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LIVER MONOAMINE OXIDASE IN LEAN AND OBESE (ob/ob) MICE

by

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A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABBREVIATIONS

CL	cardiolipin
COMT	catechol-O-methyltransferase
DPG	diphosphatidylglycerol
E_a	energy of activation
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acids
GLC	gas-liquid chromatography
i.p.	intraperitoneal
IRI	immunoreactive insulin
MAO	monoamine oxidase
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OTC	oxytetracycline
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
TC	tetracycline
TLC	thin layer chromatography
Tris	tris hydroxymethylaminomethane
T_t	phase transition temperature

SUMMARY

Studies were carried out on monoamine oxidase (MAO) in genetically obese (ob/ob) mice and their lean controls. Monoamine oxidase is believed to be located on the outer mitochondrial membrane in the liver and other tissues. It has been suggested that the activity of the enzyme may be regulated by the lipid environment in the membrane, and that, *in vivo*, enzyme activity might be affected by diseases that alter lipid metabolism. In this context, the ob/ob mouse provided an interesting model for studying the effect of lipid environment on the activity of MAO. It has been used extensively as an experimental model for obesity and maturity-onset diabetes, and in addition, has been reported to have several membrane-related processes that are defective.

The first part of this thesis focuses on studies done in non-treated lean and obese mice. Whereas no significant differences were observed in the brain and heart, a study of tissue distribution showed enhanced activity in the pancreas, white adipose tissue, and liver of obese mice. Abnormalities in the lipid metabolism of obese-mouse liver are widely documented. This tissue was therefore chosen for further studies on the effect of lipid environment on monoamine oxidase activity. A check on the activities of two other mitochondrial enzymes, cytochrome oxidase and

kynurenine hydroxylase, revealed no significant differences between lean and obese mice, suggesting that the greater activity of obese-mouse MAO was not a generalized effect on all mitochondrial enzymes.

The current view concerning the heterogeneity of MAO is that this enzyme exists in two forms, MAO-A and MAO-B, the distinction being based on substrate specificity and differing sensitivities to substrate-selective inhibitors. Studies with various substrates (serotonin, benzylamine, and tyramine) and inhibitors (clorgyline and deprenyl) indicated that unlike rat liver mitochondria, mouse liver mitochondria contained a predominance of the B-form of the enzyme. Whereas the K_m values for lean and ob/ob mice were the same for serotonin, benzylamine, and tyramine, the V_{max} values for all substrates were at least 50% greater in obese than in lean mice.

Extraction of liver mitochondria with acetone/water and acetone/water/NH_3 to remove lipids decreased enzyme activity more in obese- than in lean-mouse preparations, but residual activity was the same in both preparations, suggesting that the greater specific activity observed in obese mice may be due to an alteration in membrane lipid composition of liver mitochondria in these animals.

Results published by other investigators have implied a structural or functional interaction between MAO and mitochondrial phospholipids. A study of phospholipid species

profile did not reveal any significant differences in composition between lean and obese-mouse mitochondria. PC and PE were found to be the major components of both types of mitochondria. In the analyses of the fatty acid profile of liver mitochondrial phospholipids, arachidonic acid was found to be approximately twice as great in the PC, PE + PG and PI + PS fractions of obese-mouse liver mitochondria, than in the corresponding lean samples.

Since a lipid dependency was suggested by the results obtained with lipid-depleted mitochondria, and significant differences were observed in the fatty acid composition of mitochondrial phospholipids, two procedures were used to attempt to alter the lipid environment of the enzyme by manipulating the fatty acid composition of mitochondrial phospholipids. These treatments involved chronic cold exposure (14 ± 1°C for two weeks) and treatment with the antibiotic oxytetracycline (100 mg/kg I.M. daily for 10 days).

Oxytetracycline (OTC) has been previously reported to alter lipid metabolism in the obese mouse. Since another antibiotic, chloramphenicol has been shown to alter the acylation of phospholipid precursors in cultured BHK-21 cells, it was thought possible that treatment with OTC would also significantly alter the fatty acid composition of liver mitochondrial phospholipids in the obese mouse. The specific activity of mitochondrial MAO towards tyramine and

benzylamine, but not towards serotonin, was significantly decreased in treated lean and obese animals. The specific activity of cytochrome oxidase, a mitochondrial marker enzyme, was not altered, suggesting that mitochondrial enzymes are not all affected in the same manner by oxytetracycline treatment.

The effect of treatment did not appear to be due to direct inhibition by OTC itself, or a metabolite produced by chronic treatment of the intact animals with the drug. No significant changes were observed in such enzyme properties as inhibitor sensitivity or the pattern of substrate specificity. Treatment did not alter enzyme affinity towards tyramine and benzylamine, but decreased V_{max} values for both substrates. While changes in other fatty acids were observed which did not seem to be physiologically significant, treatment did not significantly alter the levels of arachidonic acid which was found to be present in greater amounts in liver mitochondrial phospholipids of the non-treated obese mice. It appears then that the decrease in MAO activity following treatment may not be attributable to a mechanism involving the alteration of the fatty acid composition of mitochondrial phospholipids.

It has been previously reported that the temperature of ob/ob mice drops to 31°C on chronic cold exposure to 12 ± 1°C. It was considered possible that this reduction

in core temperature would result in an adaptive change in the fatty acid composition of liver mitochondrial phospholipids. Such a response to a decrease in ambient temperature has been observed in membrane phospholipids in other organisms.

As was expected, no significant change in MAO activity was observed in lean mice, since these animals are reported to be capable of maintaining their body temperature during chronic cold exposure. Treatment, however, did not produce any significant changes in MAO activity in the cold-exposed obese mice. An analysis of the fatty acid composition of the obese-mouse liver mitochondrial phospholipids did not show an increase in unsaturated fatty acid content. Arrhenius plots of MAO activity at temperatures from 7° to 37°C revealed that the transition temperature was at 23°C, which is considerably below physiological temperature in both groups of animals. This may suggest that in spite of the reported lower body temperature following cold exposure, monoamine oxidase of obese mouse liver mitochondria was functioning in vivo in a still largely fluid environment, thus making an adaptive increase in desaturation of fatty acids unnecessary.

Since a high degree of unsaturation favors the disordered fluid state of the phospholipid matrix of biomembranes, and the fluidity of phospholipids has been reported to be essential for the functioning of some membrane-bound enzymes, it was thought that the increased

arachidonic acid in obese mouse liver mitochondria might be responsible for the enhanced MAO activity in these animals. The similar values obtained for transition temperature and Arrhenius activation energy between lean and obese-mouse MAO suggest that arachidonic acid does not appear to enhance the fluidity of mitochondrial lipids in obese mice. The observation that lean-mouse MAO activity was not altered in experiments involving the incorporation of arachidonic acid into liver mitochondria appears to support this view.

CHAPTER 1: INTRODUCTION

I. REVIEW OF LITERATURE

Life is, by the simplest definition, a manifestation of the numerous interlocking chemical reactions of the cell, and enzymes are the biological catalysts that accelerate these reactions in the living organism. Many of these changes are not only accelerated by enzymes, but without the latter, would not occur to any appreciable extent at body temperature. It can be said, therefore, without exaggeration that nearly all functions of the cell depend, directly or indirectly, on the action of enzymes.

This thesis is a report on studies done on the enzyme monoamine oxidase [monoamine : oxygen oxidoreductase (deaminating), E.C. 1.4.3.4., MAO] in the obese-hyperglycemic mouse and its lean controls. The literature review is divided into sections corresponding to the subject areas dealt with in the study. A summary of the present information on monoamine oxidase is followed by a more detailed discussion of the possible lipid dependency of this enzyme. The relevant characteristics of the ob/ob mouse are then reviewed. Finally, a background is given of the actions of the antibiotic, oxytetracycline, which relate to this study.

I.A. Monoamine oxidase

The term amine oxidase is generally used for two types of enzymes: 1) mitochondrial monoamine oxidase which catalyzes the oxidative deamination of biogenic monoamines such as tyramine, catecholamines and indoleamines, as well as benzylamine and kynuramine, and 2) plasma amine oxidase which catalyzes the deamination of benzylamine, kynuramine, spermine and spermidine (220). The latter is a soluble enzyme. A similar enzyme is found in tissues such as skin (195), bone (264) and dental pulp (223), where it is supposed to participate in the cross-linking reactions of collagen (34) and elastin (230).

Mitochondrial monoamine oxidase was first discovered in 1928 when Hare (124) found it to be capable of carrying out the oxidative deamination of tyramine. Subsequently, Blaschko and his co-workers (32) demonstrated that it could also oxidize other monoamines including catecholamines. Since then the enzyme has been designated 'monoamine oxidase'. The reaction involved, in schematic form is as follows:

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

An aldehyde is produced by the oxidative deamination of a monoamine substrate, with ammonia and hydrogen peroxide as by-products. The aldehyde formed is converted either to an acid with aldehyde oxidase or to an alcohol with alcohol dehydrogenase.

Bernheim (29) proposed that the reaction catalyzed by monoamine oxidase proceeds by way of an imine intermediate, and indirect evidence has been provided to support this view (321). The enzyme has been shown to be a flavoprotein and it is believed that flavin is reduced or partly reduced during the reaction (108, 312).

Solubilized monoamine oxidase preparations from ox thyroid (82), pig brain (315), and ox liver (231) have been shown to obey a double-displacement kinetic mechanism when acting on amine substrates. The enzyme reacts with the amine substrate to form the aldehyde product and a reduced form of the enzyme. This enzyme form in which the flavin component of the enzyme is reduced, then reacts with oxygen to regenerate the free enzyme and form hydrogen peroxide.

The membrane-bound enzyme from rat liver has also been shown to obey a double-displacement mechanism (146), but a comparison between the kinetics of this enzyme preparation and a solubilized preparation from the same source showed certain differences in the details of the kinetic pathway followed (148). These differences have been interpreted in terms of a change in the conformational stability of the enzyme, occurring when it is freed from its membrane-bound environment.

I.A.1. Properties:

a) Flavin prosthetic group:

The first definite indication that the prosthetic

group of the enzyme is a flavin was made in 1966 (226). An FAD-like substance was identified in the beef-liver enzyme and evidence indicating that the flavin group is involved in the oxidation of substrate was presented by Igaue et al. (154). FAD was found to be covalently linked to the peptide chain of the enzyme through the 8-α-CH_3 group of riboflavin (12). Subsequently, it was demonstrated that the enzymes solubilized from bovine kidney (85), rat liver (362), pig brain (314), beef brain (200) are also flavoproteins.

I.A.1. b. Copper content:

Pig brain MAO has been reported to contain 1 g-atom of copper/590,000 g protein (314, 316), however, no increase in enzyme activity was observed by preincubating the enzyme with various concentrations of Cu^{+2}.

The copper content of the beef brain enzyme was less than 0.01 µg/mg protein and copper chelating agents did not inhibit the enzyme. Beef liver MAO contains about 0.07% copper and is inhibited by known copper chelating agents, such as 8-hydroxyquinoline and sodium diethyl dithiocarbamate (225). However, when subsequent purifications of the enzyme decreased its copper content to <0.15 µg Cu/mg of protein, the characteristics of its inhibition by known copper chelating agents were almost unchanged. Most likely, copper is not an essential prosthetic group.

I.A.1. c. Requirement for iron:

The initial findings that monoamine oxidase contains a metal were based on the loss of enzymic activity in the presence of chelating agents. While the results of such experiments using purified preparations are by themselves inconclusive, Symes and his co-workers (307) have observed that the *in vitro* activity of liver MAO in iron-deficient rats was significantly lowered, whereas it was unaltered in copper-deficient rats. The results have been confirmed by measuring total MAO activity *in vivo* (306).

Several roles have been suggested for iron, namely, that it participates in the synthesis of MAO apoprotein or active MAO, or that it acts as a cofactor for MAO, and like flavin, may be tightly bound to the protein (307). There has been no conclusive evidence for or against any of these roles.

I.A.1. d. Molecular weight:

The molecular weight of rat liver enzyme was estimated by gel filtration as 300 - 400,000 whereas ultracentrifugation studies showed an estimated molecular weight of 100 - 150,000. It was therefore suggested that dimerization took place in the course of gel filtration (362).

I.A.2. Tissue distribution:

Monoamine oxidase is widely distributed in animal tissues e.g., brain, heart, kidney, and liver; and the liver has

generally the highest specific activity towards various monoamine substrates. In man, the parotid and submaxillary glands have the highest specific activity (122).

In several sources, the enzyme has been localized in the mitochondria (21, 61, 125). However, the microsomal fraction of liver (21) also contains some enzyme activity.

I.A. 3. Physiological role:

Monoamine oxidase has an important physiological role in the oxidative deamination of biologically active monoamines such as catecholamines and serotonin (65). While serotonin is metabolized solely by MAO (Fig. 1), catecholamines are inactivated by MAO and COMT. Fig. 2 shows the catabolism of the catecholamines, adrenaline and noradrenaline. The degradation of noradrenaline involves the oxidation of the side chain by the successive action of MAO and aldehyde dehydrogenase, and methylation of one of the phenolic groups by COMT. Degradation can begin with either the oxidation or the methylation in either the neurons or the liver. Adrenaline, on the other hand is first methylated, and the methoxy derivative secreted as such or attacked by MAO to give the same derivatives as noradrenaline.

