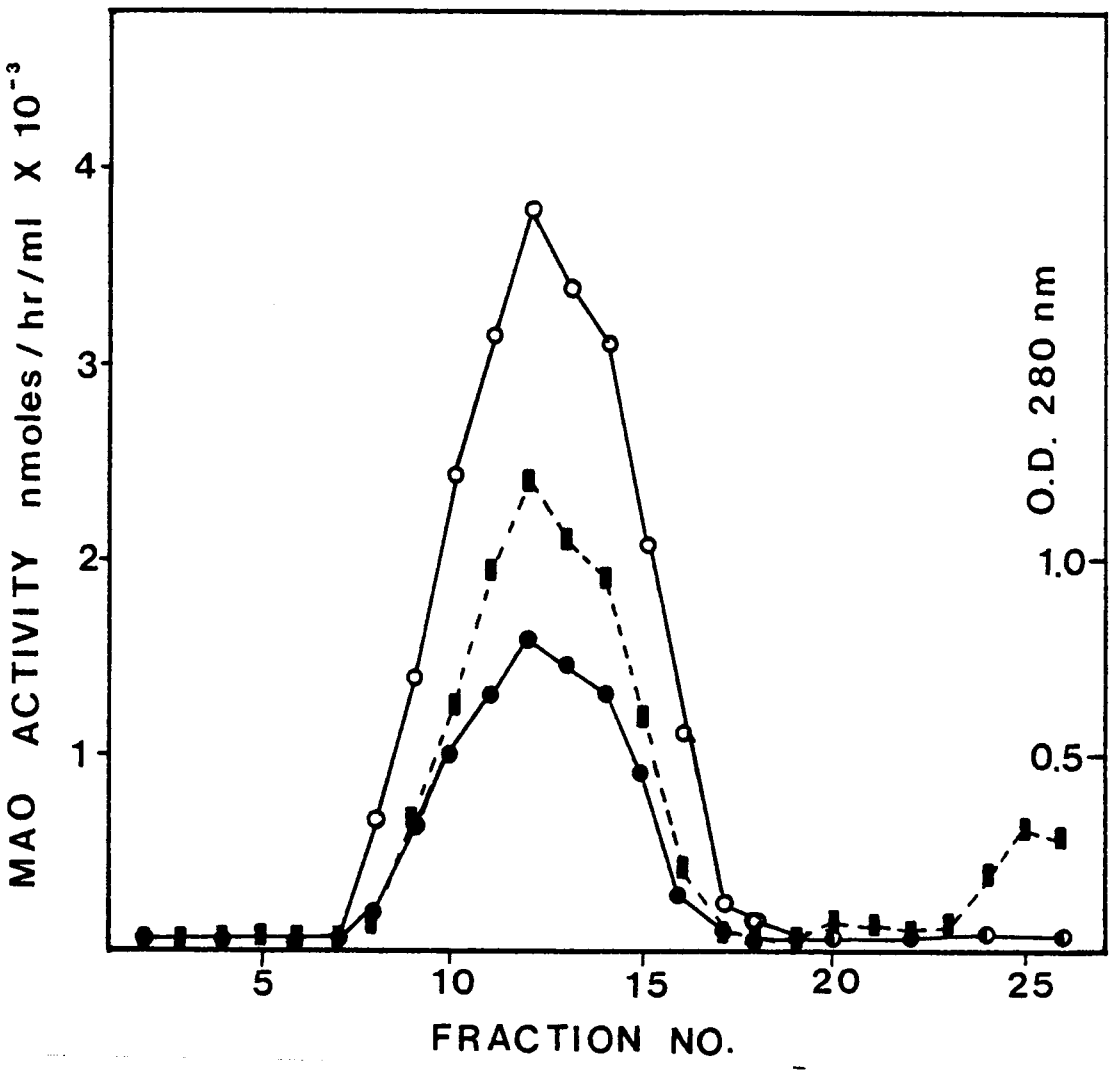

Figure 15. Equilibrium sedimentation of outer mitochondrial membranes treated with digitonin. Outer membranes treated with 2.0% digitonin were applied to a 5 to 20% continuous sucrose gradient and centrifuged at 150,000 x g for 16 hours in a SW 50.1 rotor. Monoamine oxidase activity was determined by the radiochemical method with benzylamine (O) and 5-HT (●). Optical density is also shown (--■-).

TABLE 13

Effect of phospholipase A_2 incubation on monoamine oxidase activity in outer membranes treated with digitonin

Experiment†	Sample	% Monoamine Oxidase Activity* 5-HT	Benzylamine
I	Outer membrane (O.M.)	98.6	99.3
	O.M. + phospholipase A_2	97.2	98.4
II	O.M. treated with digitonin (O.M.-dig)	45.9	95.4
	O.M.-dig + phospholipase A_2	18.1	85.7
III	O.M.-dig	54.5	96.4
	O.M.-dig + phospholipase A_2	27.1	93.5

*Activity was determined after one hour preincubation at 37° in Tris buffer, 20 mM pH 8.6, and expressed as a percentage of activity in this buffer at ice bath temperature

†Experiments I and II were carried out with 500 units of phospholipase activity per mg of outer membrane protein. Experiment III was done with 250 units of phospholipase activity per mg of protein

phospholipase A_2 to the incubation mixture resulted in additional inactivation of monoamine oxidase activity metabolizing 5-HT to 18 and 27% of control activity in separate experiments. Monoamine oxidase activity metabolizing benzylamine was not inactivated by more than 15% of control activity by incubation with phospholipase A_2.

Cholate was added to outer membranes solubilized in digitonin and unsolubilized outer membranes, after which monoamine oxidase activity was assayed. Figure 16 shows the effect of monoamine oxidase activity when outer membranes and solubilized outer membranes were preincubated with increasing concentrations of cholate at 4° for 15 minutes. Monoamine oxidase metabolizing benzylamine in solubilized outer membranes increased in activity slightly after preincubation with 0.5% cholate and then decreased with preincubation of increasing concentrations of cholate to 62% of control activity after preincubation with 4% cholate. Corresponding preincubation of outer membranes with 4.0% cholate resulted in a reduction of monoamine oxidase activity metabolizing benzylamine to 88% of the control activity. Rates of 5-HT metabolism were reduced to 15% of control activity in outer membranes solubilized in digitonin and 70% of control activity in unsolubilized outer membranes by preincubation in 2% cholate.

Figure 17 shows the effect of monoamine oxidase activity when outer membranes and outer membranes solubilized in digitonin were preincubated with cholate at 37° for 15 minutes. Monoamine oxidase activity metabolizing 5-HT or benzylamine was reduced to less than 20% of the original activity in both samples by preincubation with 2.0% cholate at 37°. This figure also shows the effect of cholate on monoamine

Figure 16. Effect of preincubation with cholate on monoamine oxidase activity in outer membranes and outer membranes treated with digitonin at 4°. Indicated concentrations of cholate were preincubated with outer membranes (——) and digitonin treated outer membranes (----) for 15 minutes at 4°. Monoamine oxidase activity was then determined by the addition of an equal volume of substrate, benzylamine (Δ) and 5-HT (O).

