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The Effect of Lipid Environment on the Mitochondrial Monoamine Oxidase of Bovine Liver

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Date

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January 26, 1996
Date

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ABSTRACT

The aims of this investigation were the isolation of bovine mitochondrial MAO-B, its reconstitution into vesicles of defined lipid composition, and examination of the influence of different lipid environments on its enzymatic activity

MAO-B was isolated from bovine liver mitochondria Out of several isolations, one preparation was purified to homogeneity and two others purified to >80% with high specific activity, while other preparations were less pure Enzymatic activity was assayed spectrophotometrically with kynuramine as substrate Triton X-100 was included in the assay of the membrane-free ("solubilized") enzyme to prevent aggregation Several methods of reconstitution were examined for the incorporation of this enzyme into model membranes of defined lipid composition, before adopting detergent dialysis with octylglucoside as the method of choice On average, 96% of the protein recovered on glycerol gradients was vesicle-incorporated in this system The influence of phospholipid headgroup and fatty acyl chain composition on the enzymatic activity and stability of membrane-incorporated MAO-B were investigated Phosphatidylethanolamine (PE) is a polymorphic lipid with the potential for forming the hexagonal phase (H_{II}) MAO-B was reconstituted with a range of concentrations of dioleoylPE (DOPE) in dioleoylphosphatidylcholine (DOPC) Activity increased with increasing DOPE content, reaching a maximum at about 40 mole% Stability of the incorporated enzyme was maximal at about 50 mole% DOPE Under the experimental conditions, DOPE was most likely in the lamellar rather than the hexagonal phase for all concentrations except 60 mole% Proteoliposomes composed of 100% DOPC and

DOPC/DOPE (70/30 mole%) were selected for further study. This concentration of DOPE was chosen because it is comparable with the concentration of PE in mitochondria. The effects of membrane surface charge due to the anionic lipids, dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidic acid (DOPA), were examined. Proteoliposomes (PRLs) were prepared containing the anionic species either as the sole lipid or as 10 mole% with DOPC or the DOPC/DOPE mixture. Whereas 10 mole% DOPS did not directly affect MAO-B activity, the stability of the enzyme reconstituted in this system was decreased. In contrast, this concentration of DOPA significantly enhanced the enzymatic activity. The effects of 10 mole% anionic lipid were reduced when 30 mole% DOPE was also present. The degree of unsaturation of the 18 carbon fatty acyl chains of PC did not alter activity of MAO-B reconstituted with these lipids, stability was marginally improved in the di-unsaturated system. The kinetics of kynuramine oxidation were compared for the membrane-free enzyme and the DOPC/DOPE (70/30 mole%) reconstituted system. The K_M was unaltered; however, the V_{max} for the PRLs was 60% of the value for the solubilized enzyme.

In summary, there was no dramatic increase in MAO-B activity upon reconstitution into DOPC vesicles. However, the stability of the reconstituted MAO-B was better than that of the membrane-free preparation, at least for the spontaneously incorporated enzyme. Relative improvements in activity upon inclusion of other phospholipids in the DOPC vesicles did not achieve the activity of the unreconstituted enzyme. It may be that this lack of sensitivity to its lipid environment is important for the function of MAO-B. The membrane may serve as a regulating mechanism for this enzyme, limiting its activity so as to prevent oxidative stress from a build-up of the toxic products of the MAO-B reaction.

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This thesis is dedicated to the memory of my parents, James and Annie Deery, and my late husband, Seymour Posner, who encouraged me to pursue my dreams.

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ABBREVIATIONS USED

BA	Benzylamine
BCA	Bicinchoninic acid
BR	Bacteriorhodopsin
Ch	Chymotrypsin
CL	Cardiolipin
COMT	Catechol-O-methyltransferase
DA	Dopamine
DMPC	Dimyristoylphosphatidylcholine
DOC	Deoxycholate
DOPA	Dioleoylphosphatidic acid
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOPG	Dioleoylphosphatidylglycerol
DOPS	Dioleoylphosphatidylserine
EDTA	Ethylenediamenetetraacetic acid
FAD	Flavin adenine dinucleotide
GSH	Glutathione, reduced
GSSG	Glutathione, oxidized
H_{II}	Hexagonal phase
HK	50mM Hepes/100mM Kcl buffer, pH 7.4

5HT	5-Hydroxytryptamine (serotonin)
K_M	Michaelis constant
L/P	Lipid to protein (weight ratio)
L/P_{dimer}	Lipid to protein dimer (mole ratio)
LUVs	Large unilamellar vesicles
LPs	Liposomes
MAO	Monoamine oxidase
MAO-A	Monoamine oxidase, type A
MAO-B	Monoamine oxidase, type B
MAOI	Monoamine oxidase inhibitor
MLVs	Multilamellar vesicles
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-Methyl-4-phenylpyridinium ion
NaP	50mM sodium phosphate buffer, pH 7 2
NE	Norepinephrine
OG	Octylglucoside
OMM	Outer mitochondrial membrane
PA	Phosphatidic acid
PAO	Plasma amine oxidase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEA	Phenylethalamine

PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PK	Protease K
PL	Phospholipid
PL-A	Phospholipase A
PL-C	Phospholiase C
PMSF	Phenylmethylsulfonyl fluoride
POPC	Palmitoyloleoylphosphatidylcholine
SM	Sphingomyelin
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SUVs	Small unilamellar vesicles
T_{BH}	Temperature of the lipid bilayer to hexagonal phase transition
T_c	Temperature of the lipid gel to liquid crystalline phase transition
TEA	Triethanolamine
TRIS	Tris(hydroxymethyl)aminomethane
Tx	Triton X-100
Tyr	Tyramine
ULVs	Unilamellar vesicles
V_{max}	Maximal velocity

INTRODUCTION

I. AIM OF THIS INVESTIGATION

Monoamine oxidase [monoamine O_2 oxidoreductase, deaminating, flavin-containing, EC 1 4 3 4] (MAO) is an integral protein located in the outer mitochondrial membrane of most mammalian tissues (Tipton, 1975) There are two forms of the enzyme, designated A and B, which have different inhibitor and substrate specificites (Johnston, 1968) Isolation of separate cDNAs for these two types of MAO (Bach *et al* , 1988) confirmed that the A and B activities arise from different enzymes

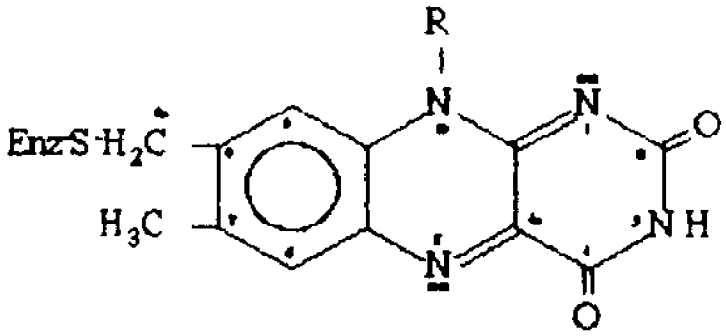
The role of the lipid environment in the activity of the two forms of MAO has not been fully assessed Phospholipid headgroups and acyl chains have the potential of affecting integral membrane protein function headgroup charge can potentially influence the accumulation of substrate in the vicinity of the enzyme's binding site and the physical state of the bilayer, as influenced by acyl chain unsaturation, can effect conformational changes consequent to substrate binding and catalysis Furthermore chemical heterogeneity of the lipid bilayer results in membrane compartmentalization or 'domain' formation, which is expected to have profound influence on the functioning of intergral membrane proteins, and in particular, enzymes

The aim of this investigation is to first isolate MAO-B from bovine liver mitochondria, and then to reconstitutue the functional protein into vesicles of defined lipid composition in order to examine MAO-B activity in different lipid environments. It is hoped that such studies will provide a more detailed understanding of the role the membrane bilayer plays in the functioning of mitochondrial MAO-B

II. MITOCHONDRIAL MONOAMINE OXIDASE

a *Molecular Properties*

MAO catalyzes oxidative deamination of biogenic and exogenous amines to their corresponding aldehydes, and is an important regulator of levels of monoamine neurotransmitters. Both the A and B forms contain flavin adenine dinucleotide (FAD) as a coenzyme, bound covalently to the enzyme active site through the 8-α-carbon to a cysteine residue (Kearney *et al*, 1971).



R = ribitol-pyrophosphate-ribose-adenine

Mitochondrial MAO (both forms) is distinct from the soluble amine oxidase of plasma (PAO), which catalyzes the same reaction, and which has overlapping substrate and inhibitor specificity with MAO. However, PAO differs from the mitochondrial enzymes in several important aspects, including sensitivity to the carbonyl reagent, semicarbazide, the lack of a flavin cofactor (the PAO active site moiety is a modified amino acid residue, trihydroxyphenylalanine (topa)), and whereas PAO is a copper-dependent enzyme, both forms of MAO are reported to be devoid of metals or other electron carriers (Erwin & Hellerman,

1967; Ichinose *et al.*, (1982), Weyler & Salach (1985), Straher (Ph D thesis, 1987); Nara *et al.*, (1966), Yasunobu *et al.*, (1966), Tipton (1975))

The pale yellow flavin coenzyme absorbs maximally in the wavelength region 445-450nm, and is non fluorescent in the native proteins However, on denaturation of the protein, the flavin exhibits characteristic emission at 520nm (Norstrand and Glantz, 1973) Anaerobic reduction of the enzyme with substrate or sodium dithionite prior to SDS treatment results in quenching of this observed fluorescence (Norstrand and Glantz, 1973)

Primary amino acid sequences of the two MAO proteins (A and B) from *human* liver have been deduced from their nucleotide sequences (Bach *et al* , 1988), and monomeric molecular weights of the A and B forms are 59 7 and 58 8kDa, respectively, with about 70 percent sequence homology Type A comprises 527 amino acid residues, while type B reveals 520 residues Similarly, amino acid analysis of type B from *bovine* liver by Weyler (1989), indicated 507 amino acids and a molar mass of 57 3kDa Most recent evidence suggests that the MAOs exists as functional dimers, each subunit having one associated FAD coenzyme Earlier investigations suggested a shared coenzyme for the subunits of the dimer (Chuang *et al* , 1974, Miniamura and Yasunobu, 1978a,b, Hellerman and Erwin, 1968) However, from cloning studies by Bach *et al* (1988), the genes for the MAO A and B forms were observed to code for a single subunit In addition, expression of the cDNAs, reported by Lan *et al* (1989a) and Weyler *et al* (1990) yielded catalytically active enzyme, which was shown to contain a covalently attached FAD with stoichiometry of protein to coenzyme of 1 1 It is thought that the earlier stoichiometric results of 2 1, arose from impure enzyme preparations Spectroscopic evidence (Yue *et al* , 1993, Woo and Silverman, 1994) suggests that the FAD

is distributed between two states oxidized and anionic semiquinone radical

In addition to the cysteine residue involved in binding the flavin moiety (Cys^{406} in MAO-A and Cys^{397} in MAO-B from *human* liver), other essential cysteines have been proposed (Gomes *et al*, 1976) These cysteine residues have been implicated in assisted binding of amine substrate (Oi *et al*, 1971) Indeed, for *human* placental MAO-A, kinetic studies of enzyme inactivation by dipyridyldisulfide, indicated modification of at least two SH groups (Weyler and Salach, 1985) Additionally, Silverman and Zieske (1986) concluded that the mechanism-based inhibitor 1-phenylcyclopropylamine formed an adduct with an active site cysteine in MAO-B The role of other amino acids in the enzyme active site is unclear Hiramatsu *et al* (1975) reported two essential histidines for the *bovine* liver MAO-B dimer which, according to Oi *et al* (1971), may be required for substrate cleavage No further mention of essential histidine has occurred in the literature

b *Forms of MAO*

By convention, the two forms of MAO are distinguished by their sensitivity to inhibition by specific acetylenic, mechanism-based inhibitors Type A is inactivated by low concentrations of clorgyline (Johnston, 1968), type B is similarly sensitive to deprenyl (Knoll *et al*, 1968) and pargyline (Fuller *et al*, 1970) The two forms are also distinguished by their specificity toward endogenous catecholamine and indoleamine substrates, although this specificity is not absolute Generally, 5-hydroxytryptamine (serotonin) and norepinephrine (NE) are considered A-selective substrates, while 2-phenylethylamine (PEA) and benzylamine are considered B-selective substrates

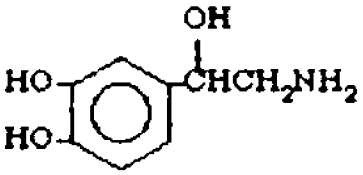
Table 1

Representative MAO Substrates

A-Selective:

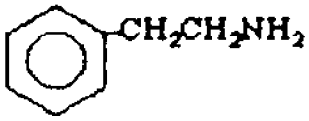
5-Hydroxytryptamine (5HT)

Norepinephrine (NE)

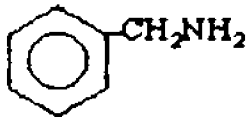


B-Selective:

2-Phenylethylamine (PEA)

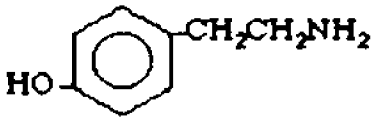


Benzylamine (BA)

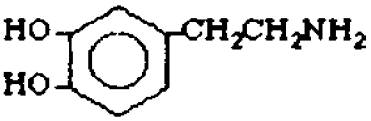


A/B Substrates:

Tyramine (Tyr)



Dopamine (DA)



Tyramine, dopamine and kynuramine are among the several substrates oxidized to a significant degree by both forms of the enzyme (Table 1) According to Tipton *et al.*, (1987), specificity is relative, and depends on the concentration, affinity, and turnover rate of a given substrate For example high concentrations of the B-selective substrate PEA can be metabolized by MAO-A from the brain and liver mitochondria of rat and also from several other species (Suzuki *et al* , 1981) In addition, at very high concentrations, MAO-B of rat brain can contribute up to 15 percent of the total activity toward serotonin, however, the Michaelis constant (K_M) is greatly elevated and the maximal velocity (V_{max}) is much lower than with the A form of the enzyme (Fowler and Tipton, 1982) For these reasons, and because of considerable variability in methodology as reported in the literature, a simple rank order of preference for the substrates of the two enzymes provides a deceptive impression However, from comparison of the ratios of turnover number to substrate binding affinity (k_{cat}/K_M) for several amines, some measure of substrate preference is obtained For MAO-B isolated from bovine liver, the apparent order of preference is PEA>kynuramine>benzylamine>tryptamine, tyramine (Yamasaki and Silverman, 1985, Tan and Ramsay, 1993) For human MAO-A (both the enzyme isolated from placenta and that derived from expression of the gene from liver in yeast), the apparent order of substrate preference is tryptamine>serotonin, kynuramine>tyramine>dopamine (Tan *et al* , 1991)

A representative table of tissue distribution of MAO forms is shown below The table (from Yasunobu and Tan, 1985) is modified as indicated (*) to show revisions or additions, and is not intended to be complete Percent types were averaged from several of the authors' sources and, consequently, do not necessarily total 100% for a given tissue

Table 2

Distribution of MAO-A and MAO-B in Several Mammalian Species

(modified from Yasunobu and Tan, 1985)

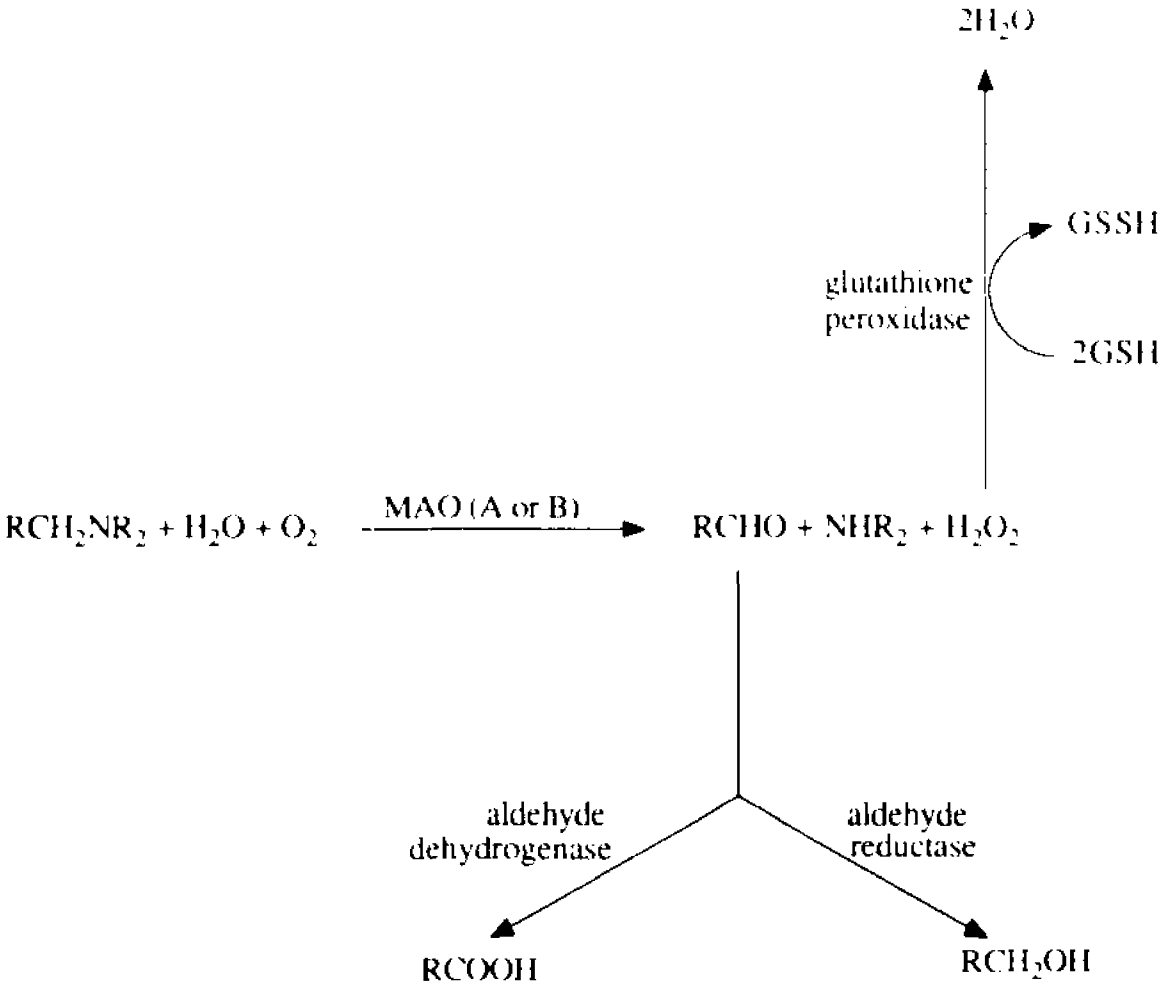
Animal	Organ	%A	%B	Animal	Organ	%A	%B
Rat	Liver	40	60	Pig	Brain	0	99
	Spleen	95	4		Liver	0	99
	Brain	55	45	Cat	Liver	8	94
	Lung	50	50		Kidney	16	87
	Kidney	70	30		Brain	31	83
	Intestine	70	30		Intestine	54	38
Mouse	Liver	2	96	Dog	Liver	0	94
	Kidney	66	34		Kidney	8	92
	Brain	44	54		Brain	31	83
	Intestine	72	31		Intestine	69	34
G Pig	Liver	39	62	Bovine	*Liver	0	100
	Kidney	58	47		Brain	15	85
	Brain	20	79	Human	*Placenta	100	0
	Intestine	84	21		*Platelets	0	100

c *Biological Importance of MAO*

Monoamine oxidase is present in several tissues, where it functions in the catabolism of biogenic amines (*i.e.*, neurotransmitters and their precursors) and exogenous amines and is thus an important regulatory and detoxification enzyme As shown in Figure 1, neurotransmitter amines are oxidatively deaminated by the mitochondrial MAOs to their

Figure 1

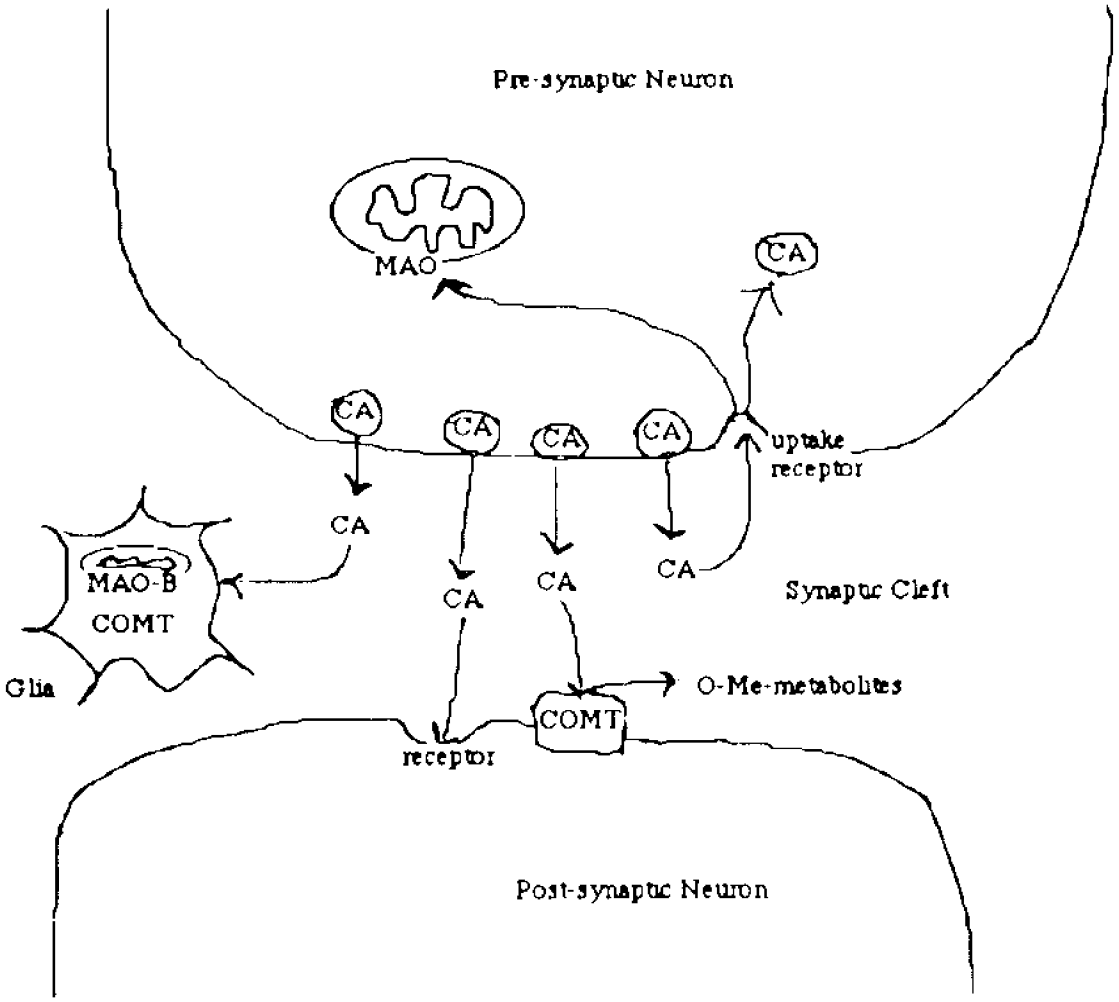
Monoamine Neurotransmitter Metabolism



corresponding aldehydes (Cooper *et al* , 1986; Roth *et al* , 1984) Further metabolism of the aldehyde occurs rapidly, either by oxidation to the corresponding acid by aldehyde dehydrogenase, an outer mitochondrial membrane protein (Smith and Packer, 1972), or by reduction to the alcohol or glycol by aldehyde reductase In brain, the resultant peroxide produced is reduced mainly by glutathione peroxidase (Youdim *et al* , 1990), an enzyme present in both the cytosol and the mitochondrial matrix (Tyler, 1992)

The amine neurotransmitters are stored intraneuronally in vesicles, and released at the nerve terminal upon stimulation Preferential release of newly synthesized amine has been reported for dopamine (Besson *et al* , 1969, Javoy and Glowinski, 1971) and norepinephrine (Kopin *et al* , 1968), although the mechanism by which this might occur has not been elucidated Deamination by the mitochondial MAOs thus regulates the concentrations of transmitters available for release, thereby playing a supporting role in the events that contribute to terminating neurotransmission As shown in Figure 2, reuptake of the amines via receptors on the pre-synaptic cell is the principal means of terminating the signal Neuronal reuptake (Iversen, 1975, Trendelenburg, 1990) is a 2-part process first, transport across the axonal membrane into the neuron; second, uptake into storage vesicles The axonal transporter is specific, having high affinity for the transmitter released by that neuron NE, DA or 5HT This specificity is relative since many structurally related amines can be taken up as well, and, indeed, are competitive inhibitors of the transmitter amine Reuptake occurs against a transmitter concentration gradient, is temperature-dependent, and is inhibited by metabolic inhibitors and by oubain, an inhibitor of Na^+/K^+-ATPase Axonal transport of these three neurotransmitters is saturable, obeys Michaelis-Menten kinetics, and is coupled

Figure 2
Diagram of a Catecholaminergic Synapse



with the sodium gradient across the neuronal membrane. The three transporters exhibit different inhibitor sensitivities. 5HT is sensitive to N-methylated tricyclic antidepressants, while NE is sensitive to their desmethylated derivatives, DA lacks sensitivity to the tricyclic antidepressants and is potently inhibited by benztropine and D-amphetamine, both catecholamine transporters are inhibited by cocaine.

Within the neuron, repackaging into storage vesicles protects the transmitter from the MAOs which degrade free neuroamines in the cell. This intraneuronal uptake (discussed by Iversen, 1975) is not sodium-dependent, but is dependent on high (>1mM) concentrations of ATP and Mg^{2+}, and is also temperature-sensitive. Catecholamine storage is stoichiometrically related to hydrolysis of extravesicular ATP by a Mg^{2+}-dependent ATPase in the vesicle membrane. Catecholamines are bound in a complex with ATP in the storage granule. Like neuronal transport, vesicular transport also obeys Michaelis-Menten kinetics. However, the affinity for catecholamines is 1000-fold lower than with axonal transport. Structurally related amines competitively inhibit vesicular uptake, and may themselves be accumulated in the storage granules, displacing the neurotransmitters. Vesicular uptake is insensitive to many of the inhibitors which act on neuronal uptake. Reserpine is a potent inhibitor of vesicular transport, having an affinity for the uptake system that is 10,000-fold higher than NE or epinephrine.

Free catecholamines in the synaptic cleft are subject to metabolism by catechol-O-methyltransferase (COMT) which occurs as a membrane protein of the post-synaptic neuron as well as in soluble form in astroglia (Roth *et al*., 1984). This enzyme transfers methyl groups from S-adenosyl methionine to the 3-hydroxy position of the

catecholamine ring Both the acid and alcohol metabolites of MAO-A and -B activity are substrates for COMT, as the O-methylated metabolites are deaminated by the MAOs Sulfation of the catecholamines and their metabolites by phenolsulfotransferase is a minor contribution to their degradation Oxidative deamination serves as the sole degradative route for serotonin, except in the pineal gland (Cooper *et al* , 1986) This organ has two enzymes involved in converting 5HT to melatonin the product of 5HT-N-acetylase is N-acetylserotonin, the preferred substrate of 5-hydroxyindole-O-methyltransferase The final product is 5-methoxy-N-acetyltryptamine (melatonin)

Neurotransmitter amine that is not cleared from the synaptic cleft diffuses into the extracellular space where it can be taken up by glial cells and metabolized by MAO-B and COMT in this tissue The glia share with other nonneuronal tissues an extraneuronal monoamine uptake mechanism, called uptake-2 to distinguish it from uptake-1 of the axons (Iversen, 1975, Trendelenburg, 1990) Like neuronal transport, uptake-2 is saturable and dependent on temperature and external sodium ions In contrast to neuronal transport, uptake-2 has low affinity but high capacity for monoamines Neurotransmitter analogs are also accumulated by uptake-2, but the structural features which favor extraneuronal uptake are different from those which are accumulated by the neuronal transporters Exogenous monoamines are also taken up by this process Amines accumulated by uptake-2 are not stored, but degraded by COMT and the MAO forms present in the extraneuronal tissue However, the specific high affinity transport of 5HT into blood platelets resembles neuronal transport rather than uptake-2, in platelets, the accumulated 5HT is bound in storage granules

In addition to the neurotransmitter amines, the MAOs metabolize a number of other substrates including primary, secondary and tertiary aliphatic monoamines with carbon chain length of 3 or more, diamines and ω-amino acids of carbon chain length of at least seven, N-*tele*-methylhistamine, certain N-acetylated polyamines, the pro-toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and many of its analogs (Tipton, 1975, Youdim and Finberg, 1991) The MAO enzymes also play an important role in the detoxification of certain aryl monoamines, such as tyramine, phenylethylamine, and their β-hydroxylated derivatives, octopamine and phenylethanolamine (Kopin, 1968) Of these tyramine is the most important, being present in several foods Unless deaminated, these substances can be taken up by the storage granules in sympathetic nerve terminals, replacing part of the neurotransmitter On stimulation of noradrenergic nerves and exocytotic discharge of the synaptic vesicle contents, these amines are released together with NE causing diminished sympathetic response due to their lower (*e.g* ,octopamine) or absent (*e.g* ,tyramine) agonistic potency This results in a *hypotensive* effect Similarly, detoxification of dietary amines follows their transport (uptake-2) into intestine and liver where they are deaminated by the MAO enzymes of these tissues Entry of these amines into the circulation and their transport (uptake-1) into sympathetic nerve endings and neurotransmitter storage vesicles is thereby prevented In patients treated with monoamine oxidase inhibitors (MAOI), ingestion of foods high in tyramine (*e.g* , cheese, red wine, beer) precipitates release of NE with consequent *hypertensive* result (referred to as the 'cheese effect') This tyramine-caused release of NE doees not involve exocytosis from the storage granules, but rather inhibition of NE reuptake into the neuron (thus increasing the NE

concentration in the synapse) as well as displacement of NE from the synaptic vesicles into the cytoplasm where the MAOI prevents deamination of both amines (Baldessarini, 1975) In humans, MAO-A in the intestine is the primary detoxifier of tyramine (Magyar, 1993) Consequently, nonselective MAOI, as well as irreversible inhibitors selective for the A form, precipitate the "cheese effect" Inhibitors which are selective for the B form, or which reversibly inhibit the A form, are now the MAOI of choice due to their greater safety in this regard, permitting degradation of tyramine

d *Clinical Importance of MAO*

Although the MAOs are not the sole metabolizing enzymes for the monoamine neurotransmitters, their absence causes abnormalities resulting in certain diseased states mainly assessed by determining the MAO activity of blood platelets (exclusively the B form) and by measuring levels of neurotransmitter amine metabolites in both the urine, cerebrospinal fluid (CSF), and also by assay of *post mortem* brain Brunner *et al* (1994) has reported mild retardation and impulsive aggression associated with complete MAO-A deficiency The syndrome was expressed in several males of a large family, who suffered from a point mutation of a glutamine codon, located on the X-chromosome, to a termination codon in the structural gene for MAO-A

An alternate (hereditary) MAO deficiency, described by Murphy *et al* (1990) as an X chromosome deletion (Norrie disease) that included both the MAO-A gene and at least part of the MAO-B gene resulted again in profoundly retarded male patients

Fowler and Saaf (1985) summarized the major effects reported for platelet MAO-B

activity in relation to disease states Conditions where increased enzyme activity was found include nonalcoholic liver disease, megaloblastic anemia, Huntington's chorea, Alzheimer's disease, epilepsy and anxiety states Conditions with decreased enzyme activity include migraine, insulin-dependent diabetes, Down's syndrome, alcoholism, alcoholic liver cirrhosis and iron deficiency anemia Contradictory results reported for schizophrenia may be due to differences in diagnosis and/or the effect of neuroleptic drugs used in treating these patients

The increased brain MAO-B activity found in aging and certain neurodegenerative diseases has evoked interest in a possible contributory role for this enzyme The finding that MAO-B metabolizes the pro-toxin MPTP to the neurotoxic species, 1-methyl-4-phenylpyridinium ion (MPP^+), that induces a Parkinsonian syndrome led to speculation that, departing from its detoxification role, MAO-B may occasionally metabolize an endogenous or environmental amine to a product which induces pathology Alternatively, hyperactivity of the enzyme may result in accumulation of its other products, ammonia and hydrogen peroxide Production of superoxide and hydroxyl radicals from peroxide may lead to oxidative stress, an imbalance between the generation of oxidants and the cell's antioxidant defenses (Gerlach and Riederer, 1993)

Parkinson's disease (PD) is characterized by the destruction of the dopaminergic nigrostriatal system (Gerlach and Riederer, 1993) Biochemical features include large decreases in the enzymes of dopamine synthesis (tyrosine hydroxylase and aromatic amino acid decarboxylase), small changes in the enzymes of DA catabolism (increase for MAO-B and decrease for COMT), and decreased activity of certain mitochondrial respiratory chain enzymes (NADH cytochrome c reductase and succinate cytochrome c reductase) Treatment

is designed to boost the dopamine content. L-DOPA, the immediate precursor, is converted by the remaining decarboxylase. L-Deprenyl inhibition of MAO-B prevents both metabolism of DA by this enzyme, and the consequent production of H_2O_2.

III MAO: INTEGRAL MEMBRANE PROTEINS

As integral membrane proteins, MAO-A and -B are extremely hydrophobic in nature and difficult to isolate in pure form without first treatment with membrane solubilizing agents which can significantly affect protein function. Hydropathy plots of human liver MAO-A and B sequences show great similarities and reveal a tight 'interweaving' of each protein with the membrane (Bach *et al.*, 1988), with seven hydrophobic regions (residues 15-30, 110-130, 170-200, 265-270; 295-315, 400-450 and 485-C-terminus) that may be responsible for anchoring the protein within the OMM. In both proteins, a pentapeptide, Ser-Gly-Gly-Cys-Tyr, associated with the covalently bound flavin coenzyme, is located near the C-terminal region. The nucleotide moiety of the coenzyme is oriented in a binding site near the N-terminal end of the sequence.

A range of subunit molecular weights for each MAO form (as determined by SDS electrophoresis) is found in the literature. For human placental MAO-A, values from 60 to 67kD have been reported (Callingham and Parkinson, 1979, Brown *et al.*, 1980, Cawthon *et al.*, 1981, Weyler and Salach, 1985, Riley *et al.*, 1989). Similarly, for bovine liver MAO-B, the reported range is 52 to 63kD (Miniamura and Yasunobu, 1978a, Salach, 1979; Weyler and Salach, 1981; Sagaro and Ito, 1982). That these differences in subunit sized reflect experimental variation is suggested by the ranges reported for these enzymes by Salach and

Weyler (1987) with several preparations of each enzyme isolated by their own procedures 60-64kD for human placental MAO-A and 58-62kD for bovine liver MAO-B No reference is found in the literature which attributes these MAO-A and -B subunit size variations to varying amounts of bound membrane lipid

Using immunological approaches, Russell *et al.* (1979) conducted a vectorial analysis and showed that MAO-A activity was associated with the inner side of the outer mitochondrial membrane (OMM) facing the intermembrane space MAO-B activity was localized to the cytosolic side of the OMM In contast, proteolytic treatment of intact and hypotonically disrupted rat liver mitochondria revealed differences in relative rates of inactivation of MAO-A and MAO-B, leading Buckman *et al* (1983a) to conclude that the two enzyme forms do not reside on opposite faces of the membrane

a *The MAO Membrane Environment*

For reconstitution studies, bilayer phospholipids and steroids, comparable with the protein's native membrane environment, both in headgroup and fatty-acyl chain saturation were chosen The total phospholipid (PL) composition of complete liver mitochondria from several species (Table 3), including bovine, has been summarized by Daum (1985) In table 4, information on the lipid composition of outer liver mitochondrial membranes (OMM) from mouse (Ardail *et al* , 1990), rat and Guinea pig (Daum, 1985), and pig heart (Comte *et al* , 1976) is summarized No corresponding published data on the bovine liver OMM is available, based on the similarity of lipid composition of whole mitochondria, it is not unreasonable to expect similarities with other species

Table 3

% of Total Phospholipids in Liver Mitochondria (from Daum, 1985)

	Bovine	Ovine	Guinea Pig	Rat
phosphatidylcholine	43	48	40	34-55
phosphatidyl-ethanolamine	35	19	28	22-36
phosphatidylglycerol	--	--	2	1
phosphatidylinositol	3	4	7	5-8
phosphatidylserine	0.1-0.3	1	--	1
phosphatidic acid	--	2	--	1-2
cardiolipin	17	14	23	12-17
lysophosphoglycerides	--	27	--	1-2
sphingomyelin	--	--	--	1

Table 4

% of Total Phospholipid in Liver Outer Mitochondrial Membranes from Different Tissues

	Mouse[a]	Rat[b]	Guinea Pig[b]
phosphatidylcholine	41	44-59	55
phosphatidylethanolamine	27	20-35	25
phosphatidylglycerol	--	(3)[c]	3
phosphatidylinositol	9	5-20	14
phosphatidylserine	<0.1	(2)[c]	--
phosphatidic acid	--	(1)	--
cardiolipin	4	4	3
lysophosphoglycerides	0.5	2-3	1
sphingomyelin	2	2-4	5

[a]Ardail *et al.*, (1990), [b]Daum (1985), unless otherwise noted, [c]Jain (1988)

Justification for the composition of the reconstituted lipid systems studied was as follows Phosphatidylcholine (PC) is the predominant phospholipid in bovine liver mitochondria, and in the outer mitochondrial membranes of all mammalian species While phosphatidylethanolamine (PE) represents a smaller proportion of the total phospholipid in the outer as compared with inner mitochondrial membranes, it is the second most prevalent phospholipid Additionally, PE (a polymorphic lipid) can preferentially form nonbilayer (hexagonal (H_{II}) phase) structures under physiological conditions (Cullis *et al*, 1985) The H_{II} phase consists of lipid cylinders with polar headgroups facing inward lining an aqueous core, each lipid cylinder is surrounded by six others The presence of nonbilayer lipid may contribute to compartmentalization of the membrane Resultant protein domains may allow improved access of substrate to the enzyme active site, or favor protein conformational changes that may be associated with catalysis Together, PC and PE represent approximately 80% of the total phospholipid content of the OMM Other phospholipids (Table 4), where determined, are present only in very small (sometimes trace) amounts This does not, however, preclude the possibility of their having a modulatory role in the membrane with direct effects on the activities of the MAO forms

The fatty acyl chains of mitochondrial phospholipids exhibit diverse heterogeneity with respect to chain length and degree of unsaturation (Table 5) Although the saturated fatty acyl chains of palmitate (16 0) and stearate (18 0) predominate in the outer membrane of mouse (Ardail *et al*, 1990) and rat liver (Daum, 1985) and in pig heart (Comte *et al*, 1976), unsaturated fatty acyl chains, including oleoate (18 1) and linoleoate (18 2), constitute a substantial component of these membranes (Table 5)

Table 5

% of Total Fatty Acids in Mouse Liver OMM
(from Ardail *et al.*, 1990)

Fatty Acid	% Total Fatty Acids
C16:0	36.3
C18:0	20.5
C18:1	18.0
C18:2	16.6
C18:3	<0.1
C20:4	7.1

Table 6

Phase Transition Temperatures of Dioleoylphosphoglycerides
(from Silvius, 1982)

Phosphoglyceride	T_c(°C)
dioleoylphosphatidylcholine (DOPC)	-22
dioleoylphosphatidylethanolamine (DOPE)	-16
dioleoylphosphatidylglycerol (DOPG)	-18
dioleoylphosphatidylserine (DOPS)	-11

Phospholipids chosen for the reconstitution studies are shown in Table 6, along with their corresponding lipid phase transition temperatures (T_c). Thus at physiologically relevant temperatures, the proteoliposomes investigated existed in the fluid (L_α) phase. Although

palmitate is represented to a greater extent than oleate in the OMM (Table 4), phospholipids with palmitoyl chains have high T_c values, *e.g.*, 41°C for dipalmitoylPC (Silvius, 1982). Their use would necessitate maintaining the enzyme at elevated temperatures (above the phospholipid T_c) following reconstitution with these lipids. MAO-B is unstable at higher temperatures, showing decreased activity above 40°C (Pohl and Schmidt, 1983) and complete inactivation within 5 minutes at 50°C (Baker and Hemsworth, 1978).