Both MAO and COMT are considered important for the metabolism of catecholamines. A study of labelled metabolites appearing in the urine after intravenous injection of radioactive adrenaline in the rat established

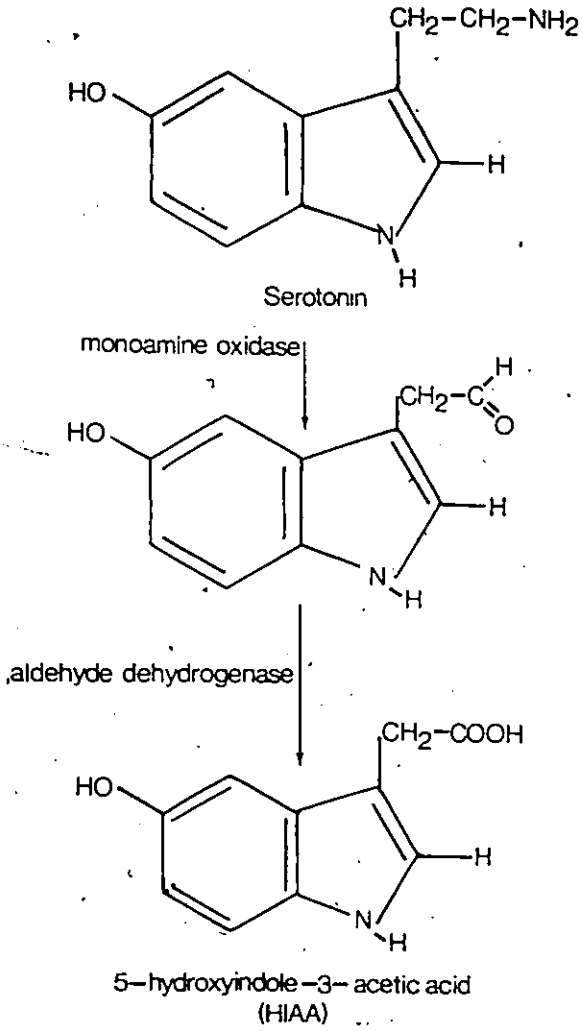

Fig. 1. Catabolism of Serotonin

[From McGilvery (216)].

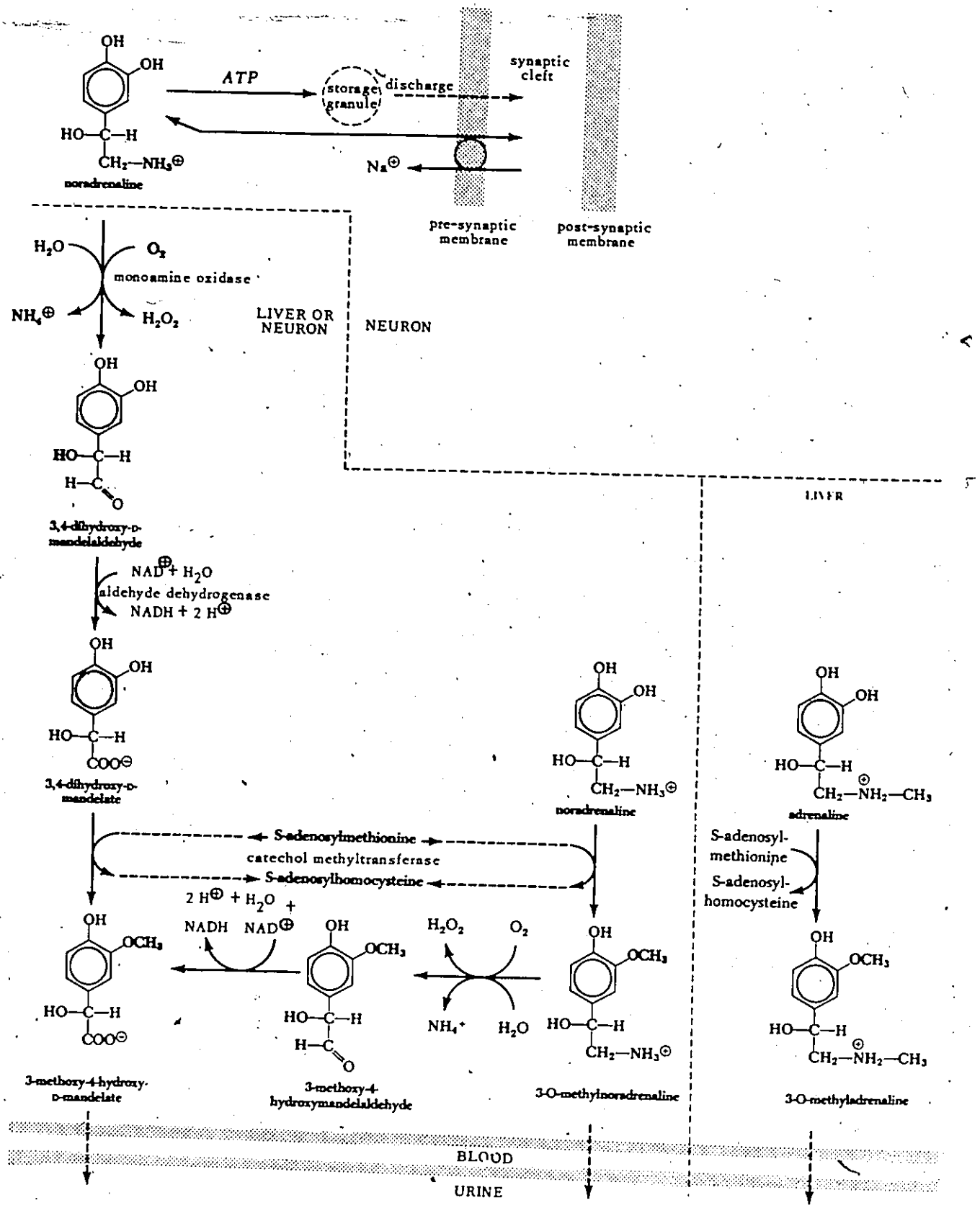

Fig. 2 THE DEGRADATION OF NORADRENALINE AND ADRENALINE
[FROM McGILVERY (216)]

that approximately 70% of circulating adrenaline initially undergoes O-methylation rather than deamination (11). Circulating noradrenaline is also metabolized predominantly by COMT (11). It should be noted, however, that information obtained from the study of infused catecholamines is applicable, in the physiologic sense, only to amines which are normally released into the bloodstream, as from the adrenal gland. Since most noradrenaline is released directly into individual tissues from the sympathetic nerve endings (87), considerable metabolism of this amine may occur before it ever reaches the circulation. In this respect, Crout et al. (62) demonstrated that endogenous noradrenaline accumulates in the brain and heart of the rat after administration of an MAO inhibitor. On the other hand, a COMT inhibitor did not produce this effect. Crout et al. (62) also observed that the liver was by far the richest source of COMT in the rat, and that O-methylating activity in this organ greatly exceeds that of monoamine oxidase. It was therefore suggested by these investigators that while COMT is of greater significance in the liver, monoamine oxidase plays the greater role in the initial metabolism of catecholamines in the brain and heart of the rat.

The relatively high levels of monoamine oxidase activity in the liver would suggest a protective function in the degradation of dietary amines. In 1966, it was suggested by

Fischer and his co-workers that thyroid MAO may be a significant source of thyroidal hydrogen peroxide, and may play a role in iodothryronin synthesis.

In 1967, Gorkin and Orekhovitch (113) reported another interesting observation on a possible new physiological role for MAO. The effects of the products of oxidative deamination of tyramine catalyzed by rat liver mitochondrial MAO on the oxidation of succinate in mitochondrial fragments suggested a possible participation of MAO in the regulation of tissue respiration. They observed a correlation between the chemical structure of monoamines and inhibition of succinate dehydrogenase activity after the incubation of monoamines with mitochondria. Thus inhibition of succinate dehydrogenase was much more pronounced in mitochondria pre-incubated with tyramine and dopamine than with phenylethylamine. Pretreatment of mitochondria with powerful MAO inhibitors, e.g., pargyline, completely prevented the inhibition of succinate dehydrogenase activity by all amines except dopamine when used in "saturating" concentrations. The succinate dehydrogenase activity was not affected by ammonia, hydrogen peroxide, or some acids and alcohols (vanillylmandelic and 5-hydroxyindoleacetic acids and tryptophol). But indoleacetaldehyde or p-nitrophenylacetaldehyde inhibited the activity of mitochondrial succinate dehydrogenase, and these effects were not prevented by pretreatment of mitochondria with the

monoamine oxidase inhibitors. These data suggested that the products of oxidative deamination of monoamines which exerted regulatory effects on the activity of mitochondrial succinate dehydrogenase are fatty aromatic aldehydes. This concept appears to be in agreement with subsequent data on the pharmacological activity of the fatty aromatic aldehydes (240, 265, 266) which suggest that the monoamine oxidases may be considered not only as participants in the detoxification of neurotransmitters but also as enzymes that catalyze the biosynthesis of new pharmacologically active compounds important for the regulation of other physiological functions (166).

I.A. 4. Subcellular localization:

With slight modifications in various tissues, in different species, or under specific metabolic conditions, the basic organization of the mitochondrion has been considered to consist of two compartments partitioned by two membrane systems, the inner membrane and the outer membrane. At the present time, more than 100 enzymes which catalyze reactions not related to oxidative phosphorylation *per se* have been assigned sites of localization within this structure. Additions to the list are being continually reported. It is also well established that at least 85% or more of the proteins comprising the mitochondrion are synthesized on the cytoplasmic ribosomes and only a small

fraction, 15% or less, by the mitochondrial protein synthesizing system itself (126). In the case of MAO, 70-85% of the total activity in adult rat liver homogenates sediments with the mitochondria. However, a significant percentage of the total activity is consistently found in the microsomal fraction (21).

Evidence has been presented in the literature which suggests the existence in mitochondria of a multiplicity of enzymes or forms of the enzyme that oxidize various monoamines (112, 251, 295, 358). Greenawalt's studies on the mitochondrial localization of MAO indicate that kynuramine oxidase activity is about equally distributed in the outer membrane and inner membrane plus matrix fractions, whereas the activity oxidizing other monoamine substrates, e.g., benzylamine, serotonin, and tyramine was largely concentrated in the outer membrane.

Whereas some investigators have assigned the localization of MAO activity to the inner membrane (117) and others (251, 329) have reported results that may suggest double localization of the enzyme in the outer and inner membranes of rat liver mitochondria, a majority of investigators assign MAO activity unequivocally to the outer mitochondrial membrane. The finding that mitoplasts, also referred to as the inner-membrane-matrix fraction, can undergo respiration-dependent ultrastructural changes and yet retain essentially no MAO activity, supports the interpretation that MAO is localized

in the outer membrane of rat liver mitochondria (115). Schnaitman et al. (265) showed that the release of MAO in association with small vesicles was correlated with the loss of the outer membrane from isolated rat liver mitochondria. The small vesicle fraction, which could be sedimented only by high-speed centrifugation, contained a high percentage of the total MAO activity of the mitochondrial fraction and exhibited a greatly increased specific MAO activity. On the other hand, enzyme markers for the matrix and inner membrane were largely absent from the small vesicle fraction. From these observations, it was concluded that mitochondrial MAO is localized on the outer membrane of rat liver mitochondria, and further, that this enzyme serves as a valid marker for this mitochondrial component.

I.A. 5. Multiple forms of mitochondrial monoamine oxidase:

Although the oxidative deamination of monoamines was once generally considered to be effected by a single enzyme, evidence has accumulated which seems to suggest the existence of multiple forms of MAO. Alles and Heegard (2) tested a large number of monoamine substrates against liver enzyme from different species and found marked differences as judged by relative rates of oxidation. Werle and Rouver (339) reported the separation of enzymes from animal sources that were capable of oxidizing either only aliphatic or only aromatic monoamines. Hagen and Weiner (119) have reported

differences in substrate specificity of enzyme from various species and organs in the oxidation of pharmacologically active amines.

Ostwald and Strittmatter (241) observed markedly different relative rates of activity with different substrates in various tissues of rat and guinea pig as well as different K_m values in different tissues. Severina and Gorkin (278) studied the influence of heat; pH, and chelating agents in rat mitochondrial MAO activity towards benzylamine and tyramine. They reported that with each of these experimental conditions the MAO activity with one substrate was different from that observed with the other substrate.

Van Woert and Cotzias (333) found the degree of inhibition by various anions, depended on whether tyramine or serotonin was used as the substrate. Gorkin and his collaborators (115) reported further evidence of the selective inhibition of serotonin in rat liver mitochondria. Maitre (201) testing the effects of a nonhydrazine MAO inhibitor, N-methyl-N-2-propynyl-1-indanamine, found that this substance inhibited brain MAO more than liver MAO when injected, and as with other inhibitors the degree of inhibition depended on the substrate used in measuring MAO activity. This author considered his data as further evidence that various tissues of the same animal contain more than a single MAO. Johnston (161) studying the effect

of clorgyline, found that tyramine was inhibited in a biphasic manner as the concentration of clorgyline was increased. Johnston interpreted these results as indicating the presence of two forms of MAO. Hall, Logan, and Parsons (120) extended the observation to a variety of mammalian species and organs and concluded that two enzymes are present in the brain of rat, man, rabbit, ox, dog and cat, and also in the liver of rat and man, whereas the MAO from the brain of the pig and liver of all species examined other than rat and man, seem to be homogeneous. Evidence for the existence of several forms of MAO in the mouse was provided by inhibition and thermal inactivation studies carried out by Squires (295). Further evidence for the existence of multiple forms of MAO in rat liver mitochondria was given by Tipton (313).