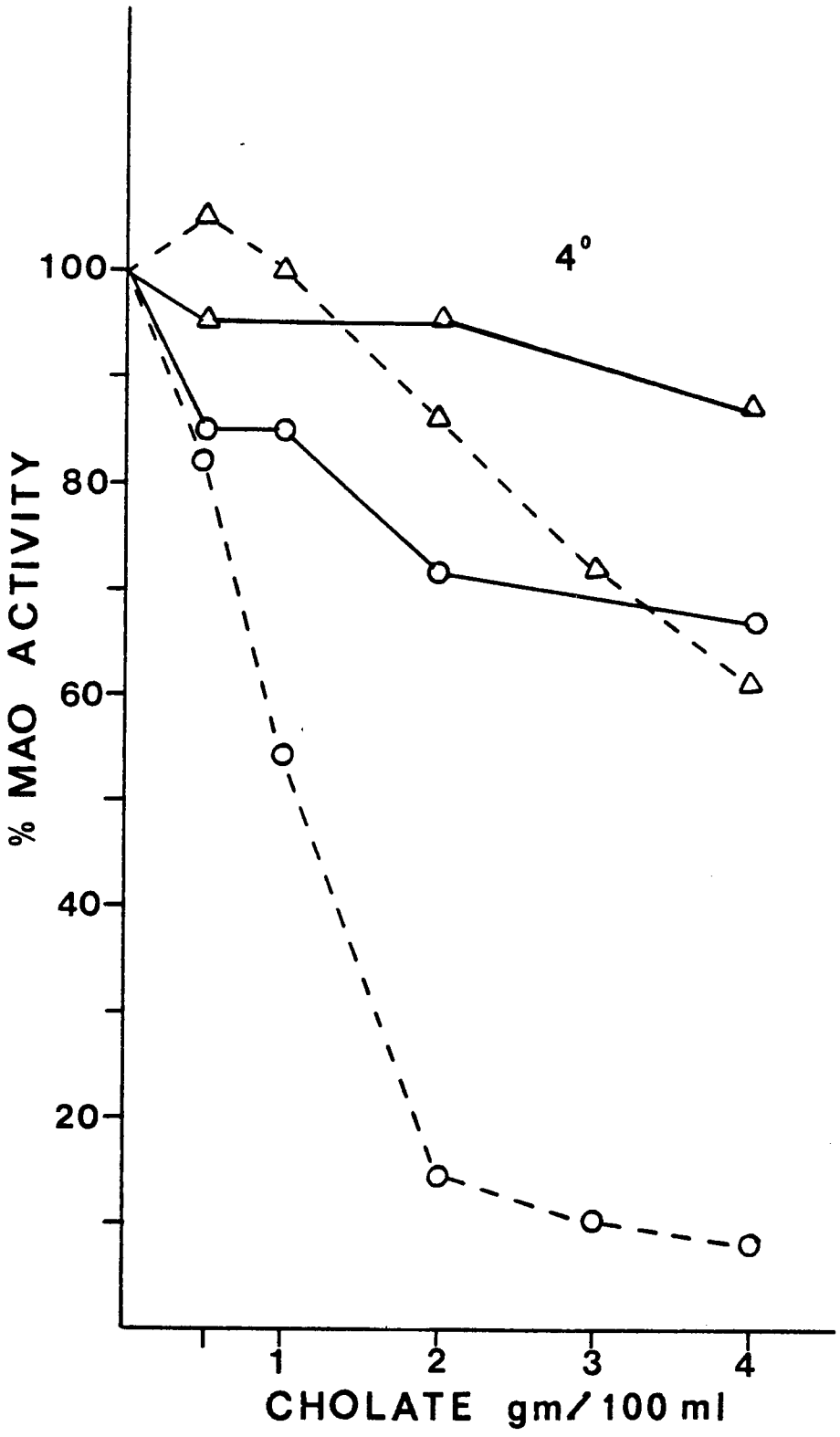

Figure 16

Figure 17. Effect of preincubation with cholate on monoamine oxidase activity in outer membranes and outer membranes treated with digitonin at 37°. Experiment was carried out as described in legend to Figure 16. Monoamine oxidase activity with benzylamine (▲) and 5-HT (●) was assayed in outer membranes (——) and outer membranes treated with digitonin (----). Activity with benzylamine by the spectrophotometric method without preincubation is also shown (□).

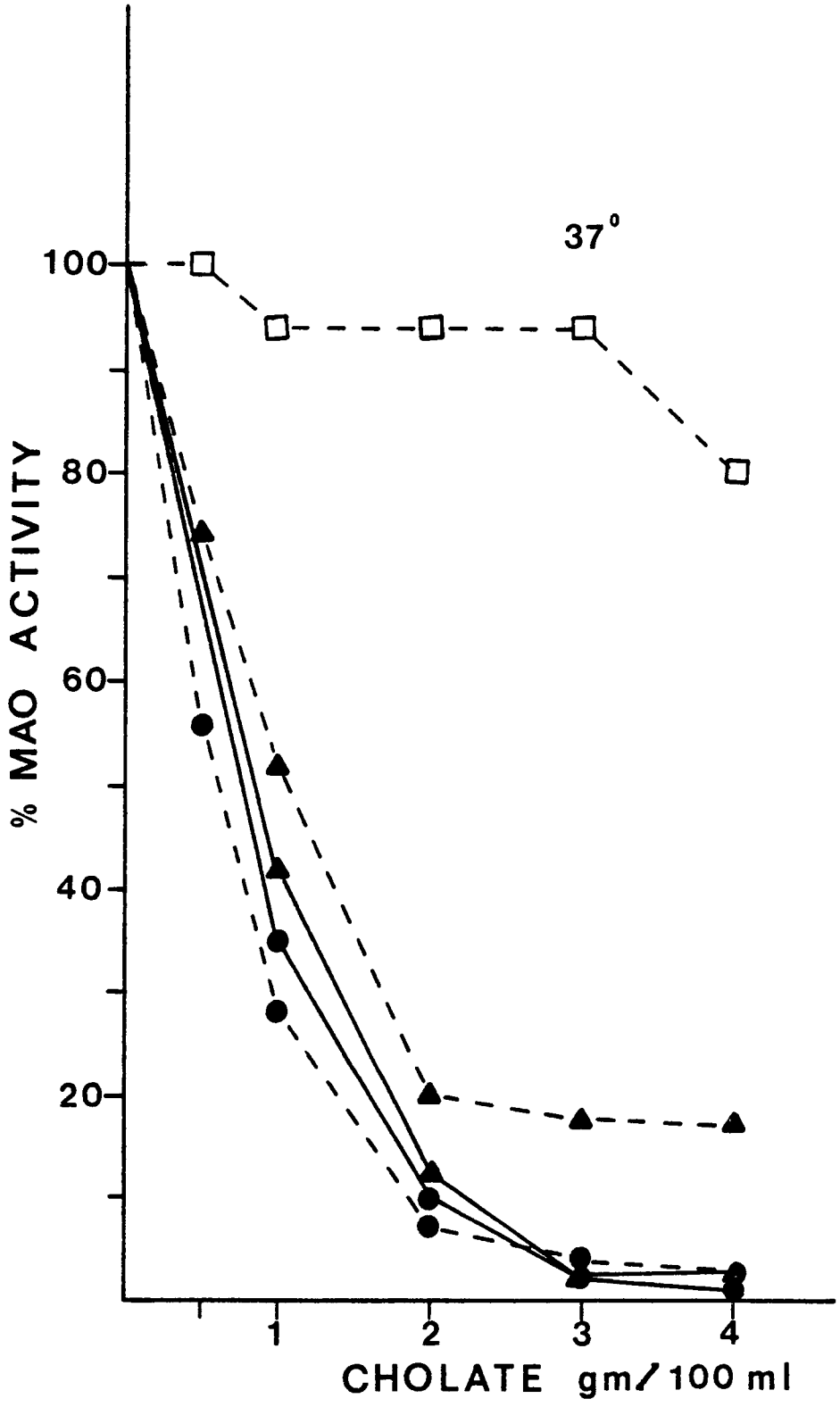

Figure 17

oxidase activity metabolizing benzylamine in the outer membrane when cholate was present during the assay only. Cholate reduced this activity less than 10% up to a concentration of 3.0% cholate in the assay mixture. The cholate concentration in the assay mixture of the other experiments in this figure and Figure 18 were one half of the indicated preincubation concentration.

Effect of Cholate on Monoamine Oxidase Activity

Monoamine oxidase activity metabolizing benzylamine was observed to decrease in activity by 54% when outer mitochondrial membranes solubilized in 1.0% cholate were concentrated from 25 ml to 3 ml using an Amincon XM 100 filter. All monoamine oxidase activity metabolizing 5-HT was recovered after this procedure. Figure 18 shows the activity of monoamine oxidase after this material was diluted with buffer not containing cholate. Monoamine oxidase activity metabolizing benzylamine was increased to 83% of the initial activity when the concentrated solution was diluted 10 fold. Activity metabolizing 5-HT was not affected by the dilution of the concentrated solution.

Figure 19 shows the increase in the rate of benzylamine deamination determined by the radiochemical method as the cholate concentration of a partially purified and delipidated monoamine oxidase preparation is reduced by dilution. Dilution with phosphate buffer containing 1.0 mg cardiolipin per ml to a concentration of 0.5% cholate resulted in a 5.8 fold increase in the rate of benzylamine deamination. Dilution with buffer without lipid resulted in a 3.9 fold increase in the rate of benzylamine deamination. The sample was solubilized in 10% cholate and

Figure 18. Effect of dilution on monoamine oxidase activity in concentrated cholate solution. Outer membranes solubilized in cholate were concentrated from 25 ml to 3 ml by an Amincon XM-100 filter. Activity was determined by the radiochemical method with benzylamine (●) and 5-HT (■) after the solution was diluted with buffer.

Figure 19. Effects of cholate and lipid on type B monoamine oxidase activity. A partially purified and delipidated enzyme preparation in 10% cholate was diluted with phosphate buffer (○) and buffer containing 1.0 mg cardiolipin (△). Activity was determined by the radiochemical method. Each point is the mean of three determinations ± standard error.