Whilst other 18-carbon fatty acids with higher unsaturation are without question biologically significant (see Table 5), these fatty acids are more susceptible to air peroxidation than the corresponding monounsaturated form. Hence, for practical purposes, the dioleoylphospoglycerides proved the most convenient phospholipid class of study.

Cholesterol constitutes only 7% of the total lipid of mouse liver OMM (Ardail *et al.*, 1990), and has been reported to represent less than 5% in rat liver (Jain, 1988). The presence of cholesterol in vesicles prepared from phospholipids at $T>T_c$ is expected to have a rigidifying effect on the physical state of the bilayer membrane (Bloch, 1991) which can have profound influence on the activity of integral proteins in these membranes.

b *Lipid Effects on Activity of the MAO Forms: A Review*

Previous investigations of the role of lipid in the functioning and regulation of the MAO forms have focussed on nutritional studies, the effects of delipidation and relipidation of mitochondrial membranes, effects of supplementing mitochondria with exogeneous lipids, and studies in which the enzyme is removed from its native membrane and reconstituted with phospholipids. MAO-A and -B, as tightly bound membrane proteins, are difficult to isolate

Harsh treatments employed in the course of delipidation experiments or isolation procedures have affected the nature of the previous investigations into lipid effects

(i) *Nutritional Studies*

In vivo lipid deficiency studies have shown significant effects on the activity of MAO forms. A fat-free diet was observed to reduce rat hepatic activity for MAO-A (70%) and for MAO-B (50%), with return to near normal levels following supplementation with 5% corn oil, a source of essential fatty acids (Kandaswami and D'Iorio, 1979). Both mitochondrial phospholipid proportions and the PL/protein ratios were unaltered by the fat-free diet However, the mitochondrial lipids were deficient in both essential fatty acids and those derived from them, *e.g.*, linoleate (18:2) and arachidonate (20:4), while the saturates palmitate (16:0) and stearate (18:0) were unchanged. Other unsaturates (16:1, 18:1 and 20:3) were substantially increased

In a study by Crane and Greenwood (1987), rats were stabilized on a diet containing 20% fat, of either all soybean oil or 90% lard with 5% each soybean and sunflower oils, to ensure against essential fatty acid deficiency. The activity of the rat brains (which contain both MAO forms) was assayed with the non-selective substrate, tryptamine. The soybean oil group was found to have V_{max} nearly 30% lower than the lard group but without significant difference in K_M, suggesting fewer enzyme molecules rather than altered substrate binding. The soybean oil diet provided twice as much poly-unsaturated fatty acids, with almost equal reductions in unsaturates and monounsaturates. Although mitochondrial lipids were not analyzed in this study, there remains the possibility that altered membrane composition plays

a role in modulating the MAO activities

In a study by Wojtczak *et al* , 1988, incubation of rat hepatocytes (which contain both MAO forms) with oleate caused reduction in the K_{M} with tyramine (a non-selective substrate) without altering the V_{max} Earlier studies by these investigators (Wojtczak and Nalecz, 1979), showed that rat liver MAO activty was inhibited by cationic surfactants and activated by anionic agents, the K_{M}, but not the V_{max}, for oxidation of the non-selective substrate dopamine was altered The anionic stimulants used were oleate, palmitoyl coenzyme A, and dodecylsulfate The authors proposed surface charge density as a possible *in vivo* controlling mechanism for membrane-bound enzymes According to this hypothesis, enzymes having cationic substrates, such as the MAOs, will have an elevated local substrate concentration as a result of the increased negative surface charge of their microenvironment This suggests the possibility that charged lipids may influence membrane-bound enzymes through such surface charge effects

(ii) *Delipidation and Relipidation*

Delipidation in the course of enzyme isolation has been reported to have various effects on MAO, primarily loss of type A activity Denaturation by organic solvent extraction (methyl ethyl ketone) of membrane lipids was the probable cause of the selective loss of MAO-A activity seen in rat tissues (Ekstedt and Oreland, 1976, Sawyer and Greenawalt, 1979), and the nearly complete loss of type B activity observed for MAO from bovine liver (Yu, 1979) Houslay and Tipton (1973) obsrved that use of perchlorate for delipidation of partially purified MAO from rat liver resulted on a loss of selectivity toward the A-selective

inhibitor clorgyline. Sonication, high detergent concentration, together with the use of Tris buffer (a noncompetitive inhibitor of both forms of MAO, Fowler *et al.*, 1977) in the activity assay probably contributed to the observed results. Ionic detergents, as well as high concentrations of the nonionic Triton X-100, have been known to decrease serotonin oxidation by rat liver (Kandeswami and D'Iorio, 1978).

Replacement of endogenous lipids of rat liver outer mitochondrial membranes with dimyristoylphospatidylcholine (DMPC) resulted in retention of type A and B substrate specificities, but loss of sensitivity to clorgyline (Houslay, 1980).

Both forms of MAO expressed in human brain and liver have been solubilized using Triton X-100 (White and Glassman, 1977). A variety of treatments were obsereved to selectively inactivate the solubilized A form, without significant effect on the B form. These include sonication with or without the ionic detergent sodium dodecyl sulfate (SDS) present, low concentrations of sulfhydryl reagents, high salt concentration, and prolonged heating in the absence of substrate. The authors suggest that the anionic phospholipids (cardiolipin, phosphatidylserine and phosphatidylinositol) prevented loss of MAO-A activity in solubilized extracts, without influencing MAO-B activity. Interestingly, phosphatidylcholine was without effect for this enzyme preparation.

Mitochondrial isolates from pig liver MAO (Inagaki *et al.*, 1986) give exclusively the B-form wheras pig spleen mitochondrial extracts displayed substrate and inhibitor selectivity indicative of MAO-A. Lipids extracted from mitochondrial extracts of both of these tissues, as well as egg yolk phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were used for lipid replacement. Their procedure produced phospholipid enrichment ranging from 2.7

to 13 times the molar lipid to protein ratio of the purified, nondelipidated enzyme The lipid-depleted enzyme exhibited increased K_M and decreased V_{max} toward both benzylamine and serotonin as determined by polarographic assay Reconstitution of the delipidated enzyme with egg yolk PE restored the K_M and V_{max} for both BA and 5HT, while reconstitution with PC was without effect A 1 1 molar ratio of PC PE decreased the K_M for BA to about one-third of the nondelipidated enzyme The mitochondrial PL from spleen were more effective in restoring the K_M toward BA than those of the liver tissue from which the enzyme was taken, and both were only minimally effective in restoring V_{max}

Phospholipase digestion is a feature of the currently standard procedures for isolation of both MAO-A and MAO-B (Salach and Weyler, 1987) Several delipidation studies were done using phospholipases as an activity-sparing alternative to organic solvents and chaotropic agents In these studies, lipases were used at mitochondrial protein to lipase weight ratios that exceeded those used in the Salach isolation procedures by 1 5- to 15-fold for phospholipase A and 25- to 50-fold for phospholipase C

Baker and Hemsworth (1978) explored the effects of phospholipase-delipidation on the A and B forms of rat liver MAO Treatment of Triton X-100-solubilized partially purified enzyme with phospholipase C (PL-C), which removes the phosphorylamine moiety, decreased the phospholipid (PL) content to 4% of the original concentration without any effect on the MAO activities Treatment with PL-A, which removes a fatty acyl chain leaving lysophospholipids, similarly decreased the PL content to about 6% of the original concentration with less than 20% loss of both MAO activities, while retarding their elution from a Biogel-A column Treatment with either lipase had no effect on the sensitivity of the

enzymes to clorgyline or deprenyl Phospholipase treatment did not change the greater thermostability of type A MAO relative to type B MAO (at 50°C MAO-B is completely inactivated within 5 minutes, while 20% of type A remains at 10 minutes), although inactivation of both forms was more rapid following PL-A treatment Similarly, the greater resistance of type B to trypsin was unchanged by lipase treatment, but both forms were inactivated more rapidly and to a greater extent with a lower dose of trypsin The results of this study suggest that the phosphorylated head groups are not required for either MAO activity, since 95% removal had no effect The accelerated thermolability and susceptibility to trypsin digestion following PL-A treatment were suggested to be due to the phospholipid hydrolysis products, as well as to the loss of a protective lipid barrier

Huang and Faulkner (1980) investigated the MAO activities of rat brain mitochondria following extensive delipidation with phospholipases Almost 75% of both MAO-A activity toward serotonin and MAO-B activity toward phenylethylamine were lost after PL-A treatment, which removed 76% of total phospholipid (90% of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and 70% of phosphatidylserine (PS) and cardiolipin (CL), the latter essentially an inner membrane lipid), causing partial disintegration of the outer mitochondrial membrane (OMM) There was no inactivation of MAO-B with PL-C delipidation, and loss of MAO-A activity did not exceed 25%, despite the loss of 42% of the phospholipids (90% of PC, 35% of PE, and almost one-half of sphingomyelin (SM), CL, PS and phosphatidylinositol (PI) were unaffected) EM revealed that the gross bilayer morphology of the OMM was retained in this case Removal of the unphosphorylated headgroup by phospholipase D (PL-D) resulted in inactivation of the B form by 75% and of

the A form by only 25% At one hour less than 20% of total phospholipids were digested (over one-half of PC and 8% of PE, with no effect on CL, PI, PS or SM, the effect on the integrity of the OMM was similar to that following PL-C digestion) The preferential removal of phosphorylated headgroups from zwitterionic lipids by PL-C reduced polar and ionic interactions at the membrane surface by 50% Removal of positively-charged headgroups by PL-D left a high negative charge on the membrane surface To introduce charge and dipolar interactions at the membrane surface, intact rat brain mitochondria were incubated with liposomes of lipid purified from bovine heart and brain prior to activity assay MAO-A was not affected by this treatment, while MAO-B activity was inhibited The enzyme was inhibited to the same degree in the presence of liposomes of PC, PE or CL, and to a greater extent by liposomes of phosphatidic acid (PA)

Huang and Faulkner (1980) conducted electron spin resonance (ESR) studies by incorporating the spin-labeled stearic acid I (12,3) [2- (3- carboxypropyl)- 4,4-dimethyl-2-tridecyl- 3-oxazolidinyloxyl] into intact and delipidated rat brain mitochondria as a probe of the hydrophobic core of the OMM This spin label reports on perturbations at the level of 3 carbon atoms from the membrane surface The increase in fluidity of the hydrocarbon core proximal to the hydrophilic surface seen following delipidation with both PL-C and PL-D was similar in extent, as was the decrease in MAO-A activity upon treatment with these 2 lipases MAO-B, on the other hand, was more profoundly affected by the increased negativity of the membrane surface consequent to PL-D digestion, as well as by the effect of charge and dipolar interactions introduced by added liposomes The greater sensitivity of MAO-B to surface charge, and of MAO-A to fluidity changes in the hydrophobic region proximal to the

polar surface, may reflect differences in the localization of the active sites of these two enzymes

A previous parallel ESR and enzymatic temperature dependence study by Huang (1980) utilized spin label I (12,3) and an additional spin-labeled stearic acid I (1,14) [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl], which has its nitroxide spin label more distal from the polar end, and consequently reports on perturbations closer to the center of the bilayer The temperature dependence of the order parameter S, indicating fluidity close to the membrane surface, had breaks at 21-22°C and 32 6-33 6°C with I (12,3) Additionaly, values for the rotational correlation time (relating to fluidity in this surface region, indicated a phase transition at 29-30°C Arrhenius plots of MAO activity over the range of 4-45°C revealed slope changes at 21-22°C and 35-36°C for MAO-A, whereas there were no inflection points for MAO-B The independence of MAO-B in intact rat brain mitochondria to phase transitions of the bulk lipids indicates a lack of influence of the hydrocarbon core on this enzyme, and is consistent with its localization at the hydrophilic surface Temperature dependence of MAO-A correlated with only one phase transition of bulk lipids, occurring in the region of the hydrophobic core proximal to the polar surface, as in the case of delipidation with PL-C and PL-D The second temperature dependence for MAO-A may reflect conformational changes in the protein itself or in its boundary lipid The other bulk lipid phase transitions reported by the two probes do not affect MAO activity

The Arrhenius plots for MAO of rat brain mitochondria differ from those for rat liver (Baker and Hemsworth, 1978) With liver mitochondria, a single inflection point at 26 8°C was seen with both the A and B forms, although the slope change for MAO-B was of a lesser

degree than for MAO-A, possibly indicating different enzyme lipid environments in the two organs.

In a further study, Huang and Faulkner (1981) delipidated rat brain mitochondria with porcine pancreas PL-A2. Prior to assay, the delipidated mitochondria were incubated with liposomes prepared from lipids purified from bovine brain, liver and heart, with average fatty acyl chain length of 18 carbons and variable degree of unsaturation. Negatively-charged lipids were the most efficacious in restoring enzyme activity to levels approaching those seen in the intact mitochondria, with PI causing an apparent activation of the A form. MAO-A activity was restored to 73% of original activity by PS, to 90% by CL, and to 185% by PI. MAO-B was restored to 90% by CL and to 100% by both PS and PI. Of the zwitterionic lipids, only PC had an effect, restoring MAO-A activity to 70%, with no effect on MAO-B activity. PE had no effect on either form of MAO. Efficacy in restoring activity declined with increasing lipid to protein dimer weight ratio (L/P). There was no further increase in activity with a L/P of more than 30 for the acidic lipids or more than 150 for PC. Huang and Faulkner (1981) proposed that this might be due to differences in lipid binding to exposed hydrophobic surfaces of lipid-depleted MAO, with negatively-charged lipids possibly binding with high affinity and low capacity (~30 molecules), suggesting local interaction at the lipid-protein interface, and PC interacting with MAO-A with low affinity and high capacity, suggesting a bulk lipid effect.

The study of phospholipid effect and rat liver mitochondrial MAO by Navarro-Welch and McCauley (1982) parallels that of Huang and Faulkner (1981), with certain exceptions. In the case of rat liver, the mitochondria were exposed to a dose of PL-A2 that was 10-fold

less than in the brain study The PL-A2 was from the same source (*Naja naja* venom) and more comparable in dose to that used in the Salach isolation procedure This treatment removed most of the phospholipid, and decreased the activities of both the A and B forms to less than 25% of intact mitochondria, apparently by decreasing substrate binding affinity without changing maximal velocity Clorgyline sensitivity or resistance of the A or B forms, respectively, were unchanged Lipid replacement via incubation of delipidated mitochondria with liposomes of PC, PE, PS or PI as in Huang and Faulkner (1981) stimulated both MAO activities, except for a slight inhibition of MAO-A by PE However, lipid addition to control mitochondria treated with EDTA-inactivated PL-A2 affected activity in the same way, and to a greater extent Subsequent separation from unincorporated lipid by centrifugation through 1 2M sucrose showed that both treated and untreated mitochondria had been enriched in phospholipid, but without any effect on the MAO activities Enzyme stimulation apparently resulted from the high concentrations of phospholipids in the medium, rather than from reconstituted lipids in the mitochondrial membrane Complex formation between PI and amine substrates had been detected through its effect on substrate-product separation in the radiochemical assay employed The authors proposed that more effective utilization by the membrane-bound enzymes of hydrophobic complexes of amine substrates with PI and the other phospholipids could explain the reactivation seen with delipidated mitochondria by both Huang and Faulkner (1981) and themselves, and the stimulation seen with nondelipidated mitochondrial MAO in this study

(iii) *Lipid Supplementation*

Huang and Faulkner (1981) and Buckman *et al.*, (1983b) supplemented isolated rat liver outer mitochondrial membranes with acidic PL by incubation with liposomes of purified lipid from bovine brain or heart or soybean The following differences in MAO activity for the two forms were noted First, in contrast to delipidation studies by Huang and Faulkner (1981), PI appeared to stimulate MAO-A activity to 124% of control, but have no effect on MAO-B In contrast, PS had no effect on the A form, and suppressed MAO-B activity by up to 55% CL was observed to have no effect on the activity of either the A or B forms Buckman *et al.*, (1983b) explained the discrepancy between the results of Huang and Faulkner (1981) with respect to PI activation, by suggesting that enzyme was released from the membrane as a result of extensive delipidation and detergent action of the added PL, and that the effects on MAO activities do not arise from relipidation of the mitochondria

In contrast to the mammalian enzymes, mitochondrial MAO in the brain and liver of the carp fish has identical sensitivity to deprenyl and clorgyline, which are selective A and B substrates, respectively (Suh *et al* , 1986) Incubation of phospholipase delipidated or intact mitochondria with liposomes of the anionic lipids, PC, PE, PI or sphingomyelin (SM) showed a significant stimulation of 5HT oxidation only for PI Again, following centrifugation of the PI-mitochondria suspension, twice as much activity was recovered in the supernatant as in the precipitated membranes In agreement with the findings of Buckman *et al* (1983b) described above for mammalian MAO, these results suggest that the enzyme had possibly been released from the membrane following delipidation processees

(iv) *Reconstitution Studies*

A few investigations have centered on the isolation of MAO activities from the native membrane, and reconstitution of purified or partially purified enzyme with phospholipids in liposomes. The earliest of these studies involved an attempt by Naoi and Yagi (1980a, 1980b) to reconstitute the beef heart enzyme with its original lipid environment. The delipidated enzyme was added to phospholipids extracted from beef heart mitochondria, and cosonicated to form liposomes, which were then separated from untrapped enzyme by Sepharose 6B column chromatography. K_M values reported for PEA and BA oxidation by the incorporated enzyme approached but did not reach normal, while the K_M for 5HT was about 33% less than with the undelipidated (native) enzyme. However, V_{max} values exceeded those of the untreated enzyme by 28 to 68%, with the larger increases seen with type B substrates. These changes were interpreted as arising from altered substrate specificity due to altered lipid environment.

Using human sources of the enzyme, attempts to restore activity to the phospholipase-treated MAO-A enzyme (from brain and liver) by incubating with liposomes of PI or PS, were performed by White and Stine (1982). Whereas both lipids increased 5HT oxidation by at least 28% in preparations not subjected to lipase treatment, the effect of PI on this lipase-treated enzyme was minimal. However, dialysis of the lipase-treated preparation before addition of these anionic lipids resulted in partial restoration of the activity. MAO-B activity was found to be unaffected by phospholipase activity.

Pohl and Schmidt (1983), using the standard Salach (1979) procedure for isolation of bovine liver MAO-B, attempted membrane enzyme reconstitution by dialysis of detergent-solubilized phosphate-buffered mixtures of protein with PC. Deoxycholate (DOC)

at 0 8% was used for the solubilization Although DOC was removed after 48 hours of dialysis, its inhibition of MAO-B was profound Homogeneous lipid-enzyme dispersions were then sonicated in the absence of detergent Suspensions of pure PS or PC in phosphate buffer were sonicated at room temperature for 1 5 or 2 hours, respectively Purified enzyme was then added at an L/P ratio of about 9000, and the lipid-enzyme mixture was further sonicated for 4 minutes Irreversible destruction of MAO-B activity by DOC was used as an assay to monitor protein reconstitution After 5 minutes of sonication, inhibition by subsequently added 0 8% DOC was minimal This was interpreted as an indication of complete reconstitution The influence of phospholipid charge on kinetic parameters of benzylamine oxidation for this reconstituted preparation were compared with purified/solubilized protein activities Reconstitution using either PC or PS decreased V_{max} by 25% The K_M was unchanged with PC, and decreased by 33% with PS Below 40°C, the temperature-dependence of reconstituted MAO-B was the same for both purified and lipid-treated preparations, with an activation energy of about 36 kJ/mol for each A greater thermal stability for the enzyme was observed in the presence of lipid The temperature for thermal degradation was raised by 10°, to ~52°C, for both PC and PS, again suggesting protection of the enzyme by the membrane environment Similarly, the effect of pH on enzymatic activity differed for purified and reconstituted MAO-B While the purified enzyme exhibited almost constant activity between pH 8 and 10, reconstitution with either PC or PS revealed a sharp optimum at pH 8

Although charge differences of the PL appeared to have little or no effect on maximal velocity of the reconstituted enzyme, differential substrate binding affinities were observed,

which were consistent with complex formation between amine substrate and anionic lipid, as suggested earlier by Navarro-Welch and McCauley (1982)

IV. METHODS OF RECONSTITUTION

The term "reconstitution" has been used in various ways, all of which refer to a recombination of membrane protein with lipid. Previous studies of reconstitution of mitochondrial monoamine oxidase have ranged from addition of lipid to whole mitochondria or to outer mitochondrial membranes, either in the native state or when partially delipidated, to recombination of purified or partially purified enzyme either with natural source lipids or with lipids defined as to headgroup, but heterogeneous as to acyl chains

In contrast, in the study described here, reconstitution is the incorporation of an isolated membrane protein into lipid vesicles of defined composition

Several methods for reconstitution of membrane proteins have been reported in the literature. These have been the subject of several reviews, including those by Racker (1979), Eytan (1982), Madden (1986), Jain and Zakim (1987), Zakim and Scotto (1989) and Silvius (1992). The principal methods used will be discussed in the light of their potential utility for MAO

Isolation of the protein from its native membrane environment generally requires solubilization with detergent, to prevent aggregation. The choice of detergent is frequently critical both for its effect on the properties and function of the protein, and for its effect on the reconstitution method chosen. The use of detergents for solubilization and reconstitution of membrane proteins have been discussed by Silvius (1992) as well as by Helenius and

Simons (1975), Helenius *et al*., (1979), Hjelmeland and Chambrach (1984), Furth *et al* (1984) and Klausner *et al* (1984) Madden (1986) proposed the following criteria for an ideal reconstituted system the components of the system should be the purified protein and a chemically defined lipid matrix, the vesicles should be unilamellar and of at least 100nm diameter to avoid lipid packing problems associated with extreme curvature of the bilayer, the vesicles should be homogeneous as to size and lipid/protein ratio, and the protein should be inserted in the bilayer with a unidirectional orientation

There are three main categories of reconstitution procedures, each with subsets

a *Detergent Dialysis Reconstitution*

(i) *Detergent dialysis* is the classical reconstitution method In this procedure, a solution of lipids in detergent is mixed with solubilized membrane protein, and proteoliposomes form on removal of detergent by dialysis Detergent removal is *slow*, typically requiring dialysis overnight or longer According to the mechanism proposed by Eytan (1982) for proteoliposome formation by this method, liposome formation and protein insertion are sequential during gradual detergent removal by dialysis, the lipid-detergent micelles coalesce to form liposomes while the protein is still in solution, the protein inserts asymmetrically into the already formed bilayer as more detergent is removed The orientation of the protein in the bilayer can be determined by a number of methods which include specific labeling with an antibody (Cardoza *et al*., 1984) and non-specific labeling of surface proteins with p-diazonium benzene sulfonate (Eytan *et al*., 1975) Several functional assays can be used to detect covert activity due to bidirectional incorporation For a reconstituted enzyme

having a membrane-impermeable substrate, additional activity detected on addition of a mild (non-inhibiting) detergent exposes active sites oriented toward the vesicle lumen (Carroll and Racker, 1977) With permeable substrates the protein orientation can be determined by its sensitivity to impermeant inhibitors, externally added or internally trapped (by inclusion in the reconstitution mixture) (Goldin, 1977), increased inhibition when the inhibitor is present on both sides of the membrane is indicative of bidirectional insertion of the enzyme. Differential inhibition by permeant and impermeant inhibitors is another method, additional loss of activity with the permeant inhibitor being indicative of inward-facing active sites (Banerjee *et al* , 1977) When ion translocation is coupled with enzyme activity, separate assay of uptake of externally added and efflux of internally trapped radioisotopic ion determines the orientation of the protein (Goldin, 1977) The detergent dialysis method has resulted in the unidirectional reconstituion of all proteins for which the orientation has been determined (Eytan, 1982) There are exceptions noted in the literature for cytochrome oxidase Bidirectional insertion was found when the oxidized (but not the reduced) form of its cytochrome c substrate was entrapped in the vesicles (Carroll and Racker, 1977, Nicholls *et al* , 1980) Also, Nicholls *et al* (1980) observed that only 75% of the cytochrome oxidase heme groups were reduced by externally added cytochrome c and ascorbate, however, since neither covert activity nor inhibition by the cholate detergent were investigated for this preparation, it may be that the enzyme was incorporated asymmetrically, but was only 75% active Detergent dialysis has been used extensively by Racker and colleagues for the reconstitution of a number of mitochondrial and other membrane proteins For example, incorporation of $^{32}P_i$ -ATP exchange protein by slow removal of cholate was effective in restoring its activity (Kagawa

and Racker, 1971, Kagawa *et al*., 1973), ATP-driven proton translocation (Kagawa *et al*., 1973), cytochrome oxidase (Hinkle *et al*., 1972; Racker, 1972a, Carroll and Racker, 1977), Ca^{2+}-ATPase of sarcoplasmic reticulum (Racker, 1972b) and purple membrane of *Halobacterium halobium* (Racker and Stoeckenius, 1974).

The advantages of this technique include insertion of the protein in a unidirectional orientation, and fusion of the proteoliposomes to form relatively large vesicles.

Disadvantages include possible deleterious effects of residual detergent on the function or properties of the protein, and a heterogeneous distribution of lipid-to-protein ratios.

(ii) *Detergent dilution* is an alternative to dialysis. Detergent dilution has been used by Stark *et al* (1985), who verified vesicle formation by light-scattering and NMR studies. In this technique, the lipid-protein-detergent solution is diluted into a relatively large volume of buffer, resulting in a *rapid* reduction in the detergent concentration. Rapid dilution into 100-fold excess of assay medium was found to be satisfactory in reconstituting cytochrome oxidase (Racker, 1972a, Carroll and Racker, 1977), and the *lac*$^-$ permease of *E. coli* (Viitanen *et al*., 1986).

Other methods which permit more rapid detergent removal include gel filtration and hollow fiber dialysis. However, these methods do not work for all proteins. For example, rapid detergent removal by Sephadex columns was observed to result in inactive $^{32}P_i$-ATP exchange reconstitutions (Kagawa and Racker, 1971, Kagawa *et al*., 1973) and hollow fiber dialysis resulted in bidirectional insertion of Na^+/K^+-ATPase (Goldin, 1977).

The advantages of this technique include rapidity and reproducibility, a stable

population of relatively small vesicles, and uniform distribution of protein.

Disadvantages include possible insertion of the protein in a random orientation, possible deleterious effects of residual detergent on function or properties of the protein, and a final lipid-to-protein ratio which is higher than physiological

b *Facilitated Incorporation*

Mechanical means used to facilitate protein incorporation into liposomes include extrusion and sonication.

Hydration of dried lipids by suspension in buffer, with or without several cycles of freeze-thawing, results in formation of multilamellar vesicles (MLVs) of heterogeneous size (Woodle and Papahadjopoulos, 1989)

Vesicles may be prepared by extrusion of the lipid suspension through filters of defined pore size under pressure with nitrogen, at a temperature above the phase transition of the lipid. Use of relatively large pore filters (200 to 500nm diameter) reduces the number of bilayers in the MLVs. Using smaller filters (*e.g.*, 100nm), large unilamellar vesicles (LUVs) are formed, with maximum size defined by the pore diameter. Multiple passes through the extruder, combined with stacking of two filters, provides a more homogeneous vesicle size distribution (Woodle and Papahadjopoulos, 1989)

Simultaneous extrusion of membrane protein with lipid (coextrusion) results in its incorporation into proteoliposomes. This method has been used in the resonstitution of gramicidin D (Williams *et al*., 1990)

Two types of facilitation procedures employing sonication are in wide use, one using

solely physical means, the other requiring the presence of an additional chemical agent. With both methods, liposomes are first prepared by sonicating a suspension of lipid in buffer, and then adding protein to these preformed liposomes. Incorporation is based on defects in the packing of acyl chains. The extreme membrane curvature of the small unilamellar vesicles (SUVs) that are produced by sonication of aqueous suspensions of lipids provides such packing defects.

(i) *Cosonication* as originally described by Racker (1973), involved sonication of a suspension of lipids and the protein, and was recommended for use when sensitivity to detergent or prolonged dialysis precluded use of the classical method. It has been applied successfully to the reconstitution of mitochondrial ^{32}Pi-ATP exchange, cytochrome oxidase, bacteriorhodopsin (Racker, 1973), Ca^{2+}-ATPase from sarcoplasmic reticulum (Racker and Eytan, 1973), phosphate transporter (P_i/OH carrier) (Banerjee *et al*., 1977), adenine nucleotide transporter (Shertzer *et al*., 1977), and gramicidin D (Williams *et al*., 1990).

However, the sonication time required for vesicle formation was long, and the treatment was too harsh for most proteins. A modification of this procedure involves brief sonication of a mixture of protein and preformed liposomes.

In the *freeze-thaw* variant of this method, described by Kasahara and Hinkle (1977) for reconstitution of the human erythrocyte D-glucose transporter, the liposome-protein suspension is quick-frozen and thawed, and then briefly sonicated. It is thought that transient defects in acyl chain packing induced by the freeze-thaw cycle probably facilitate the incorporation. This method has also been successfully used for incorporation of the plasma

membrane Ca^{2+}-ATPase (Haaker and Racker, 1979) and the adenine nucleotide transporter (Kramer and Klingenberg, 1979), and the *E. coli lac* permease (Viitanen *et al*, 1986)

Advantages of both these cosonication procedures include their rapidity, and the formation of large unilamellar proteoliposomes, driven by the the strain of the curvature of the smaller vesicles (typically 50 to 150nm (Viitanen et al, 1986)) (Woodle and Papahadjopoulos, 1989).

Disadvantages include the intolerance of many proteins to even brief sonication, the inactivation of some proteins by freeze-thawing, the insertion of protein in random orientation, and a higher than physiological final lipid-to-protein ratio

(ii) *Detergent-facilitated incorporation* involves incubation of preformed liposomes with membrane protein in the presence of a small amount of detergent Facilitating agents other than detergent have been used, *e.g.*, cholesterol, fatty acids, short-chain lecithins, lysolipids, or an impurity carried through the protein isolation It is thought that these agents may help to overcome an energy barrier to protein insertion in the membrane, or promote a more stable association of the protein with the bilayer (Jain and Zakim, 1987) Eytan (1982) has suggested that, in the slow removal of detergent by the dialysis method, reconstitution occurs finally by a process similar to direct incorporation, in which proteins adhere to the liposomes formed on reduction of the detergent concentration, and their incorporation is catalyzed by the residual detergent present

Membrane proteins which have been incorporated with the aid of such fusogens include microsomal cytochrome *b*, (Enoch *et al*, 1977), UDP-glucuronyltransferase,

cytochrome oxidase and bacteriorhodopsin (Scotto and Zakim, 1985 and 1986).

The advantages and disadvantages of detergent-facilitation are the same as those for spontaneous incorporation (discussed below), with the added disadvantage of the presence of a facilitating agent which may affect protein function. Also, facilitation by uncharacterized impurities does not allow for rigorous control of the conditions for reconstitution (Zakim and Scotto, 1989).

c *Spontaneous or Direct Incorporation*

In this procedure, preformed liposomes are incubated with membrane protein without a facilitating agent. The initial inserted protein is thought to promote the subsequent sequential incorporation of futher protein molecules (Scotto *et al*., 1987).

Spontaneous incorporation of integral membrane proteins is analogous to the assembly of these proteins into lipid bilayers in cellular membranes, and provides a means of studying the mechanism of this process (Zakim and Scotto, 1989). As in facilitated incorporation, defects in the packing of acyl chains in the lipid vesicles are believed to be the basis for spontaneous insertion of proteins. Eytan *et al*. (1976) and Eytan and Racker (1977) have described the spontaneous incorporation of several inner mitochondrial membrane proteins into liposomes containing acidic phospholipids. Further reports of successful reconstitutions into defined lipid vesicles include cytochrome b_5 and cytochrome b_5 reductase (Enoch *et al*., 1977), and human erythrocyte hexose transporter (Carruthers and Melchior, 1984).

Scotto and Zakim (1988) have investigated the conditions favoring fusion of

protein-free and protein-containing liposomes, which allows the vesicles to approach the size of biological membranes. They proposed two steps for the reconstitution process: first, the initial insertion of proteins into a small portion of vesicles, and then the subsequent fusion between protein-free (LPs) and protein-containing liposomes (PRLs). In these studies, unilamellar vesicles (ULVs) of DMPC were prepared by sonicating aqueous suspensions of lipid in the fluid phase. The vesicles were cooled to 4°C (well below the phase transition temperature for DMPC) and resonicated for various lengths of time, or not at all. The sonication resulted in reduced vesicle size, with consequent increased curvature and packing defects, which facilitated the rapid insertion of bacteriorhodopsin (BR) or UDP-glucuronyltransferase into gel phase vesicles, whether or not the vesicles had been resonicated. The rate of vesicle fusion was enhanced by resonication. Scotto and Zakim (1988) suggested that the energy barrier for protein insertion was lower than for vesicle fusion. Annealing of the packing defects by warming the vesicles above the phase transition temperature slowed the rates for both the insertion and fusion events (with greater effect on the fusion rate), showing that the energy barrier was related to the extent of packing defects in the bilayer. Rates for both protein insertion and vesicle fusion were much faster in the gel phase than the fluid phase. In contrast, both BR and UDP-glucuronyltransferase incorporated spontaneously into ULVs of DOPC above the T_c (-23°C) for this lipid. Indeed, vesicle fusion in the BR-DOPC system was faster at 30°C than at 4°C, despite presumed fewer packing defects at the higher temperature.

LUVs were also shown to spontaneously incorporate proteins (Scotto and Gompper, 1990). Both detergent-solubilized BR and purple membrane sheets were reconstituted into

LUVs of DMPC or DOPC in either the gel or liquid phase, with growth by fusion with protein-free vesicles In the DMPC system, vesicle growth in the fluid phase was independent of lipid concentration, possibly due to lipid transfer Growth in the gel phase was more rapid, and dependent on lipid concentration, suggesting a growth mechanism other than lipid transfer from liposomes to proteoliposomes Reconstitution of cytochrome b_5 into LUVs of palmitoyl-oleoyl-phosphatidylcholine (POPC) has also been reported (Williams *et al* , 1990)

Advantages of this procedure include its rapidity, the mild conditions which are gentler to proteins, absence of detergent or other impurity, non-random orientation of the inserted protein, fusion of protein-free and protein-containing vesicles to form large proteoliposomes, which are more representative of biological structures, and a more physiological final lipid-to-protein ratio

The only disadvantage of the direct incorporation method is the more heterogeneous distribution of vesicle sizes obtained in comparison with detergent dialysis or cosonication

EXPERIMENTAL PROCEDURES

The procedure developed by Salach (1979) and refined by Salach and Weyler (1987) is reported to yield the enzyme with highest specific activity, and has consequently become the standard isolation method for this enzyme This isolation procedure was used in this investigation to obtain MAO-B from bovine liver mitochondria

I. MATERIALS

A. Tissues

Livers were obtained from fresh-killed black Angus steers (Carteret Abattoir, Inc , Carteret, NJ)

B. Chemicals and Reagents

All chemicals used were reagent grade where available Phospholipids were obtained from Avanti Polar Lipids, Birmingham, AL, and used as supplied Kynuramine dihydrobromide, pargyline hydrochloride, *Naja naja kaouthia* venom, phospholipase C (from *Clostridium perfringens)*, sucrose, ethylene diamine tetraacetic acid (EDTA), Triton X-100, TRIS (Tris(hydroxymethyl)aminomethane), Folin-Ciocalteu reagent (2N phenol), copper sulfate pentahydrate, sodium potassium tartrate, disodium tartrate, bicinchoninic acid, bovine serum albumin (fraction V) were obtained from Sigma Chemical Co , St Louis, MO, and used without further purification Hepes (4-(2-hydroxethyl)-1-piperazine ethane sulfonic acid) buffer, sodium and potassium phosphates, phosphoric acid were obtained from JT Baker Chemical Co , Phillipsburg, NJ

AG 1-X8 ion exchange resin was obtained from Bio-Rad, Richmond, CA. Q-Sepharose was obtained from Pharmacia Bioprocess Technology in Piscataway, NJ. Octylglucoside was obtained initially from Boehringer Mannheim, Indianapolis, IN, a subsequent lot was obtained from Calbiochem, San Diego, CA. Other detergents used were obtained from Calbiochem. Purity was greater than 99% for octylglucoside, the zwittergent series, and Mega-8, greater than 98% for Mega-9, and 95% for dodecylmaltoside.

Preparation of Kynuramine for the MAO Activity Assay

Kynuramine, the preferred substrate for the MAO activity assay, is commercially available as the dihydrobromide salt. Because of the potential for addition of HBr to the double bond of the unsaturated acyl chains in the phospholipids employed, the substrate was converted to its chloride salt by ion exchange column chromatography using AG 1-X8 resin, (chloride form) 200–400 mesh. The resin has an exchange capacity of 3.2 milliequivalents (mEq) per dry gram or 1.4 mEq per mL of column bed. Aqueous solutions of kynuramine (10 or 50mM) were applied to AG 1-X8 columns (10-14 cm high) prepared in 50mM sodium phosphate buffer, and fractions were eluted with the same buffer. Conversion from the bromide to the chloride form was determined from a standard test for halide ions. On addition of 2 to 3 volumes of chlorine bleach to the sample, followed by extraction with 1 volume of carbon tetrachloride, a brown color in the organic layer is indicative of bromide anion, whereas chloride is colorless. The extinction coefficient for kynuramine was determined to be 4.42 ± 0.03 $mM^{-1}cm^{-1}$ at 358nm.