In view of the indirect evidence provided by the above investigations, experiments were carried out by various workers in an attempt to separate the multiple forms of MAO. MAO is tightly bound to the mitochondrial membrane (118, 293), therefore, only after successful solubilization of the enzyme would it be possible to investigate directly the possible existence of multiple forms. Kim and D'Iorio (173, 174) reported the first electrophoretic separation of MAO activity on cellulose polyacetate membrane. These authors reported four bands of MAO activity in homogenates of rat liver. Two of these bands seem to correspond to the microsomal fraction, and the other two to the mitochondrial fraction. Similar

results were obtained with mitochondria isolated from rat brain and kidney (285). Kim and D'Iorio did not discount the possibility that these bands could be due to incomplete solubilization of the enzyme or to association with sub-particles or membrane debris of different sizes, although solubilization of the enzyme by detergents or sonication seems to produce similar results (174).

Youdim and Sandler (350) and Collins, Youdim and Sandler (58) working with a partially purified preparation of human liver and placental mitochondria, and of rat liver mitochondria observed MAO activity in several bands after polyacrylamide gel electrophoresis. These investigators demonstrated that the different bands of activity isolated from rat liver have significantly different pH-activity curves, thermostability, substrate specificity, and susceptibility to inhibitors both in vitro and in vivo.

Partial separation of the MAO activity towards kynuramine, tyramine, and serotonin in liver mitochondria of beef, rat, and rabbit was reported by Ragland (251). The mitochondria were solubilized with detergent and separated by chromatography on a Sephadex G-200 column equilibrated with the same detergent. The recovery of MAO activity applied to the column varied from 40 to 80% according to substrates and species.

Shih and Eiduson (281) did polyacrylamide gel electrophoresis on chick and hen brain, and obtained several bands

with some differences in relation to age of animals and substrate specificity. Multiple forms have since been confirmed for MAO of beef liver (108), human and rat brain (56, 359), and rat uterus (57).

Sierens and D'Iorio (285) solubilized rat liver mitochondrial MAO with deoxycholate and fractionated these activities via gel electrophoresis. These investigators obtained two fractions one of which acted on benzylamine and the other on both benzylamine and serotonin. It was observed that serotonin activity is strongly diminished in the presence of deoxycholate. Diaz-Borges and D'Iorio (74) using Lubrol and sonication to solubilize rat liver mitochondrial MAO followed by sucrose density gradient centrifugation, reported at least two different enzyme activities, one for serotonin, and the other for benzylamine and kynuramine.

The demonstration that soluble preparations of monoamine oxidase could be resolved into a number of discrete bands of enzyme activity by electrophoresis has led to the suggestion that there are either more than one enzyme or several forms of the enzyme.

Although these observations have been made in separate laboratories, the phenomenon of enzyme multiplicity has not met with universal acceptance. Since the number of bands of activity varied with solubilization and electrophoretic methods used, the view has been put forward that these bands of activity may be artifacts of the procedures employed (150).

Comparison of results reported by various investigators is impossible, since each research group used different methods of solubilization and electrophoretic separation.

Tipton et al. (145, 310, 312) have shown that the phospholipid content of different electrophoretically separated forms varies and this characteristic may be responsible for the apparent multiplicity of the enzyme, since the solubilization procedure can cause enzyme molecules to be liberated with different amounts of membrane lipid material bound to them. These workers have reported that treatment of solubilized preparations of the enzyme with chaotropic agents, e.g., sodium perchlorate to remove bound lipid material, resulted in an MAO preparation which was homogeneous and manifested a single band of electrophoretic activity (145, 317). This appears to implicate lipids as an important modulating factor, as will be discussed below.

Hartman and Udenfriend (135) using immunological techniques have shown that the various separable forms of bovine liver mitochondrial MAO are antigenically identical. They attributed the differences in electrophoretic mobility on acrylamide gels to aggregation of the same protein unit or the presence or absence of detergent. With bovine brain mitochondrial MAO, they also observed that the bulk, i.e., 80%, was antigenically identical to liver MAO. The rest of the MAO activity in the crude mitochondrial fraction, however, did not cross-react with the antibody to liver MAO. This

'brain-specific' MAO was shown to have different substrate affinity and sensitivity towards inhibitors demonstrated by liver or total-brain MAO.

As already mentioned above, studies with membrane-bound preparations of the enzyme have indicated that many tissues contain two major forms of MAO activity that differ in their substrate and inhibitor specificities but show little similarity to any of the bands of activity that can be separated electrophoretically from solubilized preparations of the enzyme. In the absence of any reliable procedure for the separation of these two forms, the main way in which they have been investigated involves the use of selective inhibitors of the enzyme.

A numer of irreversible inhibitors of monoamine oxidase have been shown to inhibit the activity of the enzyme towards certain substrates at considerably lower concentrations than are required to inhibit its activity towards other substrates. Using tyramine as substrate, Johnston (161) described the stepwise inhibition by clorgyline of mitochondrial MAO from the liver and brain of the rat, dog, rabbit, and man. He designated the fraction inhibited by lowest concentrations as fraction A while the remaining fraction inhibited by much higher concentrations of clorgyline was called fraction B. Hall (120) also investigated the effect of clorgyline on mitochondrial MAO from brain and liver of rat, cat, dog, rabbit, pig, ox, and man using tyramine as substrate, and

noted whether inhibition occurred in one step or two steps. In studies on different organs of the mouse, Squires (296) observed that harmine, harmaline and ethyl tryptamine were all highly selective inhibitors of the same fraction of MAO inhibited by clorgyline and has referred to them as A inhibitors. On the other hand, deprenyl proved to be a highly selective inhibitor of the B fraction in the same mouse organs. Fig. 3 gives the structures of some of these inhibitors.

Squires also observed that kynuramine, tyramine, and tryptamine belong to a group of amines which are deaminated by both A and B fractions of MAO in many species. Similar studies by Houslay and Tipton (147) have shown that in rat liver mitochondria, adrenaline and noradrenaline and their m-O-methylated derivatives are substrates for the A-form of the enzyme in addition to serotonin, and that phenylethylamine as well as benzylamine is a substrate for the B-form.

I.A. 5. a. Distribution of the A and B forms of MAO:

Studies using tyramine as the substrate have shown that the relative proportions of the two forms vary widely among different organs of the rat (318), the A-form comprising over 95% of the total in spleen, 40% in liver, 55% in brain and only 15% in the pineal gland. A number of organs from other species including pig liver and brain, and ox liver appear to possess only the B-form of the enzyme as judged by selective inhibition towards tyramine.

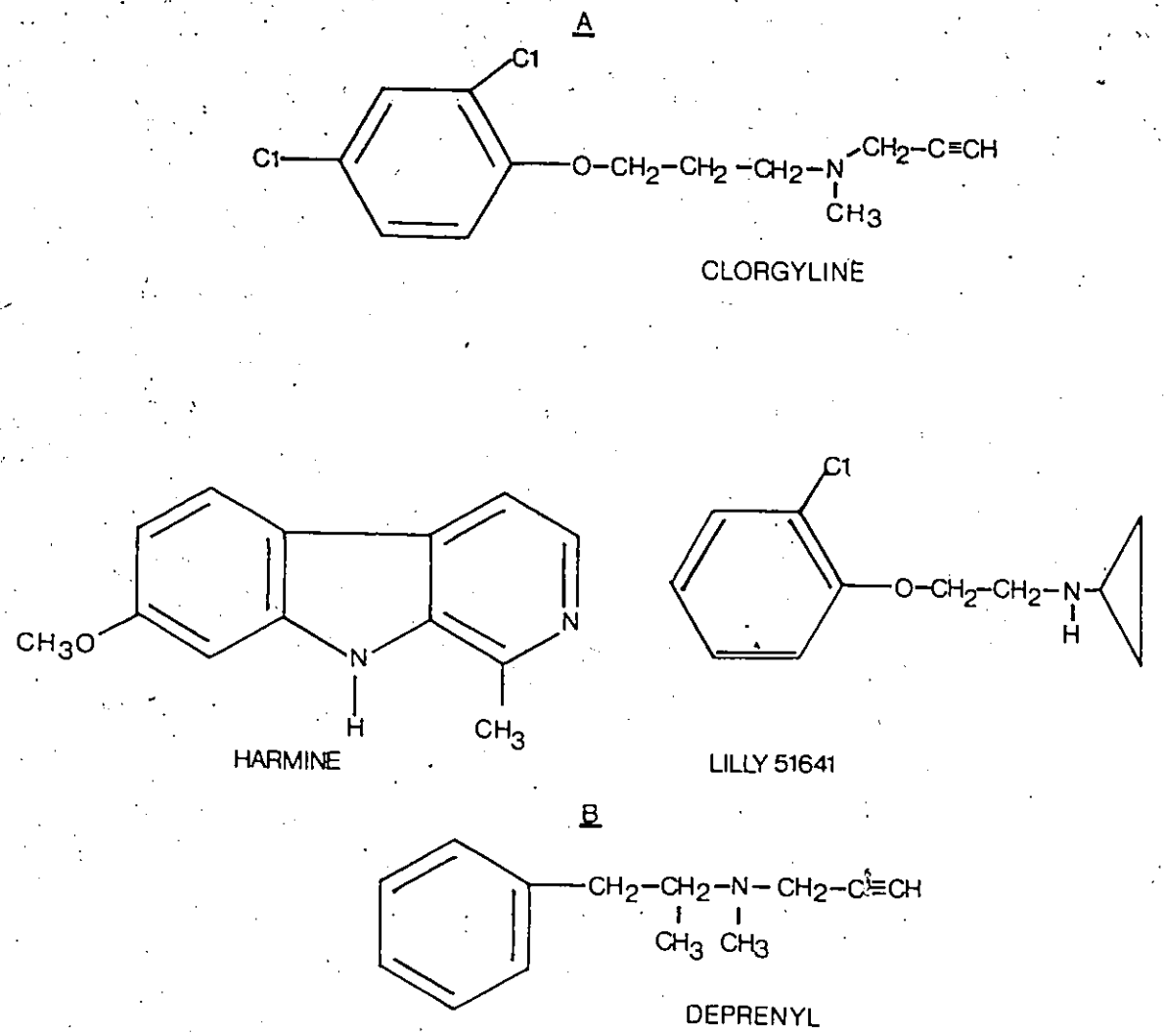

Figure 3. Structural formulas of some selective MAO inhibitors.

Since the A-form of the enzyme is active towards the amines that are normally recognized as being neurotransmitters, it has been suggested that this form might be located in neuronal tissue, the B-form being largely extraneuronal. Denervation studies have suggested that this may, indeed, be the case in the rat pineal gland (110). However, similar studies with rat vas deferens have indicated that both forms of the enzyme are present intraneuronally (158). Both forms have also been found to be associated with mitochondria prepared from purified rat brain synaptosomes (242). Their presence in isolated hepatocytes (318) would suggest that both forms of the enzyme can coexist in the same cell type. Table 1 shows the distribution of the two forms in a number of organs from the rat. Note that denervation of rat liver by 6-hydroxy-dopamine treatment does not affect the A/B ratio, suggesting that the contribution of neuronal MAO to total rat liver activity is slight.

I.A. 5. b. Nature of the multiple forms of MAO:

Unlike the forms of the enzyme separated by electrophoresis, there is good evidence that the two forms that can be detected by the use of selective inhibitors are not artefacts of the method used to prepare the enzyme. The two forms may be detected in intact mitochondria and in outer-membrane preparations derived from them, as well as in crude tissue homogenates. Evidence has also been provided (31, 101, 103, 353) that differential inhibition of the enzyme occurs after

Table 1

The distribution of the two major species of monamine oxidase activity in the rat. [From Houslay and Tipton (150)].

	Species A Activity (Percent of total)	Species B Activity (Percent of total)
Liver	40	60
Liver Parenchymal Cells	50	50
Denervated Liver	40	60
Kidney	70	30
Intestine	70	30
Intestinal Mucosa	60-70	30-40
Spleen	95	5
Lung	50	50
Testis	90	10
Brain	55	45
Superior Cervical Ganglia	90	10
Pineal Gland	15	85
Denervated Pineal Gland	5	95
Vas Deferens	50	50
Denervated Vas Deferens	35	65

in vivo administration of inhibitors.

The molecular and structural basis for MAO multiplicity appears to require further clarification. Are these enzyme forms distinct proteins, or are they variably modifiable sites on the same molecule? Are they different conformational forms of the same enzymes? Or are they the same enzyme in different chemical environments?

Using immunological techniques, Hartmann addressed himself to the problem of whether or not the multiple forms of MAO are distinct enzyme proteins (135). As discussed in section I.A. 5, he identified two types of MAO activity in beef brain. McCauley and Racker subsequently (214) carried out a similar study on beef brain enzyme, and claimed that the immuno-precipitated fraction corresponded to Type B enzyme, whereas the resistant fraction corresponded to Type A, an identification which is difficult to justify since both preparations were active towards benzylamine. Youdim (356) showed that MAO from rat liver and brain both cross-reacted with antibody prepared to the liver enzyme, but immuno-diffusion studies detected two bands with different diffusion rates. Whether these bands represented distinct proteins, or a modification of diffusion rates by the binding of lipid material is unclear at present.