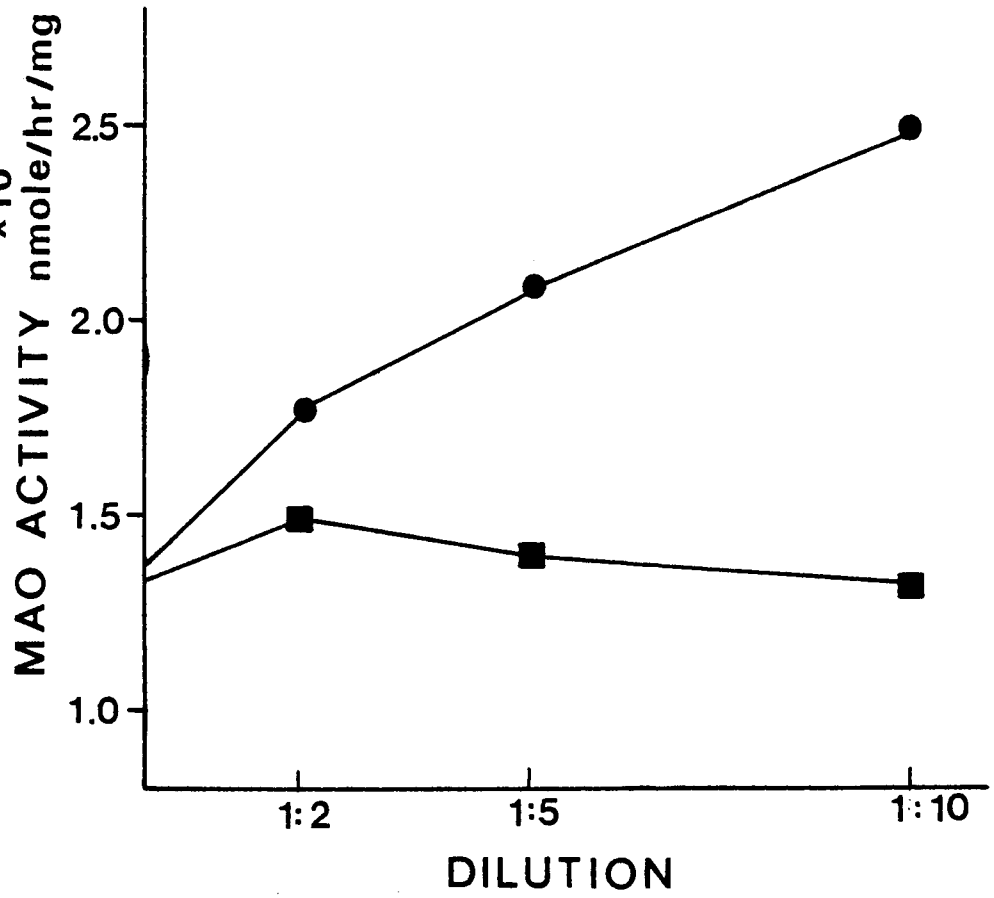

Figure 18

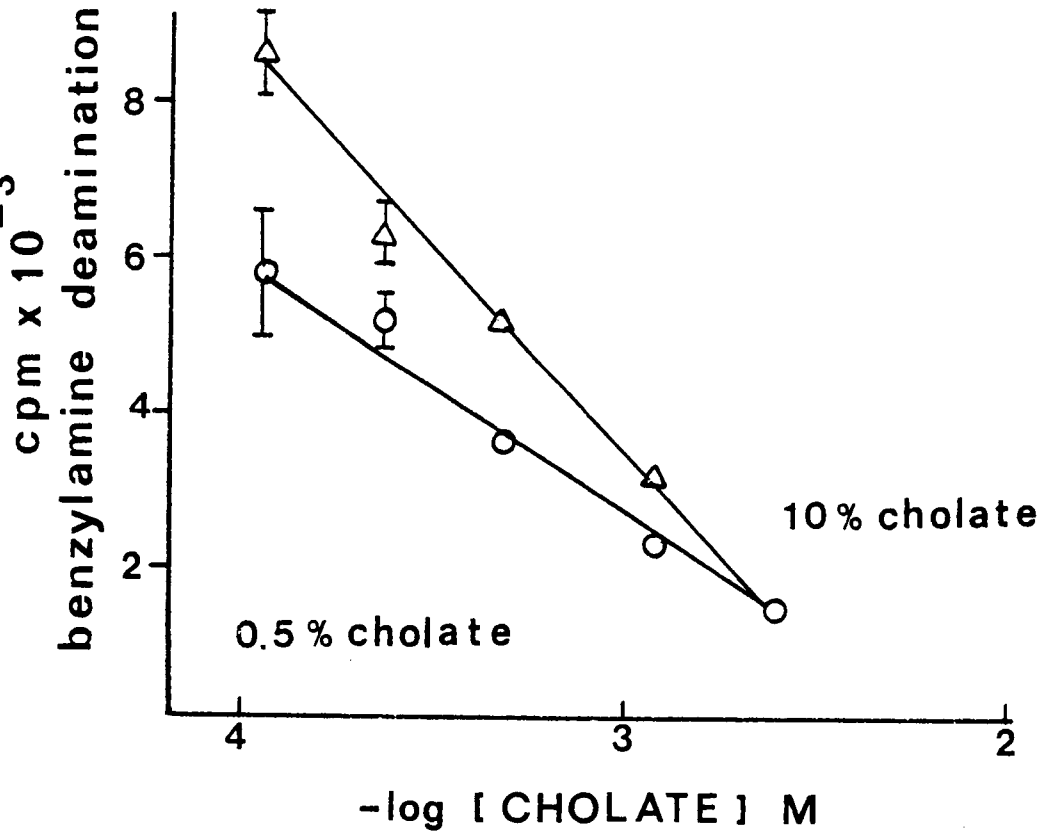

Figure 19

centrifuged into a glycerol step gradient containing 10% cholate. Monoamine oxidase used in this experiment was collected at the 30/50% glycerol interface and was equivalent to 6.8% of the outer membrane monoamine oxidase activity. Monoamine oxidase activity is restored to 26% of the outer membrane activity by dilution of the cholate concentration to 0.5% with buffer. The addition of cardiolipin to the dilution buffer increased the monoamine oxidase activity to 39% of the outer membrane activity at a cholate concentration of 0.5%. No monoamine oxidase activity metabolizing 5-HT was detected in the delipidated preparation before or after dilution.

Discussion

Monoamine oxidase activity was not solubilized to the same extent as total outer membrane protein by the detergents tested. This effect is probably due to the differential solubilization of peripheral and integral membrane proteins (93,94) and would suggest that monoamine oxidase is an integral membrane protein. Multiple types of monoamine oxidase activity are also not solubilized by detergents to the same extent. An apparently lower percentage of type A enzyme activity solubilized by all the detergents tested may be due to more inactivation of this type of activity upon solubilization than type B activity after treatment of the outer membrane with detergents. Moreover, the detergents most effective in solubilizing monoamine oxidase activity, digitonin and Triton X-100, caused the greatest inactivation of both types of enzyme activity.

Digitonin is the most effective detergent for solubilizing monoamine oxidase from the outer mitochondrial membrane by the criteria

of activity not pelleting from centrifugation at 150,000 x g for 90 minutes. However, the sedimentation behavior of outer membranes treated with digitonin in sucrose gradients and the elution profile of this material from DEAE cellulose indicate that the digitonin treatment disrupts the outer membrane into smaller outer membrane particles. Equilibrium sedimentation of outer membranes treated with digitonin indicated that the resultant outer membrane particles are all of similar density. However, DEAE cellulose chromatography of outer membranes treated with digitonin in the absence of sonication resolved two types of outer membrane particles. The ratio of type A monoamine oxidase activity to type B activity in these two fractions compared with the ratios determined in mitochondria, outer membranes and solubilized outer membranes indicates that Fraction I is greatly enriched in type B activity and Fraction II is enriched in type A activity. High recoveries of both types of monoamine oxidase activity exclude a selective inactivation of either type of activity that is solely responsible for the enrichment of a monoamine oxidase type in either fraction. These outer membrane particles are aggregates of protein, lipid and digitonin that do not represent a true solubilized outer membrane. A molecular weight of greater than 4×10^6 daltons for these particles was indicated by gel filtration on Sepharose 6-B. The unequal ratio of digitonin to protein in the two fractions of outer membrane particles eluted from the DEAE column washed with cholate containing buffer may indicate the reason two types of outer membrane particles are generated. Since digitonin binds cholesterol tightly (95), the asymmetric distribution of cholesterol or a protein with a high affinity for digitonin

could account for effect on digitonin on the outer mitochondrial membrane. Freeze-fracture elctron microscopy of rat liver mitochondrial outer membranes reveals an asymmetric location of intra-membrane particles between the cytoplasmic and inner membrane face as well as in the plane of both faces of the outer membrane (58,65). Possible asymmetry of the outer mitochondrial membrane with regard to cholesterol and phospholipid has not been studied although such asymmetry is known to exist in certain membranes (94).

Two species of outer membrane particles which have different proportions of monoamine oxidase types is not consistent with the hypothesis that the multiple types of activity are due to different active sites on the same protein. An asymmetric distribution of the two types of monoamine oxidase activity between the cytoplasmic and inner membrane face of the outer mitochondrial membrane as shown by Russel et al. (68) and possibly visualized by Chau (58,96) could account for the different ratios of enzyme types in the two species of outer membrane particles generated by digitonin. According to recent theory on incorporation of proteins into membranes (94), the asymmetric location of the two types of monoamine oxidase types in the membrane would require the existence of two different polypeptides, and therefore, two different genes. However, these two peptides could be identical after post-translational modification and incorporation into the membrane such that the two types of activity were due to the lipid surrounding the enzyme. Recent experiments by Cawthon and Breakefield (23) suggest that different polypeptides corresponding to type A

and type B monoamine oxidase activity are present in a rat hepatoma cell line. It is yet to be determined the extent that lipid is involved in the activity of monoamine oxidase.

Equilibrium sedimentation of outer membranes treated with digitonin revealed that 59% of the phospholipid was present in the outer membrane particles. Phospholipase A_2 and detergents were used to attempt to remove this lipid and examine the effect of the activity of monoamine oxidase of these agents. The more enhanced sensitivity of type A activity relative to type B activity to phospholipase digestion and incubation with cholate futher indicates that type A activity is more dependent on lipid for activity than type B. Both types of monoamine oxidase activity were inactivated by incubation with cholate at physiological temperature in both intact outer membranes and outer membranes treated with digitonin. This effect may be due to the more efficient solubilization of the enzyme in cholate at higher temperature and most likely reflects the denaturation of the enzyme. The cholate effect in this experiment was not an artifact of the radiochemical assay since: (1) the activity in outer membranes incubated at low temperature was not affected to the same extent as occurred at higher temperature, (2) cholate does not interfere with the assay below 2.0% concentration and (3) the inactivation of monoamine oxidase activity was not reversed by dilution or dialysis.

The results of concentrating outer membranes solubilized in cholate indicated that high concentration of cholate in the radiochemical assay selectively interfere with the determination of the rate of benzylamine deamination. This effect could explain the apparent reactivation of

type B monoamine oxidase activity due to the reconstitution of outer membrane material solubilized in cholate into lipid vesicles reported in an earlier chapter. Solubilization of outer membranes in 10% cholate and delipidation by centrifugation in a glycerol gradient resulted in greater inactivation of monoamine oxidase activity than expected just for the presence of 10% cholate. A larger increase in type B activity when cardiolipin was used to dilute the cholate compared to buffer alone indicates the reactivation of this activity beyond the artifact alone.