However, it should be noted that addition to double bonds requires prolonged contact

with a concentrated aqueous solution of HBr (Hickinbottom, 1957), this condition was not fulfilled in that the substrate was added to initiate the enzymatic reaction, so that there was no prolonged contact, and the concentration of substrate in the assay was only 1mM (2mM HBr) Additionally, the substrate was not added to monomeric phospholipid but to vesicle preparations wherein the unsaturated acyl chains were shielded in the bilayer from the rather large bromide anion which, presumably, as has been shown for the iodide ion (Stubbs *et al* , 1976a), is unable to enter the bilayer due to its size Therefore, it is assumed that there was no detriment incurred during activity assay with kynuramine•2HBr

C. Instrumentation

Enzyme assays, absorbance spectra and temperature dependent experiments were performed using a Cary 3E computerized spectrophotometer equipped with a temperature controller (Varian Analytical Instruments, San Fernando, CA) Absorbance measurements for Lowry and BCA protein determinations, and phospholipid-phosphorus assays were performed using an LKB Biochrom Ultrospec 4050 spectrophotometer (Pharmacia LKB Biochrom, Cambridge, UK) Fluorescamine protein assays were performed using a model SFM25 fluorimeter from Kontron Instruments, Milan, Italy A Radiometer (Copenhagen) model PHM82 pH meter with Sigma model E5759 electrode was used to monitor pH during assay of phospholipase A activity, during phospholipase digestion of mitochondria, and for buffer preparation

For enzyme isolation, low speed centrifugations were performed using Sorvall RC2B and Beckman J2-21 centrifuges, high speed centrifugations were performed using Beckman

L8-70 and L5-50B ultracentrifuges A Beckman model 12 microfuge was used for centrifugation during protein assays involving precipitation with trichloracetic acid Density gradients were prepared using a linear gradient maker from BioComp (New Brunswick, Canada) and fractionated with an ISCO Model 185 fractionator A Bausch and Lomb Abbe refractometer was used to determine glycerol content of gradient fractions.

Sonicated vessels were prepared using a Heat Systems W385 cup-horn sonicator from Ultrasonics, Inc (Farmingdale, NY), coupled to a model EN-850 Flow-thru cooler from NESLAB (Portsmouth, NH)

A 28-well Microdialysis System from Bethesda Research Laboratories (Gaithersburg, MD) was used for vesicle preparation by detergent dialysis, dialysis membranes (12-14kD exclusion limit) were obtained from the same source For complete exchange, the dialysis system was agitated using a shaking platform (Wheaton Industries, NJ).

Electrophoresis was performed using a model SE250 dual gel vertical slab apparatus (Hoefer Scientific Instruments, San Francisco, CA) A Hewlett Packard Scan Jet IIcx was used to scan a photograph of the electrophoresis gel. Densitometry was performed on the gel scan using QGEL-1D (version 1 72) software from QuantiGel Corp., Kendrick Laboratories, Inc , Madison, WI

A Con-Torque motor was used for homogenization of samples in the isolation procedure (Eberbach Corp , Ann Arbor, MI)

II. METHODS

A. Analytical Methods

1. Protein assays:

Three types of protein assays were employed. The method of Lowry *et al* (1951), the bicinchoninic acid (BCA) assay (for glycerol gradient fractions, Smith *et al*., 1985) and the fluorescamine assay (Bohlen *et al*., 1973). Bovine serum albumin (BSA) standards were used throughout. Due to the tendency of MAO-B to aggregate, solubilization of protein and any associated lipid was achieved with addition of sodium dodecyl sulfate (SDS) to ensure accurate sampling (Lees and Paxman, 1972). The concentration of SDS used in the samples and in the assay standards was 0.1% (w/v) final. Samples were assayed in triplicate, except for the BCA method, where quadruplicate assays were employed.

For most routine protein determinations, the Lowry method was used. Reagent A contained 1% SDS in order to maintain the protein in a disaggregated state (Dulley and Grieve, 1975). During the enzyme isolation procedure, rapid adjustments of protein concentration was necessary. The Schaechter and Pollack (1973) modification of the standard Lowry procedure shortened the time course of the assay by incubating at 55°C for 5 minutes instead of the usual 20-30 minutes at room temperature. However, for low protein concentrations routinely obtained for purified enzyme preparations, microestimation (1.14 mL total volume) by the Lowry method entailed smaller volumes (40µL) of sample (or standard) and reagents, together with longer incubation (45 minutes) at room temperature.

Due to interference of the Lowry method by the presence of glycerol, phospholipid, and Hepes (all used in the reconstitution process), the BCA microassay was adopted. While also susceptible to interference by lipid, protein precipitation with trichloroacetic acid (TCA) using ribonucleic acid (RNA) as co-precipitant (Polacheck and Carib, 1981) prior to BCA

assay eliminated this problem. The presence of primary amines also interfered in the Bradford and fluorescamine assays

2. Phospholipid-Phosphorus Assay:

For determination of phosphorus, aliquots of the glycerol gradient fractions were first digested by the method described in Scotto and Zakim (1985) prior to assay using nitric and perchloric acids; this procedure released the lipid phosphorus as inorganic phosphate. The resultant orthophosphate was measured using the Dittmer and Wells (1969) colorimetric microassay which entails conversion of orthophosphate to phosphomolybdic acid in the presence of perchloric acid, and then reduction with the Fiske-Subbarow reagent (1-amino-2-naphthol-sulfonic acid) to yield a blue coloration. The intensity of the blue color was determined at 830nm, and is proportional to phosphorus concentration. Standards were prepared from sodium phosphate (0-60 nmoles)

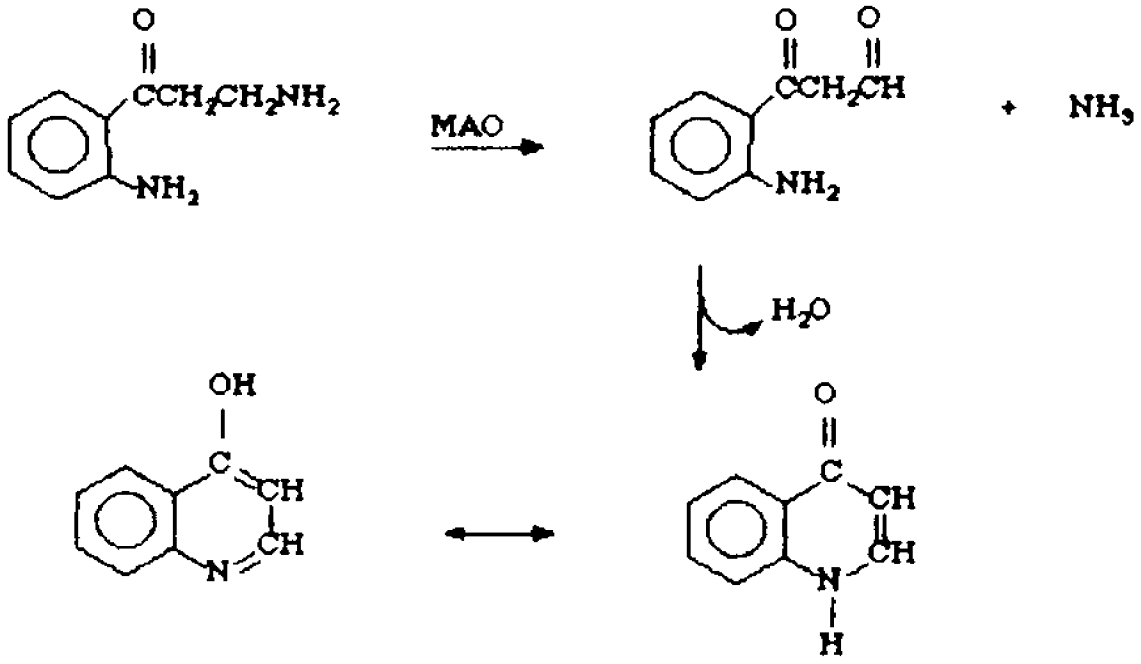
3. Enzyme Assay:

MAO-B activity was assayed spectrophotometrically by following the oxidation of kynuramine to 4-hydroxyquinoline at 314 nm using a modification of the method described by Weissbach *et al* (1960) where disappearance of kynuramine at 360nm is monitored. The molar extinction coefficient for 4-hydroxyquinoline (ϵ_{314}) is 12.3 $mM^{-1}cm^{-1}$ (Salach and Weyler, 1987). Activity is expressed in terms of nmoles of product per minute per mg protein. The assay temperature was 30°C unless otherwise noted. The assay buffer used was either 50mM sodium phosphate (pH 7.2) (NaP), or 50mM Hepes (pH 7.4) containing 100mM KCl

(HK) Triton X-100 (0 2%) was included in the buffer where noted The substrate concentration was 1mM, unless otherwise noted The reaction on which the enzyme assay is based is shown below

Figure 3

MAO-Catalyzed Oxidation of Kynuramine to 4-Hydroxyquinoline



In the Beer-Lambert relationship

$$A = \epsilon c l$$

where A is absorbance, ϵ is the molar extinction coefficient of the species being measured (in this case 4-hydroxyquinoline), c is its concentration, and l is the length of the light path (here 1cm) Experimentally the change in absorbance, measured at 314nm, with time is directly proportional to the rate of product formation $v=\Delta A/\Delta t=P$ For a unit time interval of one minute, (Δt = 1min), the change in product concentration (Δc) can be determined using a

rearrangement of the Beer-Lambert relationship

$$\Delta c = \Delta A/\epsilon l = slope/\epsilon l = slope/(12.3 mM^{-1}cm^{-1})(1cm) = (slope/12.3)mM$$

$$\Delta c = (slope/0.0123)\mu M = (slope/0.0123)\mu mol/L = (slope/0.0123)nmol/mL$$

Multiplying by the assay volume (*e.g.*, 1mL) gives the assay rate

$$1mL\ assay\ vol \times (slope/0.0123)nmol/mL = \#nmoles/min$$

Dividing by the amount of protein used in the enzyme assay gives the rate per mg of protein Simply expressed

$$(slope)(assay\ vol)/(0.0123 nmol\ mL^{-1})(\#mg\ protein) = \#nmol\ min^{-1}/mg\ protein$$

For example, typically 50µL of *purified* MAO-B enzyme isolate, containing 21.8µg of protein, was added to a cuvette containing 200µL of 10mM kynuramine and 750µL NaP buffer, where the initial (t=0 mins) substrate concentration is 1mM and the final assay volume is 2.0mL, a slope of 0.084 absorbance units min^{-1} is typically measured

$$(0.084 min^{-1})(2.0mL)/(0.0123 nmol\ mL^{-1})(0.0218mg\ protein) = 627nmol\ min^{-1}/mg\ protein$$

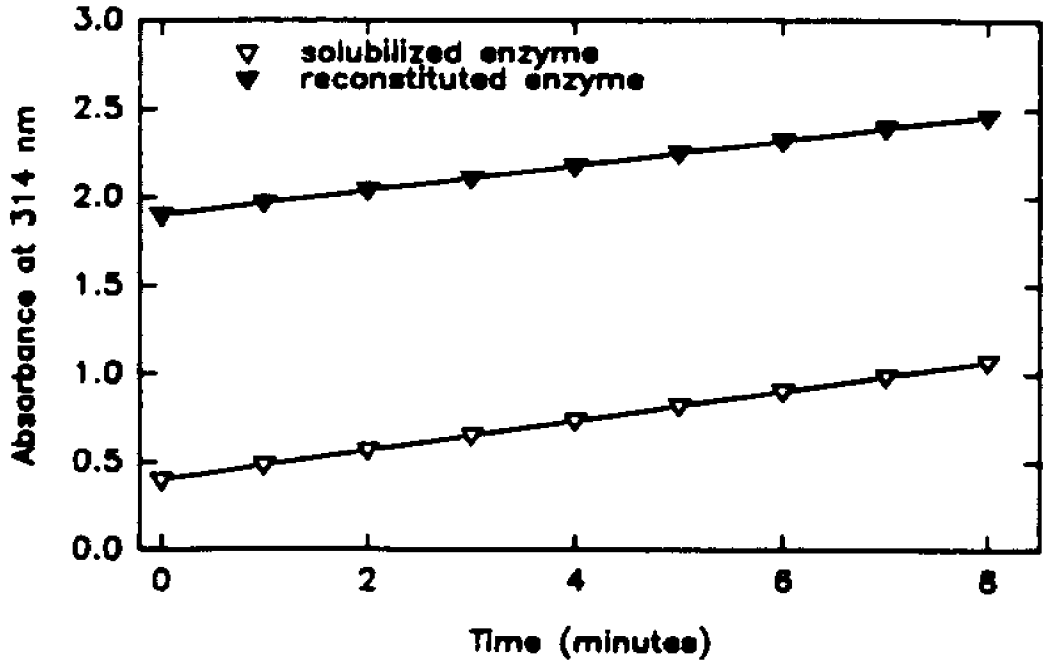
For proteoliposomes resulting from reconstitution of an aliquot of the same isolate into 70%DOPC/30%DOPE LUVs vesicles by rapid detergent dialysis typical assay conditions were as follows Vesicle suspension (250µL) containing 26.8µg of protein was added to a cuvette containing 10mM kynuramine (200µL) and NaP buffer (1.5 mL) The substrate concentration was 1mM in the 2.0mL final volume A mean slope of 0.07min^{-1} was routinely obtained

$$(0.07 min^{-1})(2.0mL)/(0.0123 nmol\ mL^{-1})(0.0268mg\ protein) = 425nmol\ min^{-1}/mg\ protein$$

A typical assay plot of absorbance (314nm) versus time, is shown below for these two assays

Figure 4

Representative MAO-B Assays



B. Isolation of Phospholipase A

Phospholipase A (required for susequent isolation of MAO-B) was prepared from the venom of the Thailand cobra, *Naja naja kaouthia*, according to the procedure of Weyler and Salach (1985). A solution of 1.0 g of dessicated venom in 10 mL deionized water was adjusted to pH 3.5 with 0.5M H_2SO_4. This was heated on a hot plate until boiling, and allowed to remain at 100°C for 10 minutes. After cooling at room temperature, the cloudy yellow-green sample was centrifuged at 150,000g for 1 hour at 4°C. The resultant pale opalescent yellow supernatant was adjusted to 50 mM Tris by addition of 1M Tris base (adjusted to pH 7.55 with 3M NH_4OH) and centrifuged as before. The clear pale yellow

supernatant was applied to a BioGel P30 column, equilibrated with 50 mM Tris HCl, pH 7 6, and eluted with the same buffer Fractions (3 6 mL each) were collected using an LKB 2112 RediRac fraction collector, and screened for protein by their absorbance at 280 nm Eluates were stored at -18°C and are stable for about 2 months

Phospholipase A activity assay was performed as described in Salach *et al* (1971) The reaction mixture consisted of 0 5 mM Tris, 0 5% Triton X-100, 2 mM $CaCl_2$, 5 8 mM NaCl and 0 71 mM EDTA at pH 8 0 To a thermostatted vessel at 25°C, 1 94 mL of reaction mixture was maintained under a slow stream of N_2 (to prevent CO_2 absorption), 3 mg of egg yolk lecithin in 50 µL ethanol was added to the mixture immediately before adding 10 mL enzyme The change in pH was monitored One unit of activity was defined as the change of 1 0 pH unit per 30 minutes (0 033 pH/min) Fractions from the Biogel P30 column, with the highest A_{280} were tested for activity

Lowry assays were carried out on the tested fractions, and activity expressed as Units/mg protein The average phospholipase A_2 activity of the active fractions (13 -29) was 9233 Units/mL or 2755 U/mg protein

C. Isolation of Monoamine Oxidase:

Monoamine oxidase type B (MAO-B) was isolated from the mitochondria of bovine liver by the method by Salach and Weyler (1987), involving a three stage purification

- preparation of mitochondria from the liver
- isolation of MAO-B in a partially purified state, and
- final purification of MAO-B by sucrose density gradient centrifugation

The isolation procedure is detailed below

1. Preparation of Mitochondria from Bovine Liver

The following reagents were used in isolating the mitochondria

Solution 1 0.25M sucrose in 10mM potassium phosphate, pH 7.0-7.2 at 0°C

Solution 2 Solution 1 containing 0.5mM EDTA

Solution 3 0.15M KCl in 10mM Tris-phosphate, pH 7.0-7.2 at 0°C

All procedures were performed at 4°C unless otherwise noted

Fresh liver was sectioned into lobes, wrapped in plastic bags and packed in ice for transport from the abattoir. In the laboratory, the liver was cut into small pieces with a razor knife and washed with several changes of solution 2 to remove blood. The tissue was ground using a food processor in 500 gram aliquots. One liter of solution 2 was added and the liver suspension or "soup" stirred. This was processed again through the food grinder, and then filtered through a single layer of cheesecloth, forcing as much tissue through the cheesecloth as possible. The liver residue was scraped from the cheesecloth, added to 200mL of solution 2 and reprocessed.

The resultant liver suspension was homogenized with two vertical passes in a 200ml. glass/Teflon homogenizer using a Con-Torque motor. About 80ml of the suspension was diluted to 200mL with solution 2 for each homogenization. Each liter of homogenate was diluted to 3 liters with the same buffer, then centrifuged at 1600rpm (400g) for 20 minutes using 3 liter capacity rotors (six 500mL bottles) in either a Sorvall or Beckman refrigerated centrifuge. The tan supernatant (S1) was decanted into a 6 liter flask through 2 layers of

cheesecloth and the red pellets discarded The supernatant was recentrifuged at 8000rpm (10,800g) for 20 minutes and the red-tan supernatant, containing the majority of the microsomes, was discarded and the mitochondrial pellets (P2) saved These pellets were resuspended in solution 1, rehomogenized and diluted to 10 liters per 3kg of liver with solution 1

The diluted mitochondria were centrifuged at 7500rpm (9500g) for 20 minutes and the tan supernatant containing microsomes and some mitochondria was discarded The pellets (P3) were resuspended in solution 3, rehomogenized, and diluted to 1200mL per 6kg liver with solution 3 The mitochondria were pelleted again at 8500rpm (11,700g) for 25 minutes in six 250ml bottles using a GSA rotor The tan supernatant containing the remaining microsomes and additional mitochondria was discarded The tan mitochondrial pellets (P4) were slurried in the residual buffer and the centrifuge bottles were frozen at an angle at -18°C

2. Isolation of MAO-B in a Partially-Purified State

Partially pure MAO-B was obtained from bovine liver mitochondria by a three step process which involved phospholipase digestion of the mitochondrial membrane, detergent solubilization of membrane protein, and polymer partitioning to isolate the enzyme The isolation procedure was carried out over a three day period

Day 1 One or more bottles of mitochondria (P4) were allowed to defrost in an ice bath in the cold room The thawed mitochondria were suspended in cold distilled water and thoroughly

homogenized with a motor-driven glass/Teflon homogenizer The homogenate was diluted 2 5-fold and centrifuged at 41,000g (23,000 rpm in a Ti45 rotor) for 15 minutes in a Beckman ultracentrifuge at 4°C

The following steps were carried out at room temperature unless otherwise noted The washed and lysed mitochondria (P5) were suspended in 0 1M triethanolamine (TEA), pH 7 2, and thoroughly homogenized as before Protein was determined by the Schaechter and Pollack (1973) modification of the Lowry method, and its concentration adjusted to 30mg/mL with the same TEA buffer

Phospholipase digestion was conducted at 30°C, using either a water-jacketed vessel or an Erlenmeyer flask located in a temperature-regulated water bath, and the contents mixed using a magnetic stirrer The protein suspension was adjusted to 25mM calcium chloride by addition of an appropriate volume of 1M $CaCl_2$ For each 500mg of protein, 670 units of phospholipase A and 1 0mg of Phospholipase C (8 units/500mg protein) were added The mitochondria were digested for one hour with stirring The acid produced from phospholipase treatment was offset by addition of 2M ammonia to maintain a pH of 7 3

The digested membranes were centrifuged for 15 minutes at 41,000g as before The pellet (P6) was thoroughly homogenized in TEA buffer Protein concentration was determined and adjusted to 15mg/mL Detergent solubilization with Triton X-100 (Tx) was accomplished using 1mg Tx for each 3mg of protein Dropwise addition of 2 5mL of 20% Tx per 100ml of P6 homogenate on a magnetic stirplate was followed by an additional 25 minutes of stirring The mixture was centrifuged at 41,000g for 15 minutes as before The golden yellow supernatant (S7) containing the Triton extract was packed in ice and stored in

the cold room overnight

Day 2 Initially, the polymers for the polymer partitioning step were suspended in TEA buffer shortly before addition to the Triton extract However, polymer hydration did not appear to be complete without prolonged mixing with buffer, and incomplete hydration might interfere with MAO recovery in the partitioning step To improve the hydration process, a stock suspension of polymers and water in TEA was prepared up to two days before use with the detergent extract This stock was based on a theoretical maximum volume for the Triton extract, overestimated to ensure an adequate amount of polymer stock would be prepared For each mL of the presumed extract volume, 0 11g dextran (M_n = 250K), 0 12g Ficoll (M_n = 400K), 0 08g polyethylene glycol (M_n = 8K) and 0 19mL distilled water were gradually added to the TEA buffer The volume of TEA used was calculated by assuming approximately 5mg Tx per mL of detergent extract and adding an amount of TEA that would reduce the Tx concentration in the extract to 3mg/mL, *i.e.*, by adding a volume of TEA that was two-thirds the presumed Triton extract volume Complete suspension of each aliquot of polymer was achieved by vigorous mixing on a magnetic stirrer before successive additions were made A fully hydrated polymer stock took (typically) up to 36 hours to prepare The stock volume was measured; the amount used was that fraction of the total equal to the actual volume of Tx extract divided by the theoretical volume used to prepare the polymer stock

The Triton extract was removed from the ice, brought to room temperature by immersing in a 25°C water bath, and its volume ascertained The appropriate volume of polymer suspension was measured, the Triton extract was added, and the mixture stirred on

a magnetic stirplate for 30 minutes The resulting emulsion was poured into 250mL volume centrifuge bottles, allowed to equilibrate for 20 to 30 minutes, and centrifuged at -20°C in an HS4 swinging bucket rotor in a Sorvall centrifuge at 6500rpm (8100g) for 30 minutes, the run was ended without the brake

The upper (yellow) and lower (almost colorless) clear phases were removed as completely as possible, retaining the beige-to-white interfacial solids The liquid phases were recentrifuged to maximize recovery of the solid material From this point, all operations were conducted at 4°C The interfacial solids were suspended in TEA buffer and homogenized thoroughly using a motor-driven glass/Teflon homogenizer Solids obtained from the recovery procedure were either suspended in buffer and homogenized with the initial interfacial material or processed separately Protein content was determined and adjusted to 10mg per ml with TEA buffer The homogenate was centrifuged at 41,000g (23,000 rpm in Ti45 rotor) for 20 minutes in a Beckman ultracentrifuge The pale yellow supernatant (S9) was packed in ice and stored in the cold room overnight

Day 3 The supernatant (S9) was centrifuged at 41,000g (23,000rpm in Ti45 rotor) The resulting clear faint yellow supernatant (S10) was recentrifuged at 252,000g (44,000 rpm in Ti45 rotor) for 2 hours The supernatant was removed as completely as possible The golden to yellow-brown pellets (P11) containing the MAO-B were collected with a minimal volume of 50mM sodium phosphate buffer (pH 7 2) containing glycerol (50%, w/v) The pellets were suspended by homogenization with a glass/Teflon vessel *At this point, the enzyme was partially pure* For some experiments, *partially purified* MAO-B was used although further

purification required sucrose density gradient centrifugation

The *partially purified* preparation was either applied to the gradient for further purification, or stored at -18°C until further purification was required, or alternatively was used for reconstitution experiments, as discussed below

3. Purification by Sucrose Gradient

Sucrose solutions were prepared as weight-to-volume percent in TEA buffer, 50mM final, pH 8 One tenth the final solution volume of 0 5M TEA, pH 8 0, was added to the appropriate amount of sucrose and the final volume made up with distilled water Discontinuous sucrose density gradients were prepared in tubes for the SW28 rotor, using 5 8mL per part One part each of 60, 55, and 50 % (w/v), two parts 45% (w/v), and one part 35% (w/v) sucrose were layered The tubes were marked to indicate each density step The gradients were cooled to 4°C

Partially purified MAO-B, either directly isolated or thawed after storage, was diluted to 4 6mL with 50mM TEA pH 8, and applied to the top of the gradient The gradients were centrifuged without the brake in a Beckman ultracentrifuge at 107,000g (27,000 rpm in a SW28 rotor) at 4°C overnight (ranging from 17 to almost 22 hours) The developed gradients had either one or two yellow bands containing flavoenzyme Where there was a single band, it was broad and generally occupied about 10mm in the middle of the 45% (w/v) concentration Where there were two bands, the upper one was located in the 45% (w/v) layer, beginning 1 to 5mm below the 35% (w/v) step The lower band was at the 45-50% (w/v) interface The bands were harvested by removing the solution from above using a

pasteur pipet or in the case of 2 bands, were harvested separately, and then combined for the next stage.

The harvested bands were diluted with 50mM TEA buffer (pH 7.2) and then centrifuged at 252,000g (62,000 rpm in Type 65 rotor) for 90 minutes at 4°C. The golden yellow pellets (P13) containing MAO-B were collected by addition of 50mM sodium phosphate buffer (pH 7.2) with 50% (w/v) glycerol, as before, and suspended by homogenization with a glass/Teflon vessel. The suspension of *purified* MAO-B was distributed over several tubes and stored under argon or nitrogen at -18°C.

D. Reconstitution Procedures:

1. Preparation of Small Unilamellar Vesicles:

Small unilamellar vesicles (SUVs) were prepared by a sonication procedure essentially according to the method of Eytan *et al.* (1976), as modified by Scotto and Zakim (1985). Generally, 1mL of a stock solution of dioleoylphosphatidylcholine (DOPC, 20mg/mL in chloroform), with or without other lipid, was dried *in vacuo*. Lipid was suspended at 10mg/ml in 50mM Hepes/100mM KCl (HK) buffer, pH 7.4, and subjected to five cycles of freezing and thawing (using an isopropyl alcohol bath, temperature nominally -89.5°C). The sample was transferred to a sonication tube, flushed with nitrogen, closed with a silicone stopper, and sealed with stretch (teflon) tape. A Heat Systems-Ultrasonic, Inc. model W-385 cup-horn sonicator was used at 50% duty cycle with microtip limit at 10, the system was water-cooled to 4 to 10°C. The sample was subjected either to continuous sonication for 10 to 15 minutes, or to 5 second pulses, until clarity was achieved (up to 45 minutes). The sample was not

centrifuged following sonication and, consequently, while consisting predominantly of small unilamellar vesicles contained possible contamination of multilamellar vesicles (Barenholz *et al*., 1977) The expected size range of the SUVs is 20 to 30 nm in diameter (Huang, 1969)

2. Reconstitution by Spontaneous Incorporation:

Reconstitution was effected by mixing *partially purified* enzyme with preformed SUVs and incubating for approximately one hour at 0°C with occasional swirling The lipid to protein weight ratio was approximately 25 1 (corresponding molar ratio of ~3740 1 based on the molecular weight mass of the MAO-B dimer (117 6kDa)) in the final 1 mL volume of sample Based on published data for the number of phospholipid molecules per 25nm radius vesicle ($3\ 5x10^5$) and the amount of protein added (72 5µg), a maximum incorporation rate of approximately 1 3 MAO-B dimers per radius vesicle can be calculated (Cullis and Hope, 1991) A control consisting of the same amount of MAO-B diluted to the same final volume in HK buffer, was incubated similarly

Conditions used for reconstituting isolated MAO-B into phospholipid vesicles varied somewhat from experiment-to-experiment and hence, specific details are described with the individual experiments in the Experimental Results section, below

3. Reconstitution by Facilitated Incorporation

560µg portions of *purified* MAO-B (preparation MAO-3a) were solubilized with either 0 5mg or 2 0mg octylglucoside (OG) in HK (200µL final volume) in an ice bath (OG to protein dimer molar ratios of 358 and 1431, respectively), corresponding to OG

concentrations for solubilizing the enzyme of 8 5mM and 34 2mM (or 0 25% and 1 0%), respectively After 30 minutes, the solubilized enzyme was added dropwise to 900µL of preformed DOPC SUVs on ice, and stirred for a further 30 minutes resulting in a lipid to protein dimer molar ratio of about 2400 1 At this point, the OG concentration was now 1 5mM or 6 2mM (or 0 045% and 0 18%), respectively, (OG to lipid molar ratio of 0 05 or 0 22, respectively) and determined not detrimental to enzyme activity Aliquots (100µL) were reserved for activity assays, and the remainder layered on 10-80% glycerol gradients and centrifuged at ~ 107,000g for 89 hours The gradients were fractionated Each fraction (250µl aliquot) was dialyzed against sodium phosphate buffer (50mM, pH 7 2) to remove glycerol for susequent activity assaying Protein and phospholipid-phosphorus assays were performed on all fractions

4. Reconstitution by Detergent Dialysis with Octylglucoside

In contrast to the procedures described above, detergent dialysis reconstitution does not utilize preformed vesicles. Reconstitution of MAO-B using octylglucoside dialysis, was a three step process.

First, an aliquot of thawed enzyme (600µg) was disaggregated in 256mM OG (15mg OG in a final volume of 200µL) Second, the solubilized enzyme was mixed with phospholipid at a lipid to protein weight ratio of 25 1 DOPC (15mg) was dissolved/suspended with either 7 5 or 15mg OG in one ml HK buffer Final concentrations in the mixture were thus 600µg protein, 15mg DOPC, and either 22 5 or 30mg OG (64 or 85mM) (OG_{cmc} = 25 mM)) in a total volume of 1 2mL Third, the resultant MAO-B/lipid/OG mixture was dialyzed to

produce proteoliposomes Initially, samples were placed in stoppered dialysis tubes from which aliquots could be removed readily for assay Samples were dialyzed against several changes of HK buffer, with the final dialysis being overnight, producing proteoliposomes (average size 140nm). Often, aliquots were reserved for enzyme stability assays, while the balance was applied to glycerol gradients and centrifuged at ~107,000g for 86 to 92 hours

Again, following gradient fractionation, an aliquot of each fraction (250µL) was dialyzed against sodium phosphate buffer to remove contaminating glycerol Dialyzed fractions were adjusted to the same volume prior to activity assay Protein and phospholipid-phosphorus assays were done on undialyzed fractions

Solubilization of DOPC in octylglucoside was examined with the aim of clarifying and hence fully solubilizing the DOPC/OG mixture A suspension of 15mg DOPC with detergent in 4 8mL HK buffer was stirred at 4°C, solid OG was added until the solution became clear At this point, the OG:DOPC weight ratio was 3 1, with OG present at 32mM This OG to lipid weight ratio was used in all subsequent detergent dialysis reconstitutions

Subsequently, a 28-well multiple sample dialysis apparatus was examined with a view to providing a uniform rate of dialysis for all samples in a single experiment, as well as providing a uniform surface for dialysis in order to equalize adsorption losses and control the dialysis rate In this procedure, the samples are dialyzed against 3 changes of buffer, the buffer is recirculated so that all samples are exposed to the same amount of detergent throughout the period of dialysis

For the test run of this apparatus, approximately 500µg of *partially purified* MAO-B was solubilized with 12 5mg OG (43mM) in a volume of one mL To this, 4mL of the

DOPC/OG solution (3 1 weight ratio) was added and mixed (final OG was 41mM) One mL aliquots were pipetted into each of four well-separated compartments in a 28-well dialysis apparatus The apparatus was set on a shaking platform set at 150rpm Recirculating HK buffer was used to make the dialysis equivalent for all positions, so that the first well would not always be exposed to fresh buffer, with subsequent wells always exposed to buffer with ever-increasing detergent concentration With recirculation, after the first pass, the first well is always exposed to detergent-containing buffer Dialysis was accomplished with three one liter changes of the standard HK buffer, the middle one running overnight After 2 hours of dialyzing, the MAO-B/DOPC/OG mixture was cloudy, and even cloudier after overnight indicating vesicle formation Following dialysis, volumes of the four samples were normalized at 1 25mL Activity assays were run on samples from positions 1, 14 and 28 As seen in Table 7 below, DOPC proteoliposomes formed upon dialysis in well-separated compartments of this apparatus did not show any significant differences in MAO-B activity Consequently, this protocol was adopted For reconstitution experiments involving several different lipid systems, the first and last well contained 100% DOPC Subsequently, the protocol was modified to reduce exposure of the enzyme to OG, after it was found that enzymatic activity declined with length of exposure to this detergent (see Results). Solubilization of MAO-B by OG was limited to 1 minute For more rapid removal of the detergent from the protein-lipid-detergent mixture, dialysis was initially performed excluding the recirculating buffer step approximately 10 liters of HK buffer was passed through the system and discarded; then, ~4 liters HK was recirculated at approximately 40ml per minute overnight; finally, ~2L of fresh buffer was recirculated before removing the vesicles from the dialysis apparatus This

Table 7

Activity of Proteoliposomes Prepared in Multi-Sample Dialysis Apparatus

Well Number	nmol min^{-1}/mg protein (±SD)
1	238±12
14	248±4
28	236±2

protocol was adopted to prepare proteoliposomes used for study of the effect of different lipid headgroups and acyl chains, and for the kinetics studies

E. Characterization Procedures

1. Glycerol Density Gradients

Lipid vesicles and proteoliposomes were separated from unincorporated protein by centrifugation on glycerol gradients Gradients used were 10-80% (w/v) glycerol in 50mM Hepes/100mM KCl, unless otherwise noted Generally, the gradients were prepared over a 0 5mL cushion of Fluorinert, a high density mixture of fluorinated hydrocarbons, used to facilitate recovery of protein driven to the bottom of the gradient The gradients were centrifuged at 40,000 rpm in a Beckman SW41 rotor (approximately 107,000g) at 4°C for periods from 15 hours to 4 days An ISCO model 185 density gradient fractionator was used to collect either 1mL or 0 5mL fractions from the top of the gradients Glycerol content was determined from the refractive indices of the gradient fractions

2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS/β-mercaptoethanol mixture (final concentrations were approximately 1% w/v for SDS and 5% v/v for β-mercaptoethanol) was added to protein samples (pI = 4.5-5, Daya *et al*., 1992) and boiled for about 5 minutes. After cooling, bromophenol blue (0.001% w/v final) was added as a tracking dye, and 10µl aliquots of sample containing ~10µg protein were applied to a vertical slab gel of 10% (w/v) acrylamide-bisacrylamide (1:1). The electrophoresis buffer was Tris-glycine, pH 8.3. The gel was electrophoresed at constant current (18-20 milliamps) until the tracking dye approached the bottom of the gel. The protein bands were stained with a 0.1% solution of Coomassie brilliant blue in 50% methanol/10% acetic acid. The gels were destained with 5% methanol/7.5% acetic acid and the positions of the bands were noted. Gels were dried on blotting paper and covered with plastic film. The gel was photographed, the photograph scanned, and densitometry was performed on the scan in order to estimate the percentage of MAO-B in each protein sample.

3. Electron Microscopy

Electron micrographs of liposomes and proteoliposomes were prepared by Ms. Lee Cohen-Gould of the Cell Biology Department of Cornell University Medical College. Vesicles, adhered to glow-discharged carbon film grids, were negatively stained with 5% uranyl acetate (Lewis and Knight, 1977). A magnification of 36,000 was used for the micrographs. Further enlargements are indicated on the figures.

F. Kinetics

1. Temperature Dependence of Kynuramine Oxidation

The oxidation of kynuramine by MAO-B reconstituted by detergent dialysis against 50mM Hepes 100mM KCl, pH 7 4 buffer, was determined over a temperature range of 15 to 45°C in 5° intervals Aliquots (250µL) of the proteoliposomes (70% DOPC/30% DOPE LUVs in 50 mM Hepes/100mM KCl, containing 26 78µg protein) were used Substrate (11 07mM) was prepared in the assay buffer (50mM sodium phosphate, pH 7 2) 181µL of substrate and buffer (1 57mL) were mixed in cuvettes ([S]=1mM in the final volume) with stirring magnets and allowed to equilibrate at each temperature The enzyme sample (250µL) was brought to temperature in a separate water bath and added to the substrate to start the reaction Triplicate assays were performed at each temperature Corresponding temperature controls, in which buffer was substituted for the reconstituted enzyme were run

From the Arrhenius equation,

$$k = Ae^{-E/RT}$$

where k is the rate constant for the reaction, A is a frequency factor related to the frequency of collisions between the reactants and the probability of a reaction occurring, E is the activation energy, R is the gas constant (8 314 J mol^{-1} K^{-1}), and T is the absolute temperature Logarithmic linearization of this expression yields

$$\log k = (-E/(2\ 303R))(T^{-1}) + \log A$$

From a plot of log k versus reciprocal temperature, the slope yields estimates of the activation energy Experimentally, the rate of a reaction may be determined directly from reaction velocities (Dixon and Webb, 1958). Hence for kynuramine oxidation, by reconstituted MAO-B, the Arrhenius plot may be reexpressed as

$$\log v = \log (\Delta A_{314}/\Delta t) = (-E/(2.303R))(T^{-1}) + \log A$$

2. Determination of K_M for Kynuramine with Solubilized MAO-B

Assays were conducted at 30°C in air ($[O_2]$ ~0 236mM according to Ramsay, 1991) for a series of kynuramine concentrations, ranging from 20μM to 2mM, using non-reconstituted *purified* MAO-B in 50mM sodium phosphate, pH 7.2, containing 0 2% Triton X-100, added to maintain the enzyme in a disaggregated state The assay volume was 1 05mL Substrate solutions were prepared in the assay buffer at 1 05 times their final concentrations Aliquots of the substrate (1mL) were added to temperature equilibrated cuvettes containing stirring magnets The stock enzyme solution was diluted 10-fold with assay buffer and pre-warmed Aliquots (50μL, containing 21 8μg protein) were added to initiate the reaction (corresponding to 20 76μg/mL final protein) For controls, buffer was substituted for enzyme

The rates of the enzyme catalyzed reactions were determined from the slope of increasing absorbance at 314nm (A_{314}), corresponding to appearance of product with time Calculated velocities were plotted against substrate concentrations (v *vs* [S]) from which a linear substrate range was ascertained

The Michaelis-Menten constant (K_M) and maximal velocity (V_{max}) were determined for the solubilized enzyme, using double-reciprocal straight-line Lineweaver-Burk plots of the inital velocity of the catalyzed reaction, as a function of substrate concentration (v_0^{-1} *vs* $[S]^{-1}$)

$$1/v_0 = K_M/V_{max}(1/[S]) + 1/V_{max}$$

Here, a narrower range of kynuramine concentrations was adopted (0 03 to 0 19mM final), corresponding to the linear portion of the (v *vs* [S]) plot

3. Determination of K_M for Reconstituted MAO-B, Using Kynuramine

A series of kynuramine solutions were prepared in standard phosphate assay buffer (*i.e.*, without Triton X-100) at 0.037mM to 0.225mM. The assay volume was 2.25mL. Aliquots (2.0mL) of each substrate solution were equilibrated in cuvettes at 30°C (fully aerated) with stirring. *Purified* MAO-B reconstituted with 70% DOPC/30% DOPE LUVs in Hepes/KCl buffer, was prewarmed to 30°C in a separate water bath. Aliquots (250µL) of reconstituted enzyme (26.8µg protein) were added to the cuvette to initiate the reaction (11.9µg protein/mL final). Six kynuramine concentrations were assayed simultaneously, using the multiple sample capacity of the Cary 3E spectrophotometer. The set was then repeated. Controls, in which buffer was substituted for enzyme were run for each substrate concentration.