The relationship between the two forms of MAO that are distinguishable through the use of selective inhibitors and the bands of activity that can be separated electrophoretically

is not clear-cut. A study of the inhibition of the latter forms by clorgyline and pargyline failed to reveal any simple correlation between their inhibitor sensitivities and the properties of crude preparations of the enzyme (59). The idea that the isoenzymes of MAO could be due to the binding of varying amounts of membrane material to a single enzyme was first advanced by Veryovkina, et al. (335). In order to investigate this possibility, the phospholipid content of electrophoretically separated bands of rat liver MAO were investigated by Tipton (145, 310), who demonstrated that the electrophoretically separated forms from rat liver MAO differed considerably in their phospholipid content, and suggested that these forms could be artefacts of either the purification or of the electrophoretic procedure itself. Similar differences have since been shown with the electrophoretically separated forms from beef adrenal medulla and cat brain (322).

Differences in thermal stability has been used to support the idea of the existence of multiple forms of MAO. The thermal stability of the enzyme has been shown to be dependent on the nature of the substrate used to assay the enzyme (296, 352, 361). Exposure of mitochondria to density gradient centrifugation caused considerable alterations in these differential thermal stabilities as a result of different degrees of protection by mitochondrial lipids. Solubilized preparations of the enzyme have been

found to be less temperature stable than membrane-bound preparations, and Oreland and Ekstedt (237) have shown that the binding of lipid material to a solubilized pig liver enzyme preparation increases its stability. Houslay (145) has also shown that differences in thermal stabilities could be eliminated by treatments which reduced the lipid content of the enzyme.

Tipton and his colleagues (319) have also investigated the possibility that the membrane-bound enzyme exists in states of aggregation that were different for the two enzyme forms. Solubilized preparations of the enzyme from rat liver have a molecular weight of 300,000 - 400,000 as determined by gel filtration, but the minimum functional molecular weight of the enzyme from a number of sources appears to be 100,000 - 150,000. The molecular weight of forms A and B as determined by the irradiation technique employed by Tipton et al. (319) were both 300,000, indicating that neither of the membrane-bound forms differed in molecular weight from the solubilized preparation of the enzyme.

Dennick and Mayer (71) prepared a purified solubilized enzyme from rat liver which they showed to be a homogeneous protein which corresponded to the B-form of the enzyme. Antibodies to this material precipitated enzyme activities towards benzylamine and serotonin simultaneously, indicating the immunological identity of the two forms of the enzyme from this source. This finding is in agreement with studies

of Chau and Hackenbrock (154) who showed that antibodies to the enzyme from this source would react with all the activity of rat liver mitochondria although, they postulate, a proportion of the enzyme was protected from the antibodies in intact mitochondria by being bound to the inner surface of the mitochondrial outer membrane.

The studies involving soluble preparations of the enzyme from rat liver and human brain, in which chaotropic agents had abolished electrophoretic multiplicity, different thermal stabilities towards different substrates, and selective effects of inhibitors, suggest that the multiple enzyme forms reported in the literature may be the result of having different amounts or types of membrane material bound to them. Chaotropic agents are known to be capable of weakening hydrophobic bonds, thus freeing protein from association with lipid material. The above results have been interpreted as indicating that the apparent multiplicity of the enzyme is a result of the binding of a single enzyme in different membrane environments. This model also suggests that the reported differences in the specificities of the A and B forms of the enzyme from different species may reflect differences in mitochondrial lipid composition.

Houslay and Tipton's (145) concept of a single kind of active site, which is influenced by its lipid environment to assume A or B properties differs from White and Glassman's concept of a single molecular entity containing multiple MAO

sites. Gel filtration of solubilized human brain and liver MAO by these investigators gave patterns of activity similar to those reported by other workers (145, 222, 282). However, no separation of substrate selective activities was achieved even in the presence of 0.05M sodium dodecyl sulfate. Their results imply that if separate forms exist these would be of similar molecular size. Similarly, isoelectric focussing which separates protein molecules on the basis of net charge in a pH gradient also failed to separate substrate-selective activities.

White and Glassman contend that if the A and B sites occur on different molecular forms of MAO, it should be possible to achieve a physical separation of these activities, and failure to achieve this separation would indicate that the different MAO sites could be part of the same molecular complex. Furthermore, they contend that if there was only one active site, it should be possible to demonstrate interconversion of substrate-binding sites, at least under some conditions. This has not been achieved by White and Glassman and other workers (80). Also, previous substrate competition experiments by White and Wu (346), and K_m determinations by White and Glassman (334) using MAO which has been selectively inhibited by low concentrations of clorgyline or deprenyl seem to provide evidence for at least two independent substrate binding sites for MAO of human brain and liver. Assuming that such multiple sites are

present, their lipid environment in the mitochondrial membrane may, however, still regulate the relative activities of A and B forms of the enzyme observed in various organs (79).

Although the issue of the molecular and structural basis for MAO multiplicity remains a matter of considerable speculation, it is noteworthy that several investigators have been able to identify the two MAO forms in vivo. Yang and Neff (353) have demonstrated that intravenous administration of clorgyline to rats induced an elevation in the brain of preferred substrates for type A enzyme; while injection of deprenyl slowed the brain metabolism of phenylethylamine, a preferred substrate of type B enzyme. Fuller (101) observed substrate-selective inhibition of phenylethylamine and tryptamine oxidation in mouse brain and heart following i.p. administration of the inhibitors pargyline or Lilly 51641. Other investigators have also demonstrated the existence of two species in the intact perfused lungs of rabbit (259) and rat (14).

I.A. 6. Physiological control:

Information on the physiological control of MAO activity has been obtained by measuring enzyme activity during various phases of the life of a normal animal. The studies have been carried out in different species, e.g., rats, mice, guinea pig, rabbits, cats, and the pig. This discussion will be restricted to studies concerning the rat

or mouse.

I.A. 6a. Hormonal regulation of monoamine oxidase activity:

Fluctuations in MAO activity during the estrus cycle in various tissues of the rat are well documented. Southgate (294) reported a fall in uterine and liver MAO after estrogen treatment. The reduction in liver enzyme activity is in agreement with the data reported by Wurtman and Axelrod (349). Holzbauer and Youdim (140) observed that in three brain regions, and the uterus, ovaries, and adrenal glands, the lowest values for MAO activity occurred in late estrus and the highest values in pro-estrus. Cavanaugh and Zeller (42) made similar observations in mouse liver. Estrogen was also implicated in the regulation of central nervous system MAO by work done by Luine et al. (197) who observed that in a number of brain regions examined in the rat, only the hypothalamus and amygdala, two regions which contain estrogen receptors, show changes in MAO activity.

It appears that the extent of any changes observed depended on the substrate used. For instance, Holzbauer (141) observed that with dopamine as substrate, progesterone produces a greater increase of activity than with any other amine, but no significant decrease was seen after 17-β-estradiol treatment. MAO activity towards serotonin was not affected by either hormone. Similar observations were reported by Southgate who observed a larger rise in rat

uterine MAO activity after progesterone treatment when dopamine was used as substrate than when tyramine was used, and a decrease in uterine activity towards benzylamine was observed after estrogen treatment but no change in activity towards dopamine.

Adrenocortical steroids can suppress MAO activity. Avakian and Callingham (10) reported an increase in cardiac MAO activity which could be antagonized by corticosteroids. Parvez and Parvez (245) made a similar observation on brain MAO activity.

Another endocrine gland which can alter MAO activity is the thyroid. Okamoto (232) found that the activity of several enzymes located in the outer membrane of liver mitochondria was decreased to the same extent in thyroxine-treated animals. Utley (331) observed an increase in cardiac MAO activity and a decrease in liver MAO activity in hyperthyroid animals when tyramine was used as the substrate. Tong and D'Iorio (323) reported that the effect of thyroxine on cardiac and liver MAO activity in young hyperthyroid rats depended on the substrate used. Ho-Van-Hap et al., (152) have demonstrated that the activity of monoamine oxidase varied with the age and sex of the animals used in their experiments.

I.A. 6b. Ontogenetic development of monoamine oxidase activity:

Holzbauer and Youdim (141) reported a steep increase in MAO activity towards dopamine, tyramine and kynuramine in

liver and various brain regions of male Wistar rats during the first one to two months of life. In contrast cardiac MAO activity increased slowly during the first month, followed by a steep rise which did not reach maximal activity even after four months. Callingham and Lyles (40) studied MAO activity in the growing heart, and observed that in the young rat (30-60 g) type B MAO was predominant, whereas in the older rat (300-400 g), type A predominated (up to 70%).

Holzbauer and Youdim (141) also studied the deamination of tryptamine and serotonin in male Wistar rats in the first three weeks following birth. In the brain, with tryptamine as substrate, a fall instead of a rise in enzyme activity was observed. In contrast, tryptamine deamination by the liver increased with age. With serotonin as substrate, there was a steep fall in enzyme activity, during the first 20 days, in livers, hearts, and brain striata and hypothalami. Similar results were reported by Bourgoin et al. for a group of Sprague-Dawley rats (35).

From the observations made with different substrates, it has been suggested that the different forms of MAO deaminating various monoamines develop separately (165). However, the observations made in the above studies may also be a consequence of differences in the rate of development of so-called allotopic factors which influence the activity of the enzyme towards a given amine.

Jourdikian and his associates (165) studied the development of MAO activities towards serotonin and p-dimethylamino-benzylamine in mice of the HS strain. These workers observed that the serotonin deaminating activity reached adult levels by 5 days after birth. These results are similar to those previously reported by Baker et al. (13) in mouse brain. On the other hand, Jourdikian observed that the dimethylamino-benzylamine deaminating activity was only 50% of adult values at 15 days, and did not develop fully until the 45th postnatal day. These authors postulate that the observed differences in rats of development of two forms of MAO may reflect the presence of two different protein moieties which accumulate at different times during brain development, or the development of phospholipid components of the brain.

I.B. The regulation of monoamine oxidase by membrane lipids

Biological membranes are organized assemblies consisting mainly of proteins and lipids. Depending on the source, the weight ratio of protein to lipid in most membranes ranges from 1:4 to 4:1 (305). Specific proteins mediate distinctive membrane functions they serve as pumps, gates, energy transducers, receptors, and enzymes. Membrane lipids through their effect on membrane fluidity, or via direct interaction with these proteins, create a suitable environment for their action. In the case of enzymes, the lipid may function in the solubilization of a non-polar or amphipathic

substrate, or the amphipathic enzyme protein itself. The motional state of the lipid phase will then determine the rotational and lateral diffusion rates of the solubilized protein, and probably, its ability to undergo conformational changes.

In animal cell membranes, phospholipids, glycolipids and cholesterol comprise the lipid phase (267). However, in some membranes, and particularly in membranes of cytoplasmic organelles in mammalian cells, the contribution of glycolipids and cholesterol is very small (90). Tissue phospholipids, on the other hand, constitute a substantial component of all membranes that have been investigated (90), and are, in fact, located mainly in the cell membrane where they contribute to both structure and function.

Monoamine oxidase has been shown to be firmly bound to the outer membrane of the mitochondrion (117, 273). The membrane environment might be expected to convey certain allotopic properties on the bound enzyme (148, 335). As cited in section I.A. 5, work done by some investigators (145, 310) suggest that the activity of this enzyme may be regulated by its lipid environment in the membrane.

The basic organization of the mitochondrion consists of two compartments partitioned by a two membrane system - the inner membrane invaginated to form cristae, and the outer membrane. Observations of freeze-fractured mitochondrial membranes indicates that the 'fluid mosaic model' proposed by Singer and Nicolson (287) is applicable to mitochondrial membranes

(336). This model suggests that membranes are two-dimensional solutions of oriented globular proteins and lipids (Fig. 4), and that the essential structural repeating unit is the phospholipid molecule in a bilayer arrangement. The other major features of the model are:

1. The lipid bilayer fulfills a dual role: it is a solvent for integral membrane protein, and it is also a permeability barrier.
2. A number of membrane lipids interact specifically with particular membrane proteins and may be essential for their function.
3. Membranes are dynamic structures in which proteins and lipids diffuse rapidly in the plane of the membrane, unless restricted by special interactions.

The proteins of membranes amount to a major proportion of the total cell protein so that membranes form the main site of phospholipid-protein interaction in biological systems. Proteins may be inserted into the predominantly phospholipid bilayer in a seemingly random fashion. Some proteins are localized at one or the other of the two surfaces of the lipid bilayer; other proteins may pass from one side of the membrane to the other; and still others may be imbedded in the hydrophobic matrix. Superimposed upon this mosaic structure, a variety of peripheral proteins may be loosely associated with the membrane as a result of the interactions with integral protein or with lipid constituents (cf. Fig. 4). Although

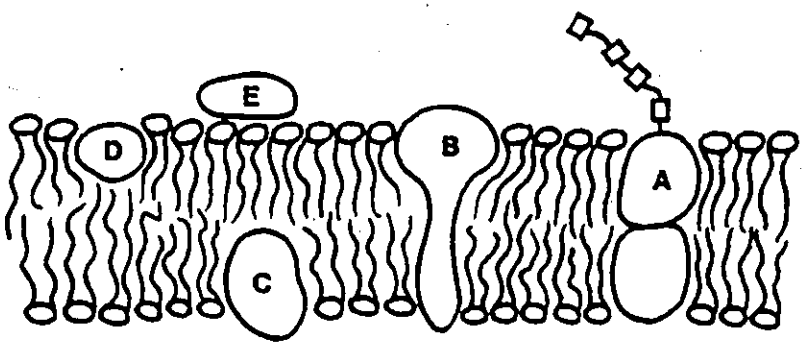

Fig. 4. THE FLUID MOSAIC MODEL OF MEMBRANE. A AND B, INTRINSIC OR INTEGRAL PROTEINS (SPANNING); C, INTRINSIC, INWARDLY EXPOSED PROTEIN; D, INTRINSIC, OUTWARDLY EXPOSED PROTEIN; E, EXTRINSIC OR PERIPHERAL PROTEIN. [FROM VIGNAIS (336)]

most of the membrane phospholipids are in the bilayer array, a considerable number may be closely associated with integral membrane proteins (267). There is no direct evidence that lipid molecules are homogeneously distributed in membranes (90). Indeed, the observation that the stability and activity of many enzymes (89, 90, 207, 277) depend on lipid association appears to indicate that there may be specific localization of some lipid components in relation to their interaction with protein.