This study leaves little doubt that monoamine oxidase is an integral membrane protein. The difficulty with which this enzyme can be solubilized has hampered the study of monoamine oxidase from certain tissues. Moreover, the definition of solubilization as that which does not sediment under defined condition is shown in this study to be inaccurate. True solubilization may better be defined as the disruption of the membrane such that one protein occupies a detergent micell. Fragmentation of the outer membrane by digitonin may be useful in further studies of this non-typical membrane. The unequal ratios of monoamine oxidase types in the two fractions of digitonin treated outer membranes strongly suggest that these two activities are asymmetrically located in the outer membrane. Inactivation of type A monoamine oxidase in digitonin treated outer membrane by detergents and phospholipase add conclusive evidence that this activity is dependent on lipid.

CHAPTER VII

MONOAMINE OXIDASE ACTIVITY IN COUPLED RAT LIVER MITOCHONDRIA

Greenawalt and Decker (16) proposed that monoamine oxidase donated electrons to the respiratory chain of the inner mitochondrial membrane. This hypothesis was based on polarographic evidence which suggested that monoamine oxidase activity was coupled to the phosphorylation of ADP and difference spectra of mitochondria which showed that the cytochromes were reduced after incubation with monoamine oxidase substrates. Smith and Reid (17) reported large changes in monoamine oxidase activity due to changes in the state of respiration of rat liver mitochondria. However, the results of these two studies are contradictory in that the first investigators observed increased monoamine oxidase activity induced by the addition of ADP while the latter investigators observed a 10 fold decrease in monoamine oxidase activity during rapid respiration stimulated by ADP.

This investigation attempted to resolve these observations and to further explore the possibility that changes in monoamine oxidase activity in response to the state of respiration might involve a transfer of electrons between monoamine oxidase and the respiratory chain. Monoamine oxidase activity was determined by the radiochemical assay in intact mitochondria plus agents known to influence the state of respiration. As shown in Table 14, monoamine oxidase activity is not changed to a large extent by ADP. However, the addition of ADP and succinate to intact mitochondria results in reduced monoamine oxidase activity. Addition of succinate alone had little effect on

TABLE 14

Monoamine oxidase activity in coupled mitochondria as a function of the state of respiration

Additions[†]	% Monoamine Oxidase Activity[*] 5-HT	Tyramine	Benzylamine
None	100	100	100
ADP	108 ± 8	102 ± 5	111 ± 6
Succinate (S)	98 ± 6	92 ± 8	73 ± 3
S + ADP	89 ± 3	72 ± 5	66 ± 6
S + ADP + oligomycin	99 ± 8	88 ± 6	67 ± 2
S + oligomycin + DNP	76 ± 7	54 ± 5	72 ± 1
S + DNP	67 ± 5	46 ± 4	70 ± 6
S + ADP + KCN	103 ± 7	101 ± 9	89 ± 5

[*]Activity expressed as the mean ± standard deviation of three determinations and normalized as the per cent activity of monoamine oxidase in mitochondria with no additions.

[†]Buffer is H' media plus 0.5mM EDTA, 2.0 mM $MgCl_2$, 2.5 mM phosphate. Additions are made to a final concentration of: ADP to 0.5 mM, Na succinate to 5. mM, oligomycin to 0.8 mM, DNP to 0.3 mM and KCN to 0.5 mM.

the rate of 5-HT or tyramine deamination but interfered with the radiochemical assay of benzylamine deamination. Oligomycin, which inhibits the phosphorylation of ADP, did not significantly affect monoamine oxidase in the presence or absence of succinate or succinate plus ADP. The uncoupler, 2,4-dinitrophenol (DNP), reduced monoamine oxidase activity in coupled mitochondria regardless of added agents. KCN did not affect monoamine oxidase in this study; in addition, this inhibitor of respiration prevented the reduction of activity due to ADP plus succinate.

Benzylamine metabolism in coupled mitochondria was measured by the absorbance of metabolites at 250 nm by split beam spectrophotometry. This assay was performed in parallel with oxygen electrode experiments under identical conditions. Figure 20 is a tracing of results obtained from this experiment. In experiment A, the measurement of respiration demonstrated that mitochondria were coupled in the presence of 1.0 mM benzylamine (acceptor control ratio was 3.7). Indicated concentrations of ADP stimulated respiration for two minutes and resulted in a decrease in the rate of benzylamine deamination to 39% of the original rate for two minutes. This rate increased to 70% of the original rate after two minutes.

Experiment B was done with a different preparation of mitochondria. Addition of succinate to mitochondria metabolizing benzylamine did not affect the rate of deamination of benzylamine. However, the subsequent addition of ADP resulted in a reduction of this rate to 62% of the rate of benzylamine deamination originally. Further addition of oligomycin and DNP resulted in an increase to 74% of the

Figure 20. Influence of respiration on benzylamine deamination in coupled rat liver mitochondria. Metabolites of benzylamine were measured at 250 nm in a split beam spectrophotometer and O_2 consumption was measured in a Clark electrode after the following additions were made to a mitochondrial suspension in H' medium: (1) Na succinate to 5.0 mM, (2) ADP to 0.1 mM, (3) DNP to 0.2 mM, (4) ADP to 0.75 mM and (5) oligomycin in ethanol to 0.8 mM. The reference cuvet and sample cuvet contained identical additions except that 1.0 mM benzylamine was present in the sample cuvet and in the oxygen electrode.

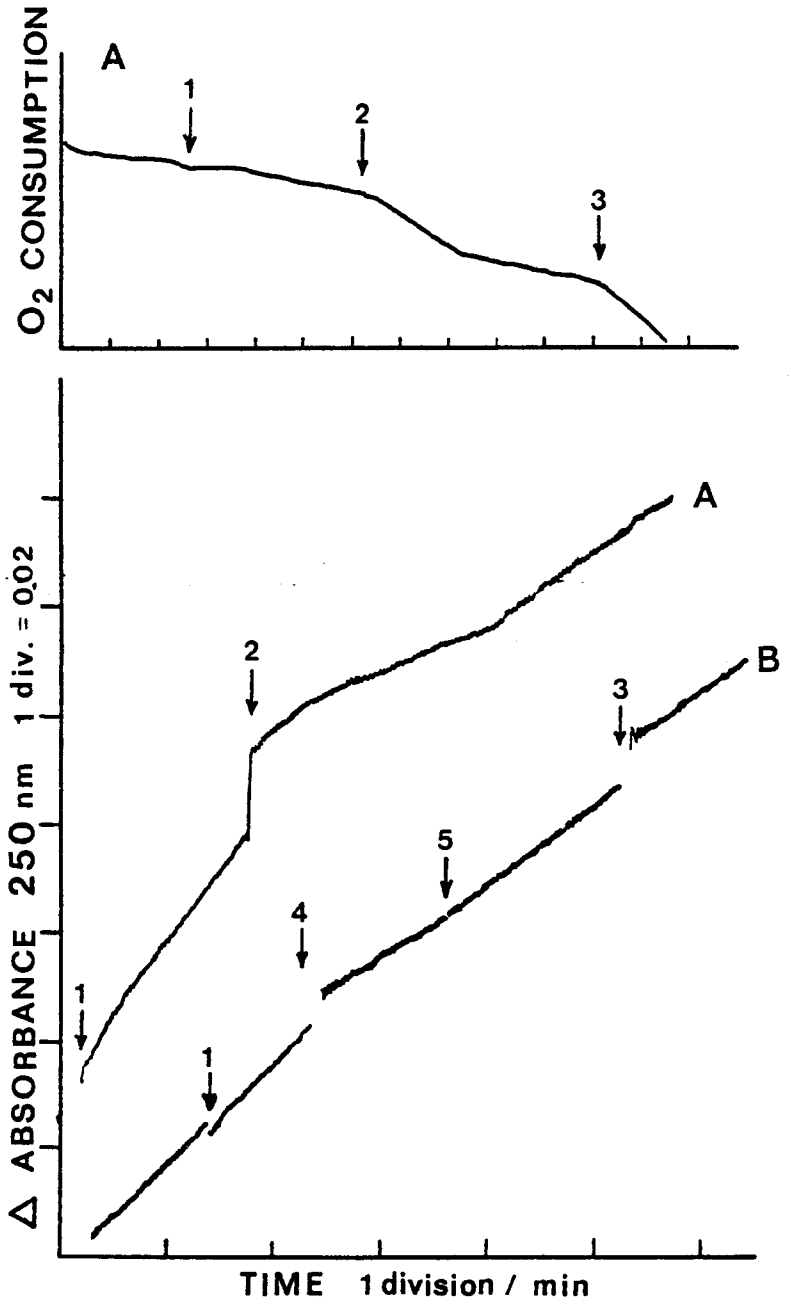
A
1
2
3
O_2 CONSUMPTION
A
B
2
3
5
4
1
1
Δ ABSORBANCE 250 nm 1 div. = 0.02
TIME 1 division / min

Figure 20

original rate and a decrease to 64% of the original rate, respectively.

Table 15 demonstrates the effect of lowering the oxygen concentration in the presence or absence of an ADP generating system on monoamine oxidase activity in coupled mitochondria. Metabolism of 5-HT was reduced more than the corresponding deamination of benzylamine when the atmosphere of the assay was air or argon. The presence of a hexokinase-glucose ADP producing system had little effect on monoamine oxidase activity in this experiment.

Discussion

These experiments agreed with the observation of Smith and Reid (17) that monoamine oxidase activity is reduced during rapid respiration of isolated rat liver mitochondria. Reversibility of the effect implies that the accumulation of inhibitory mitochondrial products is not responsible for the inhibition of monoamine oxidase activity. Rather, a rapidly equilibrating state due to the rate of respiration must influence monoamine oxidase activity. Although the nature of this mechanism is not known, the non-selective effect on the two types of monoamine oxidase activity may suggest that a general phenomena, such as lower oxygen concentration or pH, rather than a specific control of the enzyme, results in the reduction of activity.