As described above, initial reaction rates were calculated from the linear slopes obtained from the change in A_{314} *vs* time. The Michaelis-Menten (K_M) constant and maximal velocity (max) for the reconstituted enzyme, were determined from Lineweaver-Burk plots.

4. Inhibition of Solubilized MAO-B by Pargyline

The time course for pargyline inhibition of kynuramine oxidation by solubilized *purified* MAO-B was examined. Standard phosphate solution with 0.2% Tx-100 added was the assay buffer and this was used as the diluent for both substrate and inhibitor. All solutions were equilibrated at 30°C. Cuvettes with stirring magnets were used for the incubations. Enzyme (0.4 nmoles) was incubated with 0.4µM pargyline. At various time points ranging from 0-60 minutes, 100µL of this mixture was removed and transferred to cuvettes containing

1 4mL of 1 07mM kynuramine equilibrated at the assay temperature (30°C) and a final pargyline concentration of $2x10^{-4}$M Each assay contained 26 5µg protein Controls, in which buffer was substituted for the pargyline solution, were run in order to correct for time-dependent denaturation of the enzyme From plots of time versus percentage inhibition by pargyline, it was found that a 20 minute incubation of enzyme with inhibitor, resulted in about 40% inhibition of solubilized MAO-B activity All subsequent inhibition studies were thus carried out under these timed conditions

Enzyme inhibition curves, using pargyline, were performed using three separate *purified* solubilized MAO-B isolates As above, enzyme and inhibitor were incubated for 20 minutes, at 30°C in cuvettes with stirring magnets, the incubation volume was 100µL Enzyme concentration varied with isolate and a range of 17 4 to 37 0µg protein per assay was used (volume = 1 5 mLs) Pargyline concentrations (10 in total) ranging from 0 03 to 3 0µM weres used for the incubation of enzyme with inhibitor After incubation, 1 4mL of 1 07mM kynuramine was added to initiate the reaction, resulting in a 15-fold dilution of the pargyline concentration The rate of reaction was followed from the change in absorbance at 314nm per unit time, as described above Dose response curves were constructed from the data, as percent inhibition (%I) versus log([pargyline]) The final protein concentration in the activity assay was 8 72μg/mL with the isolate which was also used for examining the effect of pargyline on reconstituted enzyme Controls were performed by substituting the assay buffer for pargyline

5. Inhibition of Reconstituted MAO-B by Pargyline

The effect of pargyline on the oxidation of kynuramine by reconstituted *purified* MAO-B in 70mole% DOPC/30mole% DOPE LUVs in Hepes/KCl was examined Using standard phosphate buffer and a final substrate concentration of 1mM, a range ($5x10^{-8}$ to $5x10^{-6}$ mM) of pargyline concentrations prepared in the assay buffer were studied

Prewarmed (30°C) proteoliposomes (250µL) were incubated with 25µL of prewarmed pargyline (or buffer, for controls) in cuvettes equipped with stirring magnets for 20 minutes, at 30°C Addition of 1 083mM substrate (3 3mL) initiated the reaction, simultaneously diluting the inhibitor concentration 13-fold in a final volume of 3.575mL. The final protein concentration in the assay was 7 49µg/mL Four pargyline concentrations together with 2 controls were run simultaneously

Rates of enzyme catalyzed reaction were determined from the slopes of the change in absorbance at 314nm (ΔA_{314}) *versus* time (minutes) A dose-response curve of percent inhibition (%I) of MAO-B versus log [pargyline] was constructed

EXPERIMENTAL RESULTS

I. PURIFICATION OF MONOAMINE OXIDASE

Purification of MAO-B was classified as either *partially purified* or *purified*. The latter classification involved a further high-speed centrifugation. Final specific activities for the various enzyme preparations varied (Table 8), and, in general, higher activity preparations were achieved using maximally hydrated polymers (see Methods C.2 Day 2). The purification table for preparation number MAO-8 is shown in Table 9, and represents one of the more typically active isolates.

A. Partially Purified Enzyme

The pellet (P11) resulting from the high-speed centrifugation following the polymer partitioning step was termed *partially purified*. At this stage of preparation, the enzyme is not highly pure, as can be seen from the gel electrophoresis (described below). The *partially purified* preparation was used routinely for establishing methodology in early reconstitution experiments. The sedimentation profile versus the activity of *partially purified* enzyme (MAO-2) was determined from a 10-80% (w/v) glycerol gradient (Figure 5). For assay, aliquots (0.25mL) of the gradient fractions were dialyzed (see Methods D.4) against sodium phosphate buffer containing 0.2% Triton X-100 detergent used to disaggregate and prevent protein aggregation in the absence of lipid vesicles. Figure 5 shows that enzyme activity was detected at the bottom of the gradient, while phosphorus (representing the small amount of phosphate buffer in which MAO-B was suspended before dilution into Hepes/KCl for

Table 8

Summary of MAO-B Isolations from Bovine Liver

	Mitochondria	*Partially Purified*		*Purified*	
Liver 1	**SpA[a]**	**Ptn[b]**	**SpA**	**Ptn**	**SpA**	**Ptn**
MAO-1	23.3	4722	70.0	21.0	-	-
MAO-2	11.4	5886	39.0	8.7	-	-
MAO-3	ND	ND	448	4.3	402	3.4
MAO-4	ND	ND	-	-	11.0	12.0
MAO-5	ND	ND	-	-	9.0	7.4
MAO-6			142	12.9	-	-
Liver 2						
MAO-7	5.3	6804	262	19	278	2.5
MAO-8	11.6	6205	333	42.5	634	10.4
MAO-9			260	15.8	319	5.7
MAO-10			237	23.3	404	6.5
MAO-11			149	48.2	636	6.5

[a]Specific activity (expressed as nmol min^{-1} per mg protein) was determined by oxidation of 1mM kynuramine at 30°C in 50mM NaP buffer (pH7.2) containing 0.2% Triton X-100.
[b]Total protein in mg.

Table 9

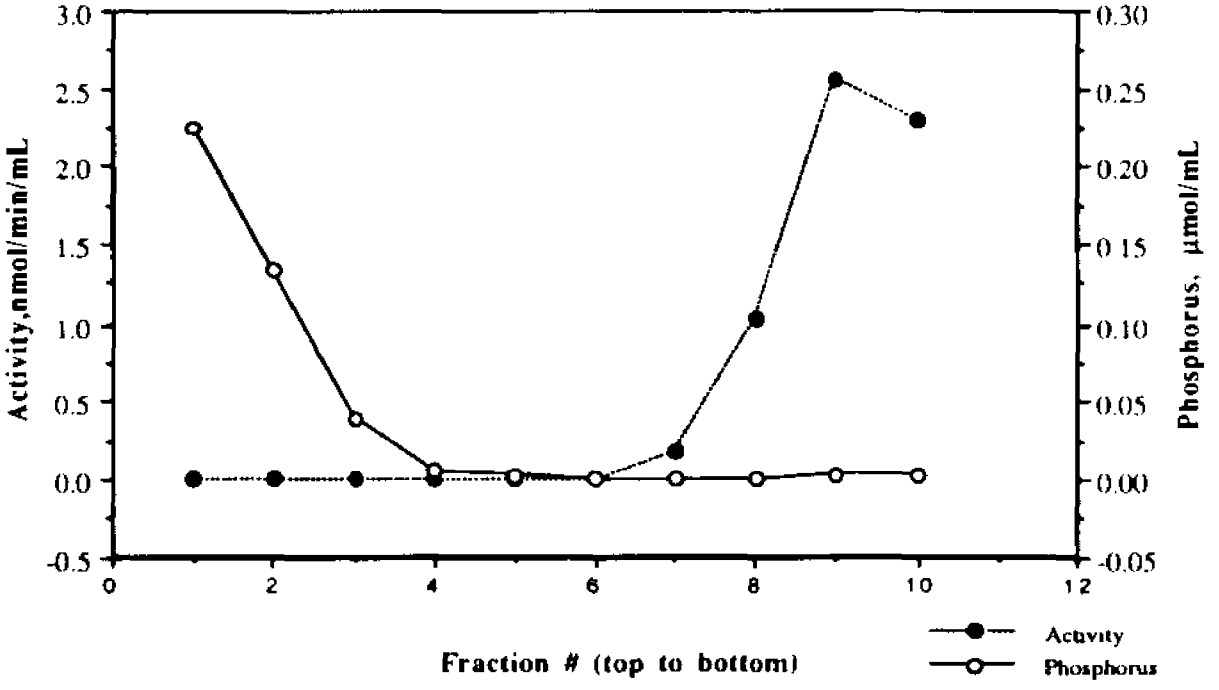
MAO-B Purification Chart

Sample	Volume (mL)	Total Activity (units)*	Protein (mg)	Specific Activity**	% Yield	Purification
Mitochondrial Homogenate	425	71,978	6205	11.6	100	1
Phospholipase digest	203	---	5786	—		
Polymer interface	168	38,148	3108	12.3	53	
High-speed pellet[a]	2.4	14,153	42.5	333	19.7	28.7
Sucrose gradient isolate[b]	1.4	6,594	10.4	634	9.2	54.7

*One unit is defined as oxidation of one nmol kynuramine per minute per mg protein at 30°C.
**Units per mg protein
[a]Defined as *partially purified* enzyme preparation
[b]Defined as *purified* enzyme preparation

Figure 5

Sedimentation Profile of *Partially Purified* MAO-B in 10-80% Glycerol Gradient[a]



[a]MAO-2 (1.45mg protein) in 100µL of 50mM Na phosphate, pH7.2 (NaP) was mixed with 900µL of 50mM Hepes/100mM KCl, pH 7.4 (HK); 50µL of this was reserved for activity assay, and the balance (1.38mg protein) applied to 10-80% linear glycerol gradient and centrifuged in SW41 rotor at 39,000rpm at 4°C for 17 hours. One mL gradient fractions were obtained. For activity assay, 250µL aliquots of these fractions were dialyzed against NaP containing 0.2% Triton X-100 (NaP + Tx); the dialyzed aliquots were adjusted to the same volume prior to assay with 1mM kynuramine•2HBr in NaP + Tx, 1.0mL assay volume. Phosphorus assay was performed on undialyzed gradient fractions as described in the Methods to localize the NaP buffer on the gradient.

application to the gradient) remained at the top. Recovery of applied activity from the glycerol gradient centrifugation was about 27%

B. Purified Enzyme

Purified MAO-B represents the pellet (P13) from the high-speed centrifugation step following the usual sucrose density gradient centrifugation. Although not homogeneous (see SDS-PAGE below), this preparation was used for most of the reconstitution experiments discussed below. Kinetic experiments using both solubilized and bilayer incorporated enzyme were performed using the *purified* MAO-B preparation.

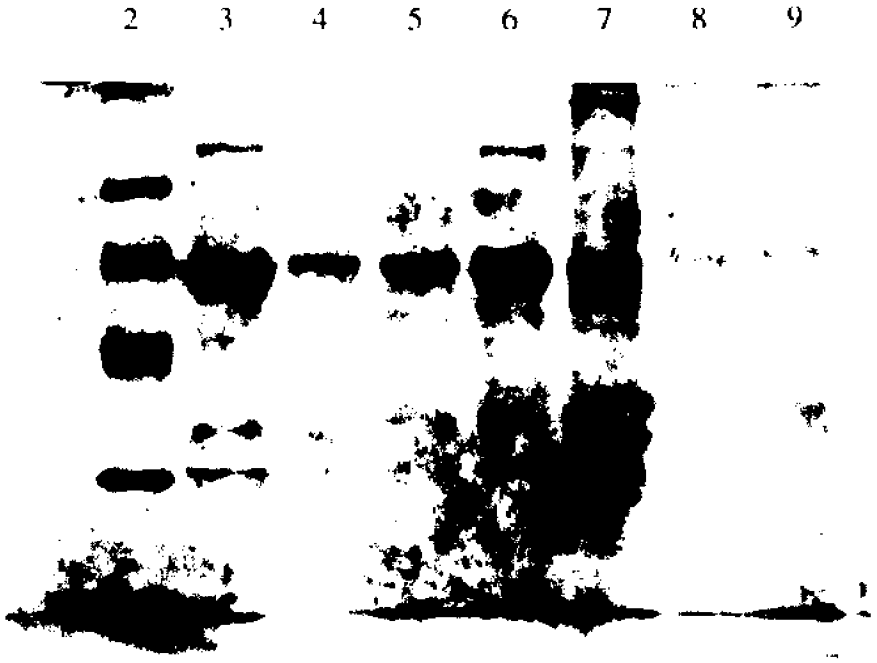
C. SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to ascertain the purity of MAO-B preparations and verify subunit size. Aliquots from several isolations were examined; these included both sucrose gradient isolates (*partially purified* enzyme) and high speed protein pellets (*purified* enzyme). Protein (~10μg) from 7 isolates (5 *purified* and 2 *partially purified*) was used for electrophoretic analysis. The MAO-3 isolate was not included, all of the sample having been consumed in experiments. The following molecular weight markers were run on the same gel: rabbit muscle phosphorylase b (97.4kD), bovine serum albumin (66.2kD), hen egg white ovalbumin (42.7kD), bovine carbonic anhydrase (31.1kD), and soybean trypsin inhibitor (21.1kD). Gels were stained only with Coomassie blue. A typical electrophoretogram is shown in Figure 6.

The mobility of the MAO-B monomer corresponded to a molecular weight (MW) of

Figure 6

SDS-PAGE of Several MAO-B Preparations



Left to right, lanes:

2, MW standards*

3-9, MAO-B isolates as indicated (µg protein applied to gel)

3, MAO-11 (10.9µg); 4, MAO-10 (10.6µg); 5, MAO-9 (10.2µg); 6, MAO-8 (11.1µg) (all *purified* preparations)

7, MAO-6 (11.1µg); 8, MAO-2 (14.5µg); 9, MAO-1 (13.0µg) (all *partially purified* preparations)

*MW standards, top to bottom:

97.4kD, rabbit muscle phosphorylase b

66.2kD, bovine serum albumin

42.7kD, hen egg white ovalbumin

31.1kD, bovine carbonic anhydrase

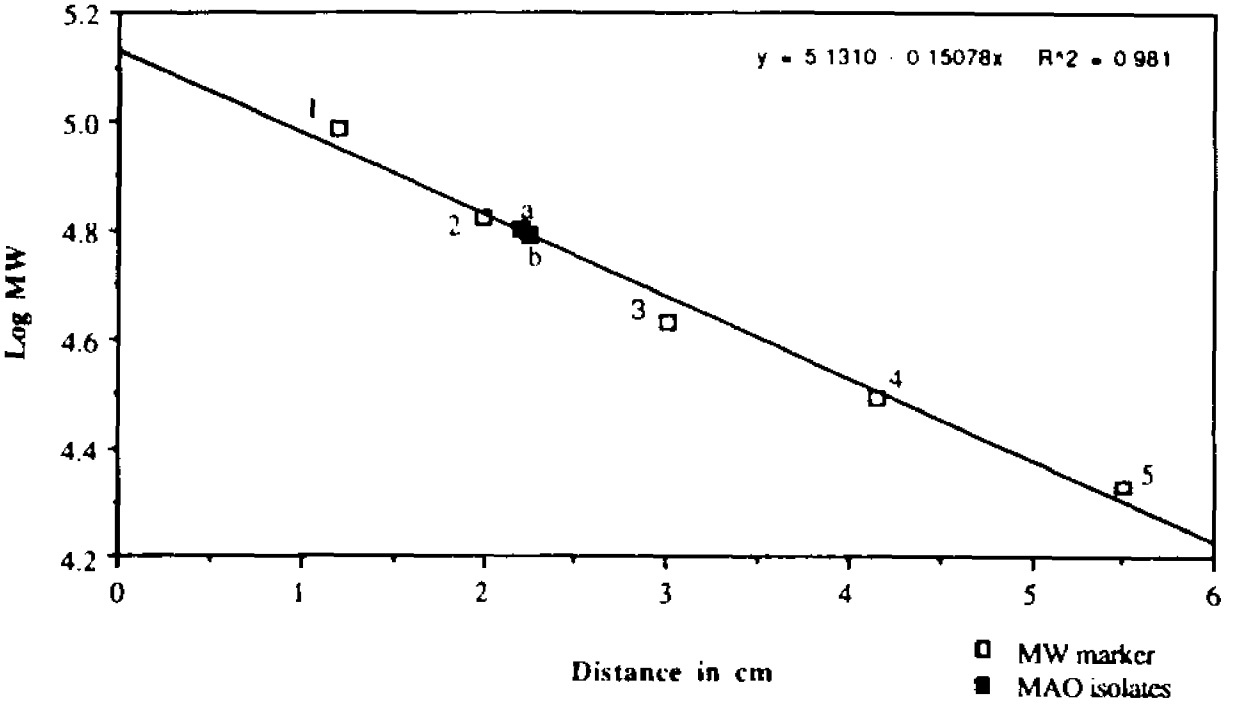
21.5kD, soybean trypsin inhibitor

62.4kD (Figure 7), in good agreement with the 58-62kD range reported by Salach and Weyler (1987) and migrating slightly ahead of the 66.2 kD bovine serum albumin standard used here. For both *purified* and *partially purified* preparations, there consistently appeared a light-staining band, with slower migration than the 97.4kD marker and corresponding to a MW of ~102.3kD. Weyler and Salach (1981) also found a slower moving band on their 0.1% SDS gels with MAO-B isolates after the polymer partitioning step, but not following sucrose density gradient centrifugation. Further, Weyler (1989) obtained a consistently slower-moving band on SDS-PAGE; however, with a MW of ~130kD, this suggests dimer formation of MAO-B despite the presence of 0.1% SDS in the gel.

In most preparations, sample overloading revealed the presence of additional protein bands for both the *purified* and/or *partially purified* enzyme preparations, arising presumably from the presence of low concentrations of other contaminating mitochondrial proteins: one migrated very slightly behind the 31.1kD protein standard; a second appeared between the 31.1 and 42.7kD standards; and a third ran ahead of MAO-B monomer, but behind the 42.7kD standard. Of the *purified* preparations, only in MAO-10 was MAO-B the only protein present, as determined by densitometry performed on Coomassie staining. The percent MAO-B in the other isolates was as follows: MAO-11 (84%), MAO-8 (80%), MAO-9 (64%), and for the *partially purified* MAO-6 (56%). The other *partially purified* preparations, MAO-1 and MAO-2, were more than one year old when the SDS-PAGE was run; these samples appeared to have degraded and, hence, their purity could not accurately be determined. However, in general, for all preparations examined, MAO-B monomer was the predominant protein. Thus MAO-B was determined to have been isolated in a highly, if

Figure 7

Determination of Bovine Liver MAO-B Subunit Molecular Weight by SDS-PAGE



MW markers:
1) Rabbit muscle phosphorylase b, MW = 97.4kD
2) Bovine serum albumin, MW = 66.2kD
3) Hen egg white ovalbumin, MW = 42.6kD
4) Bovine carbonic anhydrase, MW = 31.1kD
5) Soybean trypsin inhibitor, MW = 21.5kD

MAO-B isolates:
a) MAO-8, subunit MW = 62.9kD
b) MAO-11, subunit MW = 61.9kD

not completely pure state and was used to further investigate the reconstitution of MAO-B into phospholipid vesicles, and the effect of membrane composition on its enzymatic activity

D. Effect of Buffer and Detergent on Solubilized Enzyme Activity

1. Buffer Composition

The effect of buffer composition on the activity and stability of MAO-B was examined due to the different buffers required for the assay and reconstitution procedures. A comparison was made between 50mM sodium phosphate, pH 7.2 (ionic strength μ=0.1; osmolarity = 125mOsm$_{calc}$) (NaP), in which the enzyme has maximal activity, and 50mM Hepes/100mM KCl, pH 7.4 (ionic strength μ=0.121; osmolarity = 271mOsm$_{calc}$) (HK), the buffer system preferred for reconstitution. As shown in Table 10, the activity of *partially purified* MAO is dependent on buffer composition. The specific activity for the enzyme is two-fold in NaP, although stability of the protein appeared to be comparable in both buffer systems examined, with activity declining by 78% in HK and 83% in NaP following overnight incubation at 0°C. Lower activity in Hepes was due, at least in part, to the presence of KCl, since addition of 100mM KCl to the phosphate buffer (resultant μ=0.2; osmolarity = 325 mOsm$_{calc}$) caused a decrease in the enzymatic activity by 33% compared to NaP buffer alone.

However, while the enzyme activity was much higher in phosphate buffer, many of the assays were performed in the HK reconstitution buffer in order to maintain the reconstituted enzyme in the buffer in which the proteoliposomes were prepared.

2. Effect of Triton

Table 10

Effect of Buffer Composition on Activity and Stability of *Partially Purified* MAO-B[a]

	Specific Activity	
Buffer	**Zero Time**	**Overnight**
HK	30.5 +/- 3.2 (n=6)	6.6 +/- 2.4 (n=6)
NaP	60.6 +/- 9.5 (n=3)	10.5 +/- 0.8 (n=3)

[a]Buffers examined were 50mM Hepes/100mM KCl, pH 7.4 (HK) and 50mM sodium phosphate, pH 7.2 (NaP). Aliquots of MAO-1 (5μL containing 60μg protein) were mixed with 295μL of the indicated assay buffer at 0°C. Immediately (zero time), 0.68mL assay buffer and 20μL kynuramine 2HBr solution (final concentration in assay, 1mM) (both equilibrated at 30°C) were mixed with the enzyme sample and ΔA_{314nm} monitored against a control consisting of 1mM substrate in assay buffer. Duplicate sets of enzyme samples in these buffers were maintained at 0°C for 16-17 hours prior to assay as above (overnight). Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD; n=number of experiments.

Since the presence of Triton-X (Tx) was necessary for successful assay conditions for the solubilized enzyme, its effect on enzyme activity was also assessed (Table 11). The presence of 0.2% Tx in either of the buffer systems examined (NaP and HK) resulted in no significant decrease in activity measured at "zero time" (*i.e.*, assayed immediately after diluting the enzyme in buffer). Overnight incubation at 0°C of *partially purified* MAO-B in buffer containing no Triton showed that the enzyme was unstable losing 76 to 83% of its activity, and loss of activity was not reversed by including Tx in the assay mixture at the time of assay. However, when the enzyme was incubated overnight with buffer containing 0.2% Tx, only 17 to 26% of the activity was lost. Furthermore, better reproducibility of replicate assays was observed for those samples containing Triton (Table 11).

However, while the activity of *solubilized* MAO-B is stabilized by the presence of Triton X-100, this detergent is incompatible with proteoliposome formation via direct protein incorporation due to lipid solubilization effects, and hence unsuitable for assay of membrane-reconstituted (and hence stabilized) protein. Thus, addition of Triton was restricted to the assay of solubilized enzyme only and not used for reconstituted protein preparations.

3. Effect of Octylglucoside on the Activity of MAO-B

Octylglucoside (OG) has previously been successfully used for detergent dialysis reconstitution procedures of membrane proteins (rhodopsin by Stubbs *et al.*, 1976b; glycophorin by Mimms *et al.*, 1981; cytochrome P_{450} monooxygenase system by Schwarz *et al.*, 1984). Initially, OG was used to examine the detergent-facilitated incorporation method

Table 11

Effect of Triton X-100 on Activity and Stability of *Partially Purified* MAO-B[a]

	Specific Activity[b]	
Buffer	**Zero Time**	**Overnight**
NaP[b]	60.6 +/- 9.5	10.5 +/- 0.8
NaP + Tx[c]	57.2 +/- 2.2	44.7 +/- 1.1
HK[d]	28.8 +/- 2.6	6.9 +/- 0.7
HK + Tx[e]	25.6 +/- 1.4	21.2 +/- 1.9
HK	32.1 +/- 3.2	6.2 +/- 3.7
HK ; Tx in assay only[f]		2.9 +/- 0.7

[a]Aliquots of MAO-1 (5µL containing 60µg protein) were mixed with 295µL of the indicated buffer (defined below) at 0°C. Activity was assayed in the same buffer, unless otherwise noted. Immediately (zero time), 0.68mL assay buffer and 20µL kynuramine 2HBr solution (final concentration in assay, 1mM) (both equilibrated at 30°C) were mixed with the enzyme sample and ΔA_{314nm} was monitored against a control consisting of 1mM substrate in assay buffer. Duplicate sets of enzyme samples in these buffers were maintained at 0°C for 17-18 hours prior to assay as above (overnight). Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD.
[b]NaP: 50mM sodium phosphate, pH 7.2
[c]NaP + Tx: NaP + 0.2% Triton X-100
[d]HK: 50mM Hepes/100mM KCl, pH 7.2
[e]HK + Tx: HK + 0.2% Triton X-100
[f]HK; Tx in assay buffer only: enzyme was incubated overnight in HK buffer without Tx, but assayed in Hk + Tx

wherein the detergent is not removed, and so the effect of the presence of OG on the stability of *purified* MAO-B was tested (Table 12). The activity was determined to be more stable for the detergent to enzyme weight ratio of 25:1 (mole ratio ~10,000:1 with [OG] of 5.8mM) over a 10:1 weight ratio (mole ratio ~4000:1 with [OG] of 2.3mM), both at "zero time" and after overnight incubation on ice.

Subsequently, the detergent dialysis reconstitution method was adopted. During the series of detergent dialysis reconstitutions (described later), weight ratios of OG to protein, used for solubilizing MAO-B, decreased from 25:1 to ~18:1, with the OG concentration fixed here at 59mM (10 fold higher than discussed above). Under these conditions, the enzyme was exposed to OG for 30 to 60 minutes before addition to lipid and subsequent removal of detergent by dialysis. On examination of the activity of *purified* MAO-B exposed to this elevated detergent concentration, an inhibitory effect was found (Figure 8). Nevertheless, reconstitution with phospholipid always resulted in return of some portion of the activity (see section III.A.3). Rapid dialysis (see Methods II.D.4) of the OG-solubilized enzyme in the absence of phospholipid also resulted in return of activity. With the MAO-8 isolate this reactivation was 60-69% of that achieved with lipid; whereas with MAO-9 more activity was recovered than with lipid reconstitution (68% vs 46-61%), and with MAO-10 dialysis in the presence or absence of lipid resulted in a similar degree of reactivation. The MAO-9 and MAO-10 isolates which experienced a greater return of activity without lipid reconstitution were initially less inactivated by OG than the MAO-8 isolate.

Several other solubilizing (surfactant) agents, previously used for solubilization of membrane proteins were examined for their effect on MAO-B stability (Table 13) *(e.g.*,

Table 12

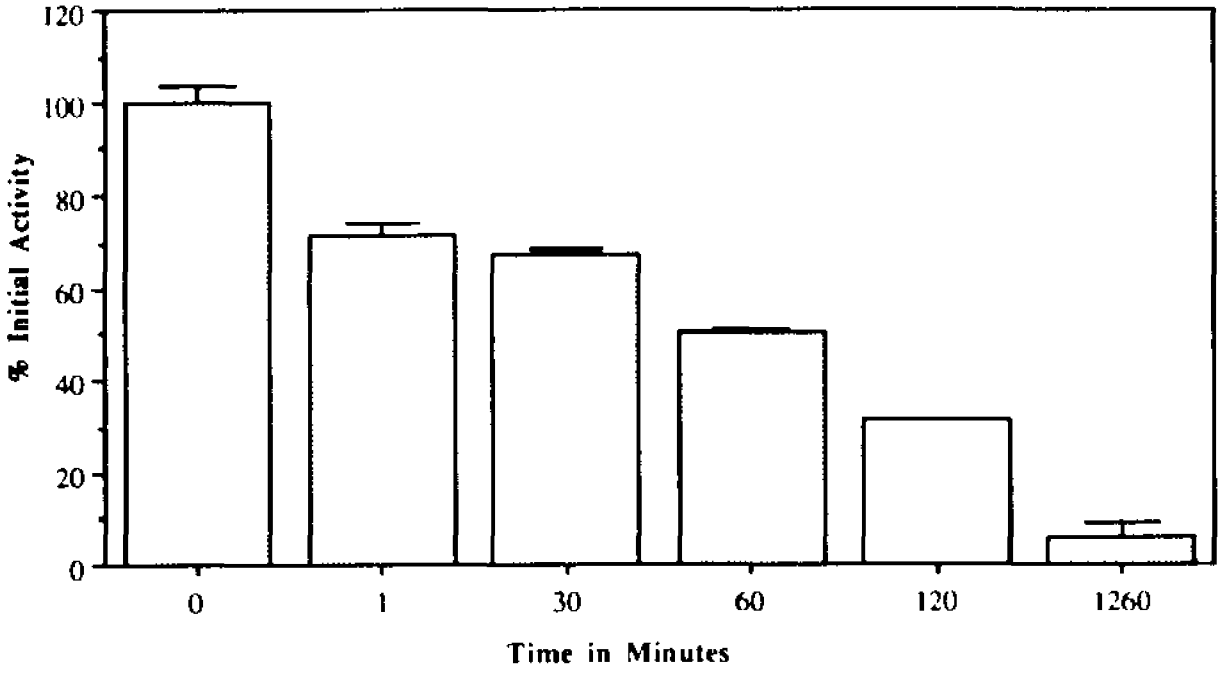
Stability of *Purified* MAO-B in Octylglucoside[a]

		Specific Activity	
		Zero Time	**Overnight**
Basal Activity:		277.7 ± 4.8	189.7 ± 36.6
Octylglucoside:			
mM OG	**OG/MAO (weight ratio)**		
2.3	10:1	239.0 ± 4.1	187.5 ± 21.6
5.8	25:1	262.8 ± 10.0	219.4 ± 38.6

[a]Aliquots of MAO-3 (3µL containing 20.4µg protein) were mixed either with 297µL of 50mM Hepes/100mM KCl, pH 7.4 (HK) (basal) or with 297µL of the indicated octylglucoside solution in HK. Immediately (zero time), 0.68mL assay buffer and 20µL kynuramine 2HBr solution (final concentration in assay, 1mM)(equilibrated at 30°C) were mixed with the enzyme sample and the reaction rates monitored as ΔA_{314nm} against controls consisting of 1mM substrate in the corresponding [OG] in HK. Duplicate sets of enzyme samples in these buffers were maintained at 0°C for 18 hours prior to assay as above (overnight). Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD.

Figure 8

Effect of 59mM Octylglucoside on the Activity of *Purified* MAO-B[a]



[a]MAO-11 (1.67mg) in 225µL of 50mM Na phosphate, pH 7.2 (NaP) was added to 1.58mL octylglucoside (19.5mg/mL in 50mM Hepes/100mM KCl, pH 7.4) (59mM final [OG]). The sample was stirred at 4°C. At the indicated times, 50µL aliquots (containing 46.3µg protein) were removed and assayed for enzymatic activity by oxidation of kynuramine•2HCl (1mM final in NaP) at 30°C in 2.0mL assay volume.

Table 13

Effect of Detergents of Different Alkyl Chain Length on Stability of *Purified* MAO-B[a]

Detergent	CMC[b]	D/Pdimer[c]	Specific Act[d]	Relative Rate[e]
None			482.3 ± 8.3	1.00
Polyoxyethylene Ether				
Triton X-100	<1	~3300	437.2 ± 7.8	0.91
Alkylglucosides				
Octylglucoside	25	7501	26.8 ± 0.9	0.05
Dodecylmaltoside	<1	4195	395.4 ± 5.2	0.82
Sulfabetaines				
ZW3-08	330	7755	368.5 ± 5.3	0.76
ZW3-10	25-40	6992	0.2 ± 0.2	0.00
ZW3-12	2-4	6484	30.2 ± 0.1	0.01
Methylglucamides				
Mega-8	58	6738	433.3 ± 10.0	0.90
Mega-9	19-25	6484	40.4 ± 1.1	0.08

[a]MAO-8 (25µL containing 185µg protein) was mixed either with 175µL of 50mM Hepes/100mM KCl, pH 7.4 (HK) or with 175µL of a 19.5mg/mL solution in HK of the indicated detergent. Samples were maintained at 4°C overnight. Aliquots (50µL containing 46.3µg protein) were removed and assayed for enzymatic activity by oxidation of kynuramine·2HCl (1.0mM final in 50mM Na phosphate, pH 7.2) at 30°C in 1.0mL assay volume.

[b]Critical micellar concentration in mM

[c]Detergent to MAO-B dimer molar ratio

[d]Specific activity expressed as nmol min^{-1} per mg protein

[e]Rate relative to that obtained in the absence of detergent

methyl glucamides, Hildreth (1982); sulfabetaines, Navarette and Serrano (1983); alkyl maltosides, Alpes *et al.* (1988) and Triton X-100, Ashikawa *et al.* (1994)). Surfactants with shorter alkyl chain lengths tend to have high critical micellar concentration (CMC) values which makes them readily dialyzable, an attribute favorable to detergent dialysis reconstitution. The various detergents were dissolved in HK buffer at concentrations of 1.95% (w/v). *Purified* enzyme was then added at a detergent to protein weight ratio of ~18 to 1, comparable to that shown for octylglucoside. Detergent to MAO-B dimer mole ratios were similar to that obtained with OG for all except Triton X-100 and dodecylmaltoside, for which the mole ratios were about half those used for the other detergents. Following overnight incubation at 4°C, enzyme activity was measured and compared for the different types of detergent. As shown in Table 13, the activity of the enzyme decreased with all the detergents tested, but to varying extents. The enzyme was best stabilized with Triton X-100, Mega-8, dodecylmaltoside, and Zwittergent 3-08. However, at the concentrations required for solubilzation, both Triton X-100 and dodecylmaltoside, having very low CMC values (well below 1mM), readily form micelles making them unsuitable for dialysis reconstitution protocols. Similarly, while Mega-8 and ZW 3-08 were promising in terms of preserved enzymatic activity, the concentrations used proved insufficient for solubilizing the lipid. Desired higher concentrations would result in micellar formation.

II. RECONSTITUTION OF MAO INTO PRE-FORMED VESICLES

A. Spontaneous Incorporation

Initially, direct incorporation was investigated using *partially purified* enzyme (high

speed pellet). Reconstitution was effected by mixing the enzyme with preformed SUVs and incubating for approximately one hour at 0°C with occasional swirling.

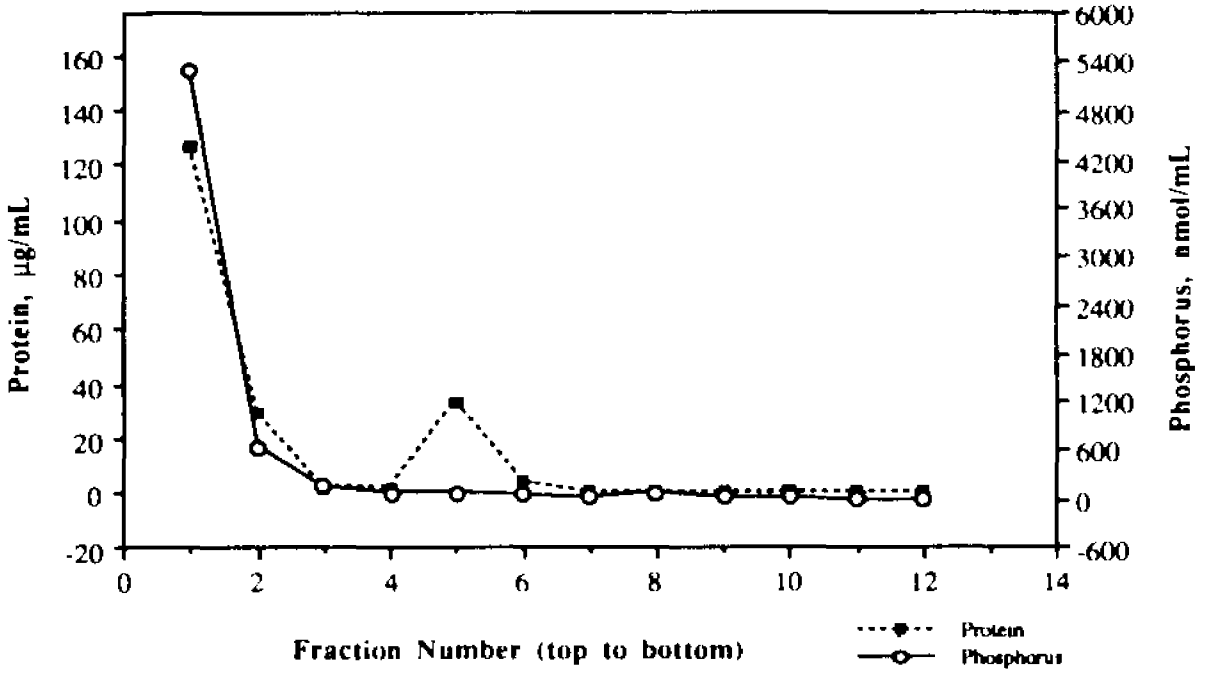
1. Proof of Reconstitution

Reconstitution of the enzyme by this method can be demonstrated by separating the products of reconstitution by glycerol density gradient centrifugation. The unincorporated enzyme aggregates in the absence of lipid or detergent, and so will sediment to the bottom of the gradient. The incorporated enzyme will be associated with lipid fractions. Results of the glycerol gradient centrifugation for reconstituted *partially purified* enzyme (MAO-1) are shown in Figure 9, and may be compared with a control gradient of MAO-B alone (Figure 10). In Figure 9, the coincidence of protein and phospholipid-phosphorus at the top of the gradient in fraction 1 suggests successful reconstitution. Additionally, a smaller protein peak in fraction 5 was observed which may represent more efficient incorporation of enzyme molecules per liposome (and therefore, greater density). The control gradient with MAO-B in phosphate buffer (Figure 10) shows that in the absence of DOPC liposomes, the protein (presumable aggregated) sediments to the bottom of the gradient.