Some membranes proteins can be dissociated from the rest of the membrane by relatively mild means such as extraction by a solution of high ionic strength. In contrast, other membrane proteins are bound more tenaciously and can be separated only by using a detergent, a chaotropic agent, or an organic solvent. This difference in the ease of dissociation has been used by some investigators (267) to distinguish peripheral proteins from integral proteins. Since peripheral proteins can be dissociated by the addition of salts, it is inferred that they are bound to the surface of the membranes by electrostatic and hydrogen-bond interactions. In contrast, it is presumed that integral proteins interact to a large extent with the hydrocarbon chain of membrane lipids because they are solubilized by detergents, which could compete for these non-polar interactions.

Monoamine oxidase is firmly bound to the membrane and cannot be extracted into aqueous media (111), unless use is made of detergents (85, 108, 362) or sonication, freezing, and thawing (314). The finding that the enzyme can be rendered soluble after extraction with organic solvent (139) indicates that it interacts with membrane phospholipids. In this connection, as has been cited earlier (cf. section I.A. 5.b.) Tipton (145, 310, 317, 322) has demonstrated that the electrophoretically separated forms of rat liver MAO differed considerably in their phospholipid content, and treatment of solubilized preparations with a chaotropic agent, which removes bound lipid material, results in a loss of multiple forms and released a single band of electrophoretic activity which did not respond in a biphasic manner to selective inhibitors. Ekstedt and Oreland (80) treated rat liver mitochondria with aqueous methyl ethyl ketone which removed about 90% of the phospholipids, without liberation of the enzyme. After extraction, only 7% of the serotonin-oxidizing activity (from-A MAO) and 80% of the β-phenylethyl-amine-oxidizing activity (form-B MAO) were recovered. These findings would suggest that MAO does exhibit functionally important interactions with membrane phospholipids.

Oreland and Olivecrona (238) extracted pig liver mitochondria with methyl ethyl ketone in the presence of 0.05M ammonium sulfate. This treatment preferentially extracted acidic phospholipids, and resulted in the liberation of a substantial

amount of enzyme into buffer. The amount of enzyme remaining in the lipid-depleted residue was observed to correlate with the amount of acidic phospholipids remaining on the mitochondrial residues. It has been suggested (66, 116) that the acidic phospholipids in mitochondria are involved in both ionic and nonionic interactions with mitochondrial proteins. This suggestion seems to be supported by the requirement for ammonium salt which would decrease the strength of ionic interactions between the acidic phospholipids and the proteins, and allow the organic solvent to extract these phospholipids efficiently by breaking their nonionic interactions with the proteins (238). Oreland and Olivecrona's observations suggest that the charged parts of the enzyme molecule interact electrostatically with the negative polar heads on the acidic phospholipids and that other parts of the enzyme molecule interact nonionically with the hydrophobic parts of phospholipids. The above evidence is in favor of the view that MAO is an integral membrane protein.

However, Singer (286) classifies monoamine oxidase as a peripheral protein based on the observations made by Erwin and Hellerman (85) that the beef kidney enzyme could be released from the membrane by digitonin, and that the purified enzyme was not inhibited by phospholipase A. Digitonin is known to interact with cholesterol on the outer mitochondrial membrane (273) and it can be argued therefore

that digitonin may have acted as a hydrophobic bond-breaking agent in the enzyme solubilization process. Further, resistance to the action of phospholipase A does not always constitute adequate evidence for the absence of direct phospholipid dependence. For instance, treatment of E. coli membranes with phospholipases did not reduce the activity of a membrane-bound ATPase (247), because the amount of lipid remaining in the membrane was sufficient to sustain activity. Moreover, Erwin and Hellerman detected digitonin in the purified enzyme sample, and did not rule out the possibility that the residual digitonin may have fulfilled a nonspecific lipid requirement for enzyme activity.

The activity of membrane-bound enzymes has frequently been associated to the fluidity of the membrane (252). As cited earlier, in the fluid mosaic model, the predominantly phospholipid matrix constitutes a "solvent" for integral proteins. The fluidity of this matrix depends on the chemical nature of the phospholipids, i.e., the polar head groups, and in large part, the fatty acid chains. The fatty acyl chains in bilayer membranes can exist in an ordered rigid state or in a relatively disordered fluid state. The transition from the rigid to the fluid state occurs as the temperature is raised above a characteristic 'melting' temperature (T_t). This temperature depends on the length of the fatty acyl chains and on their degree of unsaturation. The rigid state is favored by the presence of saturated fatty acyl residues

which, because of their straight hydrocarbon chains, can interact very favorably with each other. On the other hand, a cis double bond produces a bend in the hydrocarbon chain, which interferes with the optimal packing of fatty acyl chains. Furthermore, a cis double bond enhances rotation about the carbon-carbon single bonds on either side. An unsaturated fatty acid chain is therefore, intrinsically more flexible than a saturated one. This effect is enhanced by the presence of more than one double bond.

The length of the fatty acid chain also affects membrane fluidity. Long hydrocarbon chains interact more strongly than do short ones. Specifically, each additional methylene group makes a favorable contribution of about -0.5 Kcal/mol to the free energy of interaction of two adjacent hydrocarbon chains (305).

In living cells, the T_t of membrane lipids is usually lower than the physiological temperatures (234). Below the T_t the phospholipids are in a gel-like ordered structure with the hydrocarbon chains lying parallel, closely packed and fully extended. When the temperature is increased in the region of T_t, hindered rotation is possible around the C-C bonds giving rise to transgauche rotational isomers or kinks (275). Highly mobile kinks are formed at the order-to-fluid transition of the lipid phase observed at T_t. This endothermic process, also called "chain-melting", is

accompanied by shortening of the chains producing a reduction of bilayer thickness and a lateral expansion of the bilayer. In a heterogeneous system such as a real biological membrane, the transition from the rigid to the fluid state is spread over a range of temperatures (234). Within the temperature range, there is a coexistence of rigid and fluid regions (283) accompanied by a lateral compressibility of the phospholipid bilayer.

Factors other than changes of temperature can also produce structural changes. Since the density of packing of hydrocarbon chains varies with the relative arrangement of polar groups, factors which modify polar group interactions could lead to changes in chain packing, and hence in the transition temperature. It has indeed been reported (129, 324, 334) that changes in pH, ionic strength, concentration of divalent cations, or interactions with basic proteins able to bind the negative charges of the head groups, can alter T_t.

That changes in the activity of membrane-bound enzymes can be associated with thermal transitions is well documented in the literature. Breaks in the Arrhenius plot of ATPase activity of S. cerevisiae, for example, were observed at the temperature producing a phase transition in the extracted mitochondrial phospholipids (198). The activity of the succinate oxidase of mitochondria from the livers of rat, rabbit and guinea pig showed a discontinuity in the Arrhenius

plot with an increase in E_a at 23°C (253). In most cases, the temperature at which the change in slope occurs is determined by the fatty acid composition of the biomembrane (299) and the break in kinetics occurs within the temperature range circumscribed by the bulk phase transition defined by calorimetry (299).

Since phospholipids account for over three quarters of the total lipid content of mitochondria and are responsible in large part for the internal mobility of these membranes, these lipids are assigned an important regulatory role for the activity of membrane-bound enzymes (262). Membrane-bound enzymes can be viewed as structural elements in an array of these molecules which make up the fluid mosaic membrane. In some cases, phospholipids can bring about conformational changes in protein which lead to increased enzyme activity as shown in β-hydroxybutyrate dehydrogenase (93).

Quite often membrane-bound enzymes require specific phospholipids for maximal activity (260, 261, 272). In fact, at times, the interaction between the protein and phospholipid may require a specific fatty acid chain or a particular polar head group (167, 263, 272). The sugar specific phospho-transferases of E. coli membranes, for example, exhibit an absolute requirement for phosphatidyl glycerol (181), while other E. coli phospholipids have little or no activating potential. Guinea-pig kidney Na^+,K^+-ATPase is stimulated by

the addition of sonicated anionic liposomes of single purified phospholipids, e.g., phosphatidylserine, phosphatidylinositol, and cardiolipin. Mitochondrial D(-)β-hydroxybutyric acid dehydrogenase has an absolute specificity for lecithin and is not activated by other phospholipids (167).

The degree of unsaturation of the fatty acyl moiety in mitochondrial phospholipids has also been reported to affect the degree of activation of phospholipid-dependent membrane-associated enzymes. For instance, optimal activation of mitochondrial D(-)β-hydroxybutyric acid dehydrogenase occurs with an acyl unsaturated lecithin, whereas only partial activation is achieved with totally saturated lecithins such as dimyristoyllecithin (167). In the case of the E. coli membrane enzyme which transfers galactose from UDP-galactose to galactose-deficient lipopolysaccharide, synthetic dioleyl phosphatidylethanolamine is a potent activator, whereas dipalmitoyl phosphatidylethanolamine is without effect.

Although lipids have been implicated in the activity and specificity of mitochondrial MAO, the contribution of membrane lipids in the modulation of mitochondrial MAO activity is still a matter of debate and speculation. Tipton (310) observed that prolonged dialysis of a partially purified rat liver MAO preparation against Triton X-100, as well as treatment of the enzyme with phospholipase A, resulted in loss of activity. Naoi and Yagi (224) have reported that after subjecting beef heart mitochondrial MAO to lipid-

depletion by treatment with sodium perchlorate, enzymatic activity was reduced, the decrease in benzylamine-oxidizing activity being more remarkable than that towards serotonin. The activity was partly restored by incorporation of the enzyme into liposomes composed of phosphatidylcholine. As cited earlier, Ekstedt and Oreland (80), extracting rat liver mitochondria with aqueous methyl ethyl ketone, found that about 80% of the β-phenylethylamine oxidizing activity was retained by the ketone-extracted mitochondria whereas only 7% of the serotonin-specific activity was recovered after extraction. In further experiments by these authors in which mitochondria were labelled with the irreversible MAO inhibitor (^{14}C)-pargyline and then extracted with aqueous methyl ethyl ketone, the major part of the radioactivity was recovered in the lipid-depleted residue and less than 10 percent was found in the ketone extract. This excluded the possibility that the decrease in activity previously observed was due to extraction of the enzyme into the solvent, and suggested that the enzyme is dependent on membrane lipids for its activity.

It has been shown in various systems (17, 19) that the fatty acid composition of biological membranes can be altered by changing dietary lipid. In this respect, Century and Horwitt (43) reported that liver MAO activity was highest in rats fed 7% cod liver oil or linseed oil, in comparison with rats fed beef fat. Kandaswami and D'Iorio (169) reported a decrease in activity of liver MAO in rats fed fat-free diets.

Bersohn and Spitz (30) have also reported a 57% decrease in brain MAO activity in lipid-deprived rats. These two groups claim a recovery of activity on supplementation of the diet with essential fatty acids.

Aithal et al. (1), however, contend that MAO is not directly dependent on membrane lipids for the manifestation of its full activity. These authors claim to have detected MAO activity in rat liver outer mitochondrial membrane only after the preparation had been treated with Triton X-100. This preparation was found not to undergo diphasic Arrhenius kinetics, a rationale used by Raison (253) to classify MAO as a peripheral protein. It is pertinent, perhaps, to recall at this point, that detergents like Triton X-100 disrupt membrane lipid-protein interaction (330), and may have been the cause for the failure to detect discontinuities in the Arrhenius plot of detergent-treated liver MAO.

From the above, it appears that the role of lipids in the regulation of MAO activity invites further investigation. In this context, the ob/ob mouse, with its reported membrane-related defects (37, 95, 153, 168) seems to be an excellent model for the study.

I.C. The obese-hyperglycemic mouse:

The evidence that the multiple forms of the enzyme monoamine oxidase may result from differences in membrane-lipid binding (cf. section I.A. 5. b.) suggests that its

activity in vivo may be affected by drugs and diseases that affect lipid metabolism. In this respect, certain features of the obese-hyperglycemic syndrome make the ob/ob mouse an interesting model for MAO studies.

I.C. 1. Genetic development:

In 1949, a few animals in the V strain at Jackson Laboratory in Bar Harbor, Maine were observed to be plump early in life and to become markedly obese (up to 90 g) late in life. Breeding experiments revealed that the obesity syndrome was caused by a single autosomal recessive gene, obese (gene symbol ob) (155). The mutation has been transferred into the C57BL/6J and C57BL/KsJ strains for studies involving the effect of inbred background. The mice used for the experiments described in this thesis are of the C57BL/6J strain which is commercially available from the Jackson Laboratory. In contrast, to heterozygous obese (ob/+) or homozygous lean (+/+) mice, all homozygous obese mice develop moderate hyperglycemia, marked hyperinsulinemia, and obesity coupled with hypertrophy and hyperplasia of the islets of Langerhans.