Linkage of monoamine oxidase to the electron transport chain might allow the enzyme to operate more efficiently under low oxygen concentrations. A Km value for oxygen utilization by monoamine oxidase of approximately 300 μM has been measured in purified rat liver

TABLE 15

Dependence of monoamine oxidase activity in coupled mitochondria on oxygen

	Assay Atmosphere	% Monoamine Oxidase Activity* 5-HT	Benzylamine
No Additions	Oxygen	100	100
	Air	80	95
	Argon	67	80
ADP Generator†	Oxygen	92	103
	Air	74	89
	Argon	64	72

*Activity expressed as the percentage of activity in mitochondria assayed in the absence of additions and under an atmosphere of oxygen.

†ADP generator is 0.05 mg hexokinase and 12.5 mM glucose in the assay system (0.1 ml).

enzyme (9). The corresponding Km value for oxygen utilization by cytochrome oxidase was determined to be approximately 0.5 μM for the purified enzyme although the Km for oxygen consumption by the respiratory chain under in vivo conditions has been estimated to be less than 0.05 μM (97). However, lowering the oxygen concentration in coupled mitochondria in the presence or absence of ADP (to facilitate electron flow) resulted in decreased activity indicative of a high Km for oxygen. This suggests that electrons are not transferred from monoamine oxidase to the respiratory chain.

A reduction in monoamine oxidase activity due to rapid respiration of the mitochondria is not consistent with the hypothesis of electrons flowing from monoamine oxidase to the respiratory chain. It is hard to imagine that a direct connection of the respiratory chain and monoamine oxidase flavin exist in the mitochondria. Inhibition of monoamine oxidase activity during state III (rapid) respiration when the electron carriers are less reduced does not support the idea that electrons from the respiratory chain might reduce the flavin of monoamine oxidase and thus inactivate the enzyme. Furthermore, the lack of an effect by KCN on monoamine oxidase activity does not support any connection of the enzyme with the respiratory chain. KCN blocks the respiratory chain at cytochrome oxidase and thus fully reduces the electron carriers. There is no simple model to explain how the reduction of electron carriers relates to monoamine oxidase activity.

The molecular logic of monoamine oxidase occurring on the outer mitochondrial membrane is unclear. If the electrons from monoamine

oxidase were flowing into the respiratory chain, the enzyme could function with increased efficiency at intracellular oxygen concentrations and H_2O_2 would not be produced. However, the inhibition of monoamine oxidase activity during rapid respiration has no apparent physiological significance in the liver cell. It is conceivable that this phenomena could be involved in the regulation of amine levels in cells which are synthesizing monoamine since an increased respiration might be required for synthetic energy. However, state III respiration rates (ADP stimulated) of isolated mitochondria may be much higher than typical respiration rates in vivo. Electron microscopic analysis of the condensed (indicating rapid respiration) and orthodox (indicating resting respiration) conformations of mitochondria in tissue slices indicates that changes in the rate of respiration in vivo are small. Thus, this effect could be only an artifact of a rapid pH drop induced by rapid respiration in vitro.

CHAPTER VIII

STUDIES ON PIG LIVER MONOAMINE OXIDASE: PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION

Purification of Pig Liver Monoamine Oxidase

A purification method to purify monoamine oxidase from pig liver outer mitochondrial membranes was adapted from the method developed for mitochondria by Hollunger and Oreland (98). Extraction of mitochondria with methyl ethyl ketone (MEK) in two steps (the second step in the presence of ammonium sulfate to extract cardiolipin) liberates monoamine oxidase activity into the buffer used to wash the residue after the second extraction. Outer mitochondrial membranes from rat liver, bovine kidney and pig liver were extracted with MEK. One extraction with this solvent inactivated most of the rat liver and bovine kidney enzyme. However, the pig liver enzyme was stable toward the MEK extraction but was not solubilized after repeated extractions. Increasing concentrations of cholate were added to the buffer extract of the delipidated outer membrane residue. Monoamine oxidase activity was not solubilized until 5% cholate was present in the washing buffer. A maximum solubilization was achieved with 10% cholate in the washing buffer. A 56 fold purification of monoamine oxidase activity over that found in the mitochondria was obtained by this solubilization procedure as shown in Table 16. This procedure using the outer membrane was not significantly better than the procedure developed in Oreland's laboratory since that method gave a 30 fold purification of the enzyme from the mitochondria directly.

TABLE 16

Purification of monoamine oxidase from pig liver mitochondria

Step	Total Protein (mg)	Total Activity unit x 1000	Specific Activity units/min/mg	Recovery %
Washed Mitochondria	2600	152.1	58.5	100
Outer Membranes	330	136.3	413	89.6
Cholate Extract*	17	57.6	3,390	37.8
G-200 Sephadex† Chromatography	2.78	19.8	7,151	13.0
20 to 50% Saturation Ammonium Sulfate ppt.	0.88	15.1	17,140	9.9

*10% cholate in 100 mM phosphate buffer, pH 7.2, extract after MEK extraction as described in Methods chapter.

†Chromatography first in 100 mM phosphate buffer, pH 7.2, then rechromatography in the same buffer plus 2.0% cholate.

Table 16 shows the purification of the enzyme solubilized from the pig liver outer mitochondrial membranes by standard techniques. A 300 fold purification was obtained by these techniques. The maximum purification of monoamine oxidase from pig liver mitochondria reported was a 500 fold purification (47). Polyacrylamide electrophoresis in the presence of SDS resulted in one major band and one minor band. The final enzyme preparation was subjected to phospholipid analysis. Lipid phosphorus was undetectable after correcting against the background level present in the cholate.

Immunological Studies on Pig Liver and Bovine Kidney Monoamine Oxidase

Greenawalt and others have developed an antiserum against bovine kidney monoamine oxidase purified by the method of Hellerman (50). These workers also purified an IgG fraction from this serum (anti-MAO IgG) which precipitated monoamine oxidase activity from solubilized bovine kidney and rat liver mitochondria (unpublished). This anti-MAO IgG was used to test the immunological cross reactivity of monoamine oxidase from pig liver and bovine kidney by Octerlony double immunodiffusion. Figure 21 shows an immunodiffusion plate illustrating the antigenic relationship of the two enzymes. Only one precipitin line is formed by the antibody diffusing from the center well and the enzyme diffusion from the outer wells. This is indicative of a single immunologically recognized population of proteins (antigens) in each enzyme preparation. The precipitin lines formed from these two enzymes fused completely. This type of result demonstrates that the pig liver and bovine kidney monoamine oxidase are immunologically identical (99).

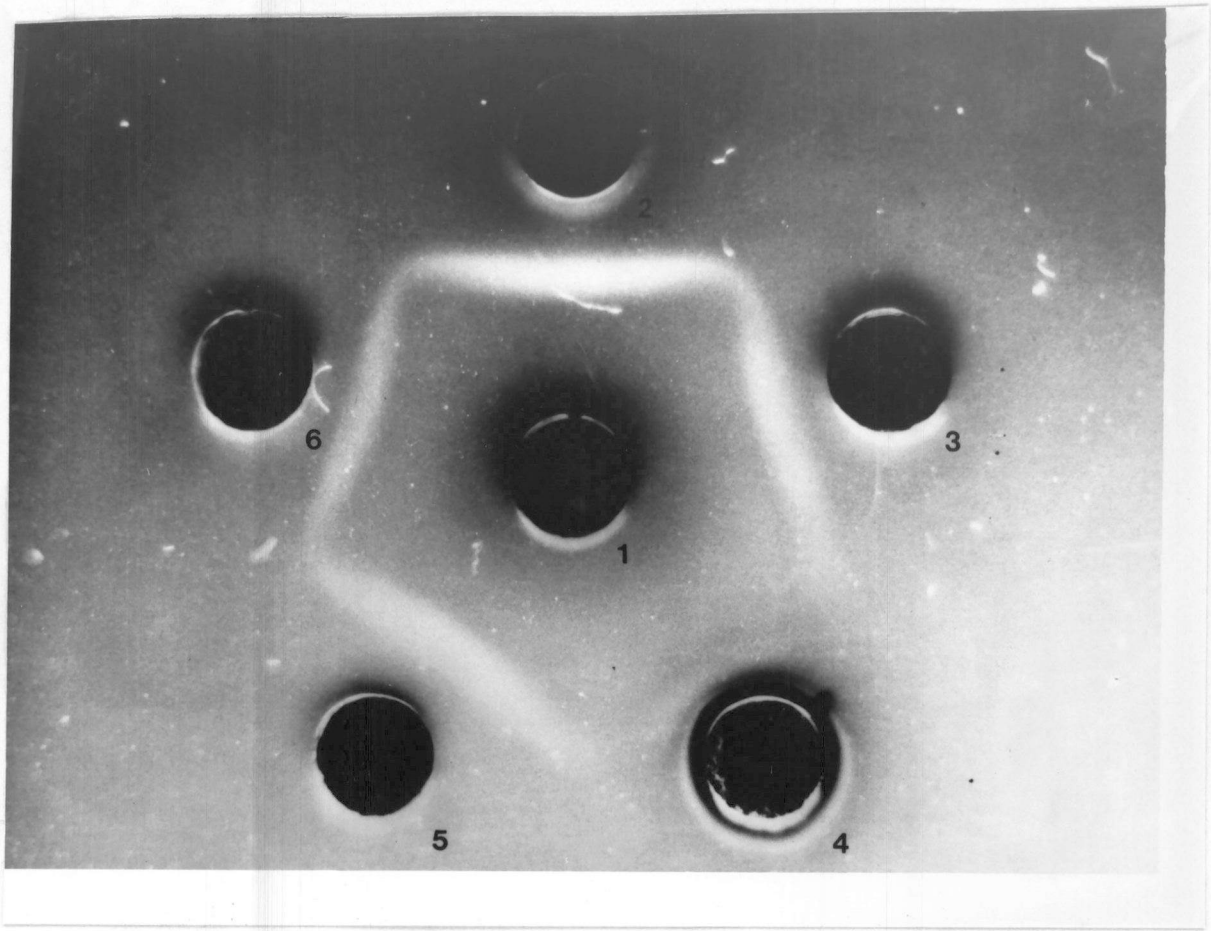

Figure 21. Immunodiffusion of pig liver and beef kidney monoamine oxidase. Well 1 contains 10 µl of IgG antibody against beef kidney monoamine oxidase. Wells 2 and 5 contain 50 µl of beef kidney monoamine oxidase. Wells 3 and 6 contain 50 µl of pig liver monoamine oxidase. Well 4 contains 50 µl of partially purified pig liver monoamine oxidase.