Attempts to assay enzymatic activity of the reconstituted preparation were unsuccessful. It was found that glycerol from the gradient was inhibitory to kynuramine oxidation by this enzyme (Table 14), and that the loss of activity was not due to the presence of Hepes buffer used for the reconstitution procedures. In all future gradient experiments, aliquots for activity assay were dialyzed first to remove glycerol. Subsequent assays of reconstituted enzyme were accomplished in either Hepes or phosphate buffer, containing no

Figure 9

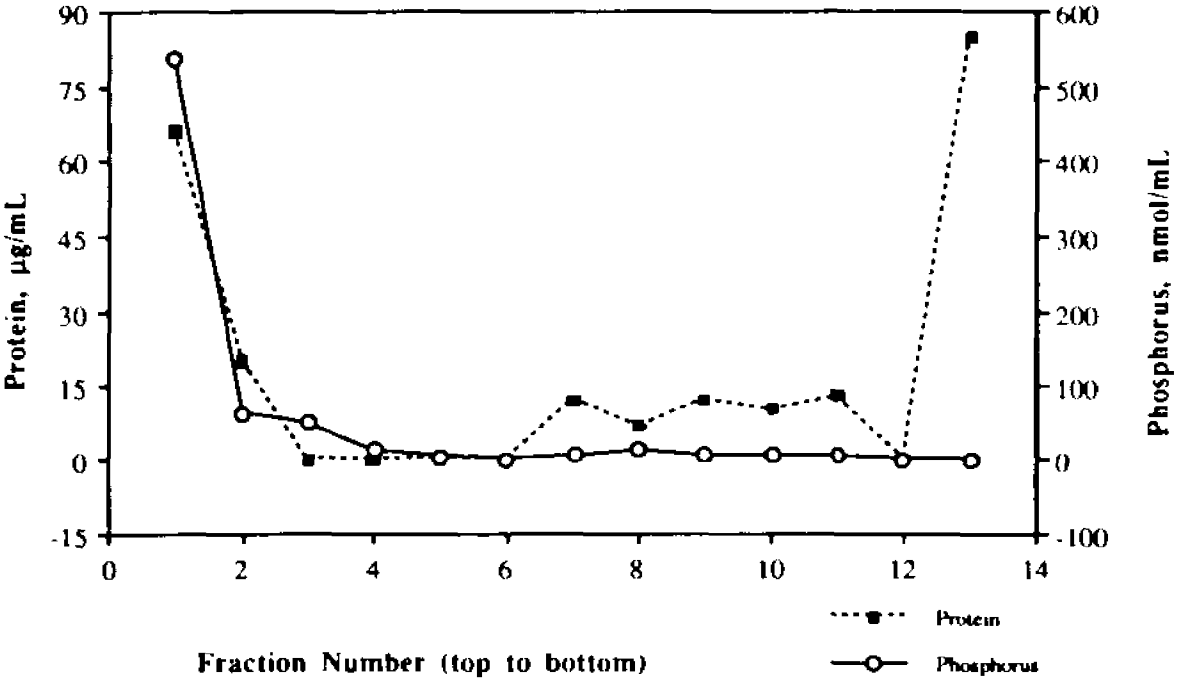
Characterization of a Spontaneous Incorporation of *Partially Purified* MAO-B with DOPC Liposomes on 10-80% Glycerol Gradients[a]



[a]MAO-1 (50µL containing 600µg protein) was mixed with DOPC SUVs (5mg lipid) in 50mM Hepes/100mM KCl, pH 7.4 (HK) (total volume, 1.0mL) and incubated for 1hr at 0°C. The sample was applied to 10-80% linear glycerol gradient containing HK buffer and centrifuged in SW41 rotor at 39,000rpm at 4°C for 15hrs. Gradient was fractionated from the top into 1mL aliquots which were assayed for protein and phospholipid-phosphorus content.

Figure 10

Sedimentation Profile of *Partially Purified* MAO-B on Glycerol Gradients in the absence of DOPC Liposomes[a]



[a]MAO-1 (600µg protein) in 50µL of 50mM Na phosphate, pH 7.2 (NaP) was mixed with 950µL of 50mMHepes/100mM KCl, pH 7.4 (HK). The sample was applied to 10-80% linear glycerol gradient and containing HK buffer and centrifuged in SW41 rotor at 39,000rpm at 4°C for 15hrs. The gradient was fractionated from the top into 1mL fractions which were assayed for protein and phosphorus content.

Table 14

Effect of Glycerol on Kynuramine Oxidation by *Partially Purified* MAO-B[a]

% Glycerol (w/v)	Specific Activity nmol min^{-1} per mg protein
0	32 ± 1.3
10	19 ± 4.0
20	16 ± 7.1
30	17 ± 3.0
40	12 ± 5.0
50	11 ± 4.0
60	13 ± 4.0

[a]Aliquots of MAO-1 (5µL containing 60µg protein) were mixed with 250µL of the indicated glycerol solution in 50mM Hepes/100mM KCl, pH 7.4 (HK), and immediately transferred to cuvettes containing 0.725µL HK and 20µL kynuramine2HBr (final concentration in assay, 1mM) (equilibrated at 30°C). Reaction rates were monitored as ΔA_{314nm} against controls consisting of 1mM substrate in HK.

Triton X-100.

2. Optimal Lipid to Protein Ratio

In order to determine the optimal lipid to protein (L/P) ratio to use for reconstitution, *partially purified* enzyme (MAO-1) was incubated with different amounts of DOPC liposomes. Lipid to MAO-B dimer molar ratios of approximately 1500, 3750 and 7500 to 1 were used, corresponding to weight ratios of 10, 25 and 50 to 1, respectively. The reconstituted enzyme was assayed in HK buffer (without added Tx) and the activity compared with that of corresponding unincorporated MAO-B, both at "zero time" and after overnight incubation at 0°C. The concentration of enzyme in the assay was the same for the incorporated and the unincorporated protein. Results are summarized in Table 15. At "zero time" no significant differences in MAO-B activity as a function of lipid to protein molar ratio, were observed; reconstitution at any of the L/P_{dimer} ratios used was neither stimulatory nor inhibitory. After overnight incubation on ice, a considerable decrease in the basal (unincorporated) enzyme activity was observed. Protein activity was significantly stabilized by incorporation and apparently at higher lipid concentration (less protein per vesicle). As shown in Table 15, at L/P_{dimer} molar ratio of ~3750:1(corresponding to 25:1, w/w) there appeared to be a saturating effect.

3. Stability of Spontaneously Incorporated MAO-B

Partially purified enzyme (MAO-1) was directly incorporated into DOPC liposomes at an L/P weight ratio of 25:1. The stability of the proteoliposomes was determined by

Table 15

Effect of Lipid to Protein Weight Ratio on Kynuramine Oxidation by *Partially Purified* MAO-B[a]

	Specific Activity	
	Zero Time	**Overnight**
Basal (no lipid)	28.8 ± 2.6	6.9 ± 0.7
DOPC Liposomes (L/P)		
10:1	28.0 ± 3.6	12.6 ± 1.5
25:1	33.0 ± 1.5	21.7 (n=2)
50:1	34.0 ± 3.7	19.7 ± 5.3

[a]Aliquots of MAO-1 (5µL containing 60µg protein) were mixed either with 295µL of 50mM Hepes/100mM KCl, pH 7.4 (HK) (basal) or with 295µL of DOPC SUVs in HK buffer at the indicated lipid to protein (L/P) weight ratio, *i.e.* weight of lipid in the liposome sample relative to the weight of protein in the enzyme sample. Immediately (zero time), 0.68mL assay buffer and 20µL kynuramine 2HBr solution (final concentration in assay, 1mM)(equilibrated at 30°C) were mixed with the enzyme sample and the reaction rates monitored as ΔA_{314nm} against controls consisting of 1mM substrate in in HK. Duplicate samples were maintained at 0°C overnight prior to assay as above (overnight). Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD.

assaying the enzymatic activity daily over a three day period. Assays were performed in the HK reconstitution buffer and the activity was compared with that of the unincorporated enzyme (defined here as the basal activity). Both the proteoliposomes and the stock solubilized MAO-B were maintained at 0°C during this period. As seen from Table 16, no significant differences in activity between the reconstituted and the non-reconstituted enzyme were observed. For both preparations, there was a small decrease in rate over time, which was marginally less for the incorporated form of the enzyme, than for the basal activity.

The basal activity reported in Table 16 was fairly stable, in contrast to the dramatic loss of activity overnight seen in the experiment summarized in Table 15. Considerable variability in the stability of different MAO-B isolates, as well as the effect of multiple thawings on the stability of a particular isolate, appeared to contribute to such activity differences.

4. Varying Lipid Headgroup Composition

The effect of the lipid headgroup on the activity of reconstituted *partially purified* enzyme (MAO-2) was investigated. Four types of phospholipid vesicles were prepared: DOPC; DOPC/DOPE; DOPC/DOPG; DOPC/DOPE/DOPG. The base lipid in all was DOPC. DOPE and DOPG were included, separately or together, at 25 mole% each. For PE this mole% falls within the reported range present in the outer mitochondrial membrane, as discussed previously (see Introduction, section IIIa). In contrast, phosphatidylglycerol (PG) represents a much lower fraction of the OMM phospholipid with 3 mole% typically reported for Guinea pig liver (Daum, 1985). However, in this study, for a more direct comparison with

Table 16

Stability of *Partially Purified* MAO-B/DOPC Proteoliposomes prepared by Spontaneous Incorporation[a]

Length of Incubation	Specific Activity	
	Basal	Reconstituted
Zero Time	35.4 ± 6.2	32.9 ± 1.5
1 Day	26.3 ± 2.0	25.2 ± 0.2
2 Days	21.4 ± 0.9	20.5 ± 2.2
3 Days	21.0 ± 1.4	23.7 ± 1.2

[a]Aliquots of MAO-I (5μL containing 60μg protein) were mixed either with 295μL of 50mM Hepes/100mM KCl, pH 7.4 (HK) (basal) or with 295μL of DOPC SUVs in HK buffer at a lipid to protein (L/P) weight ratio of 25:1. Samples were maintained at 0°C. At the indicated times, 0.68mL assay buffer and 20μL kynuramine 2HBr solution (final concentration in assay, 1mM)(equilibrated at 30°C) were mixed with the enzyme or enzyme/lipid sample. Reaction rates were monitored as ΔA_{316nm} against controls consisting of 1mM substrate in HK or 1mM substrate plus DOPC liposomes. Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD.

PE, the same mole% DOPG was added. In addition cholesterol (17 mole%) was added to a duplicate set of the four vesicle types described. While the range of cholesterol content in the OMM is somewhat lower than used in this study (~6 mole% versus 17 mole%, respectively), the higher molar content was used in an "all-or-nothing" attempt to determine any membrane modulating role of cholesterol on the reconstituted MAO-B activity.

Bilayer incorporation was carried out by adding the enzyme to the mixed lipid liposome preparation in an ice bath (as discussed in Methods D.2). The L/P weight ratio used was 25:1. Kynuramine oxidation was measured in sodium phosphate (NaP) buffer (50mM, pH 7.2) for maximal enzymatic activity, and the rates compared with the activity of the enzyme in the absence of liposomes (basal activity). The eight reconstituted preparations and the unincorporated enzyme were subsequently maintained at 0°C and the activity re-assayed after 24 hours to assess effect of the headgroup on enzyme stability. Results are summarized in Table 17. At zero time, no significant effect on enzyme activity was determined for any of the lipid vesicles examined, with or without cholesterol, when compared with basal activity. Preservation of enzyme activity (estimated by a loss not greater than 8% of the original activity) was best for liposomes comprising 100% DOPC, than for other lipid compositions examined.

5. Reconstitution of the *Purified* Enzyme

So-called *purified* MAO-B (taken through the sucrose density gradient as a final purification step to remove the last traces of Triton X-100 and polymers) resulted in a bright yellow enzyme preparation, with high specific activity and a characteristic flavin absorption

Table 17

Effect of Liposome Composition on the Activity of *Partially Purified* MAO-B Reconstituted by Spontaneous Incorporation[a]

"Mol %" Lipids ± 17 mol%Cholesterol	Specific Activity	
	Zero Time	Overnight
Basal (0% Lipid)	24.7 ± 4.7	16.6 ± 4.7
100% DOPC	21.1 ± 2.3	19.4 ± 0.2
75% DOPC/25% DOPE	19.8 ± 3.1	17.6 ± 0.8
75% DOPC/25% DOPG	21.1 ± 1.6	16.4 ± 1.3
50%DOPC/25%DOPE/ 25%DOPG	20.8 ± 2.4	17.1 ± 0.7
(100% DOPC) + Cholesterol	19.7 ± 2.1	16.8 ± 0.6
(75% DOPC/25% DOPE) + Cholesterol	22.2 ± 2.1	16.3 ± 1.0
(75% DOPC/25% DOPG) + Cholesterol	19.0 ± 5.9	17.2 ± 0.2
(50%DOPC/25%DOPE/ 25%DOPG) + Cholesterol	21.7 ± 0.9	16.4 ± 0.8

[a]Aliquots of MAO-2 (3µL containing43.5µg protein) were mixed either with 297µL of 50mM Hepes/100mM KCl, pH 7.4 (HK) (basal), or with 297µL of SUVs of the indicated composition at a lipid to protein (L/P) weight ratio of 25:1. Immediately (zero time), 0.68mL of 50mM Na phosphate, pH 7.2 (NaP) and 20µL kynuramine·2HBr solution (final concentration in assay, 1mM)(equilibrated at 30°C) were added. Reaction rates were monitored as ΔA_{314nm} against controls consisting of 1mM substrate in NaP or 1mM substrate plus liposomes in NaP. Assays were performed in triplicate. Specific activity is expressed as nmol min^{-1} of substrate oxidized per mg of protein ± SD.

(Figure11). Spontaneous incorporation was performed using *purified* MAO-3 and DOPC liposomes at a L/P weight ratio of 25:1. A 10-80% (w/v) glycerol gradient was run for 82 hours to resolve the products of the reconstitution. From visual inspection it appeared that the bright yellow flavoenzyme was at the bottom of the gradient; the protein was thus estimated not to have incorporated into the preformed DOPC SUVs.

The failure of *purified* MAO-B to spontaneously incorporate under these conditions may be due in part to the absence of residual Triton and/or polymers, or to the removal of some trace contaminant. Consequently, stable protein aggregates may have formed after the removal of the residual detergent and polymers. In the previous reconstitutions in this study, the partially purified enzyme was either not in an aggregated state or the incorporations were facilitated by a trace contaminant.

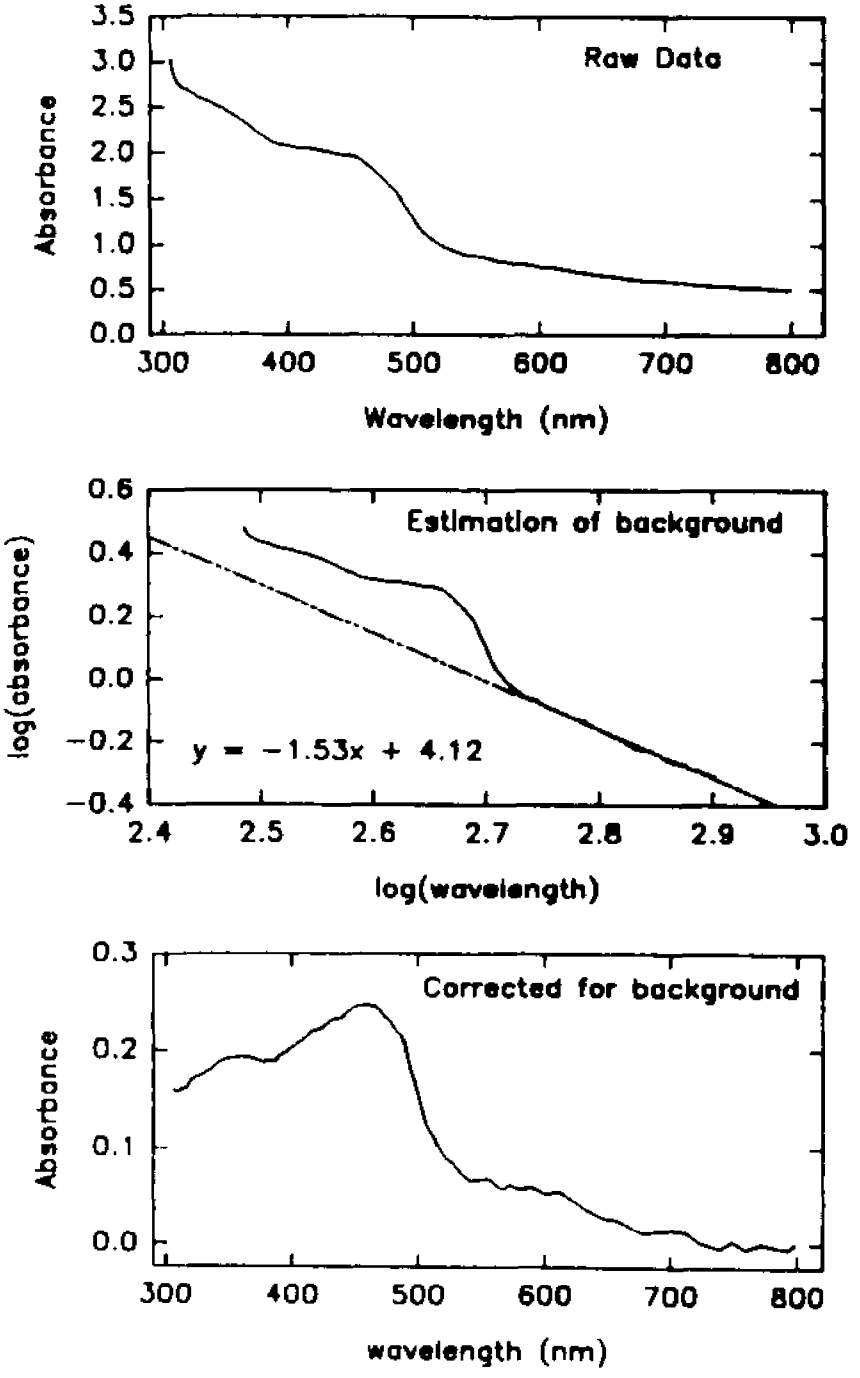
Results of the spontaneous incorporation studies suggest that purified MAO-B may require the presence of a small amount of detergent either for facilitating the incorporation or disaggregating the enzyme. Hence, other methods of reconstitution were pursued.

B. Facilitated Incorporation

Facilitated protein incorporation involved incubation of pre-formed liposomes with membrane protein in the presence of a small amount of detergent. Turbidity measurements (Figure 12) suggest that titration of DOPC liposomes with octylglucoside, at a molar ratio of up to 6:1 OG/DOPC, does not destroy the integrity of the vesicles. However, with higher detergent concentrations (molar ratios of 9:1), a dramatic decrease in the turbidity monitored at 400nm was observed, suggesting vesicle disintegration.

Figure 11

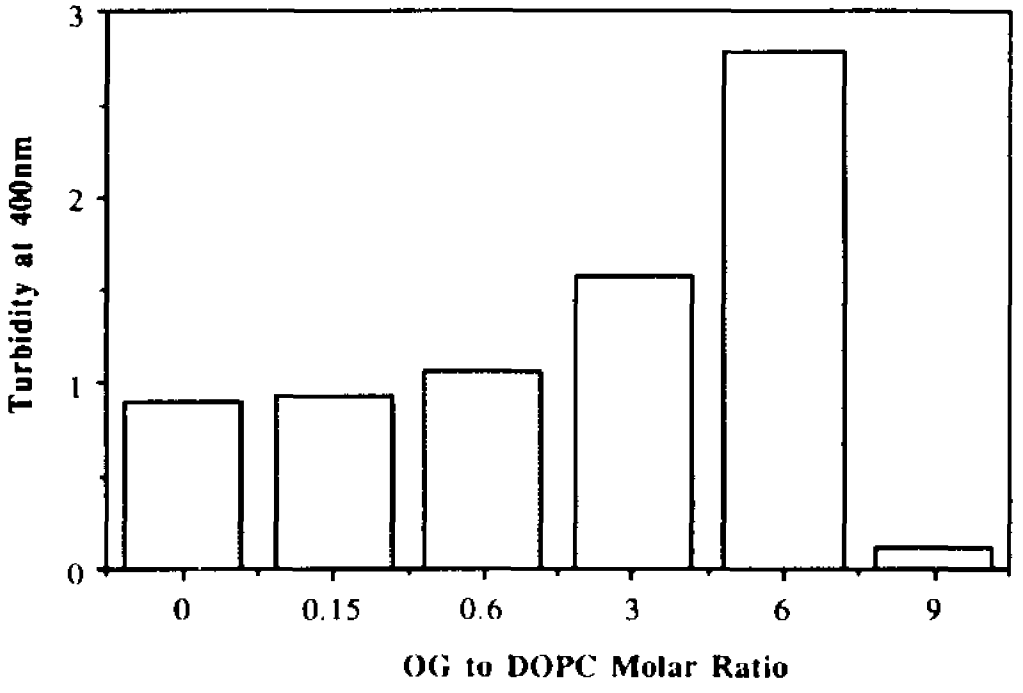
Absorption Spectrum of a *Purified* Preparation of MAO-B



Raw data is the spectrum contaminated with scatter. The scatter component (background) was subtracted to yield the corrected spectrum.

Figure 12

Effect of Octylglucoside to Lipid Molar Ratio on Turbidity of DOPC Liposomes[a]



[a]DOPC SUVs were prepared by sonicating (at 4°C) a suspension of 100mg DOPC in 10mL of 50mM Hepes/100mM KCl, pH 7.4 (HK). Octylglucoside (OG) solutions (various concentrations) were prepared in HK buffer. To 0.9mL liposomes (9mg DOPC), 0.1mL OG solution was added, and optical density at 400nm was measured. Final concentrations of OG were 0, 1.7, 6.8, 34.2, 68.4 and 102.6mM. The test was perfored at 30°C.

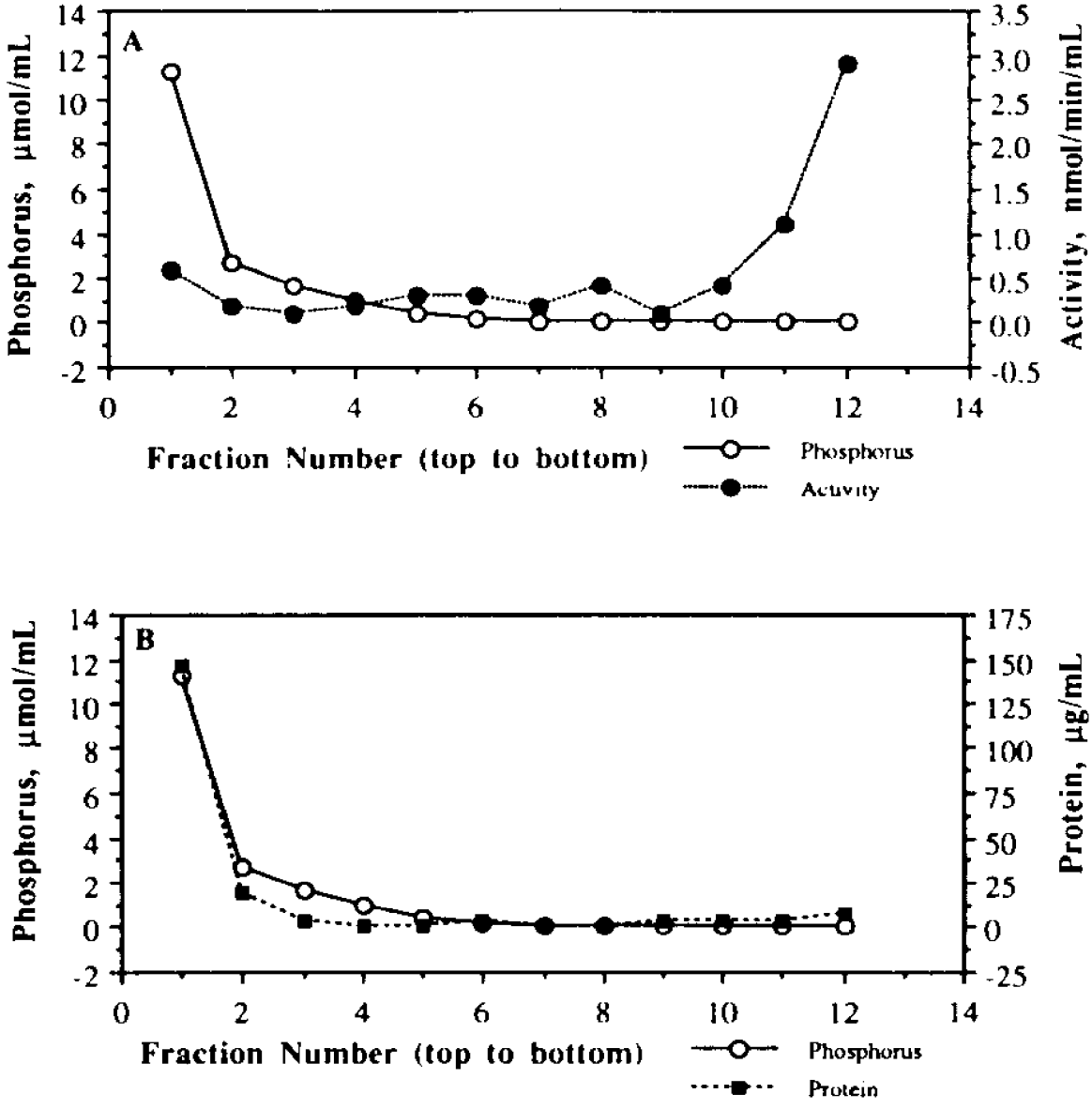
Facilitated reconstitutions using two different octylglucoside/MAO-B dimer molar ratios (350:1 and 1400:1) were performed. Subsequent analysis by glycerol gradients revealed phospholipid-phosphorus only at the top of the gradient with both detergent concentrations whereas the bulk of the enzyme activity was found at the bottom of the gradient (fraction 12) with 0.5mg OG (Figure 13). Qualitatively, similar results were obtained using 2.0mg OG (Figure 14). Both these detergent concentrations fall well within the limits for maintenance of pre-formed vesicle integrity, as discussed above. However, for the lower concentration ([OG]=0.5mg), 78% of the total protein and 9% of the enzyme activity appeared in fraction 1. In contrast, for 2.0mg OG, 42% of the protein and 2.5% of the activity were found present in fraction 1. In general, the bulk of the enzyme activity was located at the bottom of the gradient, while only a small amount of the activity was associated with phospholipid-phosphorus and protein located at the top of the gradient. These data suggest that any MAO incorporated by this system was not reconstituted in an active/native state. The greater buoyancy observed from the glycerol gradient runs of MAO-B with 2.0mg OG, despite no associated lipid, may reflect a greater dispersion of the protein in the higher detergent concentration.

III. RECONSTITUTION BY DETERGENT DIALYSIS

Reconstitution by detergent dialysis involves mixing the membrane protein with a solution of lipids in a given detergent, followed by slow removal of the detergent by dialyzing overnight or longer. The protein inserts into the vesicles which form as the detergent concentration is reduced. The main advantage of this method is that aggregated protein can

Figure 13

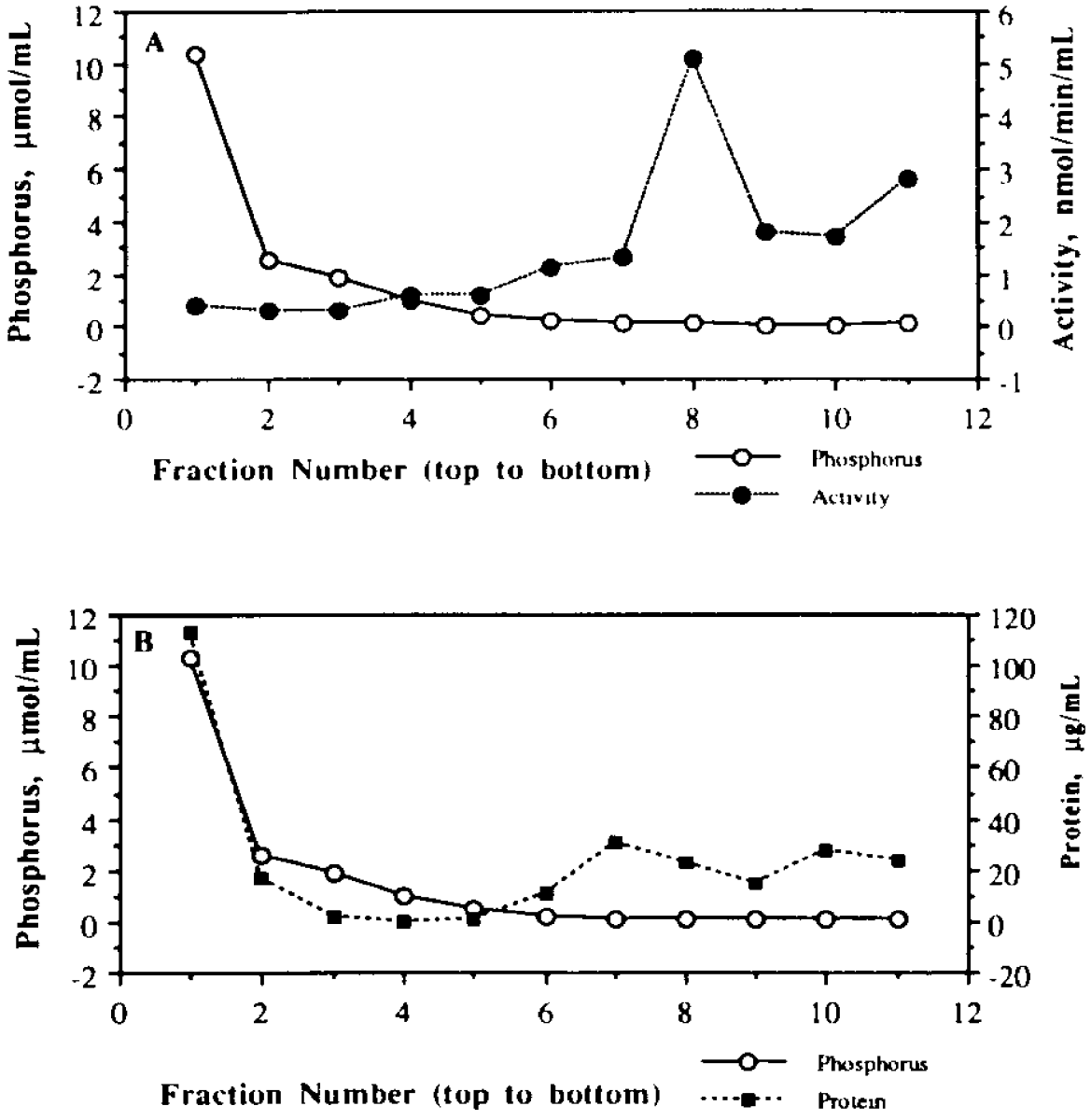
Detergent Facilitated Reconstitution of *Purified* MAO-B with 0.5 mg Octylglucoside[a]



[a]MAO-3a (564µg protein) in 140µL 50mM Na phosphate, pH 7.2 (NaP) was treated with 0.5mg OG in 60µL 50mM hepes/100mM KCl, pH 7.4 (HK) for 30mins at 0°C. MAO-OG solution was then added dropwise to 0.9mL DOPC SUVs (10mg lipid/mL in HK) and stirred at 0°C for 40mins. One mL of MAO-OG-DOPC suspension was applied to 10-80% linear glycerol gradient in HK and centrifuged in SW41 rotor at 39,000rpm at 4°C for 89hrs. Gradient was fractionated from the top into 1mL fractions. Aliquots (250µL) were dialyzed against NaP prior to activity assay with 1mM kynuramine•2HCl in NaP. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

Figure 14

Detergent Facilitated Reconstitution of *Purified* MAO-B with 2.0 mg Octylglucoside[a]



[a]MAO-3a (564µg protein) in 140µL 50mM Na phosphate, pH 7.2 (NaP) was treated with 2.0mg OG in 60µL 50mM hepes/100mM KCl, pH 7.4 (HK) for 30mins at 0°C. MAO-OG solution was then added dropwise to 0.9mL DOPC SUVs (10mg lipid/mL in HK) and stirred at 0°C for 40mins. One mL of MAO-OG-DOPC suspension was applied to 10-80% linear glycerol gradient in HK and centrifuged in SW41 rotor at 39,000rpm at 4°C for 89hrs. Gradient was fractionated from the top into 1mL fractions. Aliquots (250µL) were dialyzed against NaP prior to activity assay with 1mM kynuramine•2HCl in NaP. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

be first disaggregated in detergent prior to reconstitution of the proteoliposomes. However, disadvantages of this method are that residual detergent may affect function or protein properties and that a broad range of lipid to protein ratios is obtained.

A. OG Dialysis in Hepes/KCl Buffer

1. Buffers Used

Hepes and phosphate were the two buffers of choice for liposome preparation. Hepes offers the advantage over phosphorus buffer, that organic solvent extraction of phospholipids prior to the phosphorus assay is not necessary. However, monoamine oxidase, a mitochondrial protein has a preference for phosphate anion, expressing maximal activity in *in vitro* assay. For this reason, reconstitution of MAO-B in an all-phosphate system was attempted. Visual inspection of the protein/phospholipid sample resulting from OG dialysis against phosphate buffer however, showed no significant increase in sample turbidity associated with vesicle formation following overnight dialysis. An additional 2 to 3 days of dialysis was required to obtain turbidity *(i.e.*, vesicle formation), at which time the enzyme showed only negligible activity. Thus, it was necessary to use Hepes buffer for subsequent detergent dialysis reconstitution.

2. Vesicle Formation by Detergent Dialysis

Evidence for vesicle formation in Hepes by detergent dialysis using octylglucoside was obtained from examination of the turbidity of liposomes prepared without protein, and from electron microscopy of both liposomes and proteoliposomes.

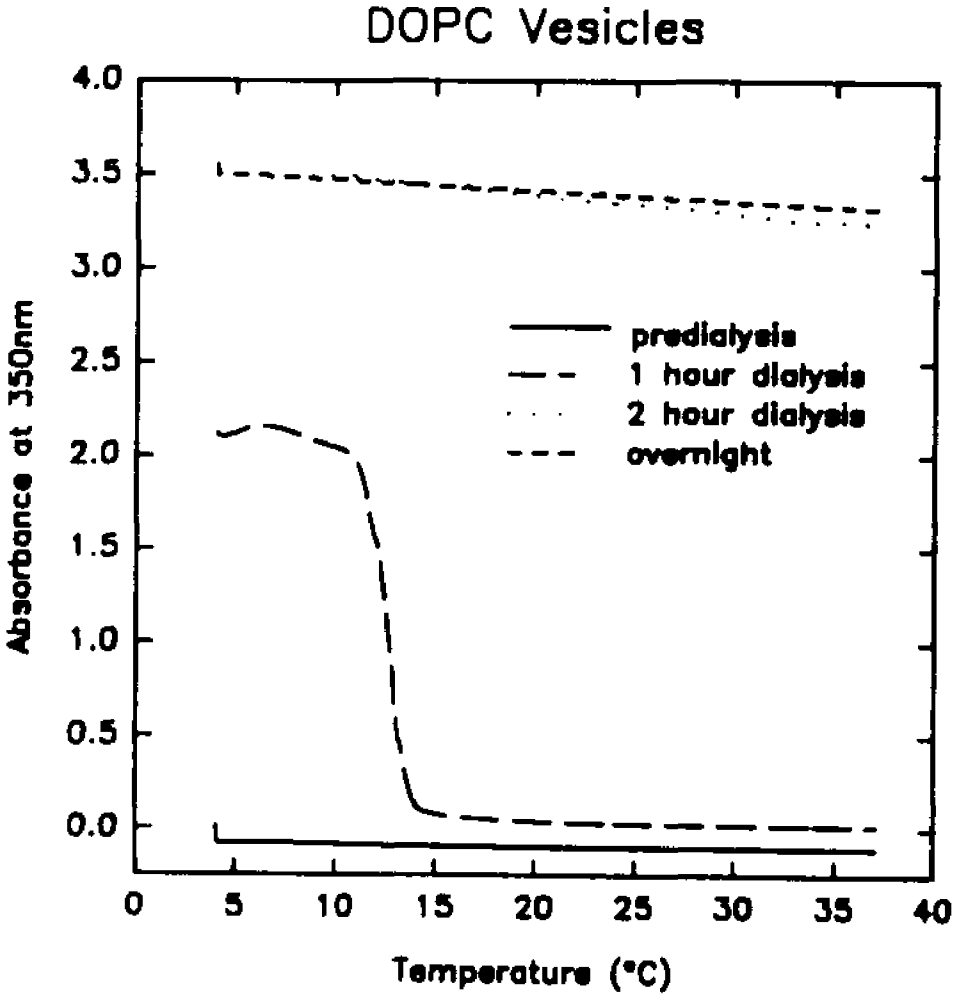
The turbidity of OG-solubilized DOPC shows a broad transition in its scatter profile over a period of 20 hours, suggesting a corresponding change from small micellar to highly scattering vesicular structures (Paternostre *et al*., 1988; Miguel *et al*., 1989). Light scattering by these structures was further investigated as a function of temperature (Figure 15). The sample consisted of 12.5mg DOPC solubilized with 44.4mM OG in HK, representing an OG/Lipid molar ratio of 8.4. The sample was dialyzed against HK buffer in a membrane tube from which aliquots were removed at various times in order to measure the optical density at 350nm. At this wavelength, no interference from the absorbance of OG (λ_{max} at 260nm) was present. The optical density at 350nm was monitored, relative to a blank of HK buffer, as a function of increasing temperature from 4° to 37°C. Over this temperature range, the corresponding CMC for OG decreases from 31 to 16mM (Miguel *et al*., 1989). Prior to dialysis, the sample exhibited no light scattering over this temperature range. After dialyzing for one hour, the optical density observed was approximately 2.0 at 4°C, suggesting formation of vesicular structure. With increasing temperature from 11 to 14°C, the observed scattering declined sharply, suggesting disintegration of vesicular structure. In contrast, after 2 hours incubation at 4°C, the optical density increased to 3.5. Now with increased temperature, only a slight decrease in turbidity was observed. After overnight dialysis, the turbidity level remained constant and unchanged, and again stable with increasing temperature. This data suggest that stable vesicles are formed as early as 2 hours into dialysis; however, overnight dialysis was used routinely to ensure vesiculation.

Similarly, turbidity was examined as a function of time and temperature in the dialysis

Figure 15

Effect of Temperature on Turbidity of DOPC Pre- and Post-Dialysis[a]

Transition from Micellar to Vesicular Structure during Dialysis with Octylglucoside



[a]DOPC (12.5mg) was dissolved in 2mL octylglucoside solution (19.5mg/mL in 50mM Hepes/100KCl, pH 7.4 (HK)) at 4°C. The lipid solution was diluted with 1mL HK, and the optical density at 350nm scanned over the temperature range 4-37°C (pre-dialysis T-ramp). The lipid solution was dialyzed against HK buffer at 4°C. At time intervals, a 1mL sample was removed from the dialysis tube and scanned as above, then returned for contined dialysis. T-ramps were performed after 1, 2, and 20 hours of dialyzing.

of an OG solubilized sample of mixed lipids (75%DOPC/25%DOPE) (Figure 16). For this vesicle system, after dialyzing for 1 7 hours, a gradual decrease in turbidity from 7 to 35 °C was observed, unlike the sharp decline observed previously for 100% DOPC As determined from maximal turbidity, temperature stability of the formed vesicles occurred after 3 hours of dialysis.

Liposomes comprised of 70%DOPC/30%DOPE, prepared by OG dialysis, were examined by electron microscopy. Electron micrographs provided conclusive evidence for formation of vesicles (Figure 17). With inclusion of the protein, similar results were obtained (Figure 18). That these were indeed proteoliposomes was shown by glycerol gradient characterization of another 70%DOPC/30%DOPE reconstitution identically prepared. The electron micrographs show that both the protein-free and protein-containing liposomes were predominantly single-walled vesicles. The size ranges for both types of vesicles are shown in Figure 19; the average diameter was 139 ± 35 nm for the proteoliposomes (59 PRLs) and141 ± 32 nm for the liposomes (90 LPs).