I.C. 2. Time course for the manifestiation of the obese-hyperglycemic syndrome:

The earliest demonstrable defect in these mice is a decrease in thermogenesis. A fall in core temperature and a

reduction in oxygen consumption are apparent as early as 10-18 days of age (33, 99, 170, 326). This impaired thermogenesis may account for the increased fat cell size observed from day 14 to 20 (162, 171) and the observed increase in body weight. Shortly after, (17-21 days), there is a small increase in serum insulin with a consequent hypoglycemia (181). While insulin levels rapidly increase, there is a gradual transition from hypoglycemia to hyperglycemia. Obesity, as indicated by carcass fat or Lee index (28, 76, 162, 191) is detectable by 17-21 days, but the animals are not visually detectable as 'obese' until 25-28 days or even later. Obesity progresses until about 6 months of age at which time weight gain slows or stops. Hyperglycemia reaches a peak level by 12 weeks of age (213), while elevated levels of circulating insulin reach a maximum by 6 months (341) and decline in very old animals (105). Marked resistance to exogenous insulin has been observed in these animals (213) and develops after hyperinsulinemia becomes evident (38).

I.C. 3. Energy balance:

The accumulation of fat implies that energy intake exceeds expenditure. The regulation of food intake in the adult ob/ob mouse is abnormal, showing incomplete compensation of intake in response to dilution of the diet with indigestible substances (244) and to quinine adulteration (100). A number of investigators (49, 102, 191, 244) have reported an increase

in food intake by ob/ob mice which appears to be accompanied by an increased efficiency of energy utilization (36, 191). Such an increase in efficiency may be related to either an increase in food energy intake or to a decrease in energy expenditure, i.e., defective thermogenesis, reduced physical activity or decreased heat loss. The hyperphagia alone cannot be entirely responsible for the excess energy deposition in ob/ob mice since caloric restriction, exercise or the maintenance of a normal body weight through a combination of these two treatments will prevent adiposity (48, 138).

All reports agree that there is a reduction in physical activity of ob/ob mice both during the dynamic postweaning phase of the obesity and during the more static adult phase (53, 164, 354). Energy utilization, measured by oxygen consumption and expressed on a surface-area basis is decreased in adult ob/ob mice (213, 215). Body temperature of obese mice is likewise depressed at ambient temperatures of 20-25°C (163, 326). As stated earlier, a decrease in body temperature can already be detected at 10-14 days of age, particularly after brief exposure to a cold environment (309, 326), and depressed oxygen consumption is demonstrable a few days later. The inability to thermoregulate results from the lack of cold-induced thermogenesis and not from the lack of any physical response (64). A reduction in energy expenditure on thermoregulatory processes would lead to an increase in efficiency of food utilization (38). Hypoactivity, together

with hyperphagia, and a more efficient utilization of ingested food results in the storage of excess energy as fat.

I.C. 4. Lipid metabolism:

Enhanced fatty acid synthesis in the liver and adipose tissue of ob/ob mice has been demonstrated in both *in vivo* and *in vitro* experiments with a number of different isotopes (7, 8, 9, 82, 131, 194). This phenomenon has been related to the increased activity of all lipogenic enzymes in the adipose tissue and liver (7, 36, 99, 206, 268).

Assimacoupoulos-Jeannet *et al.* (9) have reported that in male ob/ob mice, 8 to 11 weeks of age, basal lipogenesis was much higher in livers of ob/ob than in those of control mice. The addition of various substrates resulted in increases in lipogenic rates that were always greater in ob/ob mice (9).

The increase in hepatic lipid content in ob/ob mice results not only from the increase in hepatic fatty acid synthesis and consequent esterification, but also from the increased availability of circulating free fatty acids (9, 82, 268). This preferential reesterification of hepatic free fatty acid in the ob/ob mice results in an enhanced secretion of lipoproteins into the circulation and a reduced capacity for the production of ketone bodies (82, 300). Despite this increased secretion of triacylglycerol, plasma triacylglycerol levels are not markedly increased in ob/ob mice (268) since there is a compensatory increase in lipo-

protein lipase activity in adipose tissue, heart, and skeletal muscles (68, 83, 254). As a result, triacylglycerol biosynthesis in adipose tissue of ob/ob mice is markedly elevated to store this increased supply of fatty acid (156).

Treatment of obese mice with either streptozotocin or anti-insulin serum suppresses both hepatic and adipose tissue lipogenesis to close to normal values (193, 194) and increased ketone body production to values found in lean mice (159). Thus, these studies suggest that the increased lipogenesis in liver and adipose tissue of obese mice is a response to the chronic insulin stimulation to which these tissues are subjected.

As mentioned above, in obese mice hepatic oxidation of free fatty acids to ketone bodies is diminished when compared with the controls (9, 300). However, obese mice adapt to prolonged fasting by shifting from glucose oxidation to free fatty acid and ketone body oxidation. Still, even in fasted animals, lipogenesis is accelerated in the liver and adipose tissue of obese mice when compared with the controls (18, 20, 212).

I.C. 5. Pathogenesis of the obese-hyperglycemic syndrome:

The manifestation of the obese-hyperglycemic syndrome in the ob/ob mouse involves several possible causative factors.

I.C. 5a. Role of the pancreas:

The role of a pancreatic abnormality in obese mice was

first recognized in morphological studies. The obese pancreas is characterized by marked hypertrophy and hyperplasia of the islets of Langerhans (106). Hyperplasia of both α and β cells have been described in the pancreas of obese mice, although the greatest increase occurs in the number of β cells. The histological changes in the β cell show a great reduction or complete absence of granules (348), an enlarged Golgi apparatus, large and misshapen mitochondria, and an enlarged endoplasmic reticulum, all of which suggest enhanced synthetic and secretory activity.

As expected on the basis of morphological findings in the pancreas, insulin secretion in obese mice differs from that of the lean controls. In obese mice, plasma insulin-like activity and plasma immunoreactive insulin are increased compared with the controls, both in the fed and fasted states (51, 297). Plasma insulin concentrations in obese mice markedly decrease with fasting but do not return to normal values even with prolonged starvation (51, 297). Plasma IRI, like blood glucose concentrations, also changes with the age of the mice. Plasma IRI concentrations in 7-month-old obese mice are four times those seen in 5 to 6 week old obese mice, whereas plasma IRI levels in lean controls remain constant with age (105, 203). The high plasma insulin levels in obese mice might suggest decreased insulin degradation. However, there were no differences in insulin degradation rates <u>in vitro</u> in liver samples from obese and lean mice; and in

epididymal adipose tissue homogenates, the insulin-degrading activity in obese mice is 8-fold higher than that observed in lean animals (340, 342).

Numerous investigations have been done on insulin secretion in vitro using isolated pancreata or islet preparations. In 5 to 6 week-old mice, insulin output in response to glucose or glucose and theophylline is twice that seen in the controls (202). Insulin secretion, however, is not only controlled by circulating metabolites such as glucose or amino acids, but is also affected by adrenal corticoids, growth hormone, and the hypothalamus (36). It may therefore, be argued that the hypersecretion of insulin in obese animals does not necessarily reflect a primary genetic defect in the pancreas. A strong case can be made, however, for a major role for the pancreas in the development of the obese-hyperglycemic syndrome in the ob/ob mouse. The necessity of increased insulin secretion for weight gain in ob/ob mice has been demonstrated by Brosky and Logothetopoulos (39). The pancreas of the ob/ob mouse responds in a unique manner to certain agents known to affect islet function. Mannoheptulose, an inhibitor of insulin secretion, appears ineffective in suppressing insulin release (142). Caffeine, an insulin secretagogue, produces increased β-cell degranulation in ob/ob but not in lean mice (180). The altered islet composition is not remedied by food restriction (213). Data for the role of the pancreas in the development of the ob/ob

syndrome has been presented by Strautz (303, 304), who showed that transplantation of either pancreas or isolated islets from lean into obese mice prevented further weight gain and reduced plasma insulin levels to normal, whereas pancreas from ob/ob mice transplanted into lean mice did not affect body weight. These observations would appear to suggest that the pancreas of normal mice releases a factor which inhibits either directly or indirectly, insulin secretion, and that this factor is absent in obese mice. These data still await confirmation from other laboratories.

I.C. 5b. Insulin resistance:

In the obese mouse, hyperinsulinemia is accompanied by hyperglycemia, with blood glucose levels rising above 400 mg/dl in animals fed ad libitum (341). Both hyperinsulinemia and hyperglycemia, which are first evident within 4 to 5 weeks of birth, are maintained throughout life, but may decrease in very old animals (132).

The high blood glucose concentration could be due to elevated glucagon secretion (211) and peripheral insulin resistance. The lack of response of the gluconeogenic and glycogenic pathway to insulin both in vivo (179) and in vitro (276) suggests that decreased insulin sensitivity is a characteristic of the obese mouse.

Insulin resistance has been further demonstrated from studies of the effect of exogenous insulin, from glucose

tolerance tests, and from investigation of insulin sensitivity of muscle and adipose tissue. Stauffacher and his co-workers (298) observed that basal incorporation of glucose into muscle glycogen and lipids of adipose tissue was low in lean animals, but maximally stimulated by small doses of insulin (300 μU/ml). In contrast, although basal incorporation of glucose into ob/ob muscle and adipose tissue was normal, both tissues were resistant to the stimulatory effects of insulin, the diaphragm showing greater resistance than adipose tissue. These authors suggested that the preferential resistance of muscle to the action of insulin would lead to an increased conversion of glucose to lipids by adipose tissue, resulting in obesity.

Some authors (9, 158) observed diminished lipogenesis in perfused livers of streptozotocin-treated obese mice, and have concluded that lipid metabolism of the liver is markedly influenced by insulin. This was confirmed by studies in vivo on lipogenesis in mice treated with anti-insulin serum (194). This conclusion also provides the explanation for the distinctly diminished lipogenesis in mice during the static phase of obesity (156) when insulin levels spontaneously decline (cf. section I.C. 2).

I.C. 5c. Role of adrenals

The adrenals of ob/ob mice are hypertrophied (130), the hypertrophy being absent in young animals and increasing with age. The increased adrenal weight results from a distinct

hyperplasia of the cortex in the obese mouse. The adrenal hyperactivity of these animals presumably results from an excess ACTH secretion (115). This may also reflect an increased adrenal sensitivity to ACTH (41).

Adrenalectomy to 2-month old obese mice lowers blood glucose and restores insulin sensitivity and is followed by a weight gain smaller than that of non-operated mice (292).

I.C. 5. d. The brain and hypothalamus:

There is considerable data available to suggest that the genetic defect of obese mice may be in the hypothalamus. The accumulation of the adipose tissue mass in obese mice is due, at least in part, to hyperphagia (210). Investigations on hyperphagia in obese mice suggest defects in satiety mechanisms. Their response to dietary manipulation are similar to those documented for rats (301) and mice (5) with hypothalamic injury. Whereas normal mice can compensate for the dilution to their food with cellulose, so that they continue to gain weight at a normal rate, obese mice do not increase their intake sufficiently and their body weight levels off (244). The association of obesity with hyperphagia and hypoactivity also suggests that the genetic defect in the obese mouse is in the hypothalamus.

A recent report on anatomic studies measuring neuronal size and brain weight has indicated a generalized reduction in most areas of the brain of ob/ob mice (27). The brain

weight in male ob/ob mice was 14% less than in lean age-matched controls. The cross-sectional area of neurons in the ventromedial nucleus, cingulate cortex, medial amygdaloid nucleus, ventrobasal nucleus of the thalamus, and dorsomotor nucleus of the vagus were significantly smaller in tissues from the ob/ob mice (27). Only the lateral hypothalamus neuronal area was the same. The generalized reduction in volume of neurons may well be the anatomic basis for the hypothalamic defects in this strain of mice (38).

Lorden et al. (192) have reported increased concentrations of noradrenaline in the hypothalamus of 2 and 5-month-old obese mice of both sexes. Using older animals, Nemeroff et al. (229) could detect no difference in the hypothalamic level of noradrenaline. By 8 months of age, however, the ob/ob mouse is no longer rapidly gaining weight, suggesting that the findings in the younger animals may be more relevant to the development of the obese-hyperglycemic syndrome.

I.C. 6. Defective membrane-related processes:

A number of membrane-related processes in the ob/ob mouse have been reported to be defective. For instance, decreased binding of insulin in ob/ob mice has been demonstrated in liver plasma membranes and isolated hepatocytes (168, 291), adipocytes and adipocyte plasma membrane (98, 235), isolated kidney cells (45), cardiac muscle (95), soleus muscle (185), and lymphoid tissue (290). The

diminished binding has been attributed to the loss of receptor sites rather than to any change in the affinity of receptors (167, 291), and may reflect a more generalized change in membrane structure (45). Recent data have also suggested that the regulation of Na^+, K^+-ATPase in a number of tissues of ob/ob mice is defective. The activity of this enzyme is low in liver, kidney, and muscle of ob/ob mice (37, 355, 190).

The response of ob/ob mouse adipose tissue adenylate cyclase to β-agonists such as isoproterenol has been shown to be severely impaired by a number of investigators (67, 280). One of these groups, Hyslop and York (153), have reported that the microviscosity of adipocyte plasma membranes from ob/ob mice was significantly reduced at physiological temperatures, and that after housing obese mice at 34°C for 7 days to normalize gross membrane fluidity, the adipose tissue adenylate cyclase showed dose-response to isoproterenol that was comparable to the response of lean mice.