Complete cross reactivity of the two enzymes was not expected. It is doubtful that these two enzymes from different species share a common amino acid sequence even though both enzymes have exclusively type B monoamine oxidase activity. It is interesting that the two enzymes are antigenically similar since the beef kidney enzyme has not been well characterized. Molecular weights of both enzymes are around 150,000. Both enzymes have eight sulfhydryl groups and have covalently bound flavin (47,50). These two enzymes may also be glycoproteins. Oreland reported that pig liver monoamine oxidase contains sialic acid and hexoamine (47), and Greenawalt showed that the beef kidney enzyme reacted with periodic acid-Schiff reagent in SDS-polyacrylamide gels (unpublished). No other monoamine oxidases have been shown to contain carbohydrate. The carbohydrate content of these two enzymes could explain the antigenic similarities of these enzymes shown in this study.

CHAPTER IX

CONCLUSIONS AND SPECULATIONS

This chapter is intended to bring together the conclusions and possibilities discussed in earlier chapters concerning the molecular nature of the multiple forms of monoamine oxidase. In addition, a model will be presented to explain the results of this study. Data from the literature are selected to support the model.

The concept that multiple forms of monoamine oxidase are due to differential lipid effects on a single polypeptide was based on the inability to conclusively demonstrate two polypeptides by biochemical and immunological techniques. Differential effects on the two types of activity by detergents, organic solvents and chaotropic agents were interpreted as the interconversion of the multiple forms of monoamine oxidase activity. The recent demonstration that two monoamine oxidase polypeptides are responsible for the multiple forms of activity undoubtedly proves this hypothesis to be invalid.

In this light, the experiments designed to test for the interconversion of multiple forms of activity in earlier chapters always showed that inactivation rather than transformation of multiple forms was responsible for the changes in the ratios of multiple forms of activity upon delipidation. Furthermore, the similarity of the phospholipid composition of the mitochondria from the Novikoff hepatoma and rat liver is inconsistent with the idea that lipid is solely responsible for the two forms of activity. Reconstitution of monoamine oxidase into liposomes of defined lipid also failed to

demonstrate that lipid modulated the ratio of multiple forms of activity.

Multiple forms of monoamine oxidase activity are not entirely due to differential binding of outer membrane lipid, but the activity of the multiple forms is affected differently by lipid. Type A activity is more sensitive to delipidation with detergents, phospholipase and organic solvents than type B activity. Furthermore, the reactivation of type A activity is very dependent on the type of phospholipid used in the reconstitution, while the type B activity seems to be rather indiscriminate in the requirement for a hydrophobic environment. Type B activity does vary in proportion with the fluidity of added lipid. Arrhenius plots of monoamine oxidase activity show that the multiple forms of monoamine oxidase activity are associated differently with lipid in the intact outer membrane. Type B activity is sensitive to a bulk lipid phase transition of the outer membrane. This is indicative of the indiscriminate interaction of this enzyme with outer membrane lipids. The untypical reduction of the energy of activation of type B monoamine oxidase at temperatures below the phase transition may indicate the shift of this enzyme to a more fluid lipid environment. Type A monoamine oxidase is probably associated with a specific lipid which does not undergo a phase transition in the range of temperatures tested.

Figure 22 shows a model of how the multiple forms of monoamine oxidase may be situated in the outer mitochondrial membrane. A specific lipid (phosphatidylserine) is proposed to be associated with type A monoamine oxidase. This lipid is probably not required for activity but may interact with the enzyme such that the enzyme's

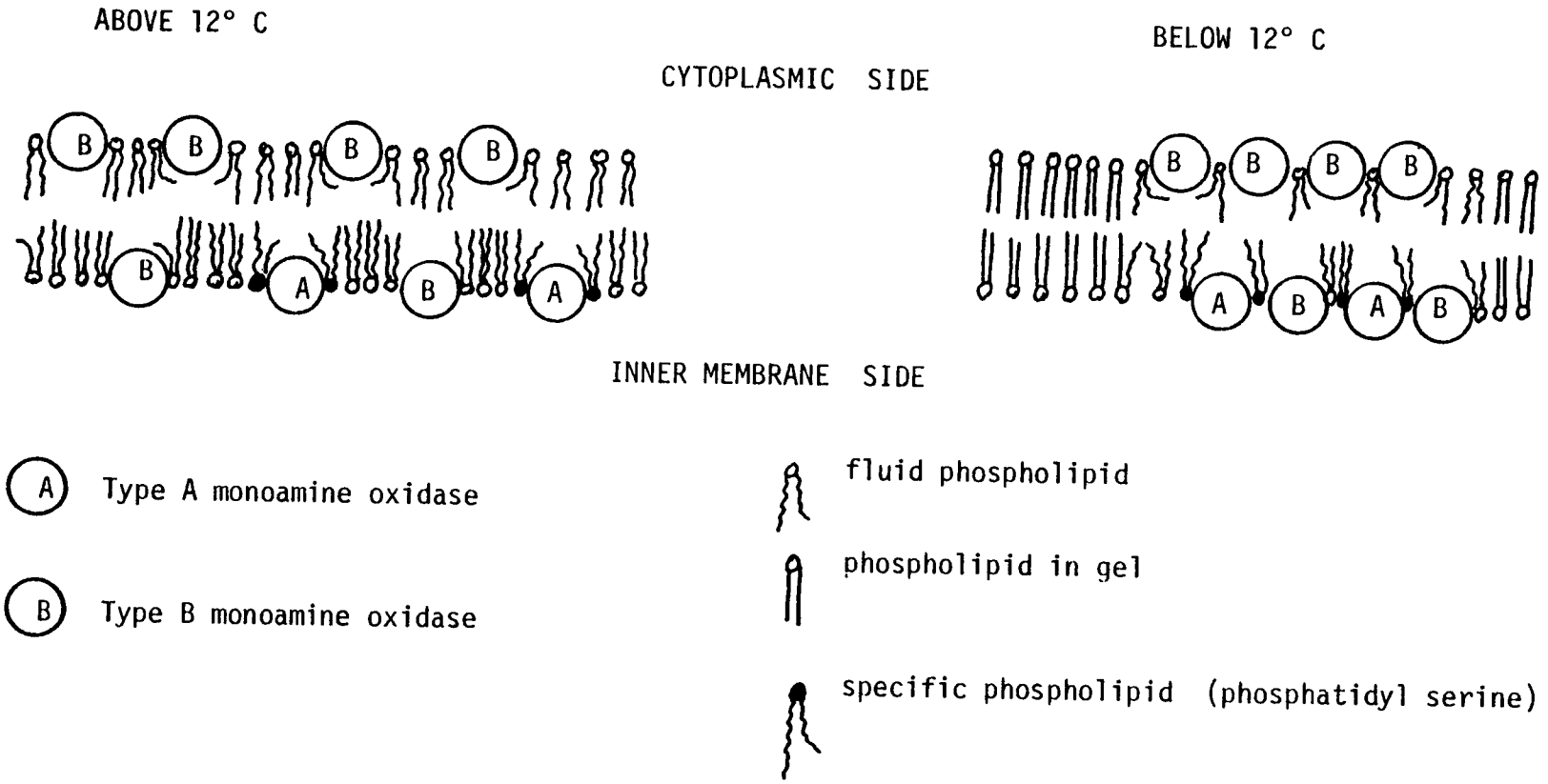

Figure 22. Model of the molecular organization of monoamine oxidase in the outer mitochondrial membrane.

conformation is optimal for highest activity. A model of the membrane at temperatures below the phase transition is also shown. Phase separation of the outer membrane has been shown by freeze fracture electron microscopy (65), and it is proposed that monoamine oxidase migrates to the fluid region of the membrane. The lipid associated with type A enzyme may migrate with the enzyme such that the enzyme has a constant environment.

The location of the multiple forms of the enzyme in this model are taken from the literature. Russel et al. (69) showed by immunological methods that type B monoamine oxidase was located on the cytoplasmic surface while type A enzyme was on the inner membrane surface of the outer membrane. Edward and Pak (24) measured three type B enzymes for every type A enzyme by titration with labeled inhibitors. Chau (58,96) visualized an equal distribution of monoamine oxidase molecules on either surface of the outer membrane. Thus, this model incorporates all of the above features. Some type B enzyme was placed on the inner membrane surface, in contrast to Russel's observations, to account for Chau's observation of an equal distribution of monoamine oxidase between the two surfaces of the outer membrane. However, freeze fracture studies show more membrane particles in the outer leaflet of the membrane.