Physical separation of the proteoliposomes from any protein-free vesicles formed by OG dialysis is not achieved by glycerol density gradient centrifugation. One might expect to be able to separate these on the basis of charge because of the negative charge contributed by MAO-B. Attempts were made to use the anionic exchange medium Q-Sepharose for this purpose. However, no conclusive data resulted from this method and it was not pursued.

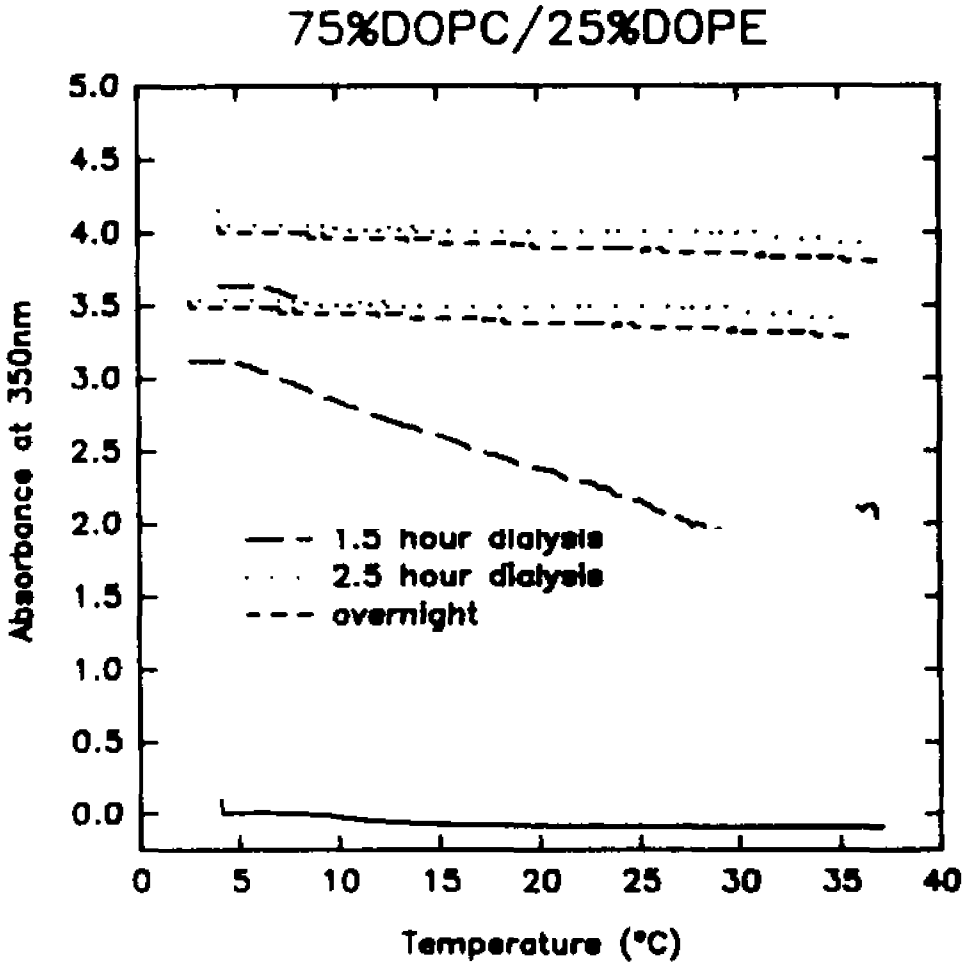
3. Gradient Characterized OG Dialysis Reconstitutions

Gradient profiles (10-80% glycerol) for the products of reconstitution obtained with

Figure 16

Effect of Temperature on Turbidity of 75% DOPC/25% DOPE Pre- and Post-Dialysis[a]

Transition from Micellar to Vesicular Structure during Dialysis with Octylglucoside



[a]DOPC (9.4mg) and DOPE (3.1mg) were dissolved in 2mL octylglucoside solution (19.5mg/mL in 50mM Hepes/100KCl, pH 7.4 (HK)) at 4°C. The lipid solution was diluted with 1mL HK, and the optical density at 350nm scanned over the temperature range 4-37°C (pre dialysis T-ramp). The lipid solution was dialyzed against HK buffer at 4°C. At time intervals, a 1mL sample was removed from the dialysis tube and scanned as above, then returned for continued dialysis. T ramps were performed after 1.5, 2.5, and 20 hours of dialyzing.

Figure 17

Electron Micrograph of 70%DOPC/30%DOPE Vesicles[a]

Liposomes prepared by detergent dialysis in the absence of protein

Magnification = 49,000x

[a]DOPC and DOPE were dissolved at 70/30mole% in 1.95mg/mL octylglucoside (OG) in 50mM Hepes/100mM KCl, pH 7.4 (HK) (6.25mg lipid/mL). Two mL of the lipid solution was diluted with 1mL HK prior to rapid dialysis against HK to form LUVs. All steps were performed at 4°C. The vesicle preparation was diluted 1:1000 with HK, and sample was adhered to carbon-coated Formvar on copper grids (glow-discharged). Following negative staining with 5% uranyl acetate, vesicles were examined using a JEOL-100 CX-II electron microscope at a magnification of 36,000. Prints of the electron micrographs were enlarged to a final magnification of 49,000.

Figure 18

Electron Micrograph of 70%DOPC/30%DOPE Vesicles Containing *Purified* MAO-B[a]

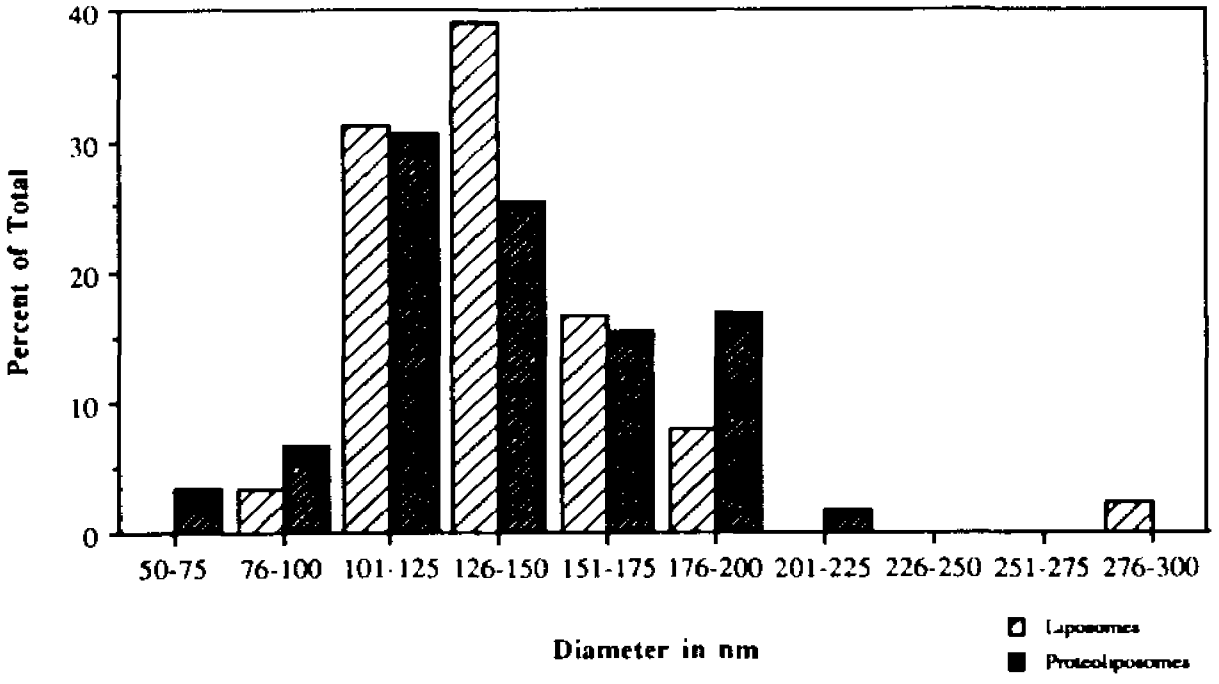
Proteoliposomes prepared by detergent dialysis in the presence of MAO-11

Magnification = 73,400x

[a]MAO-11 (1.7mg protein) was stirred for 1min with octylglucoside (1.95mg OG/mL in 50mM Hepes/100mM KCl, pH 7.4 (HK)). DOPC and DOPE were dissolved at 70/30 mole% in OG solution (6.25mg lipid/mL). The enzyme and lipid solutions were mixed, then diluted with HK (1mL per 2mL sample), prior to rapid dialysis against HK to form LUVs. All operations were performed at 4°C. The vesicle preparation was diluted 1:1000 with HK, and sample was adhered to carbon-coated Formvar on copper grids (glow-discharged). Following negative staining with 5% uranyl acetate, vesicles were examined using a JEOL-100 CX-II electron microscope at a magnification of 36,000. Prints of the electron micrographs were enlarged to a final magnification of 73,400.

Figure 19

Size Range of Protein-Free and Protein-Containing 70%DOPC/30%DOPE Vesicles Prepared by Detergent Dialysis[a]



[a]Liposomes and proteoliposomes were prepared as described in Figures 17 and 18, respectively. Vesicle diameters were measured from the electron micrographs.

different octylglucoside concentrations are shown in Figures 20 and 21. The coincidence of enzyme activity and phospholipid-phosphorus is unequivocal evidence of reconstitution of the *purified* enzyme (MAO-3) in DOPC liposomes.

This coincidence of MAO-B activity and lipid was found in fraction 7 in Figure 20 (7.5mg OG) and at the top of the 10-80% glycerol gradient in Figure 21 (15mg OG). It may be expected that the greater buoyancy of the latter proteoliposomes as compared to the former may arise from a greater incorporation of protein into the former vesicles, since the vesicle density would increase with higher protein content. Indeed, calculated lipid to protein dimer (L/P_{dimer}) molar ratios reveal 3974 versus 3527, respectively. Similar reconstitutions were carried out for a *partially purified* enzyme preparation (MAO-6). A 3-60% glycerol gradient was used to characterize the dialysis reconstitution in which 45mg OG had been used to solubilize the lipid (OG/ DOPC weight ratio of 3:1). Again, enzyme activity was found in the top two fractions along with phospholipid-phosphorus (Figure 22), indicating successful reconstitution.

Although the activity of the OG-solubilized enzyme was only 3% of untreated enzyme samples, reconstitution with phospholipid (DOPC, DOPE, DOPS and DOPA, in various mole% combinations) effected reactivation of 22 to 30% of the control activity.

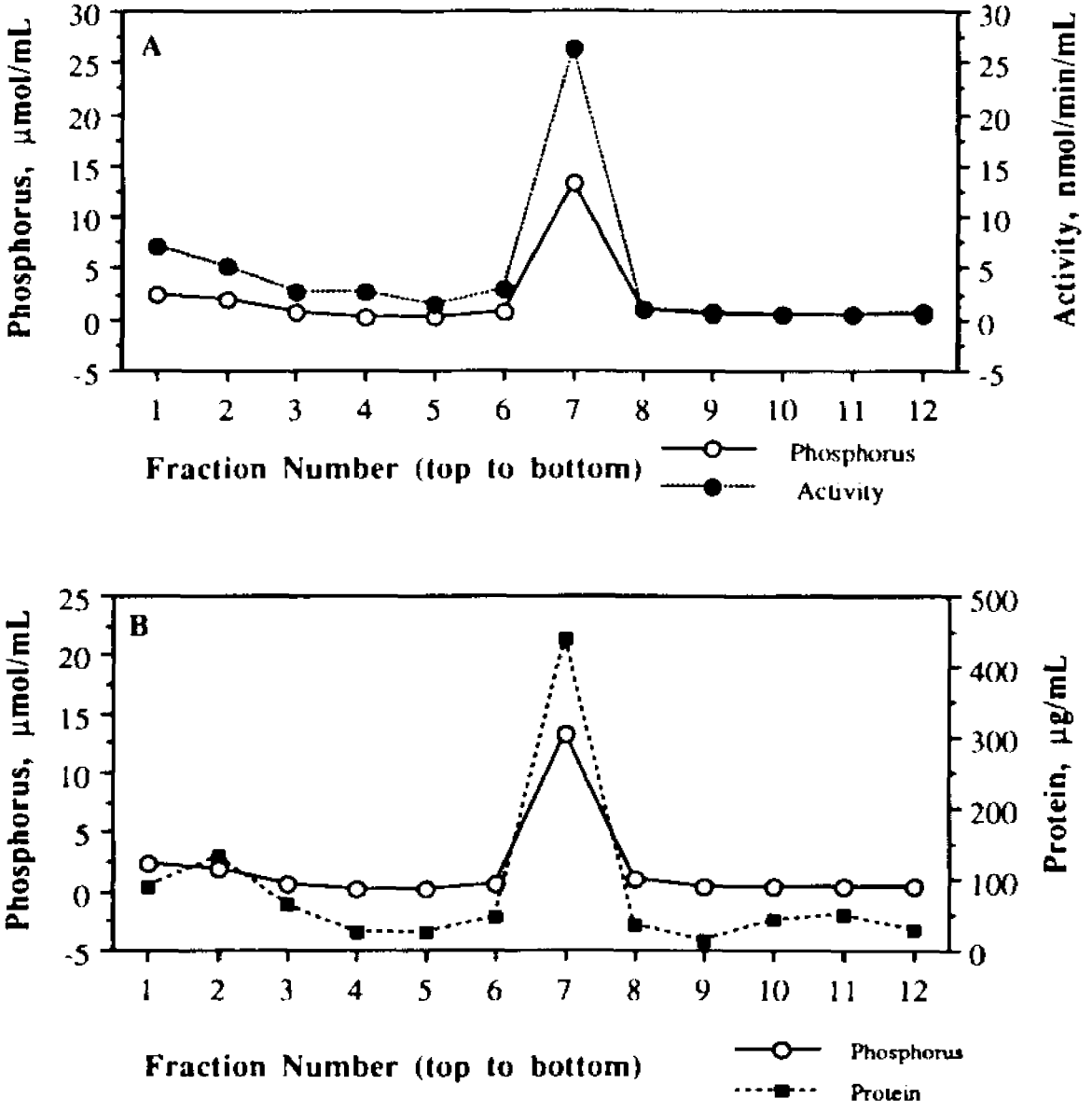
B. Stability of MAO-B Pre- and Post-Incorporation

1. Comparison of Limited OG Solubilization of MAO-B and Rapid Dialysis

Based on the observation using octylglucoside discussed above (and in section I.D.3), it was necessary to minimize exposure of the enzyme to this detergent where [OG] was

Figure 20

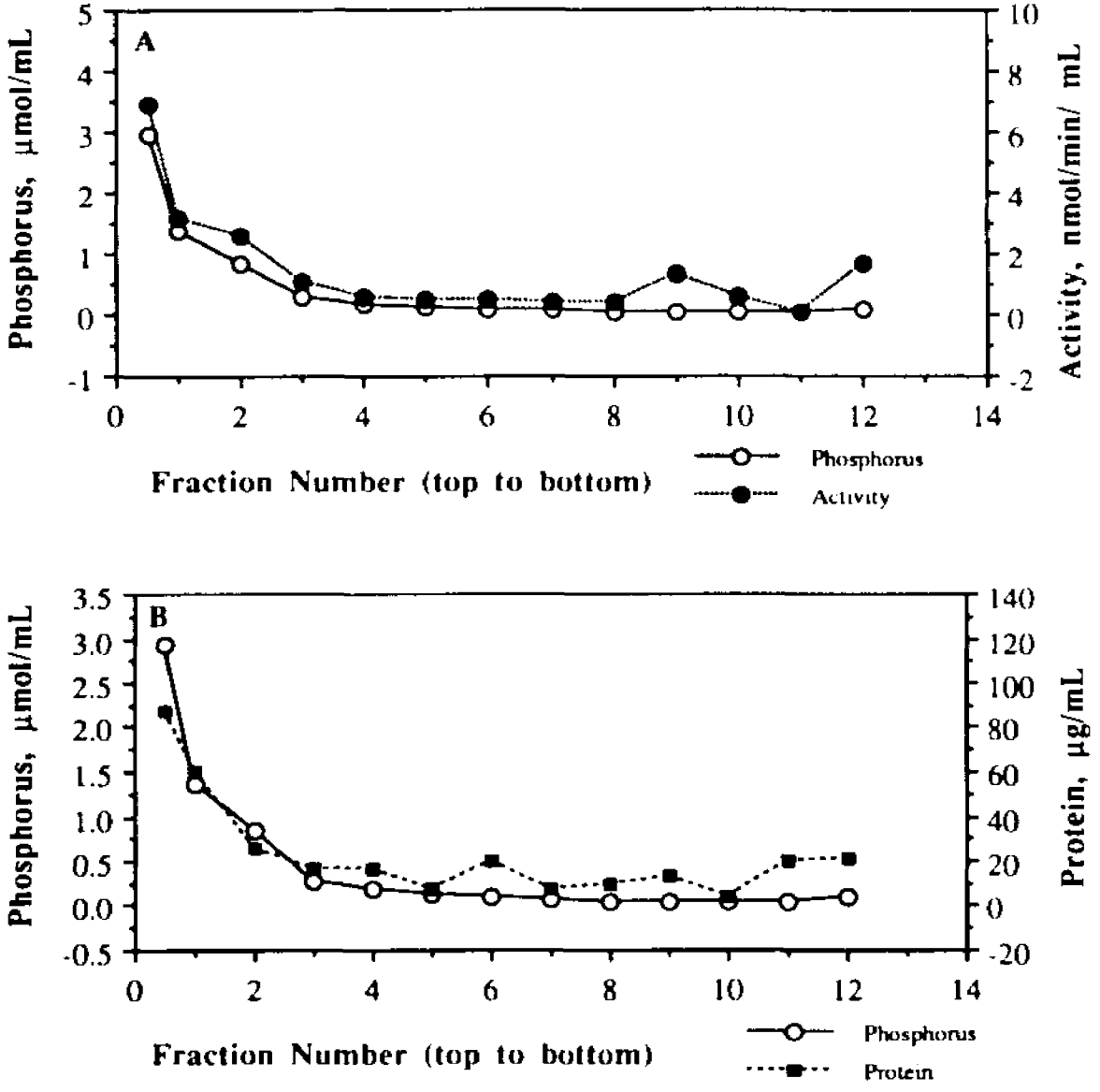
Reconstitution of *Purified* MAO-B by Detergent Dialysis with 7.5mg Octylglucoside[a]



[a]DOPC (15mg) was suspended in 1mL of 7.5mg/mL solution of octylglucoside (OG) in 50mM Hepes/100mM KCl, pH 7.4 (HK). Lipid sample was vortexed and subjected to 5 cycles of freeze/thaw. MAO-3 (600µg) was treated with 15mg OG in HK (0.2mL volume) for 30 mins at 0°C. Lipid and enzyme samples were mixed, then dialyzed against HK for 24hrs at 4°C. One mL of dialysate was applied to 10-80% linear glycerol gradient in HK and centrifuged in SW41 rotor at 39,000rpm at 4°C for 90hrs. Gradient was fractionated from the top into 1mL fractions. Aliquots (250µL) were dialyzed against 50mM Na phosphate, pH 7.2, prior to activity assay with 1mM kynuramine•2HCl. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

Figure 21

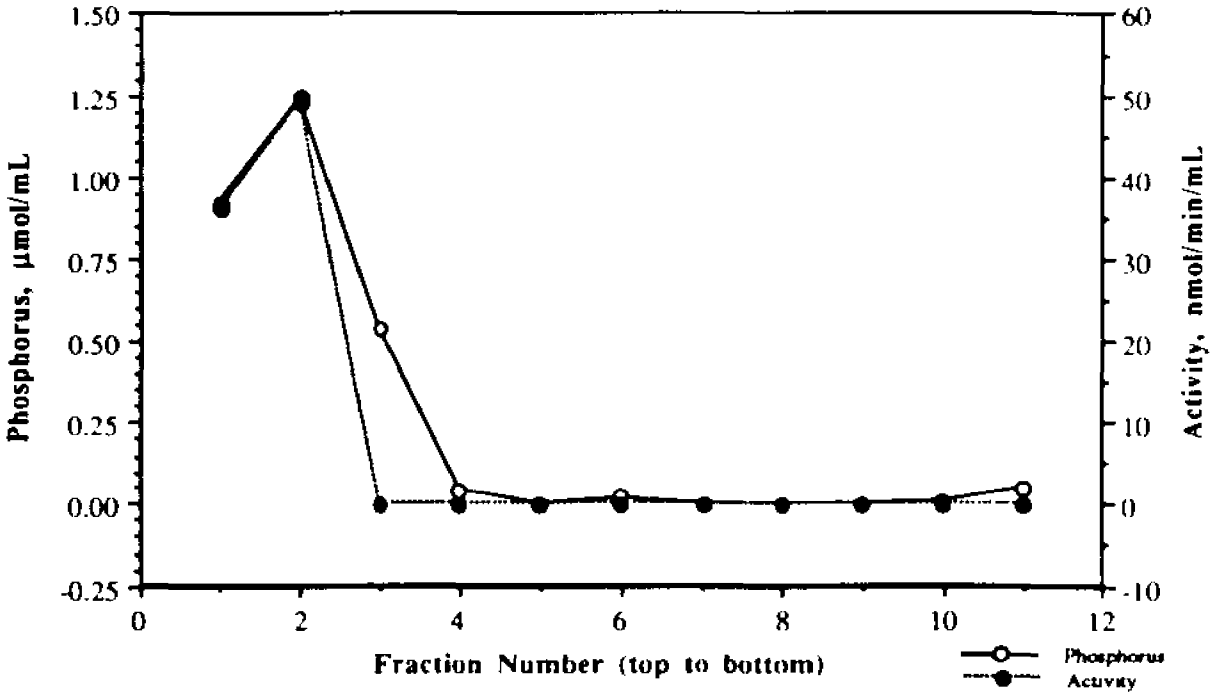
Reconstitution of *Purified* MAO-B by Detergent Dialysis with 15 mg Octylglucoside[a]



[a]DOPC (15mg) was suspended in 1mL of 15mg/mL solution of octylglucoside (OG) in 50mM Hepes/100mM KCl, pH 7.4 (HK). Lipid sample was vortexed, subjected to 10 cycles of freeze/thaw, then sonicated to clarity (20mins). MAO-3 (600µg) was treated with 15mg OG in HK (0.2mL volume) for 1hr at 0°C. Lipid and enzyme samples were mixed, then dialyzed against HK for 21hrs at 4°C. Dialysate (995µL) was applied to 10-80% linear glycerol gradient in HK and centrifuged in SW41 rotor at 39,000rpm at 4°C for 92hrs. Gradient was fractionated from the top; 0.5mL was collected for the first 2 fractions, 1mL for the rest. Aliquots (250µL) were dialyzed against 50mM Na phosphate, pH 7.2, prior to activity assay with 1mM kynuramine•2HCl. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

Figure 22

Reconstitution of *Partially Purified* MAO-B by Detergent Dialysis[a]



[a]DOPC (15mg) was solubilized with 45mg octylglucoside (OG) at concentrations of 3.1mg DOPC and 9.3mg OG per mL 50mM Hepes/100mM KCl, pH 7.4 (HK). MAO-6 (463µg) was treated with 12.5mg OG in HK (1.0mL volume) for 30mins at 0°C. Lipid solution was mixed with the enzyme sample. Four 1mL aliquots were dialyzed against HK for 20hrs at 4°C. One dialyzed aliquot was applied to 3-60% linear glycerol gradient in 50mM Hepes/150mM KCl, pH 7.4 (modified HK) and centrifuged in SW41 rotor at 39,000rpm at 4°C for 90hrs. Gradient was fractionated from the top into 1mL fractions. . Aliquots (0.5mL) were dialyzed against 50mM Na phosphate, pH 7.2, prior to activity assay with 1mM kynuramine•2HCl. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

elevated in order to maximize lipid solubilization before dialysis. Under these conditions, protein solubilization was limited to one minute of stirring (in contrast to the previous 30-60 minutes for lower [OG]) with octylglucoside before addition to the lipid solution, dilution with HK buffer, and immediate transfer to the dialysis apparatus. PRLs prepared by the rapid dialysis procedure (see Methods II.D.4) exhibited enzymatic activity ranging from 46 to 87% (depending on the isolate) of that seen with control aliquots of solubilized MAO-B that were not exposed to OG, but were maintained at 4°C for the same length of time as the PRLs. This contrasts with PRLs prepared using the longer protein solubilization period and recirculating buffer, in which only 22 to 30% of the activity was recovered. Thus, the rapid dialysis procedure was considered superior and consequently adopted for the remainder of this study.

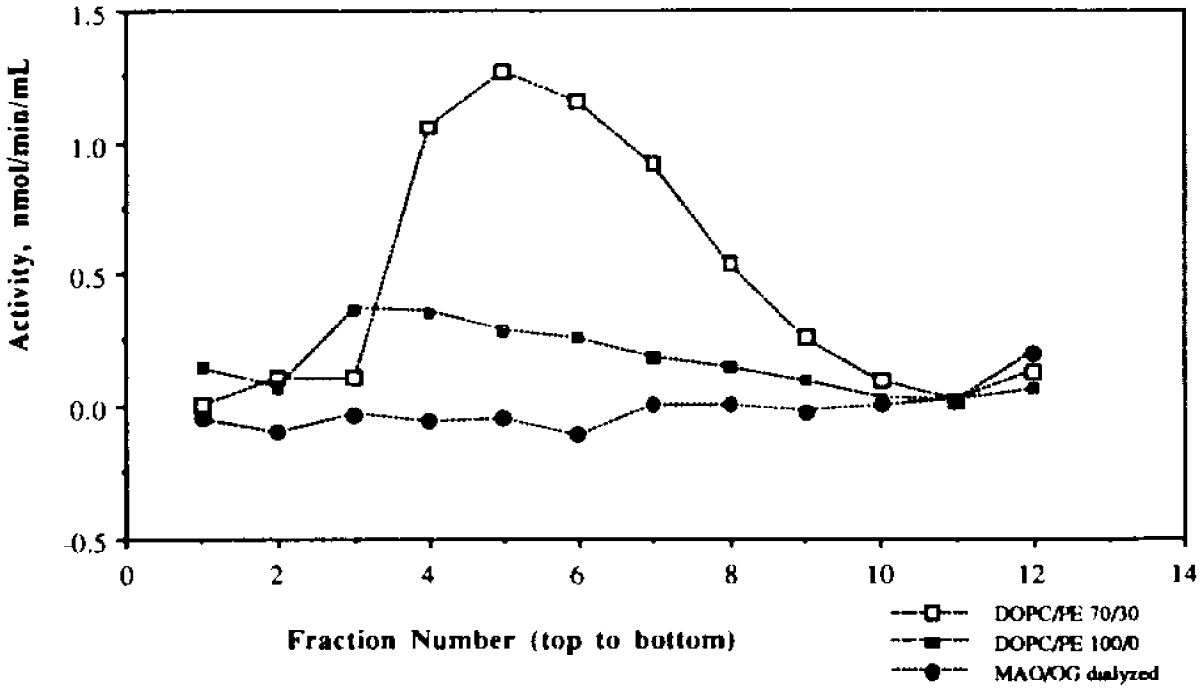
2. Gradient Characterized Rapid Dialysis Reconstitutions

Proteoliposomes (both pure DOPC and 70/30 mole% DOPC/DOPE vesicles), prepared by the rapid dialysis method, were isolated on 3-20% glycerol gradients, in contrast to the customary 10-80% glycerol, in an attempt to separate the proteoliposomes from non-protein containing vesicles. Fractions were analyzed for evidence of reconstitution. A sample of OG-solubilized MAO-B was also dialyzed and run on this gradient type as a control.

Only 1.2% of the initial activity remained in the OG-solubilized MAO-B and this was found at the bottom of the gradient (Figure 23). This contrasted sharply with the activity profiles of the DOPC and DOPC/PE proteoliposomes (Figures 24 and 25, respectively), in

Figure 23

Profile of Activity on 3-20% Glycerol Gradients of *Purified* MAO-B: Octylglucoside-solubilized and Reconstituted[a]



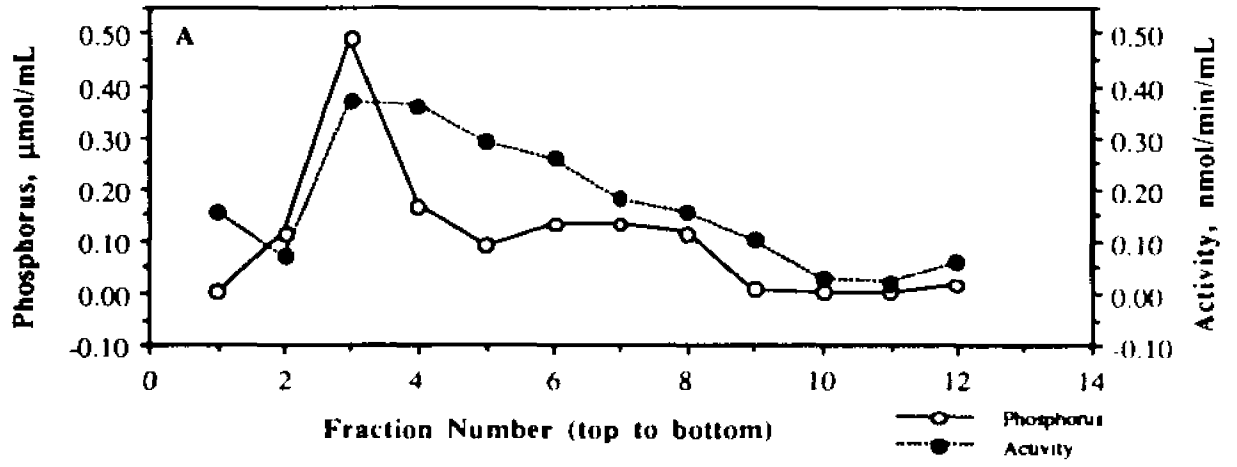
[a]*Purified* MAO was solubilized in octylglucoside and divided into three aliquots. Two aliquots were reconstituted by detergent dialysis with either 100%DOPC or 70%DOPC/30%DOPE.

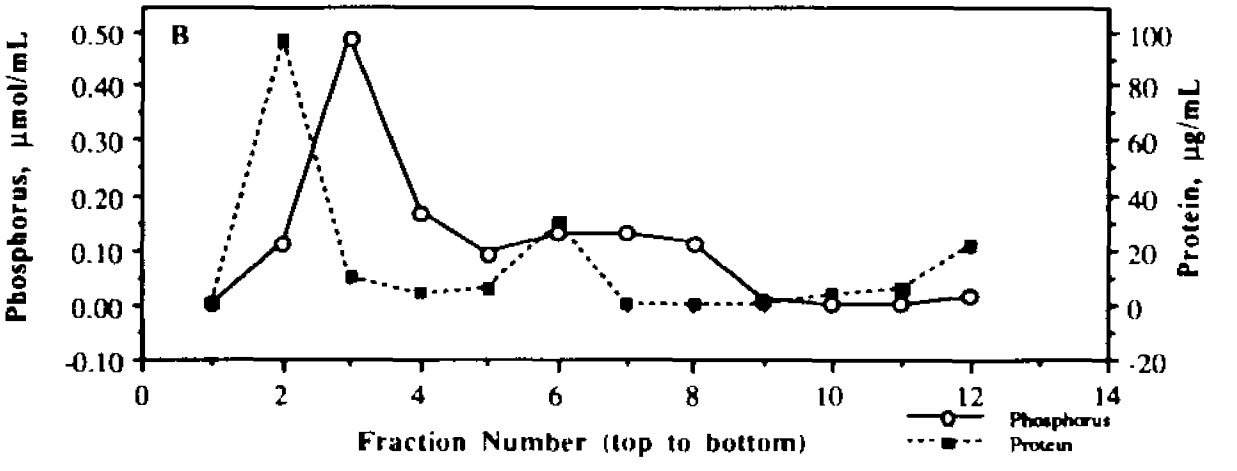
The third aliquot was dialyzed at 4°C without added phospholipid.

The three samples were run on 3-20% glycerol density gradients. The gradients were fractionated into 1mL aliquots which were dialyzed against NaP buffer to remove glycerol. The dialyzed fractions were assayed at 30°C for enzymatic activity toward kynuramine.

Figure 24

Reconstitution of *Purified* MAO-B with 100% DOPC by Rapid Detergent Dialysis[a]

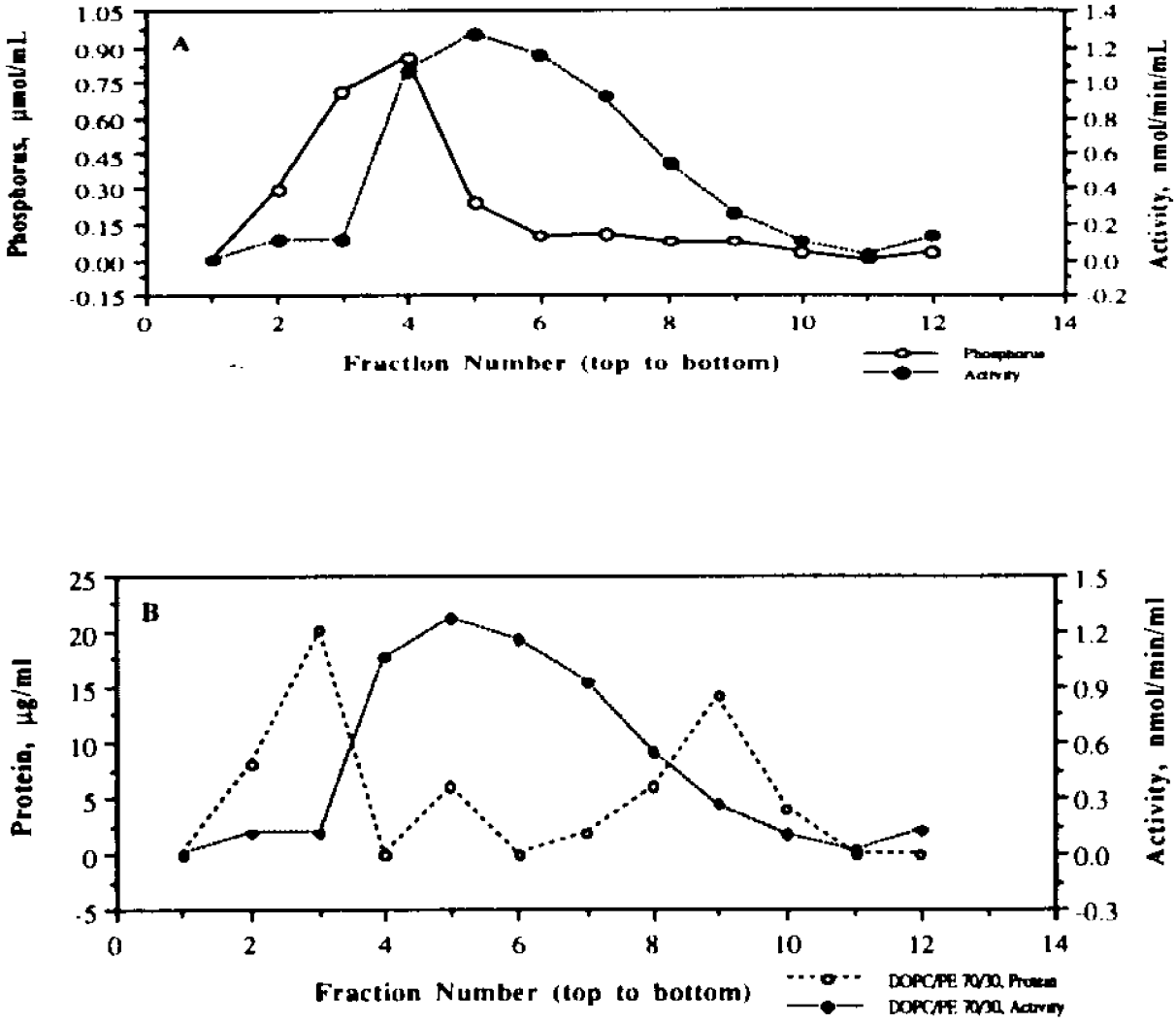




[a]Procedure for reconstitution of MAO-8 with DOPC as detailed in Figure 23 legend. Activity assay was performed on dialyzed fractions from the 3-20% glycerol gradient. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

Figure 25

Profile of Activity and Phospholipid-Phosphorus on 3-20% Glycerol Gradient of *Purified* MAO-B Reconstituted into 70% DOPC/30% DOPE Vesicles by Rapid Detergent Dialysis[a]



[a]Procedure for reconstitution of MAO-B with DOPC/DOPE (70/30 mole%), as detailed in Figure 23 legend. Activity assay was performed on dialyzed glycerol gradient fractions. Phospholipid-phosphorus and protein assays were performed on undialyzed fractions.

which the enzymatic activity appeared mainly in the upper part of the gradient. For both proteoliposome preparations, the activity moved with the phospholipid-phosphorus, although the activity peak was broader than that of the phospholipid.

For PRLs composed of 100% DOPC, coincidence of PL-P and activity occurred mainly in fraction 3, with a phospholipid tailing peak over fractions 6 to 8 and a broad band of activity dropping down to fraction 8 (Figure 24). Lipid to protein dimer molar ratios were highest for the most active fractions, 3 and 4 (5400 and 5533, respectively), which corresponded to glycerol concentrations of 7% and 8-9%, respectively. The lower L/P_{dimer} ratios found in fractions 5 and 6 may represent populations of vesicles with greater concentrations of incorporated enzyme; paradoxically, these showed less activity. The DOPE-containing vesicles were less buoyant than those without this lipid. Enzyme activity was found in a broad band, part of which coincided with peak PL-P in fraction 4 (Figure 25) at 8-9% glycerol. Again, subpopulations with greater density had lower L/P_{dimer} ratios and lower activity. It would seem from this that a high level of incorporation interferes with the enzyme's function. Perhaps, at high density the protein is not sufficiently solvated and aggregates within the bilayer with consequent reduction of activity.

Comparison of the buoyancy of PRLs prepared by rapid dialysis with those prepared with the re-circulating buffer method is difficult since different types of gradients were used to characterize the vesicles. Whereas 3-20% glycerol gradients in HK were used for the rapid method, the glycerol gradients used for the original method were either 10-80% in HK or 3-60% glycerol in modified HK (150mM KCl).

3. Stability of Proteoliposomes

The stability of *purified* MAO-B reconstituted into vesicles prepared by both methods of detergent dialysis was examined by assaying the activity over a period of several days during which the PRLs were maintained at 4°C. Enzyme activity was seen to decrease slowly over a six day period (Table 18).