The defect in the regulation of adenylate cyclase in the adipose tissue of ob/ob mice has been attributed to a defect in the interaction between the hormone receptor and the catalytic subunit of the enzyme (69, 280). Some investigators have also claimed a reduction in the number of β-receptors in ob/ob mouse adipocytes (182). There has been some evidence that the number of insulin receptors and β-receptors is inversely proportional to membrane fluidity

(3, 12). In other reports, it has been shown that although basal adenylate cyclase activity is insensitive to changes in membrane fluidity, the activity is affected when the enzyme is coupled to the glucagon or the β-receptor (12, 75). Phospholipids are believed to have an important role in this coupling mechanism (60).

I.C.7. Thermoregulation:

The cold-activated adaptive mechanisms in mammals consist of responses which decrease heat loss or increase heat production. The former group includes piloerection and cutaneous vasoconstriction, while the latter includes increased food intake, shivering and nonshivering thermogenesis. Lean mice are able to maintain their body temperature on exposure to a cold environment by a combination of physical (e.g. piloerection and shivering) and metabolic (e.g. non-shivering thermogenesis) responses (64). On the other hand, exposure of obese mice to cold (3°C) produces a rapid decrease in body temperature and death with a mean survival time of only 2.2 hours (64).

The reason for the lethal effect of cold was originally reported to be due to the absence of any metabolic changes, and not to an impaired physical response. Davis and Mayer (64) observed that oxygen consumption which rose in lean mice exposed to cold, failed to increase in ob/ob mice at 3°C, although piloerection and shivering were evident.

More recently, Trayhurn and James (325) have reported that obese mice previously adapted to 12°C for 19 days can survive longer upon exposure to 4°C. These authors studied the metabolic rates of lean and obese mice during exposure to various environmental temperatures for forty minutes, and observed that below 30°C the oxygen consumption of the lean mouse was greater than that of the obese mouse, and that between 25 and 10°C, the metabolic rate of obese mice was only 80 to 83% of that of the lean animals. The maximum oxygen consumption in the obese occurred at 10°C, but a further increase was observed for the lean when the environmental temperature was reduced to 0°C. Trayhurn and James also determined the capacity of lean and obese animals for nonshivering thermogenesis by investigating their response to noradrenaline at 31°C. The increase in the resting metabolic rate after noradrenaline administration in the lean animals was 130%. The increase observed in the obese animals, on the other hand, was only 72% of their resting metabolic rate. The injection of noradrenaline at 10°C did not produce an increase in metabolic rate in either group of mice. It appears then, that at low environmental temperature, the obese mouse, like its lean counterpart utilizes fully its capacity for nonshivering thermogenesis, but that this capacity for heat production is less than that of the lean mouse (325).

This impaired capacity for heat production was made

evident in a study made by Trayhurn and James (325) on the effect of low environmental temperatures on the rectal temperature of lean and obese mice. These investigators observed that after 2.5 hours at 4°C, the mean temperature of the obese mice had fallen to below 20°C and after an average of 3.75 hours, the obese animals died of hypothermia with a deep body temperature of between 10 and 12°C.

A reduced body temperature generally leads to a change in the nature of tissue lipids (96, 127). It was of interest, therefore, to determine how cold exposure would affect the fatty acid composition of obese mouse liver mitochondrial phospholipids, and to what extent these changes would affect liver mitochondrial MAO activity.

I.D. Oxytetracycline:

Previous studies by Bégin-Heick et al. (22, 23, 24) have shown that administration of oxytetracycline (OTC) to the obese mouse tended to correct many of its abnormalities. Oxytetracycline is an antibiotic elaborated by the actinomycete, Streptomyces rimosus. Introduced in 1950, it belongs to a group of broad-spectrum antibiotics called tetracyclines. These drugs possess activity against gram-positive and gram-negative bacteria which overlaps that of penicillin, streptomycin, and chloramphenicol (338). They are derivatives of naphthacene carboxamide, and are very much alike in their chemical, antimicrobial, pharmacological, and

therapeutic properties, which permits their discussion as a class (338).

Oxytetracycline has been reported to have varied metabolic effects. It is known to inhibit mitochondrial protein synthesis in rapidly growing tissue (73, 136). It has also been shown to influence carbohydrate metabolism in both humans (68) and experimental animals (70). Several authors have observed that in animals (134) or humans (134, 218) that have received either tolbutamide or exogenous insulin, OTC potentiated the action of insulin. In particular, OTC-treatment of the obese mouse (22, 23, 25) resulted in some weight loss, decreased levels of plasma glucose and insulin, normalized glucose and insulin tolerance, increased binding of insulin by the liver, increased insulin sensitivity in the diaphragm, and normalization of *in vivo* insulin secretion by the pancreas.

Of special interest to us were its reported effects on lipid metabolism. An earlier study by Bégin-Heick *et al.* (20) showed a tremendous reduction in the weight and lipid content of the liver in OTC-treated obese mice. Subsequent studies by the same group (21) showed that OTC-treatment decreased the *in vivo* incorporation of ^{14}C-glucose and $^{3}H_2O$ into total liver lipids of obese mice, whereas it did not appreciably affect the incorporation in lean animals. It was also observed by these investigators that the bulk of the $^{3}H_2O$ was in the fatty acid fraction of liver lipids. It

should be noted that the incorporation of ^3H_2O into fatty acids *in vivo* occurs via the reduced pyridine nucleotides, and any changes in its concentration would reflect changes in the *de novo* synthesis of fatty acids.

In addition, a number of the observed effects of OTC appear to be related to membrane function: treatment with OTC has been shown to increase the binding of insulin to liver membranes in the ob/ob mouse (20), to alter the secretion kinetics of the exocrine pancreas (23), and to increase the activity of lipoprotein lipase in rat tissues (22). Alterations in phospholipid metabolism have also been reported after treatment of rats with TC (199). Oxytetracycline was therefore chosen as a potential agent to alter lipid metabolism in the obese mouse liver, and thereby possibly alter the lipid environment of MAO in the mitochondrial membrane.

II. STATEMENT OF THE RESEARCH PROBLEM

Monoamine oxidase is believed to be associated with the outer mitochondrial membrane in the liver and other tissues, and despite a few reports to the contrary, is generally regarded as an integral membrane enzyme. The association between lipids and protein appears to be essential for the efficient functioning of such membrane-bound enzymes. Although lipids have been implicated in the activity, stability and specificity of mitochondrial MAO, much remains to be understood regarding the contribution of membrane lipids in modulating MAO activity.

The work of previous investigators in this field suggests that enzyme activity in vivo might be affected by disorders that alter lipid metabolism. In this respect, the obese-hyperglycemic mouse would present an interesting model in which to study the interaction between MAO and membrane lipids. The ob/ob mouse has been reported to have a wide range of membrane-related defects, and has been widely used as a model for obesity and maturity-onset diabetes.

In particular, the liver of the obese mouse would be an excellent system for studying the effect of lipid environment on the activity of MAO. Abnormalities in the lipid metabolism of this tissue have been reported by a number of authors. In the present investigation, various properties of mitochondrial MAO in ob/ob mouse liver were studied in parallel with those

of preparations from its normal-weight counterpart.

It has been claimed that the observed multiplicity of MAO forms is due to different amounts of bound membrane constituents, and that these forms are affected differently by the lipid environment in the membrane (cf. Review of Literature, Section I.A.5. b.). It is also generally held that the multiple forms of the enzyme are distinguishable by their relative activities towards selected substrates and by their response to specific inhibitors. The present study was therefore carried out using different substrates and inhibitors.

In order to establish if any differences between lean and obese mouse enzyme are part of a generalized effect on mitochondrial enzymes, activities of other mitochondrial marker enzymes were determined in parallel with monoamine oxidase.

An investigation of the kinetic parameters of mitochondrial MAO in lean and obese animals was carried out, as it has been reported that the reactive mechanism of a soluble rat liver MAO preparation differed from that of the membrane-bound enzyme in certain formal details (148). The results of these authors implied that the configuration of the protein changed on solubilization, and that, therefore, the membrane might impose a physical constraint on the tertiary structure of the protein; or, that a specific lipid moiety may achieve control of the protein's tertiary structure.

The effect of lipid depletion by aqueous acetone on

enzyme activity was examined. An analysis of the phospholipid species profile of liver mitochondria from the two groups of mice was undertaken, since phospholipids have often been implicated in studies on the lipid dependency of monoamine oxidase, and certain phospholipids have been reported to be more effective than others in reactivating the enzyme in lipid-depleted mitochondrial preparations (cf. Review of Literature, Section I.B.).

As discussed in Section I.B., the properties of the fatty acyl moiety of membrane phospholipids appear to be an important determinant of membrane fluidity. In this respect, it has been demonstrated that the obese-mouse liver has a high capacity to synthesize long and unsaturated fatty acids from labelled precursors (347). It was of interest, therefore, to determine the fatty acid composition of mitochondrial phospholipids of lean- and obese-mouse liver.

Two procedures were used in an attempt to manipulate the fatty acid composition of mitochondrial phospholipids. These involved treatment of the intact animal with the antibiotic, oxytetracycline (100 mg/kg I.M. daily for 10 days) and chronic cold exposure (14 ± 1°C). Oxytetracycline has been reported to alter lipid metabolism in obese-mouse liver (cf. Review of Literature, Section I.D.), and a related antibiotic, chloramphenicol, has been shown to inhibit the acylation of phospholipid precursors in BHK-21 cells (189);

whereas, chronic exposure to 12 ± 1°C has been reported to reduce the core temperature of ob/ob mice to 31°C. It was hoped that such a decrease in body temperature would result in a change in the fatty acid composition of liver mitochondrial phospholipids. Such a response to a decrease in temperature has been demonstrated in membrane phospholipids of other experimental models (96, 128).

It was the purpose of this project to compare the enzyme from lean and obese mice; to determine if the altered lipid metabolism in obese-mouse liver would give rise to significant differences in liver mitochondrial composition; and to relate any differences between lean- and obese-mouse MAO to any alteration in membrane constituents.

CHAPTER 2: MATERIALS AND METHODS

I. Chemicals:

The following chemicals were obtained from J.T. Baker Chemical Co.:

Potassium chloride, sodium chloride, sodium hydroxide, sodium phosphate (dibasic), sucrose, and Tris (Buffer).

The following reagents were purchased from Fisher Scientific Co.:

Acetic acid, acetone, ammonium hydroxide, ammonium molybdate, benzene, chloroform, hydrochloric acid, iodine, magnesium nitrate, methanol, petroleum ether, phenol reagent (Folin - Ciocalteau Reagent), potassium phosphate (dibasic), potassium phosphate (monobasic), and sodium carbonate.

Sigma Chemical Co. supplied the following: arachidonic acid, L-ascorbic acid (sodium salt), benzylamine, bovine serum albumin (fraction V), butylated hydroxytoluene (2,6-di-ter-butyl p-cresol), cytochrome c, dithiothreitol, EDTA (disodium salt), kynurenine sulfate, D-mannitol, and NADPH.

Silica Gel H (no binder) was obtained from Analabs, Inc., silicic acid (Bio-Sil A, 100 - 200 mesh) from Bio-Rad Laboratories, Amberlite CG 50(H) (100 - 200 mesh) from the British Drug House, Ltd., tyramine hydrochloride from Calbiochem, cupric sulfate and sodium potassium tartrate from Canlab, 5-hydroxytryptamine binoxalate from ICN Pharmaceuticals, Aquasol-2 (liquid scintillation counting

cocktail) from New England Nuclear Corp.; oxytetracycline (Terramycin) from Pfizer; Ficoll 400 from Pharmacia Fine Chemicals; fatty acid standards (D-104), phospholipid standards, and di-arachidonyl phosphatidylcholine from Serdary Research Laboratories; and fatty acid standards (PUFA #1) and 10% SP-2550 on 100/200 Chromosorb WAW from Supelco, Inc.

Hydrogen, nitrogen, compressed air, and hydrogen chloride were supplied by Liquid Carbonic of Canada.

[^{14}C] Benzylamine (methylene-^{14}C-benzylamine hydrochloride) was purchased from ICN Isotope and Nuclear Division; [^{14}C] hydroxytryptamine (hydroxy-2-^{14}C-tryptamine binoxalate) from New England Corporation; and [^{14}C]tyramine (p-hydroxyphenyl-2-^{14}C-ethylamine hydrochloride) was from Amersham Corporation.

II. Animals:

Male mice, strain C57BL/6J, genotype ob/ob, and their nonobese (lean) controls, genotype +/?, were obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A. These were used in experiments at 9 to 12 weeks of age.

The animals were housed four to a cage, and the cages were placed in a room with artificial lighting for 12 hours a day (from 0600 to 1800 h). Temperature in this room was maintained at 24 ± 1°C. Both obese and lean animals were given Purina Chow and water ad libitum unless otherwise specified.

Sprague-Dawley rats used in some of the experiments were obtained from Bio-Breeding, Ottawa, Canada.

III. Treatments:

A. Oxytetracycline treatment:

Lean mice were randomly divided into two groups: the oxytetracyline-treated animals and the non-treated group. Obese mice were likewise randomly divided into three groups: one oxytetracycline-treated group and two control groups, the non-treated mice and the food-restricted group. The food-restricted mice had free access to water, but were allowed to consume only 4.5 g of Purina Chow/day, the amount which was observed by previous investigators (23) to be the average daily consumption of OTC-treated mice.

All mice were housed in individual cages and were placed in a room maintained at 24 ± 1°C, and with artificial light from 0600 to 1800 h.