Fragmentation of the outer membrane with digitonin into two fractions is somewhat consistent with this model. One fraction contained almost exclusively type B enzyme while the other contained both types of enzyme. Digitonin might split the membrane bilayer such that the first fraction was composed primarily of the outer

leaflet while the second fraction was mostly derived from the inner leaflet of the outer membrane. The different net charge of the two fractions of outer membranes treated with digitonin is also consistent with the different distribution of anionic sites between the outer surface and inner surface of the outer membrane. More polycationic ferritin bound to the inner surface of the outer membrane than the outer surface (88). The more negatively charged fraction of outer membrane treated with digitonin eluted from the DEAE column is the one proposed to be derived from the inner leaflet of the outer membrane. Perhaps the charge is due to phosphatidylserine or phosphatidylinositol, which is asymmetrically located in the inner leaflet of the outer membrane. Moreover, perhaps this lipid is preferentially associated with type A monoamine oxidase.

LIST OF REFERENCES

LIST OF REFERENCES

1. Hare, M.L.C. (1928) Tyramine oxidase I. A new enzyme system in liver. Biochem. J. 22, 968-979.

2. Blaschko, H. (1939) Amine oxidases and diamines. The action of guanidine. J. Physiol. 95, 30P.

3. Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) The submitochondrial localization of monoamine oxidase. J. Cell Biol. 32, 719-735.

4. Erwin, V.G. and Simon, R.J. (1969) Occurrence of newly synthesized monoamine oxidase in subcellular fraction of the rat liver. J. Pharm. Sci. 58, 1033-1035.

5. Walker, W.H., Kearney, E.B., Seng, R. and Singer, T.P. (1971) Sequence and structure of a cysteinyl flavin peptide from monoamine oxidase. Biochem. Biophys. Res. Commun. 44, 287-292.

6. Oreland, L., Kinemuchi, H. and Yoo, B.Y. (1973) The mechanism of action of the monoamine oxidase inhibitor pargyline. Life Sci. 13, 1530-1531.

7. Youdim, M.B.H. and Sourkes, T.L. (1972) The flavin prosthetic group of purified rat liver mitochondrial monoamine oxidase. Adv. Biochem. Psychopharmac. 5, 45-53.

8. Youdim, M.B.H. and Sourkes, T.L. (1966) Properties of purified, soluble monoamine oxidase. Can. J. Biochem. Physiol. 44, 1397-1400.

9. Tipton, K.F. (1972) Some properties of monoamine oxidase. Adv. Biochem. Psychopharmac. 5, 11-24.

10. Yasunobu, K.T. and Oi, S. (1972) Mechanistic aspect of the bovine hepatic monoamine oxidase reaction. Adv. Biochem. Psychopharmac. 5, 91-100.

11. Symes, A.L. and Sourkes, T.L. (1975) Essential sulfhydryl groups of rat liver monoamine oxidase. Can. J. Biochem. Physiol. 53, 910-911.

12. Oi, S., Yasunobu, K.T. and Westley, J. (1971) The effect of pH on the kinetic parameters and mechanism of beef liver monoamine oxidase. Archs. Biochem. Biophys. 145, 557-564.

13. Hiramatsu, A., Tsurishiin, S. and Yasunobu, K.T. (1975) Evidence for essential histidine residues in bovine liver mitochondrial monoamine oxidase. Eur. J. Biochem. 57, 587-593.

14. Kielly, W.W. (1963) Preparation and assay of phosphorylating submitochondrial particles. Meth. Enz. 6, 272-277.

15. Blaschko, H. (1952) Amine oxidase and amine metabolism. Pharmacol. Rev. 4, 415-458.

16. Greenawalt, J.W. and Decker, G.L. (1976) Reduction of respiratory chain components by mitochondrial monoamine oxidase, interaction of outer and inner membranes. Fed. Proc. 35, 1435.

17. Smith, G.S. and Reid, R.A. (1978) The influence of respiratory state on monoamine oxidase activity in rat liver mitochondria. Biochem. J. 176, 1011-1014.

18. Johnston, J.P. (1968) Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochem. Pharmac. 17, 1285-1297.

19. Hall, D.W.R., Logan, B.W. and Parsons, G.H. (1969) Further studies on the inhibition of monoamine oxidase by M + B 9302 (clorgyline) - I. Substrate specificity of various mammalian species. Biochem. Pharmac. 18, 1447-1454.

20. Knoll, J. and Magyar, K. (1972) Some puzzling pharmacological effects of monoamine oxidase inhibitors. Adv. Biochem. Psychopharmac. 5, 393-408.

21. Chuang, H.Y.K., Patek, D.R. and Hellerman, L. (1974) Mitochondrial monoamine oxidase. Inactivation by pargyline. Adduct formation. J. Biol. Chem. 249, 2351-2354.

22. McCauley, R. (1976) 7[^{14}C] pargyline binding to mitochondrial outer membranes. Biochem. Pharmac. 25, 2214-2216.

23. Clawthon, R.M. and Breakefield, X.O. (1979) Differences in A and B forms of monoamine oxidase revealed by limited proteolytic and peptide mapping. Nature 281, 692-694.

24. Edward, D.J. and Pak, K.Y. (1979) Selective radiochemical labeling of types A and B active sites rat liver monoamine oxidase. Biochem. Biophys. Res. Comm. 86, 350-357.

25. Christmas, A.J., Coulson, C.J., Maxwell, D.R. and Riddell, D. (1972) A comparison of the pharmacological and biochemical properties of substrate selective monoamine oxidase inhibitors. Br. J. Pharmac. 45, 490-495.

26. Neff, N.H., Yang, H.Y.T. and Fuentes, J.A. (1974) The use of selective monoamine oxidase inhibitor drugs to modify amine metabolism in brain. Adv. Biochem. Psychopharmac. 12, 49-57.

27. Lyles, G.A. and Callingham, B.A. (1974) The effect of thyroid hormones on monoamine oxidase in the rat heart. J. Pharm. Pharmac. 26, 921-930.

28. Mantle, T.J., Garrett, N.J. and Tipton, K.F. (1976) The development of monoamine oxidase in rat liver and brain. FEBS Letters 64, 227-230.

29. Pedersen, P.L., Greenawalt, J.W., Chan, T.L. and Morris, H.P. (1970) A comparison of some ultrastructural and biochemical properties of mitochondria from Novikoff hepatomas 9618A, 7800 and 3924A. Cancer Res. 2620-2626.

30. White, M.T. and Tewari, K.K. (1973) Structural and functional changes in Novikoff hepatoma mitochondria. Cancer Res. 33, 1645-1653.

31. White, M.N., Arya, D.V. and Tewari, K.K. (1974) Biochemical properties of neoplastic cell mitochondria. J. Natn. Cancer Inst. 53, 553-559.

32. Martin, A.P., Cornbleet, P.J., Lucas, F.V., Morris, H.P. and Vorbeck, M.L. (1974) Mitochondrial membrane-associated properties of Morris hepatomas. Cancer Res. 34, 850-858.

33. Cornbleet, P.J., Vorbeck, M.L., Lucas, F.V., Esterly, J.A., Morris, H.P. and Martin, A.P. (1974) Differences in distribution pattern of marker enzymes among subcellular fractions from Morris hepatoma 16. Cancer Res. 34, 439-446.

34. Murphy, D.L., Donnelly, C.H. and Richelson, E. (1976) Substrate and inhibitor-related characteristics of monoamine oxidase in C6 rat glial cells. J. Neurochem. 26, 1231-1235.

35. Hawkins, M. and Breakefield, X.O. (1977) Monoamine oxidase A and B in cultured cells. J. Neurochem. 30, 1391-1397.

36. Hartman, B.K., Yasunobu, K.T. and Udenfriend, S. (1971) Immunological identity of the multiple forms of beef liver mitochondrial MAO. Archs. Biochem. Biophys. 147, 797-804.

37. Hidaka, H., Hartman, B.K. and Udenfriend, S. (1971) Comparison of mitochondrial monoamine oxidases from bovine brain and liver using antibody to purified liver monoamine oxidase. Archs. Biochem. Biophys. 147, 805-809.

38. Hartman, B.K. (1972) The discovery and isolation of a new monoamine oxidase from brain. Biol. Psychiat. 4, 147-155.

39. McCawley, R. and Racker, E. (1973) Separation of two monoamine oxidases from bovine brain. Mol. Cell. Biochem. 1, 73-81.

40. Youdim, M.B.H. and Collins, G.G.S. (1975) Properties and physiological significance of multiple forms of mitochondria monoamine oxidase (MAO). In Isozymes II Physiological Function (Markert, C.L., ed.). pp. 619-636. Academic Press, New York.

41. Deniick, R.G. and Mayer, R.J. (1977) Purification and immunological characterization of monoamine oxidase from rat and human liver. Biochem. J. 161, 167-174.

42. Youdim, M.B.H. and Collins, G.G.S. (1975) The binding of [^{14}C] phenylhydrazine to rat liver monoamine oxidase. Biochem. Pharmac. 24, 703-706.

43. Tipton, K.F., Youdim, M.B.H. and Spires, I.P.C. (1972) Beef adrenal medulla monoamine oxidase. Biochem. Pharmac. 21, 2197-2204.

44. Youdim, M.B.H. and Holman, B. (1975) The nature of inhibition of cat brain mitochondrial monoamine oxidase by clorgyline. J. Neural Trans. 37, 11-24.

45. Houslay, M.D. and Tipton, K.F. (1973) The nature of the electrophoretically separable multiple forms of rat liver monoamine oxidase. Biochem. J. 135, 173-186.

46. Tipton, K.F., Houslay, M.D. and Garrett, N.J. (1973) Allotopic properties of human brain monoamine oxidase. Nature, New Biol. 246, 213-215.

47. Oreland, L. (1971) Purification and properties of pig liver mitochondrial monoamine oxidase. Arch. Biochem. Biophys. 146, 410-421.