IV. EFFECT OF LIPID COMPOSITION ON RECONSTITUTED MAO-B

A. Effect of Headgroups

1. Bilayer/Nonbilayer

The influence of phosphatidylethanolamine (PE) on reconstituted monoamine oxidase was explored. Dioleoylphosphatidylethanolamine (DOPE) was substituted in increasing percentage for DOPC in the rapid detergent dialysis procedure.

The change in optical density at 350nm of the DOPC/DOPE mixtures with MAO-B prior to dialysis, compared with that of the corresponding vesicles post-dialysis, is shown in Figure 26. At 30°C, the turbidity of the vesicle preparation increased in a linear fashion with increasing DOPE content; 60% DOPE vesicles exhibited 2.3 times the turbidity of non-DOPE vesicles. All of the 0-60 mole% DOPE pre-dialysis samples were cloudy at 0-4°C, suggesting lipid/protein vesicles, but cleared when warmed to 12°C, indicating solubilization of lipid as shown previously (Figures 15 and 16). The post-dialysis samples remained turbid at 12°C due to the formation of proteoliposomes.

Enzyme activities were measured using the kynuramine assay procedure for *purified* MAO-B in proteoliposomes with DOPE content ranging from 0-60% of the total lipid. The

Table 18

Stability of *Purified* MAO-B in Vesicles Prepared by Detergent Dialysis Procedures

Enzymatic Activity as Percent of Rate on Day 1[a]

	100% DOPC (original)[b]	100% DOPC (rapid)[c]	75%DOPC/30%DOPE (rapid)[d]
Day 1	100 ± 3.6 [e]n=2	100 ± 3.9 n=3	100 ± 3.3 n=3
Day 2		85.7 ± 2.2 n=2	92.6 ± 2.4 n=2
Day 3	87.2 ± 5.8 n=1		
Day 5	81.3 ± 2.0 n=2		
Day 6		59.5 ± 2.9 n=1	71.8 ± 1.5 n=1

[a]Activity of proteoliposome samples was measured by oxidation of 1mM kynuramine2HCl at 30°C in 50mM Na phosphate, pH 7.2. Proteoliposome samples were maintained at 4°C and assayed at the indicated times. Percent of activity on day 1 was used as a measure of stability of the reconstituted enzyme.

[b]Reconstitution of MAO-3 with DOPC was performed as described in legends to Figures 20 and 21.

[c]Reconstitution of MAO-8 with DOPC was performed as described in legend to Figure 23.

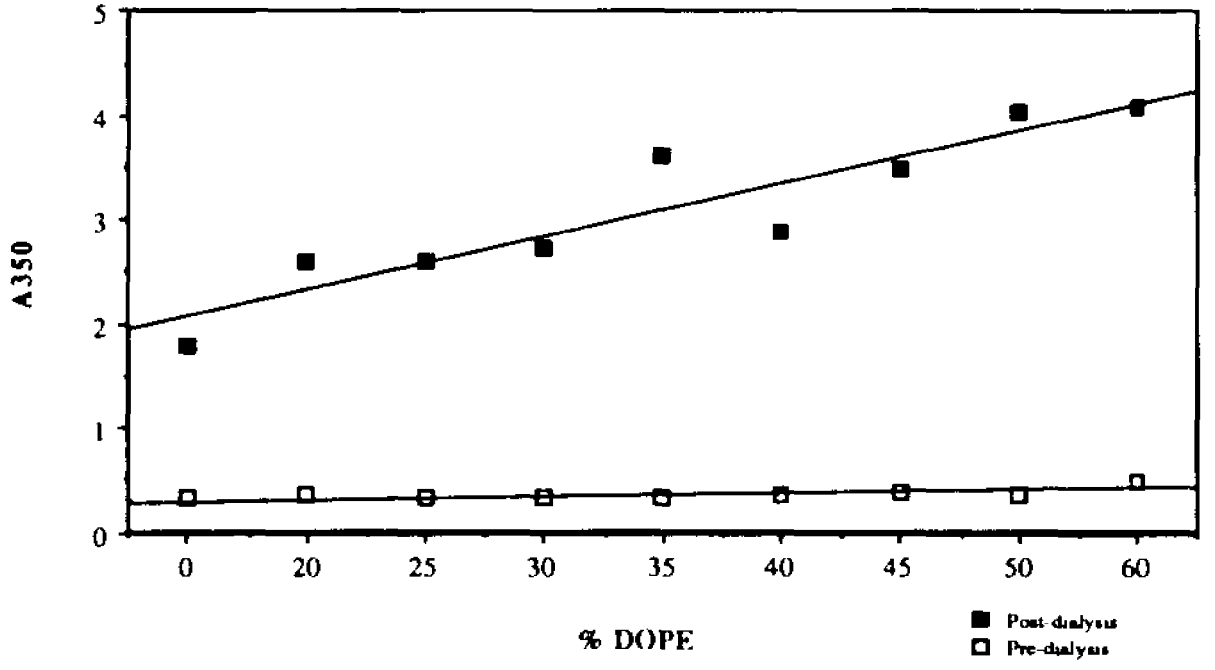
[d]Reconstitution of MAO-8 with DOPC/DOPE (70/30 mole%) was performed as described in legend to Figure 23.

[e]n=number of experiments performed, each assayed in triplicate.

Figure 26

Change in Absorbance with Increasing DOPE Content[a]

A_{350} of DOPC/DOPE/MAO-B Pre- and Post-Dialysis



[a]DOPC/DOPE mixtures (containing 0 to 60 mole% DOPE) were solubilized with octylglucoside: 6.25mg lipid and 19.5mg OG per mL 50mM Hepes/100mM KCl, pH 7.4 (HK). Lipid samples were subjected to 5 cycles of freeze/thaw. *Partially purified* MAO-6 (2.0mg) was treated with 40mg OG in HK (2.2mL volume) for 1hr. Enzyme solutions (0.2mL) was mixed with 0.8mL of each lipid solution and the samples diluted to 1.5mL with HK. All operations were performed at 4°C. Optical density at 350nm was measured for all samples at 12°C (pre-dialysis). Following overnight dialysis against HK at 4°C, optical density was again measured for all samples at 12°C (post-dialysis).

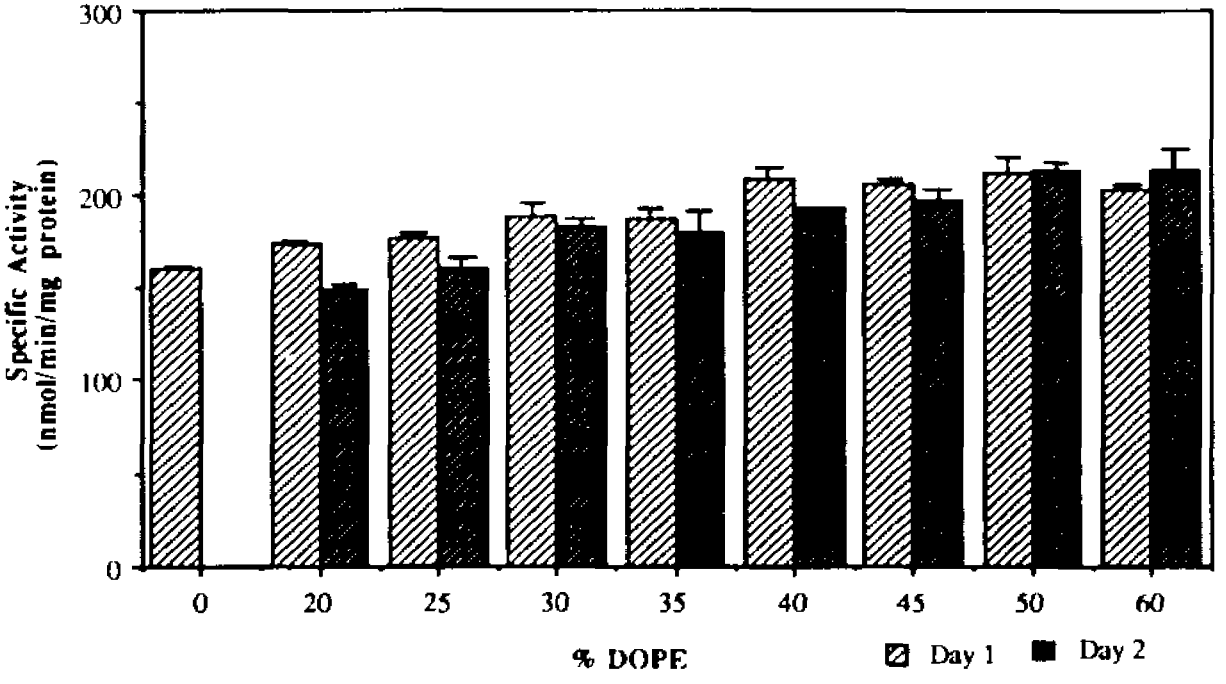
results are summarized in Figure 27. Compared with DOPC, reconstituted enzyme activity increased with all amounts of DOPE, with the higher concentrations (40 to 60 percent PE) plateauing at 28-32% above the control activity. Activity of *purified* MAO-B in the 70/30 vesicles was 18% greater than in the absence of polymorphic lipid, while activity in the 50/50 vesicles was 12% higher than with 70/30. Further, stability of the proteoliposomes was estimated by repeating the assay after overnight incubation at 4°C and the ratio of measured activities for day 2 to day 1 was used as a measure of stability. Figure 27 shows that stability tended to improve with increased DOPE, with proteoliposomes containing 50-60 mole% retaining all of their initial activity. However, lower DOPE was not as efficient at maintaining enzyme activity, unlike the higher mole ratios. A similar DOPE lipid effect was observed for two different MAO-B preparations. Results are shown in Table 19. In each case the maximum effect on enzyme activity was seen with 40 mole% DOPE, while the 70/30 (DOPC/DOPE) vesicles exhibited more modest increases of 7% and 5%, respectively.

Although both activity and stability at the higher PE concentrations were marginally improved above that obtained with the other PE concentrations, it was decided to continue the investigation with 30% DOPE, that being more physiologically relevant.

Reported differences in MAO-B activity in the presence of polymorphic lipid may reflect some degree of membrane compartmentalization; protein domains can facilitate improved access of substrate to enzyme or favor the protein conformational changes associated with catalysis. Although hexagonal phase formation is unlikely to occur with 30% DOPE (Cullis *et al.*, 1985), tighter bilayer packing due to the small PE headgroup could contribute to this. Studies have shown that PE prefers to locate on the inner leaflet of the

Figure 27

Activity and Stability of *Purified* MAO-B in Proteoliposomes with Increasing Non-Bilayer Lipid Content[a]



[a]MAO-9 (3.7mg) was reconstituted with DOPC/DOPE mixtures (0 to 60 mole% DOPE) essentially as described in Figure 26 legend, except that OG solubilization of enzyme was limited to 1min and that the rapid dialysis method was employed. Proteoliposomes (0.5mL aliquots containing 46.3µg protein) were assayed for enzymatic activity toward 1.0mM kynuramine•2HCl in 50mM Na phosphate, pH 7.2, at 30°C (2.0mL assay volume) (Day 1). Proteoliposome samples were maintained at 4°C overnight, and re-assayed as before to determine stability (Day 2).

Table 19

Relative Activity of *Purified* MAO-B in Proteoliposomes with Increasing Non-Bilayer Lipid Content

Comparison of Reconstitution Experiments with 2 different *purified* MAO-B preparations[a]

% Lipid Composition DOPC/DOPE	Rate Relative to 100/0	
	MAO-9	MAO-10
100/0	1.00 ± 0.02	1.00 ± 0.02
70/30	1.07 ± 0.01	1.05 ± 0.02
60/40	1.16 ± 0.04	1.16 ± 0.07
50/50	1.02±0.01	1.02 ± 0.05
	Rate Relative to 70/30	
	MAO-9	MAO-10
100/0	0.94 ± 0.02	0.95 ± 0.02
70/30	1.00 ± 0.01	1.00 ± 0.02
60/40	1.08 ± 0.04	1.10 ± 0.07
50/50	0.95± 0.01	0.97 ± 0.05

[a]MAO-9 (0.83mg) and MAO-10 (1.2mg) were each reconstituted with DOPC/DOPE mixtures (0 to 50 mole% DOPE) essentially as described in Figure 26 legend, with the modifications indicated in Figure 27 legend. Enzymatic activities were assayed by oxidation of 1mM kynuramine·2HCl in NaP buffer at 30°C and expressed relative to rates obtained with 100% DOPC proteoliposomes or with 70%DOPC/30%DOPE proteoliposomes.

bilayer leading to heterogeneity of lipid packing (Cullis and Hope, 1991).

2. Anionic Lipids

Anionic lipids examined were dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidic acid (DOPA). Activity as measured by kynuramine oxidation was compared with proteoliposomes composed of 100% DOPC or the physiologically relevant 70/30 mole% DOPC/DOPE to ascertain any modulatory role for acidic phospholipids.

a. Dioleoylphosphatidylserine

The effect of DOPS on the activity of reconstituted MAO-B is summarized in Table 20. Addition of small (10 mole%) amounts of DOPS to pure DOPC vesicles was associated with an insignificant decrease in enzyme activity. In contrast, enzyme activity measured for vesicles composed of 100 mole% DOPS activity was significantly lower ($p<0.01$). The negative charge of PS appears to exhibit an inhibitory effect on the enzyme activity.

For proteoliposomes containing 30 mole% PE, addition of up to 10 mole% PS resulted in enzyme activity levels identical to that of the 100% DOPC proteoliposomes. In the presence of 30 mole% PE, with 10 mole% DOPS an inhibitory effect results.

Stability of the reconstituted enzyme in vesicles containing DOPS was determined by re-assaying after storage of the vesicles overnight at 4°C. There was a significant decline in activity of all vesicles containing DOPS. In contrast, no loss of activity was determined for 100 mole% DOPC proteoliposomes (Table 21). It is clear from these data that the presence of DOPS has a destabilizing effect on preserved MAO-B activity. This destabilizing effect

Table 20

Effect of DOPS on the Activity of Purified MAO-B

Reconstituted by Detergent Dialysis[a]

% Lipid Composition DOPC/DOPS	Activity on Day 1	Activity Relative to 100% DOPC
100/0	141.9 ± 4.7	1.00
90/10	136.1 ± 1.9	0.96
0/100	109.2 ± 1.0	0.77*
DOPC/DOPE/DOPS		
70/30/0	153.2 ± 1.4	1.08
60/30/10	141.3 ± 8.1	1.00

[a]MAO-8 (2.22mg) was reconstituted with the indicated lipid mixtures by the procedure described in Figure 26 legend, with the exception that the freeze/thaw treatment of the lipid solutions was omitted. Proteoliposomes (0.5mL aliquots containing 46.3µg protein) were assayed for enzymatic activity toward 1mM kynuramine2HCl in NaP buffer at 30°C.
*$p<0.01$ for the difference in activity between 100%DOPS and 100%DOPC PRLs as determined by Student's t-test

Table 21

Stability of *Purified* MAO-B Activity in Proteoliposomes with and without DOPS[a]

% Lipid Composition DOPC/DOPS	Activity on Day 2	Relative Rates Day 2/Day 1
100/0	139.8 ± 1.5	0.98
90/10	124.3 ± 2.4	0.91*
0/100	96.5 ± 1.5	0.88*
DOPC/DOPE/DOPS		
70/30/0	141.1 ± 5.9	0.92*
60/30/10	122.6 ± 7.5	0.87**

[a]Proteoliposomes described in Table 19 were maintained at 4°C overnight and reassayed for activity as before (Day 2). The ratio of the activity on Day 2 to Day 1 (from Table 19) (relative rates) was used as a measure of the stability of the enzyme reconstituted into vesicles of each lipid composition.

*Difference in mean rates between day 1 and day 2 significant at $p<0.01$ (Student's t-test)

**Difference in mean rates between day 1 and day 2 significant at $p<0.05$ (Student's t-test)

of DOPS is less significant when DOPE is present.

b. Dioleoylphosphatidic acid

MAO-B reconstituted with DOPA was assayed with kynuramine in both sodium phosphate (NaP) and Hepes/KCl (HK) buffers. A comparison of activities in both buffer systems is shown in Table 22; assay in NaP gave higher activity in all lipid environments. Where significant enhancement (or decline) is seen in NaP assay, the change is less marked with HK.

The effect of DOPA on reconstituted MAO-B is shown in Table 22. With two different MAO isolates, one *purified* and the other *partially purified*, addition of 10% DOPA to pure DOPC gave an increase in activity that was significant at $p<0.01$ when assayed in NaP. Vesicles composed of 70% DOPC/30% DOPE exhibited activity in NaP 19-26% higher than that of the 100% DOPC controls ($p\ll 0.01$). However, with addition of 10% DOPA to vesicles containing 30% DOPE loss of activity was observed, the enzymatic rate now being indistinguishable from that of pure DOPC, except for *purified* MAO-7 where the activity in NaP is actually significantly less than in the 100%DOPC controls.

Contradictory results are seen for MAO activity in 100% DOPA vesicles: significant decline in activity in one experiment (MAO-6) and substantial activation in the second (MAO-7) (Table 22). The direction of the change is the same regardless of assay buffer employed. The mean of the two experiments indicated no significant change in activity for MAO-B when incorporated in 100% DOPA vesicles as compared to 100% DOPC vesicles. The basal activity of the MAO isolates used in these experiments was low. The activity of PRLs

Table 22

Effect of DOPA on the Activity of MAO-B Reconstituted in Different Buffers by OG Dialysis[a]

% Lipid Composition	Specific Activity[b]				Activity Relative to Mean of Both Expts[c]	
	MAO-6 *partially purified*		MAO-7 *purified*			
DOPC/DOPA	**HK**	**NaP**	**HK**	**NaP**	**HK**	**NaP**
100/0	14.6 ± 0.4	25.6 ± 0.5	35.7 ± 0.5	48.4 ± 1.1	1.00	1.00
90/10	15.0 ± 0.1	28.7 ± 0.4*	37.6 ± 1.3	54.0 ± 0.3*	1.04	1.12*
0/100	13.6 ± 0.1†	20.1 ± 0.1§	41.4 ± 4.0	58.6 ± 1.3§	1.05	1.00
DOPC/DOPE/DOPA						
70/30/10	15.6 ± 0.8	30.5 ± 0.4§	43.4 ± 1.0*	60.9 ± 1.1§	1.15	1.23*
60/30/10	15.4 ± 1.0	26.1 ± 0.5	33.8 ± 2.0	46.1 ± 0.1§	1.00	0.99

[a]MAO-6 (2.22mg) and MAO-7 (2.23mg) were each reconstituted with the indicated lipid mixtures as described in Figure 26 legend, with the omission of freeze/thaw treatment of the lipid solutions. Aliquots (0.5mL) of the proteoliposomes (46.3µg protein for MAO-6; 43.5µg protein for MAO-7) were assayed for activity toward 1mM kynuramine2HCl at 30°C, both in the reconstitution buffer (50mM Hepes/100mM KCl, pH 7.4) (HK) and in 50mM Na phosphate, pH 7.2 (NaP).

[b]Specific activities are expressed as nmol per minute per mg protein.

[c]For each MAO-B isolate the activity in each lipid mixture was expressed relative to that obtained with 100%DOPC PRLs, and the relative rates for the 2 isolates were averaged.

Comparisons are relative to activity with 100%DOPC; statistical significance: †$p<0.02$, *$p<0.01$, §$p<0.001$ (Student's t-test)

prepared from a more active isolate (*purified* MAO-10) was compared. The mean specific activities (nmol min^{-1} per mg protein) ± SD for triplicate assays were 204.9 ± 18.9 for 100%DOPC and 214.8 ± 4.6 for 100%DOPA; the 5% increase in activity with DOPA was not significant.

B. Acyl Chain Unsaturation

The effect of degree of unsaturation of the acyl chains was assessed by replacing the monounsaturated DOPC (18:1) with either the diunsaturated dilinoleoyphosphatidylcholine (18:2) or the triunsaturated dilinolenoylphosphatidylcholine (18:3). Acyl chains were identical at positions 1 and 2 of the phospholipid. In this study, the 18 carbon chain length and the phosphatidylcholine headgroup were kept constant. Since the lipids were well above their lipid transition values (T_c) for all three lipid types, the effect of altered membrane fluidity on enzymatic activity was determined to be not relevant. However, the altered extent of unsaturation may affect overall lipid packing (or ordering) in the membrane.

Enzymatic activities were measured on Day 1, and then reassayed for stability after overnight incubation at 4°C in a nitrogen atmosphere (Day 2). Rates shown in Table 23 are expressed relative to those obtained for 100% DOPC PRLs. On Day 1, there was no significant effect associated with increasing fatty acyl chain unsaturation. On Day 2, a slight, but not significant, increase in activity was found with 18:2 relative to 18:1. Stability, as indicated by the ratio of the rate obtained on Day 2 to the initial activity, is shown in the same table. MAO-B in vesicles composed of diunsaturated lipid was somewhat more stable than

Table 23

Effect of Fatty Acyl Chain Unsaturation on the Activity of *Purified* MAO-B Reconstituted into Phosphatidylcholine Vesicles by Detergent Dialysis[a]

	Specific Activity				Average
	MAO-9		MAO-10		
Acyl Chains	Day 1	Day 2	Day 1	Day 2	Day 2/Day1
Di 18 1[b]	102 2 ± 1 3	90 9 ± 0 4	201 2 ± 19 6	162 8 ± 6 3	0 84
Di 18 2[c]	97 7 ± 0 7	83 1 ± 0 7	206 9 ± 8 6	199 3 ± 10 0	0 91
Di 18 3[d]	93 9 ± 4 2	71 8 ± 3 0	210 5 ± 3 6	196 2 ± 10 6	0 85

[a]MAO-9 (1 22mg) and MAO-10 (0 37)mg) were each reconstituted in phosphatidylcholine with the indicated fatty acyl chains, as described in Figure 26 legend (excepting omission of freeze/thaw treatment of lipids) using the rapid OG dialysis procedure All solutions were first degassed, then saturated with nitrogen Proteoliposomes (0 5mL aliquots containing 46 3µg protein for both MAO-9 and MAO-10) were assayed in triplicate for enzymatic activity toward 1mM kynuramine2HCl at 30 C in NaP buffer (Day 1) Vesicle samples were maintained at 4 C overnight, and re-assayed as before (Day 2)
Ratio of activities (Day 2/Day 1) was used as a measure of the stability of the reconstituted enzyme Results obtained with the 2 enzyme isolates were averaged
[b]1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)
[c]1,2-Dilinoleoyl-sn-glycero-3-phosphocholine
[d]1,2-Dilinolenoyl-sn-glycero-3-phosphocholine

in either 18:1 or 18:3, although the differences were not significant. The enzyme stability in DOPC vesicles was lower than that seen previously.

V. Kinetics of Solubilized and Reconstituted MAO

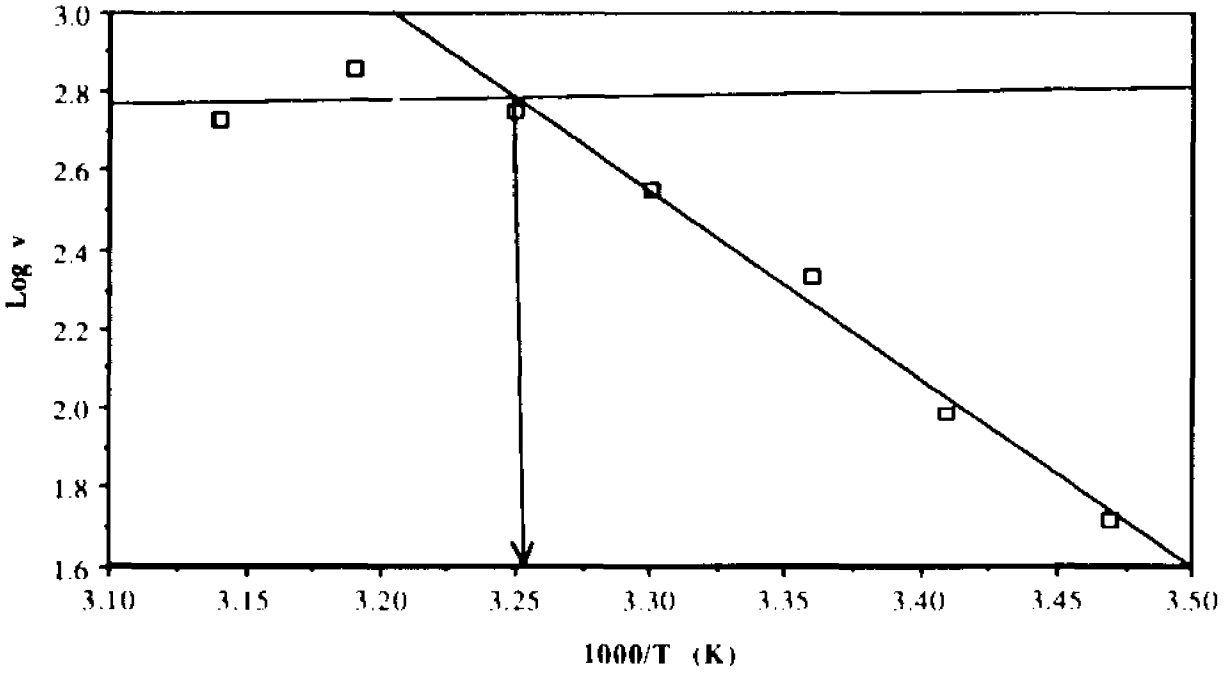
Kinetics were determined for *purified* MAO-B reconstituted into vesicles of 70/30 mole% DOPC/DOPE. The behavior of reconstituted MAO-B was compared with that of the solubilized enzyme with regard to the effects of temperature and substrate concentration on activity, and the degree of inhibition by pargyline, a mechanism-based inhibitor.

A. Arrhenius Plot

Enzyme activity was measured over the temperature range 15 to 45 °C with kynuramine as substrate. The resultant Arrhenius plot for the reconstituted enzyme is shown in Figure 28. A biphasic data fit was observed with the inflection occurring around 45° C. The observed change in enzymatic activity at 45° C is likely due to thermal denaturation of the protein rather than to any effect of the phospholipid, since both lipids in this vesicle system are in the fluid phase for the entire temperature range examined. Alternatively, a reduction in oxygen concentration at higher temperature could also contribute to decreased enzymatic activity. MAO-B activity is sensitive to $[O_2]$. At 25° C, bovine liver MAO-B has a K_I for oxygen of 0.28mM measured with benzylamine and 2.8mM measured with 2-phenylethylamine (Husain *et al.*, 1982). Oxygen partitioning into lipids is temperature dependent, and decreased $[O_2]$ at elevated temperatures may result in a slower reoxidation of the flavin coenzyme.

Figure 28

Arrhenius Plot of *Purified* MAO-B Reconstituted into 70% DOPC/30% DOPE Vesicles[a]



[a]Proteoliposomes were prepared by OG rapid dialysis against 50mM Hepes/100mM KCl, pH 7.2 (HK). Aliquots (250µL containing 26.8µg protein and 0.72mg lipid) were mixed with 1.57mL assay buffer (50mM Na phosphate, pH 7.2) (NaP) and equilibrated at each assay temperature. Kynuramine•2HCl (11.1mM in the assay buffer) was also equilibrated at each assay temperature before addition of 0.18mL to the proteoliposome suspension. Reaction rates were monitored as ΔA_{314nm} against controls consisting of 1mM substrate in NaP buffer.

Sawyer and Greenawalt (1979) also report an inflection point at 13°C in the Arrhenius plot for MAO in intact, but not in delipidated, rat liver mitochondria. This was attributed to a lipid phase transition in the intact mitochondria. Baker and Hemsworth (1978), using the same substrates, found an inflection at 26.8°C for both membrane-bound and solubilized rat liver MAO-B, which was attributed to a conformational change in the enzyme. No inflection was seen for benzylamine oxidation by rat brain mitochondria over the range of 4 to 45°C by Huang (1980), who identified lipid phase transitions in these membranes at 21-22°C and at about 33°C by electron spin resonance.

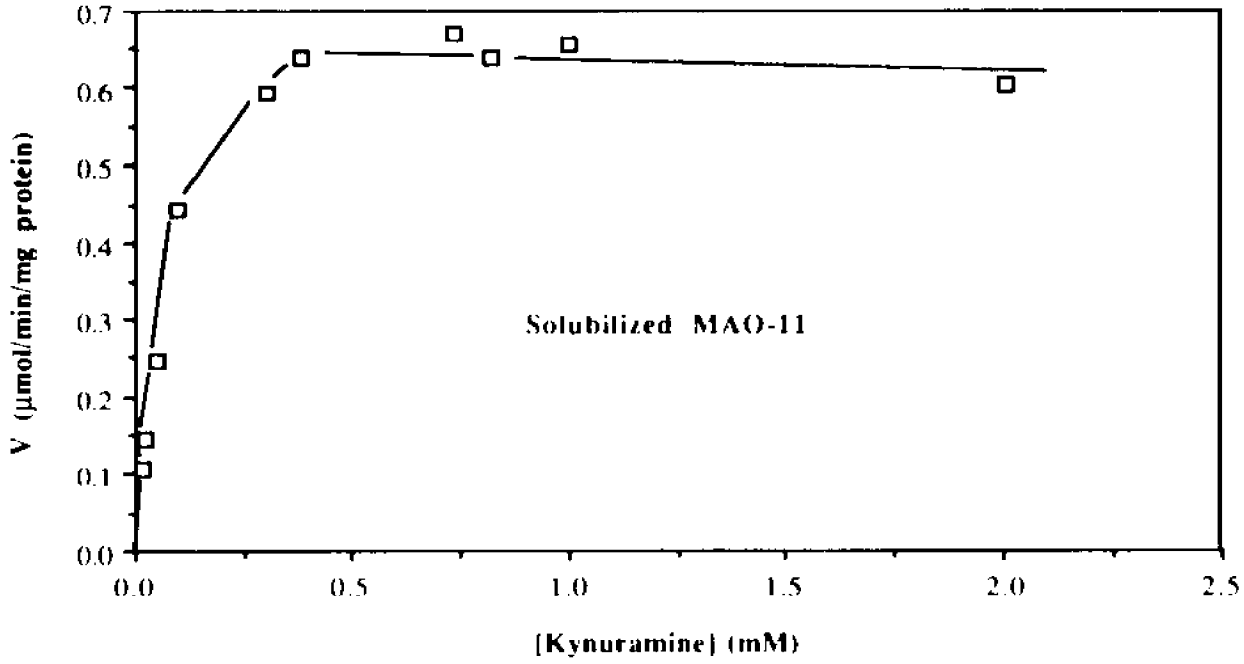
In the present study, the activation energy (E_{A-T}) determined from the slope of the line from 15 to 35°C for the *reconstituted* enzyme with kynuramine as substrate, was 91.4kJ/mol. This value is comparable with those determined previously from Arrhenius plots of rat liver mitochondria above T_c: 93.7 and 103.4kJ/mol with tyramine and benzylamine, respectively (Sawyer and Greenawalt (1979).

B. Kinetics

Plots of reaction velocity *vs* substrate concentration (v *vs* [S]) for the solubilized *purified* enzyme showed a characteristic hyperbolic relation (Figure 29). The corresponding Lineweaver-Burk plot is shown in Figure 30. Extrapolation gives a K_M value of 77μM and a Vmax of 755nmol min^{-1} per mg of solubilized protein. The value for K_M compares favorably with the previously reported value of 84μM obtained for kynuramine with bovine liver MAO-B (Tan and Ramsay, 1993), also at air saturation.

Figure 29

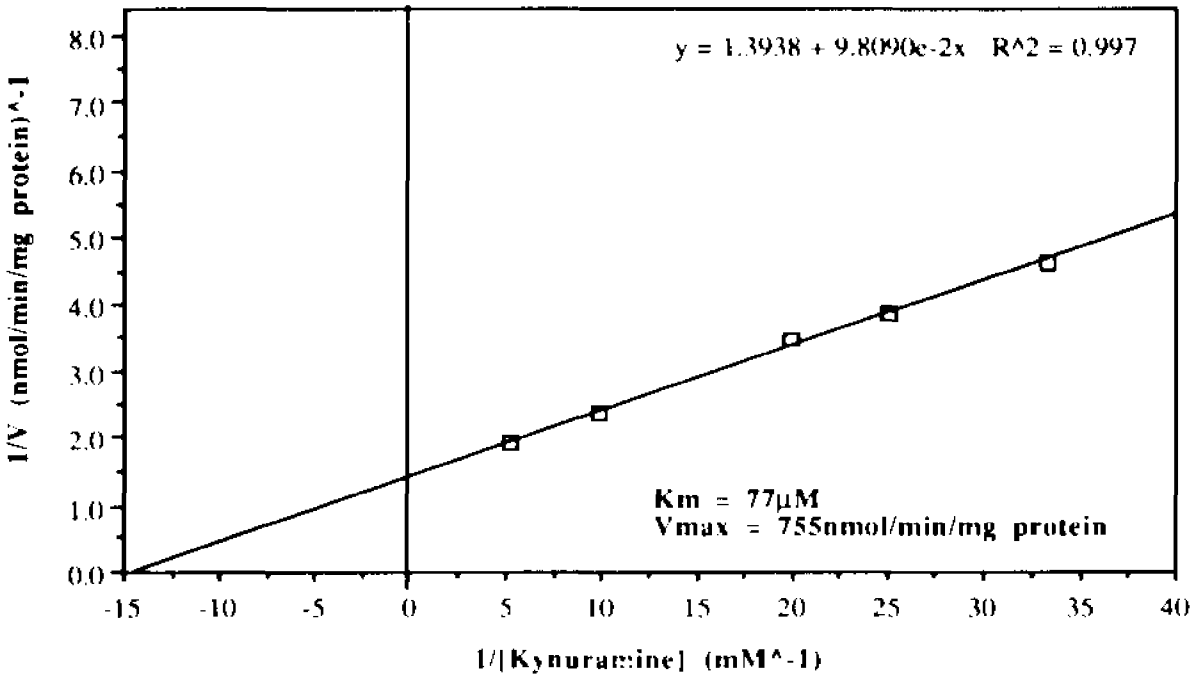
V vs [S] for Kynuramine Oxidation by Solubilized *Purified* MAO-B[a]



[a]MAO-11 (21.8µg protein) in 50mM Na phosphate, pH 7.2, containing 0.2% Triton X-100 (NaP + Tx) and kynuramine•2HCl (various concentrations in NaP + Tx) were equilibrated separately at 30°C. The reaction was initiated by addition of 1.0 mL substrate to 50µL enzyme solution, and monitored as ΔA_{314nm} against a control consisting of substrate at the same final concentration.

Figure 30

Lineweaver-Burk Plot for Kynuramine Oxidation by Solubilized *Purified* MAO-B[a]



[a]MAO-11 (21.8µg protein) in 50mM Na phosphate, pH 7.2, containing 0.2% Triton X-100 (NaP + Tx) and kynuramine•2HCl (various concentrations in NaP + Tx) were equilibrated separately at 30°C. The reaction was initiated by addtition of 1.0mL substrate to 50µL enzyme solution, and monitored as ΔA_{314nm} against a control consisting of substrate at the same final concentration.

The Lineweaver-Burk plot for the reconstituted *purified* enzyme is shown in Figure 31. The determined K_M value of 72.5μM is 94% of that obtained for the solubilized enzyme, while the V_{max} of 451nmol min^{-1} per mg protein is 60% of that obtained with solubilized MAO-B. V_{max} is defined as the product of the turnover number (k_{cat}) and the total enzyme concentration $[E]_t$ (Segel, 1975). The protein concentration in the assays of reconstituted MAO-B was 57% of that used in the assays of solubilized enzyme.

Thus, there does not appear to be any significant difference in substrate binding ability as a result of incorporation into 70/30 mole% DOPC/ DOPE vesicles. One may speculate on why a lower V_{max} value was obtained with the reconstituted enzyme. This result may be attributed to a portion of the incorporated MAO-B having a different orientation in the bilayer and, therefore, not being accessible to the substrate. Alternatively, some of the MAO-B molecules may have been incorporated in a nonfunctional state, *e.g.*, as monomers, consequent of the initial detergent solubilization of the protein. While the functional (catalytic) protein is the dimeric form, substrate binding may only require a single subunit and, thus, the finding of an unaltered K_M value.

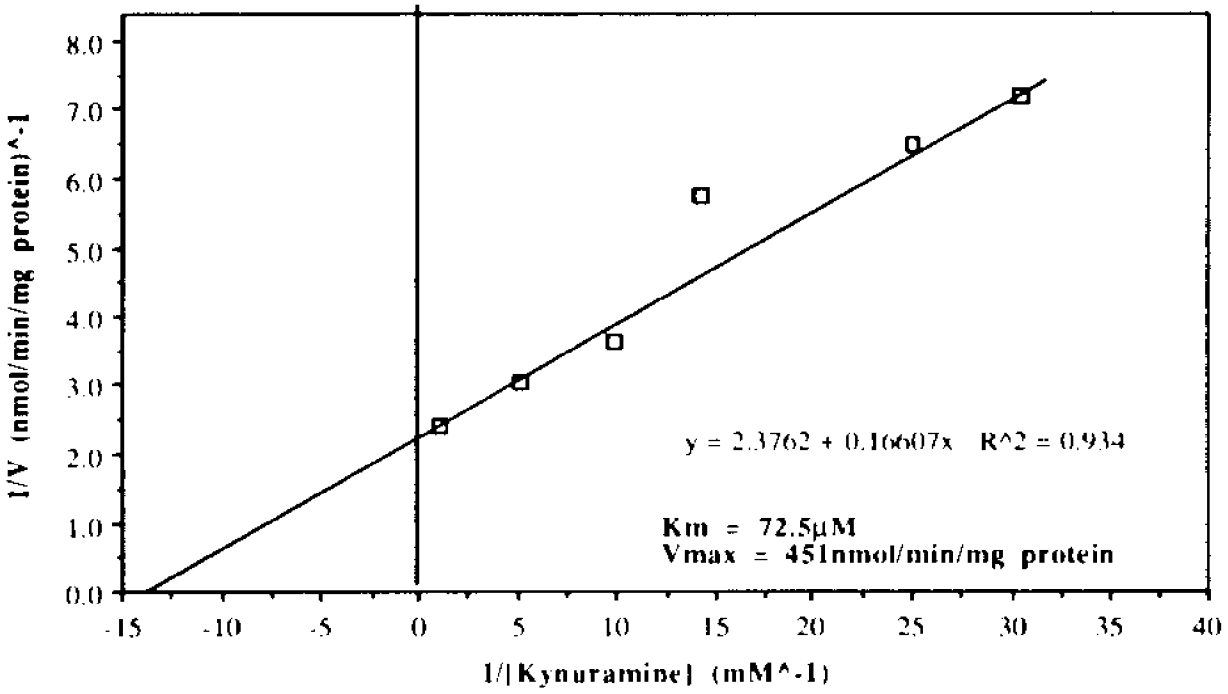
Neverless, the conditions employed for reconstitution (limited OG solubilization with rapid dialysis) appear suitable for preserving the enzymatic activity of this enzyme.

C. Pargyline Inhibition

Pargyline is a mechanism-based inhibitor of monoamine oxidase, relatively specific for MAO-B. The inhibition of the solubilized and the reconstituted enzymes were compared in

Figure 31

Lineweaver-Burk Plot for Kynuramine Oxidation by *Purified* MAO-B Reconstituted into 70% DOPC/30% DOPE Vesicles[a]



[a]Proteoliposomes (PRLs) were prepared by OG rapid dialysis against 50mM Hepes/100mM KCl, pH 7.2. Kynuramine•2HCl solutions (various concentrations) were prepared in 50mM sodium phosphate, pH 7.2 (NaP). PRLs and substrate solutions were equilibrated separately at 30°C. The reaction was initiated by addition of 250µL PRLs (containing 26.8µg protein and 0.72mg lipid) to 2.0mL substrate solution, and monitored as ΔA_{314nm} against a control consisting of substrate at the same final concentration.

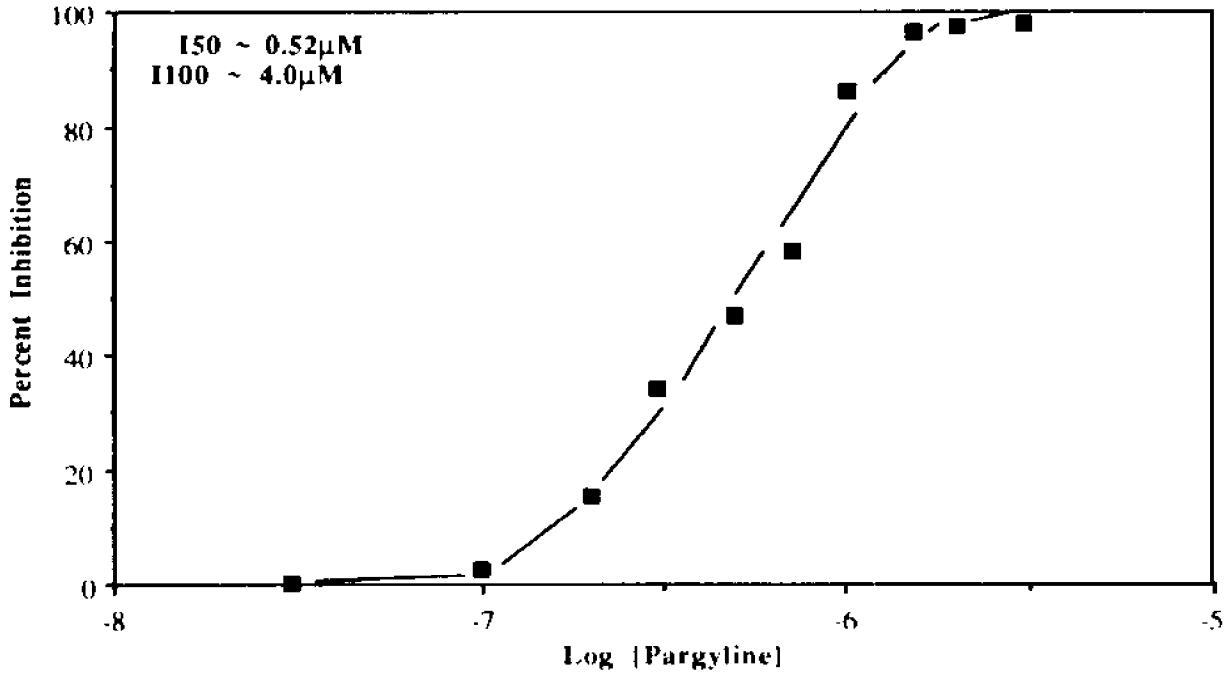
order to detect any effect of the membrane on the action of pargyline.

From the dose response curve for pargyline inhibition of kynuramine oxidation by Triton-*solubilized* MAO-B (Figure 32), an I_{50} value of 0.52μM was obtained; the curve was extrapolated to obtain an I_{100} value of 4.0μM. The dose response curve obtained for the reconstituted *purified* enzyme (Figure 33) gave an I_{50} value of 0.4μM, and extrapolation provided an I_{100} of 6.0μM. While pargyline I_{50} values have been reported for preparations of MAO-B from other tissue sources using different substrates, there has been only one report of inhibition of kynuramine oxidation by porcine brain MAO-B; in this system, an I_{50} of ~0.3μM and an I_{100} of ~5μM was found (Straher, Ph.D. thesis, 1987).

Similarity in the inhibition data for the solubilized and the reconstituted enzyme may be explained in two possible ways. Either all of the reconstituted enzyme active sites are at the surface of the membrane, or pargyline is membrane soluble and may partition through the bilayer, thus being accessible to MAO-B located within the inner bilayer leaflet. As discussed in the Introduction (section IV a.*i.*), unidirectional insertion is one of the advantages of the detergent dialysis reconstitution method. Therefore, whether permeant or impermeant, the active sites with which pargyline combines are all on either one bilayer or the other, not randomly distributed between the two leaflets.

Figure 32

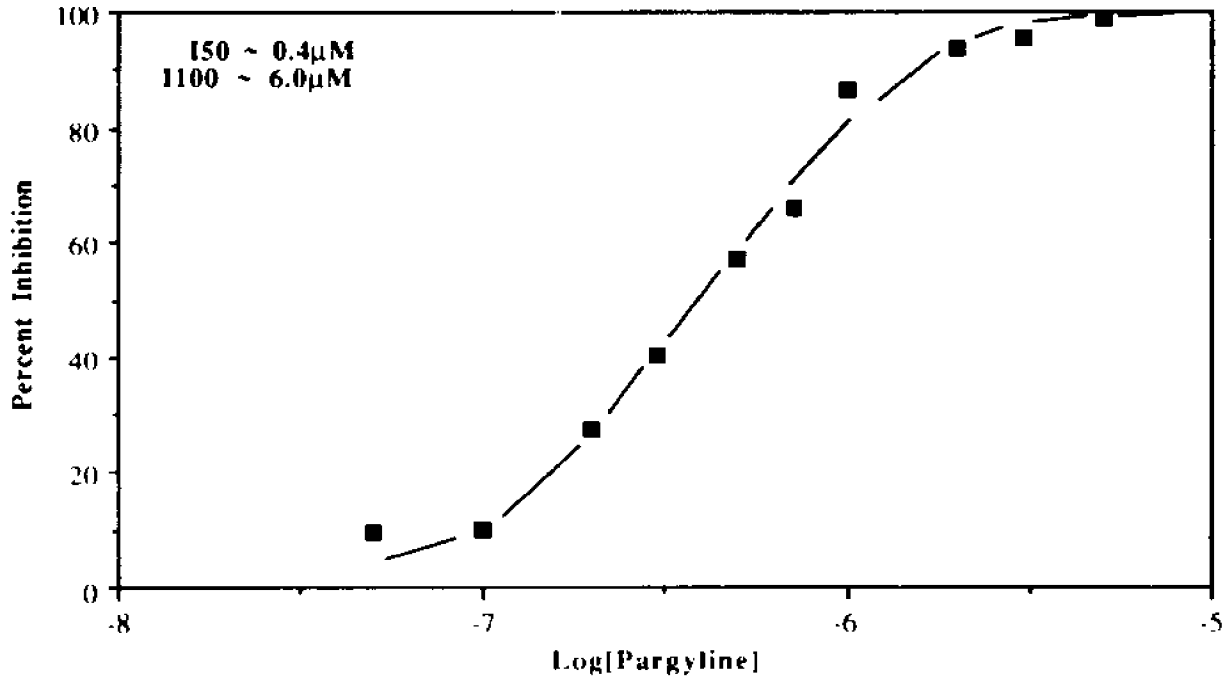
Pargyline Inhibition of Kynuramine Oxidation by Solubilized *Purified* MAO-B[a]



[a]MAO-11 (50μL containing 17.44μg protein) in assay buffer (50mM Na Phosphate, pH 7.2, containing 0.2% Triton X-100) (NaP + Tx) and 50μL pargyline solution (various concentrations in assay buffer) were equilibrated separately at 30°C. After mixing, enzyme and inhibitor were incubated at 30°C. After 20 minutes, 1.9mL of 1.05mM kynuramine•2HCl in assay buffer was added and the enzymatic reaction monitored as Δ_{A314nm} against a control consisting of 1mM substrate in NaP + Tx.

Figure 33

Pargyline Inhibition of Kynuramine Oxidaton by *Purified* MAO-B Reconstituted into 70%DOPC/30%DOPE Vesicles[a]



[a]Proteoliposomes (PRLs) were prepared by OG rapid dialysis against 50mM Hepes/100mM KCl, pH 7.4. Pargyline solutions (various concentrations) were prepared in 50mM Na phosphate, pH 7.2 (NaP). PRLs and pargyline solutions were equilibrated separately at 30°C before addition of 250μL PRLs (26.8μg protein and 0.72mg lipid) to 25μL pargyline solution. After 20 minutes incubation, 3.3 mL of 1.08mM kynuramine•2HCl in NaP (at 30°C) was added and the enzymatic reaction monitored as ΔA_{314nm} against a control consisting of 1mM substrate in NaP.

DISCUSSION

The aims of this investigation were the isolation of bovine mitochondrial MAO-B, its reconstitution into vesicles of defined lipid composition, and examination of the influence of different lipid environments on MAO-B enzymatic activity

I. Isolation of the MAO-B Enzyme

MAO-B was isolated from bovine liver mitochondria by the method of Salach and Weyler (1987) The isolated protein had an absorbance spectrum characteristic of flavin The isolated enzyme was not a single species on SDS-PAGE, even after sucrose density gradient centrifugation, and preparation of the enzyme displayed a range of specific activities The subunit weights determined by SDS-PAGE of several preparations of the isolated protein was 62 4kDa The reported range of subunit weight for MAO-B isolated by this method, as determined by SDS-PAGE, is from 58 to 62kDa (Salach and Weyler, 1987) A subunit weight of 63 8kDa per mole of FAD was determined by dithionite titration of the enzyme in the presence of SDS (Weyler 1989) In the same report, a molecular mass of 57 3kDa was determined from a revised amino acid analysis and the molar content of flavin

Although the enzyme recovered from the sucrose density gradient was not electrophoretically homogeneous, it was used for the reconstitution studies, and is herein referred to as *purified* MAO-B

Since MAO-B is an integral membrane protein, residual lipid in the isolated enzyme would be expected to support activity Alternatively, an appropriate detergent could provide

a hydrophobic environment in which the enzyme would not be aggregated beyond its functional unit (i e , dimer), in so doing, the detergent micelle would be substituting for the lipid environment of the membrane *E.g* , Triton X-100 (0 2%, w/w) used in the assay of isolated MAO-B maintains the enzyme in a disaggregated state Several integral membrane proteins have been reported to contain tightly bound lipids which are specifically required for their activity These include the inner mitochondrial membrane proteins β-hydroxybutyrate dehydrogenase (Cortese *et al* , 1982), cytochrome oxidase (1982), NADH coenzyme Q reductase (Fry and Green, 1981), and cytochrome P-450$_{sc}$ (Pember *et al* , 1983) (These proteins are discussed in section III below) If MAO-B contained tightly-bound lipids specifically required for function, their removal would abolish activity Detergent conditions which would effect this delipidation would result in loss of enzymatic activity If, rather than such tightly-bound lipid, the isolated MAO-B contained a shell of loosely bound lipids (annulus) which provided a hydrophobic environment supportive of protein function, replacement of the lipids with detergent would not necessarily result in loss of activity provided the active conformation is maintained Any detergent interaction that altered this conformation would cause loss of function Any effect of residual membrane lipid on reconstitution of MAO-B would depend both on the nature of the association of lipid with protein (tightly or loosely bound) and on the method of incorporation Annular (loosely bound) lipid would promote direct incorporation through fusion of the annulus with the liposomes Exchange of annular with vesicle lipid would be slow but would occur over a period of time, provided the vesicle lipid is an adequate substitute for the annular lipid, *i.e* , either headgroup structure or charge, or acyl chain length or unsaturation, are suitable for the

active conformation of the enzyme. Tight-binding lipid would not exchange with vesicle lipid in this system. Without exchange of vesicle lipid for residual lipid, reconstituted MAO-B will not be affected by its liposomal environment. In the detergent dialysis reconstitutions in this study, MAO-B is first solubilized with octylglucoside which would displace annular lipids and, at the high concentrations used herein, any tight-binding lipid. Thus, MAO-B would be reconstituted without any native lipid and, therefore, be subject to any influence exerted by the liposomal lipid. The MAO-B isolates used in this study were seen to sediment to the bottom of glycerol gradients without any associated phospholipid-phosphorus. Because the MAO-B isolates did not contain any residual lipid, both the spontaneous incorporation and the detergent dialysis reconstitution methods place the protein in direct contact with the vesicle lipids, allowing them to exert their effects on the enzyme.

Kynuramine is a non-selective substrate which is oxidized by both forms of MAO. The product of kynuramine oxidation (4-hydroxyquinoline) has an absorbance maximum at 314nm. Kynuramine was used in the activity assays instead of the B-selective substrate benzylamine, which has an absorbance maximum at 250nm, in order to reduce interference from protein, which has a broad absorbance with a maximum at 278-280nm, and Triton X-100, which also absorbs at 250nm, and was included in the assay medium to prevent aggregation of the unreconstituted enzyme. The enzyme isolated in this study had a K_M value of 77μM for kynuramine, which is similar to the value of 84μM reported for the oxidation of this substrate by bovine liver MAO-B (Tan and Ramsay (1993)). In both cases, activity assays were conducted at air saturation.

Selective inhibition of the enzyme activity with pargyline is indicative of type B activity. Pargyline inhibition data for kynuramine oxidation by bovine liver MAO-B has not

been reported However, the data obtained herein is similar to that found by Straher (Ph D thesis, 1987) for pargyline inhibition of kynuramine oxidation by porcine brain MAO-B

II. Reconstitution of the Enzyme

Although *partially purified* MAO-B was reconstituted using direct incorporation, this method was not successful with the *purified* enzyme Residues of Triton X-100 and polymers employed in the enzyme isolation procedure are removed by the sucrose density gradient in the final purification step These residues may have had a "facilitating" role in the spontaneous incorporation of the *partially purified* MAO-B in that the protein may have been maintained in a less aggregated state than the *purified* enzyme and, therefore, inserted more readily into the bilayer Similarly, although removal of residual cholate from UDP-glucuronyltransferase by extensive dialysis resulted in failure of the enzyme to incorporate into DMPC vesicles, addition of a small amount of cholate (insufficient to solubilize the liposomes) resulted in reconstitution (Scotto and Zakim, 1985, 1986) Although Lawaczeck *et al* (1976),found that vesicles above the T_c of their lipid components are annealed, and lack the packing defects which permit vesicle fusion, Scotto and Zakim (1986) showed that bacteriorhodopsin (BR) spontaneously incorporated into vesicles of DOPC above its T_c, and also into DMPC vesicles that had been annealed prior to addition of protein However, with both these BR reconstitutions, vesicle growth was much less than had been previously observed with unannealed DMPC liposomes

The *partially purified* MAO-B preparation was exposed to Triton X-100 during the isolation procedure, and from the Tx solubilization step onward, the enzyme remained in the soluble (supernatant) fraction The high speed centrifugation that yielded the *partially*

purified enzyme resulted in a "soft" golden pellet, not a hard precipitate Therefore, it would seem that the resulting MAO-B was in a "solubilized" or disaggregated state which could readily be dispersed in the reconstitution buffer Following removal of residues on the sucrose density gradient, the *purified* MAO-B is now in an aggregated state Re-solubilization of the aggregates with octylglucoside may be harder to achieve than maintaining an already disaggregated protein in that state In both trials of OG-facilitated reconstitution, the bulk of the enzymatic activity (*i.e.*, active protein) was found at or near the bottom of the glycerol gradients, while the top gradient fractions contained lipid and protein with only a small amount of associated enzymatic activity These findings suggest that the MAO-B incorporated in this system was mainly in an inactive state The reason for this is unclear The OG concentrations used in these trials had previously been determined to be "safe" for the enzyme over a 24 hour period Perhaps, the catalyzing amount of OG partitioned into the bilayer together with the protein and remained associated with the enzyme throughout the 89 hour centrifugation Although OG would be removed during the subsequent dialysis to remove glycerol, long exposure to this detergent may have inactivated that portion of the MAO-B that had incorporated Any MAO-B that did not incorporate would have been separated from the detergent on the glycerol gradient and, hence, activity would be found with the unreconstituted enzyme In the early detergent dialysis reconstitutions, the higher OG concentrations used to solubilize MAO-B would ensure complete dispersal of the protein and, thereby, promote its insertion into the nascent liposomes formed during this procedure

Octylglucoside dialysis was successfully used for reconstitution of the *purified* enzyme The effect of temperature on light scattering by 100%DOPC or the 70%DOPC/30%DOPE mixture was used as a guide for determining how long dialysis should

proceed for vesiculation to occur. Although this was found to be 2 or 3 hours, respectively, overnight dialysis was routinely used, and vesicle formation was verified by electron microscopy. Limiting the length of exposure to the detergent during protein solubilization, and performing more rapid dialysis, were observed to better preserve MAO-B enzymatic activity.

In fact, dialysis of the OG-solubilized enzyme in the absence of lipid also resulted in recovery of some activity. Reactivation by vesicle reconstitution following detergent-inactivation has been reported for several other membrane proteins, including the KdpD osmosensor from *E. coli* (Nakashima *et al.*, 1993), ubiquinone-cytochrome c reductase (Fry and Green, 1981), and phosphate exchange protein (Maloney and Ambudakar, 1989). Provided the protein is not irreversibly denatured, detergent removal and replacement with a more favorable hydrophobic environment allows resumption of its active conformation.

From inspection of the electron micrographs of the vesicles produced by the rapid detergent dialysis method, a mean diameter of 139±35 nm was determined for the proteoliposomes, and 141±32 nm for non-protein-containing liposomes. By extrapolation of the data used by Cullis and Hope (1991) for 100nm diameter vesicles, it may be calculated that the proteoliposomes obtained may contain on average not more than 36 MAO-B dimers per vesicle.

Glycerol gradient characterization was performed on some, not all, preparations. Sedimentation of two *partially purified* MAO-B isolates on 10-80% gradients is shown in Figures 5 and 10. The spontaneous incorporation of *partially purified* MAO-1 is demonstrated in Figure 9. The unsuccessfully incorporated *purified* MAO-3 was also run on a gradient which is not shown here because it was not fully characterized; however, this

isolate also sedimented to the bottom. The attempted reconstitutions of *purified* MAO-3a by the detergent facilitated method are shown in Figures 13 and 14. Characterization of reconstitutions with DOPC of *purified* MAO-3 by the original OG dialysis method on 10-80% gradients is shown in Figures 20 and 21, and of *partially purified* MAO-6 on a 3-60% gradient in Figure 22. Characterizations on 3-20% gradients were performed for reconstitutions by the more rapid OG dialysis procedure using *purified* MAO-8 with DOPC (Figure 24) and with the 70%/30% DOPC/DOPE mixture (Figure 25). These latter reconstitutions are compared with the sedimentation of MAO-8 that had been OG-solubilized and subsequently dialyzed in Figure 23. Protein determinations were performed on all gradient fractions except for the detergent dialysis reconstitution of MAO-6 (Figure 22). Protein and activity peaks were congruent for the detergent dialysis reconstitutions run on 10-80% gradients (Figures 20 and 21). However, this alignment does not hold as well for the subsequent reconstitutions run on 3-20% gradients (Figures 24 and 25), and the coincidence of phospolipid-phosphorus with enzymatic activity was taken as a better determinant of reconstitution than protein content. For all the glycerol gradient characterized reconstitutions, the average amounts of enzymatic activity and protein recovered at the bottom of the gradient were <3% and 4%, respectively. Uncharacterized incorporations were performed under the same conditions as characterized reconstitutions and, consequently, the extent of incorporation is assumed to be the same as in the characterized preparations (*i.e.*, all or nearly all). Incorporation was determined for the gradient characterized preparations and extrapolated to the uncharacterized preparations that were performed under the same conditions.

III. Effect of Lipid Composition on the Reconstituted Enzyme

The effect of phospholipid headgroup and acyl chain composition on the activity and stability of the enzyme reconstituted by OG dialysis was examined. There was no dramatic increase in MAO-B activity upon reconstitution into DOPC vesicles, although stability was improved, at least for enzyme spontaneously incorporated into SUVs.

Some integral membrane proteins resemble MAO-B in their relative insensitivity to their lipid environment. An example is NADH-cytochrome b_5 reductase, found on both the outer mitochondrial membrane and the endoplasmic reticulum, this enzyme is bound to the bilayer by its N-terminal hydrophobic domain and has its hydrophilic catalytic domain entirely in the cytosol (Borgese *et al*., 1993). In this respect the reductase differs from both forms of MAO which are interwoven with the membrane. Lack of sensitivity to lipid environment is not surprising for the cyt b_5 reductase since its catalytic domain is external to the membrane. However, the reductase is sensitive to membrane fluidity, since this determines access to its membrane-bound substrate. When reconstituted into dimyristoylPC vesicles together with cytochrome b_5, the sensitivity of the reductase to the phase transition is dependent on the cyt b_5 concentration, being completely insensitive at high acceptor levels (Houslay and Stanley, 1982). MAO-B, however, is reported to be relatively insensitive to membrane fluidity as determined by ESR studies (Huang and Faulkner, 1980). This is congruent with a protein whose active site is believed to be near the membrane surface, and whose substrates approach from the cytosol.

On the other hand, some integral membrane proteins have been found to contain tightly-bound lipid essential for their activity. β-Hydroxybutyrate dehydrogenase has two identical non-interacting phosphatidylcholine binding sites, the PC headgroup is necessary for

binding the pyridine nucleotide coenzyme, and neither other phospholipids nor a detergent environment can reactivate the delipidated enzyme (Cortese *et al*, 1982) Cytochrome c oxidase (cyt ox) contains 2-3 tightly-bound cardiolipin molecules (Robinson, 1982) As isolated with its boundary (annular) lipids, cyt ox retains activity in the presence of phospolipids or in a micelle with a mild detergent, whereas removal of tightly-associated CL with more rigorous detergent treatment abolishes activity Reactivation of the lipid-depleted enzyme is specific for the CL headgroup NADH-coenzyme Q reductase loses all activity on complete delipidation, but is reactivated on addition of CL or a lipid mixture containing CL (Fry and Green, 1981) Cytochrome P-450$_{scc}$, the side chain cleavage enzyme in synthesis of pregnenolone from cholesterol, has 1-2 binding sites specific for CL, this phospholipid promotes the binding of cholesterol to the enzyme, which exhibits very low activity upon delipidation (Pember *et al*, 1983)

A more general effect of lipids was seen in a study of tyrosine kinase of the rat liver plasma membrane (Gavrilova *et al.*, 1993) The effect on enzyme activity of supplementation with phospholipids was compared with the fluid state of the membrane as determined by steady-state diphenylhexatriene (DPH) fluorescence anisotropy Enzyme activity was increased by lipids with a fluidizing effect and decreased on addition of lipids which increased order in the membrane, although there was no correlation between activity and bulk membrane fluidity The order of activation was PG>DOPC>PS>PE. Activity decreased by 30% with added DPPC (T_c = 41°C) A 2- to 3-fold activation was found with PS and PE The 6- to 7-fold activation found with PG and DOPC suggests a more specific interaction of these membrane lipids with tyrosine kinase

$^{32}P_i$-ATP exchange activity required both PC and PE for functional reconstitution

(Kagawa *et al*., 1973) Heterogeneous acyl chains supported more activity than dioleoyl, dilinolenoyl, dipalmitoyl, or 1-stearoyl-2-undecenoyl phospholipids. Small amounts of anionic lipid augmented activity in the order of CL>PI>PS. Lipid titration of the Ca^{2+}-ATPase of the sarcoplasmic reticulum with dioleoylphospholipids showed that the ATPase activity is dependent on the charge distribution of the headgroup with PC>PE>PS>PG>PA (Bennett *et al*., 1978). In the same study, reconstitution of solubilized Ca^{2+}-ATPase with dioleoylphospho-lipids by cholate dialysis supported calcium uptake in the order of PC>50%PC/50%PE>PS>PA>PG. DOPE did not form vesicles and, hence, could not support the pump function.

A. Nonbilayer Lipid

Phosphatidylethanolamine is a non-bilayer forming lipid, and therefore could possibly influence the distribution of enzyme in the membrane by creating lipid domains. The decrease in light scattering observed with increasing temperature for unstable vesicular structures occurs over a broader temperature range for the mixture containing 30%DOPE than it does for 100% DOPC. Inclusion of DOPE in DOPC vesicles resulted in increased MAO-B activity and, generally improved enzymatic stability. The presence of 30% DOPE offset the small drop in activity seen in its absence with 10% DOPS, and increased the decline in enzymatic stability seen with 10% DOPS. Additionally, the significant increase in enzyme activity seen with 10% DOPA was negated when the proteoliposomes also included 30% DOPE. Perhaps, in the presence of PE the membrane partitions in such a way that the enzyme is shielded from the effects of the anionic lipids.

Phosphatidylethanolamine is a polymorphic lipid with the potential to form the H_{II}

hexagonal phase structure Factors which influence adoption of lamellar or hexagonal phase include temperature, degree of hydration, ionic strength, pH, and acyl chain unsaturation (Cullis *et al* , 1985). In a single lipid system, under physiological conditions, the temperature at which the transition from bilayer to H_{II} (T_{BH}) occurs with DOPE is at or above 10°C However, H_{II} structures can be stabilized in lamellar form when combined with bilayer-forming lipids in mixed lipid systems Non-bilayer PE has a ^{31}P NMR spectrum which is characteristically different from bilayer PC At 30°C and pH 7, equimolar mixtures of egg PC and soya PE have been observed to form a bilayer, whereas isotropic motional averaging seen with 15 or 30 mol% PC in this system indicate the presence of intermediate structures, such as inverted micelles (Cullis and Hope, 1991) The acidic phospholipids are preferentially lamellar under physiological conditions, and 15-30mol% is sufficient to stabilize PE in the bilayer form in mixed lipid systems (Cullis *et al* , 1985)

In the present study, OG dialysis reconstitution of *purified* MAO-B with lipids was performed at 4°C Since this temperature is below its T_{BH}, it is expected that DOPE is in the lamellar phase at all concentrations examined

However, while the proteoliposomes of the present study were maintained at 4°C, their MAO-B activity was assayed at 30°C At this temperature, whether DOPE shifts to hexagonal phase depends on the amount of bilayer lipid present For most mixed lipid reconstitutions, 30% DOPE was used in combination with 70% DOPC or with 60% DOPC and 10% anionic lipid In either of these preparations, bilayer structures are expected There is the possibility that the 40%DOPC/60%DOPE sample might not be completely in the lamellar phase. However, the enzyme activity and stability did not differ significantly from that seen with equimolar DOPC/DOPE proteoliposomes

Cholesterol has a bilayer destabilizing effect in mixed lipid systems (Cullis *et al* , 1985) Addition of cholesterol at 50mol% induces a shift to the H_{II} phase in 50%DOPC/50%DOPE Less cholesterol is required when less DOPC is in the mixture For 20%DOPC/80%DOPE mixtures labelled with ^{2}H on C_{11} of the acyl chains of either the PC or the PE, the presence of different amounts of cholesterol produced ^{31}P NMR spectra reflecting both lamellar and hexagonal phases, as well as intermediate structure permiting isotropic motion ^{2}H NMR spectra of the same mixtures were equivalent, Cullis and coworkers (1985) interpreted this finding as indicating that DOPC and DOPE partitioned with equal probability among the bilayer, H_{II}, and intermediate structures

In one series of experiments, *partially purified* MAO-B was spontaneously incorporated into single or mixed lipid systems comprising 25%DOPE combined with either 75%DOPC or 50%DOPC/25%DOPG, to which 17mol% cholesterol was added Cholesterol, at the concentration added, is not expected to destabilize the bilayer MAO-B enzymatic activity was unaffected by DOPE in the presence or absence of cholesterol in these systems

B. Anionic Lipids

The negative charge of the headgroups of anionic phospholipids influences the membrane surface charge Altered membrane potential may affect accumulation of charged substrates in the vicinity of the enzyme active site and, hence, the enzymatic activity Indeed, Sugawara *et al* (1994) have reported increasing DPPS content of DPPC liposomes decreased the uptake of anionic compounds, and increased the uptake of cationic substances, including tryptamine (Sugawara *et al* , 1995) In both studies, DPPS concentration, relative membrane

potential and uptake correlated well

Navarro-Welch and McCauley (1982) reported complex formation between PI and monoamine substrates Consistent with this, Pohl and Schmidt (1983) found a differential effect on MAO substrate binding affinity, with K_M toward benzylamine one-third lower with PS than PC Decreased K_M, but unaltered V_{max} , has been reported for MAO in proteoliposomes prepared by dialysis of detergent-solubilized rat liver mitochondria with acidic phospolipids (Nalecz *et al* , 1980) However, this study utilized an oxygen consumption assay (which does not provide initial rates) and the non-selective substrate dopamine which does not distinguish between effects on the A and B forms of MAO, both of which are found in rat liver

In the present investigation, under the conditions employed for both reconstitution and assay, the anionic lipids used were in the lamellar phase DOPA, at 10 mol%, had a significant stimulatory effect However, activity of 100% DOPA proteoliposomes was variable and appeared to be dependent on the enzyme preparation used In contrast, 100% DOPS proteoliposomes resulted in a significant decrease in enzymatic activity (see Table 24) This negative effect of DOPS on MAO-B activity is consistent with the findings of Buckman *et al* (1983b) In contrast to DOPS, the phosphate group of DOPA has two dissociable protons, with the second pK occurring at pH ~8 (Cullis *et al.*, 1985) Although this second ionization would occur in less than 20% of the DOPA under MAO-B assay conditions, the difference in surface charge between vesicles containing DOPS and DOPA would be significant A higher concentration of amine substrate due to the more negative surface charge of PRLs containing 10% DOPA, as opposed to DOPS, would explain the observed enhancement of activity Charge repulsion in proteoliposomes composed solely of anionic

lipid may alter the positioning of the enzyme in the membrane causing it to be less active

Table 24

Effect of Anionic Lipid

% Anionic Lipid	% MAO-B Activity
0	100
10% DOPA	112-123
100% DOPA	79-121
10%DOPS	96-100
100% DOPS	77

A requirement for anionic lipid has been reported for functional reconstitution of other membrane proteins, including the acetylcholine receptor (Sunshine and McNamee, 1994), cytochrome-c oxidase (Volwerk *et al*., 1987), Na^+/K^+-ATPase (Brotherus *et al*., 1980, 1981), and cytochrome oxidase (Racker, 1972a, Eytan *et al*., 1976, Eytan and Racker, 1977)

C. Acyl Chain Unsaturation

Acyl chain unsaturation influences the degree of flexibility in the bilayer In the mitochondrial outer membrane, the concentration of saturated and unsaturated acyl chains is approximately equal In the present study, MAO-B activity was not altered with increasing unsaturation in the acyl chains of PC (see Table 25) Since the three types of acyl chains examined (18 1, 18 2 and 18 3) are in the fluid phase at 4°C, vesicles comprised of these lipids are likely to be highly permeable, and diffusion of substrate to active site is probably not restricted

Table 25

Effect of Acyl Chain Unsaturation

PC Acyl Chain	%MAO-B Activity
Di 18:1	100
Di 18:2	99
Di 18:3	98

IV. Kinetics of Reconstituted MAO-B

A. Arrhenius Plot

The Arrhenius plot for kynuramine oxidation by MAO-B reconstituted with 70%DOPC/30%DOPE is straight for the temperature range of 15-35°C. From 40 to 45°C there is a decline in enzymatic activity. There are several possible reasons for this decline. These include thermal denaturation of the enzyme, decreased rate of flavin reoxidation due to decreased oxygen concentration at elevated temperatures, and altered membrane structure due to bilayer to hexagonal transition of the DOPE with increasing temperature. The enzyme exhibited maximal activity close to physiological temperature.

B. Kinetics

There was no significant difference in the K_M for kynuramine oxidation by MAO-B incorporated with DOPC/DOPE (70/30 mole%) as compared with solubilized MAO-B, however, the V_{max} value was found to be 40% lower in the reconstituted enzyme. Pohl and Schmidt (1983) found no effect on the K_M for benzylamine with bovine liver MAO-B when

treated with PC, but a decrease from 0 28 to 0 18 mM when treated with PS. V_{max} declined by 27% in the presence of either PC or PS in their systems The K_M for benzylamine oxidation by isolated pig liver MAO-B was increased almost 2-fold when the enzyme was cosonicated with PC, was unaffected by PE (which would not have been in the lamellar phase), and was reduced almost 3-fold with equimolar PC/PE (Inagaki *et al* , 1986) The V_{max} decreased with all three lipid treatments 86% with PC, 41% with PE, and 21% with the 50/50 mixture of PC/PE Variation in results may stem from differing enzyme assays and from different experimental conditions The cosonication method of reconstitution was used by both Pohl and Schmidt (1983) and Inagaki *et al* (1986) In *this* investigation, sonication of DOPC/DOPE (80/20 mole%) proteoliposomes for only 90 seconds resulted in a 21% loss of MAO-B activity Additional delipidation of the enzyme with the ionic detergent, cholate, prior to reconstitution by Inagaki *et al.* (1986) may have contributed to the different K_M result found by them. Furthermore, the initial rate assay employed in the study by Pohl and Schmidt (as well as *this* investigation) is more valid for kinetic studies than the polarographic assay used by Inagaki *et al* (1986)

Although there was no change in the K_M of cytochrome oxidase in heart mitochondria from hypothyroid rats, the V_{max} was found to be decreased in parallel with reduced cardiolipin content of the inner membrane (Paradies *et al* , 1993) Both V_{max} and cardiolipin levels were restored to normal following thyroid hormone treatment Apparently, as found for MAO-B in *this* study, substrate binding affinity (and, therefore, the binding site) of cyt ox is not influenced by lipid environment For cyt ox, the lower V_{max} with subnormal CL levels suggests that a conformational change in the protein induced by this lipid is essential for catalysis

The lower V_{max} found with the incorporated MAO-B in the present investigation suggests several possibilities. The orientation of the protein in the bilayer can influence activity. If the enzyme is located on the inner rather than the outer leaflet, the activity will be determined by the ability of the substrate to diffuse through the membrane. As discussed in the Introduction (see section IV.a), bidirectional insertion can be detected with an impermeable substrate. The K_M would not be affected in such a case since it is dependent on the distribution of enzyme between free and substrate-bound states, rather than the absolute number of enzyme molecules. V_{max}, however, is dependent on the enzyme concentration and, consequently, would be reduced. However, the reconstitution procedures used in this study afford unidirectional incorporation of proteins (Eytan, 1982). Thus, all the MAO-B molecules are either in an inward or an outward orientation, outward being the native form. If all the enzyme is oriented outward, its access to substrate should be the same as for the free (unreconstituted) enzyme. Inward oriented enzyme would be equally accessible to membrane permeant substrate and, thus, there would be no discernible differences in K_M or V_{max} due to "sidedness". Impermeant substrate would react only with outward facing protein, the orientation hence decided by the presence or absence of activity. Therefore, "sidedness" *per se* does not account for the lower V_{max} seen with the incorporated MAO-B. Another possibility is that some of the MAO-B may have been incorporated in a non-functional state. Disaggregation to monomers in the initial detergent solubilization would result in decreased activity if some of the protein subsequently failed to dimerize. Although the catalytic form is the dimer, substrate binding may only require a single subunit, a scenario that would result in decreased V_{max} and unaltered K_M. Perhaps the membrane environment itself imposes a constraint on MAO-B. That the fluidity of the bilayer composed of dioleoyl phospholipids

on pargyline might facilitate its partitioning into the membrane bilayer, as it does for benzyl alcohol and carbocaine (Houslay and Stanley, 1982) If pargyline were membrane-impermeable it would be possible to use inhibition by this agent to determine the orientation of reconstituted MAO-B in the bilayer (*i.e.*, outer *vs* inner leaflet), analogous to the use of the impermeant agent oubain in determining the orientation of reconstituted Na^+/K^+-ATPase (Goldin, 1977) The neurotransmitter amines bear structural resemblance to pargyline due to the presence of an aryl ring and a quaternized amine nitrogen at physiological pH Uptake of these substances into both neuronal and nonneuronal tissue involves sodium-dependent transport (Trendelenburg, 1990), suggesting that these amines (and probably pargyline) are not freely soluble in the membrane

V. Summary

In conclusion, MAO-B was isolated from bovine liver mitochondria and successfully reconstituted into lipid vesicles The influence of the lipid composition of the proteoliposomes on the enzymatic activity was explored Surprisingly, considering the tight association of this enzyme with its native membrane suggested by its hydropathy plot, the activity of MAO-B was not greatly affected by these lipids Activity was enhanced by PE at mitochondrial concentration, and also at higher levels Anionic lipids did not have their anticipated effect of *greatly* enhancing MAO-B activity through increased accumulation of its cationic substrates due to increased negative charge of the membrane surface The acidic phospholipids had mixed effects modest stimulation with a low concentration of PA, but not PS There was no distinguishable effect due to increasing unsaturation of the acyl chains, possibly because those examined were all in the fluid phase

The question now arises: what role does the membrane environment contribute to the functionality of MAO?

Mitochondrial MAO oxidation of neuroamines regulates their cellular concentration. The presence in serotoninergic neurons of MAO-B, for which serotonin is not a preferred substrate, suggests that the enzyme "caps" the cellular concentration of this transmitter, becoming important only when serotonin approaches the high K_M value this enzyme form has for this substrate. The rather broad substrate specificity of MAO-B supports its scavenger role, deaminating exogenous as well as endogenous monoamines. MAO-B is present in high concentration in the liver, the major detoxification organ. Apart from the protonated amine, the MAO (A or B) substrates are relatively hydrophobic, most having a phenyl or indole ring.

This rather broad role for MAO-B may also account for its lack of sensitivity to its lipid environment. Its activity is neither greatly enhanced nor greatly compromised by its presence in the membrane. Despite the appearance of the hydropathy plot, membrane lipid may not influence the conformation of the protein to an appreciable extent, leaving the enzyme unconstrained to interact with a wide range of substrates. From this study, it is seen that the effects of the membrane lipids examined are not highly significant. With DOPE, large effects were only seen at non-physiological concentrations. Although the solubilized and the reconstituted MAO-B appear to bind kynuramine equally well, as judged by the unaltered K_M for this substrate, the maximal velocity was reduced for the enzyme in the membrane environment. As mentioned in the Introduction (section I c), in addition to generating a specific neurotoxin by its metabolism of MPTP, the normal by-products of MAO-B metabolism are toxic, and excessive activity by this enzyme would lead to their accumulation. The membrane environment may serve as a control mechanism, regulating the activity of

MAO-B and, thereby, preventing an imbalance which could lead to oxidative stress and cell death It should be noted that the MAO mechanism involves the transfer of electrons (Silverman et al , 1980; Simpson et al , 1982) Its location within a mitochondrial membrane rather than the plasma membrane or that of some other organelle may be of significance in view of the role the mitochondrion plays in electron transport

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