48. Ekstedt, B., Olivecrona, T. and Oreland, L. (1975) Binding of cardiolipin/lecithin and monoamine oxidase to lipid-depleted mitochondrial membrane structure. Arch. Biochem. Biophys. 171, 170-175.

49. Olivecrona, T. and Oreland, L. (1971) Reassociation of soluble monoamine oxidase with lipid depleted mitochondria in the presence of phospholipids. Biochem. 10, 332-340.

50. Ekstedt, B. and Oreland, L. (1976) Effect of lipid-depletion on the different forms of monoamine oxidase in rat liver mitochondria. Biochim. Pharmac. 25, 119-124.

51. Erwin, V.G. and Hellerman, L. (1967) Mitochondrial monoamine oxidase. I. Purification and characterization of the bovine kidney enzyme. J. Biol. Chem. 247, 4230-4238.

52. Baker, S.P. and Hemsworth, B.A. (1978) Effects of phospholipid depletion by phospholipases on the properties and formation of the multiple monoamine oxidase forms in the rat liver. Eur. J. Biochem. 92, 165-174.

53. Diaz Borges, J.M. and D'Iorio, A. (1972) Multiple forms of monoamine oxidase. Adv. Biochem. Psycopharmac. 5, 79-90.

54. Shih, J.H.C. and Eiduson, S. (1973) Monoamine oxidase (E.C. 1.4.3.4). Isolation and characterization of multiple forms of the brain enzyme. J. Neurochem. 21, 41-49.

55. McCauley, R. (1978) Properties of a monoamine oxidase from rat liver mitochondrial outer membranes. Arch. Biochem. Biophys. 189, 8-13.

56. Houslay, M.D. and Tipton, K.F. (1974) Rat liver mitochondrial monoamine oxidase. A change in the reaction mechanism on solubilization. Biochem. J. 145, 311-321.

57. Enster, L. and Kuylenstierna, B. (1970) Outer membrane of mitochondria. In Membranes of Mitochondria and Chloroplast (Racker, E., ed.) pp. 172-212. Van Nostrand Rheinhold Company, New York.

58. Chau, R. (1976) The structural organization of the outer mitochondrial membrane with special reference to monoamine oxidase. Dissertation, The University of Texas Health Science Center at Dallas.

59. Parsons, D.F. (1963) Mitochondrial structure: Two types of subunits in negatively stained mitochondrial membranes. Science 140, 985-987.

60. Hackenbrock, C.R. (1972) States of activity and structure in mitochondrial membranes. Ann. N.Y. Acad. Sci. 195, 492-502.

61. Hackenbrock, C.R. and Miller, K.J. (1975) The distribution of anionic sites on the surfaces of mitochondrial membranes. Visual probing with polycationic ferritin. J. Cell Biol. 65, 615-630.

62. Levy, M., Toury, R. and Sauner, M.T. (1969) Recent findings on the biochemical and enzymatic composition of the two isolated mitochondrial membranes in relation to their structure. In Mitochondria, Structure and Function (Ernster, L. and Drahota, A., eds.) pp. 33-42.

63. Stoffel, W. and Schiefer, H.G. (1968) Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes. Hoppel-Seyler's Z. Physiol. Chem. 349, 1017-1026.

64. Melnick, R.L., Tinberg, H.M., Maguire, J. and Packer, L. (1973) Studies on mitochondrial proteins. I. Separation and characterization by polyacrylamide gel electrophoresis. Biochim. Biophys. Acta 311, 230-241.

65. Hackenbrock, C.R., Hochli, M. and Chau, R.M. (1976) Calorimetric and freeze fracture analysis of lipid phase transitions and

lateral translational motion of intramembrane particles in mitochondrial membranes. Biochim. Biophys. Acta 455, 466-484.

66. Lee, M.P. and Gear, A.R. (1974) The effect of temperature on mitochondrial membrane linked reactions. J. Biol. Chem. 249, 7541-7549.

67. Raison, J.K. (1973) Temperature-induced phase changes in membrane lipids and their influence on metabolic regulation. Soc. Exptl. Biol. 27, 485-512.

68. Aiathal, H.N., Janki, R.M., Guchulak, B.D. and Reno-Tustanoff, E. (1976) Lipid protein interactions in the outer membranes of yeast and rat liver mitochondria. Arch. Biochem. Biophys. 176, 1-11.

69. Russel, S.M., Davey, J. and Mayer, R.J. (1979) The vectorial orientation of human monoamine oxidase in the mitochondrial outer membrane. Biochem. J. 181, 7-14.

70. Greenawalt, J.W. (1974) The isolation of inner and outer mitochondrial membranes. Meth. Enzym. 31a, 310-322.

71. Callingham, B.A. and Laverty, R. (1973) Studies on the nature of increased monoamine oxidase activity in the rat heart after adrenalectomy. J. Pharm. Pharmac. 25, 940-947.

72. Tipton, K.F. (1969) Assay of monoamine oxidase. Anal. Biochem. 28, 318-320.

73. Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954) Purification of amine oxidase from beef plasma. J. Biol. Chem. 208, 645-661.

74. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

75. Morton, R., Cunningham, C., Jester, R., Waite, M., Miller, N. and Morris, H.P. (1976) Alteration of mitochondria function and lipid composition in Morris 7777 hepatoma. Cancer Res. 36, 3246-3254.

76. Ames, B. and Dubin, D.T. (1960) The role of polyamines in the neutralization of bacteriophage DNA. J. Biol. Chem. 235, 769-772.

77. Decker, G. and Greenawalt, J.W. (1977) Ultrastructural and biochemical studies of mitoplasts and outer membranes derived from French-pressed mitochondria. J. Ultrastru. Res. 59, 44-56.

78. Bruner, J., Skrabal, P. and Hauser, H. (1976) Single bilayer vesicles prepared without sonication. Physiochemical properties. Biochem. Biophys. Acta 455, 322-331.

79. Jacobs, E.E., Jacobs, M., Sandai, D.R. and Bradley, L.B. (1956) Uncoupling of oxidative phosphorylation by cadmiom. J. Biol. Chem. 223, 147-156.

80. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 243, 3588-3595.

81. Swanson, M.A. (1955) Glucose-6-phosphatase from liver. Meth. Enzym. 2, 541-543.

82. Novikoff, A.B. (1957) A transplantable rat liver tumor induced by 4-dimethyliaminoazobenzene. Cancer Res. 17, 1010-1027.

83. Novikoff, A. and Holtzman, E. (1972) Cells and Organelles, Holt, Rinehart and Winston, Inc. New York.

84. Fowler, C.J., Callingham, B.A., Mantle, T.J. and Tipton, K.F. (1978) Monoamine oxidase A and B: A useful concept? Biochem. Pharmac. 27, 97-101.

85. Tipton, K.F., Houslay, M.D. and Mantle, T.J. (1976) The nature and location of the multiple forms of monoamine oxidase. Ciba Fdn. Symp. 39, 5-33.

86. Gear, A.R.L. (1970) Inner- and outer-membrane enzymes of mitochondria during liver regeneration. Biochem. J. 120, 577-587.

87. Melchior, D.L. and Stein, J.M. (1976) Thermotropic transitions in biomembranes. Ann. R. Biophys. Bioeng. 5, 205-238.

88. Silvius, J.R., Read, B.D. and McElhaney, R.W. (1978) Membrane enzymes: Artifacts in Arrhenius plots due to temperature dependence of substrate-binding affinity. Science 199, 902-904.

89. Oreland, L. and Ekstedt, B. (1972) Thermal and protelytic inactivation of soluble pig liver monoamine oxidase. Biochem. Pharmac. 21, 2479-2488.

90. Oldfield, E. and Chapman, D. (1972) Dynamics of lipids in membranes: Heterogeneity and the role of cholesterol. FEBS Letters 23, 285-297.

91. Eytan, G.D., Matheson, M.J. and Racker, E. (1976) Incorporation of mitochondrial membrane proteins into liposomes containing acidic phospholipids. J. Biol. Chem. 251, 6831-6837.

92. DeCaldentey, M.I. and Wheeler, K.P. (1979) Requirement for negatively charged dispersions of phospholipids for interaction with lipid-depleted adenosine triphosphatase. Biochem. J. 177, 265-273.

93. Singer, J.S. (1974) The molecular organization of membranes. Ann. Rev. Biochem. 43, 805-834.

94. Rothman, J.E. and Lenard, K. (1977) Membrane asymmetry. Science 195, 743-753.

95. Cook, H.P. (1958) Cholesterol, pp. 502-512, Academic Press, New York.

96. Chau, R.M. and Hackenbrock, C.R. (1975) Localization of monoamine oxidase in rat liver mitochondria. In Abstracts of the Fifteenth Annual Meeting of the American Society for Cell Biology, Number 126, p. 63.

97. Nicholls, P. and Chance, B. (1974) Cytochrome oxidase. In Molecular Mechanism of Oxygen Activation (Hayaishi, O., ed.) pp. 479-528. Academic Press, New York.

98. Hollunger, G. and Oreland, L. (1970) Preparation of soluble monoamine oxidase from pig liver mitochondria. Arc. Biochem. Biophys. 139, 320-328.

99. Roitt, I.M. (1971) Essential Immunology, Blackwell Scientific Publications, Oxford.

VITA

Stephen T. Sawyer was born on February 20, 1952 in Dandridge, Tennessee. He is the son of Mrs. Claude Sawyer and the late Mr. Sawyer of Morristown. He graduate from Morristown-Hamblen High School East in 1970 and received a Bachelor of Science degree with honors from the University of Tennessee in 1974. He entered graduate school at the University of Tennessee in January, 1975 and entered the Ph.D. program directly. He is a member of the Phi Kappa Phi Honor Society. On September 9, 1978, he married Lynda F. Sweeney of Memphis.