For a period of 10 days, the OTC-treated mice received daily intramuscular injections of oxytetracycline suspended in olive oil. The dose was 100 mg/kg in 0.2 ml, calculated on the basis of the average weight of lean mice. The non-treated control groups received placebo injections. All injections were given between 16:00 and 16:30 h daily, and the weighed food was placed in the appropriate cages at about the same time. On the 11th day, the animals were sacrificed by decapitation between 09:00 and 09:30 h.

III. B. Cold exposure:

Obese mice were divided into groups: one group was placed for two weeks in a cold room maintained at 14 ± 1°C and will be referred to as cold-exposed mice (C), while the second group was maintained at 24 ± 1°C for two weeks and served as controls (W). The same procedure was followed with lean mice.

Mice were housed in separate cages and had unlimited access to Purina Chow and water. To monitor the amount of food consumed, pellets were weighed daily at 09:30 h throughout the two-week period. Shortly before sacrifice, animals were transported to the laboratory from their temperature-controlled rooms, and weighed. Animals were sacrificed between 09:00 and 09:30 h on the 15th day.

IV. Tissue preparation and preparation of homogenates:

Immediately after animals were sacrificed, organs (brains, hearts, white adipose tissue, pancreas, and/or livers) were removed, rinsed in ice-cold isolation medium (0.25 M sucrose containing 10 mM potassium phosphate, pH 7.5), blotted dry, weighed, and minced. All subsequent procedures were carried out at 0 - 4°C. The minced tissue was suspended in 9 volumes of isolation medium and homogenized with 4 strokes of a Potter-Elvejehm homogenizer attached to a Tri-R Stirrer motor set at 600 rpm. The homogenates were centrifuged for 10 minutes at 600 x g in the SS - 34 rotor of the Sorvall RC-2B centrifuge. The pellets were discarded, and the supernatant was either

assayed directly for monoamine oxidase activity, or used for the preparation of subcellular fractions.

V. Preparation of subcellular fractions:

A. Mitochondrial fractions:

The 600 x g supernatant obtained above was centrifuged for 20 minutes at 12000 x g (brain and liver), 17000 x g (heart and pancreas), or 27000 x g (white adipose tissue). The pellet obtained was resuspended in the isolation medium by manually homogenizing it with a Potter-Elvejehm homogenizer, and re-centrifuged as above. This washing procedure was repeated two additional times. The resulting pellets were either purified on discontinuous Ficoll gradients as described in Section VI, or resuspended in isolation medium and homogenized in a Potter-Elvejehm homogenizer to give a final concentration of 4 mg protein/ml. This suspension was assayed for enzyme activity.

B. Microsomal fractions:

Microsomal fractions were prepared by centrifugation of the post-mitochondrial supernatant at 100000 x g for 60 minutes in the #30 rotor of a Spinco Model L ultracentrifuge. The resulting pellet was resuspended in isolation medium, manually homogenized in a Potter-Elvejehm homogenizer, and assayed for enzyme activity.

C. Cytosol:

The post-microsomal supernatant was assayed for enzyme activity as the soluble fraction of the cytoplasm.

VI. Purification of mitochondrial fractions:

This method of purification was adapted from the procedure developed by Clark and Nicklas (52).

The crude mitochondrial pellet from about 7.5 g of liver tissue prepared as described in Section V.A. was resuspended in 3% Ficoll medium (3% Ficoll - 0.12 M mannitol - 0.03 M sucrose - 25 µM EDTA, pH 7.4) to a final volume of 10 ml per 7.5 g of tissue. This suspension was carefully layered onto 20 ml of a 6% Ficoll medium (6% Ficoll - 0.24 M mannitol - 0.06 M sucrose - 50 µM EDTA, pH 7.4) and centrifuged for 30 minutes at 12000 x g in the SS - 34 rotor of the Sorvall RC - 2B centrifuge. The supernatant was decanted and the slight fluffy layer removed from the pellet. The mitochondrial pellet was resuspended in isolation medium (40 ml) and recentrifuged for 10 minutes at 12500 x g. The washed mitochondrial pellet was either used for lipid extraction, or resuspended in 10 ml of isolation medium and used for assay of monoamine oxidase activity.

VII. Preparation of lipid-depleted mitochondrial fractions:

The lipid-depletion of mitochondria was carried out as described by Fleischer and Fleischer (94). This method was

developed for the removal of phospholipid from mitochondria in order to determine the contribution of such lipid to the structure and function of enzymes in beef heart mitochondria.

The method was used on mitochondrial preparations previously purified on Ficoll gradients as described in Section VI. All operations were carried out at 0 - 4°C. Three milliliters of purified mitochondria (25 mg protein per ml of 0.25 M sucrose) was added to 72 ml of an acetone-water mixture (67.5 ml of dry acetone and 4.5 ml of distilled water), and mixed in an Erlenmeyer flask. The mixture was allowed to stand 10 - 12 minutes with occasional swirling, and then centrifuged for 2 minutes at 2000 x g. The supernatant was decanted, and the pellet was rapidly mixed with 75 ml of 0.88 M sucrose - 0.01 M Tris-chloride, pH 7.5, homogenized in a Potter-Elvejehm homogenizer with a Teflon pestle and centrifuged at 25000 x g in the #30 rotor of a Spinco Model L Ultracentrifuge for 10 minutes. The washing procedure was repeated one more time, and the pellet was finally resuspended in 3.0 ml of the same buffer. This procedure has been reported (94) to extract neutral lipid as well as phospholipid, with only about 15% of the original phospholipid remaining, and the residual phospholipid consisting mainly of cardiolipin. To obtain mitochondria depleted of greater than 95% of their lipid content, the above procedure was modified by adding 12 µl of concentrated ammonia (sp. gr. 0.880) per 100 ml of acetone-water extraction

mixture (94).

VIII. Monoamine oxidase [monoamine - O_2 oxidoreductase (deaminating), EC 1.4.3.4]:

Monoamine oxidase activity was assayed using radioactive substrates as described by Robinson et al. (258). The complete reaction mixture contained the following components in a final volume of 0.1 ml: potassium phosphate buffer, pH 7.5 (10 mM); tyramine, serotonin, or benzylamine (1 mM, at a specific radioactivity of 0.5 µCi/µmol); and enzyme. The amount of enzyme was adjusted such that the reaction was linear for at least 40 minutes, and in no case was more than 25% of the substrate consumed. Blanks consisted of buffer, substrate, and boiled enzyme. After a 30 minute incubation in a shaking water bath at 37°C, a 50 µl aliquot of the reaction mixture was pipetted onto columns (0.5 x 14.0 cm Pasteur pipettes plugged with glass wool) containing Amberlite CG-50 (2.5 cm) prepared as described by Pisano (249). The reaction products were eluted with two 1-ml portions of water. The eluate was stirred on a Vortex-Genie mixer; a 1-ml portion was transferred to a counting vial; 10 ml of Aquasol-2 scintillation cocktail added, and the sample counted in a Nuclear-Chicago Mark I liquid scintillation spectrometer.

One unit of enzyme activity was taken as the amount of enzyme necessary to catalyze the formation of 1 nmol of

product per minute. Specific activity was defined as units/mg of protein.

When inhibitors were used, the enzyme was pre-incubated for 10 minutes at 37°C with the inhibitor and cooled for 10 minutes at 0 - 4°C before the addition of the substrate. The concentrations of inhibitor used are specified in the appropriate tables or figures.

IX. Cytochrome oxidase (ferricytochrome c-O_2 oxidoreductase, E.C. 1.9.3.1):

The method was adapted from the procedure designed by Smith (289).

Cytochrome oxidase activity was measured spectrophotometrically at 550 nm in a final volume of 1 ml. The reaction mixture contained 67 mM sodium phosphate buffer, pH 7, 33.5 µM cytochrome c previously reduced by dialysis for 24 hours against twice the molar concentration of sodium ascorbate, and 0.09 mM EDTA. The absorbance at 550 nm was read for 30 seconds and the reaction started by the addition of a mitochondrial preparation containing 5 - 10 µg protein. The decrease in absorbance was recorded for 60 seconds and the reaction stopped by the addition of 10 µl of a saturated solution of potassium ferricyanide.

Activity was expressed as the first-order velocity constant for the oxidation of cytochrome c per mg of protein.

X. Kynurenine hydroxylase (kynurenine, NADPH - O_2 oxidoreductase, EC 1.14.13.9)

This activity was assayed in a final volume of 3.0 ml containing 30 mM KCl, 0.14 mM NADPH, 0.1 M Tris buffer adjusted to pH 8.1 with 1 M acetic acid, and mitochondrial preparation (0.6 - 1.0 mg of protein). After temperature equilibration for 10 minutes at 24°C, 10 µl of kynurenine sulfate to give a final concentration of 0.3 mM was added to start the reaction. The decrease in absorbance at 340 nm was measured over a 10 minute period. One unit of activity was defined as the amount of enzyme necessary to produce a change in A_{340} of .001/10 min. Specific activity was defined as units/mg of protein.

XI. Lipid composition

A. Lipid extraction:

The procedure used for the extraction of lipids was adapted from that used by Colbeau et al. (54).

The mitochondrial pellet (25 - 30 mg protein) was homogenized with a Willems Polytron PT 10 homogenizer in 10 ml of a $CHCl_3$-MeOH (2:1 v/v) mixture for 2 minutes at room temperature. The homogenate was centrifuged at 800 x g in a Sorvall GLC-2 centrifuge, and the supernatant collected by filtration. The residue was re-extracted as described above.

The residue from the second extraction was homogenized

with a Polytron PT 10 homogenizer, using 5 ml of a 1:2 v/v $CHCl_3$-MeOH mixture. The supernatants from the three extractions were combined and concentrated in a Heidolph rotary evaporator at 30°C. To remove traces of water, benzene or MeOH was added, the resulting suspension stirred on the Vortex-Genie mixer, and re-evaporated in the rotary evaporator. The residue was dissolved in a suitable volume of $CHCl_3$ and stored under nitrogen at -20°C.

B. Fractionation of lipid extract:

The lipid extract obtained above was fractionated into neutral lipids and phospholipids by a method adapted from Rouser et al. as described by Kates (173).

The fractionation was carried out on a silicic acid column which was prepared as follows: silicic acid (0.6 g of Bio-Sil A, 100 - 200 mesh, previously dried for 2 hours in an oven at 120°C) was placed in a 50 ml beaker with 2 ml $CHCl_3$. The slurry was poured into a glass column (a glass tube ca. 1 cm in diameter, drawn out on one end, and blocked at that end with glass wool), and the tube tapped to dislodge air bubbles. The solvent level was allowed to drop to the top of the silicic acid. This was followed by two washes with 2 column volumes of chloroform.

With the solvent level at the top of the column, a solution of about 150 - 200 mg of total lipids in 5 ml of $CHCl_3$ was added carefully down the sides of the column with

a Pasteur pipette. The neutral lipids were eluted with 10 column volumes of chloroform. Phospholipids were subsequently collected by elution with 10 volumes of methanol. This solution was concentrated under a stream of nitrogen and dissolved in 500 µl of $CHCl_3$.

C. Separation of phospholipids by thin layer chromatography:

Glass plates (20 x 20 cm) were coated with 0.5 mm of a slurry of silica gel H in distilled water. The plates were allowed to dry in air for 1 hour, and then dried for 2 hours at 120°C. Prior to use, plates were washed with $CHCl_3$-MeOH (1:1) to remove any impurities; the solvent was evaporated in air; and the plates were reactivated at 120°C for 1 hour.

The phospholipid sample described in Section B was applied (25 µl per spot) on the TLC plate together with appropriate phospholipid standards (PC, PI, PS, PE, PG, DPG, and PA). The chromatogram was then developed in $CHCl_3$/MeOH/CH_3CO_2H/H_2O (25:15:4:2 v/v) (288) until the solvent front reached approximately 1 cm from the top of the plate. Standard spots were stained with iodine vapor by blowing through a Pasteur pipet containing iodine crystals while the rest of the plate was covered with a glass plate to protect it from the iodine vapours.

Spots were outlined with a needle, wet with a drop of water, then scraped into 15 ml conical tubes fitted with ground glass stoppers. Elution of phospholipids was carried

out according to Skipski (288) by adding 3 ml of $CHCl_3$/MeOH/CH_3CO_2H/H_2O (25:15:4:2 v/v) to each tube, the tubes agitated at 37°C for 10 minutes in a vigorously shaking water bath. The tubes were centrifuged in a Sorvall GLC-2 centrifuge at 400 x g and the supernatant transferred to centrifuge tubes. This elution was repeated once with the same solvent system. Two more elutions were carried out at room temperature, one with 2 ml of MeOH followed by a final elution with 2 ml of MeOH/CH_3CO_2H/H_2O (94:1:5 v/v).

The pooled supernatants were concentrated under nitrogen to a final volume of 0.5 ml. This was used for the following assay of lipid phosphorus content.

D. Determination of phospholipid phosphorus content:

The procedure used for this assay was adapted from Ames (4).

Sixty microliters of 10% $Mg(NO_3)_2$ in ethanol were added to each of the above phospholipid samples, and the solutions taken to dryness over a strong flame until brown fumes were no longer produced. Hydrochloric acid (0.5 N, 0.3 ml) was added to each tube; and the reaction mixture was boiled for 15 minutes. After cooling to room temperature, 0.7 ml of a mixture composed of 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate tetrahydrate in 1 N H_2SO_4 was added and the reaction mixture incubated for 1 hour at 37°C. Absorbance was read at 820 nm.

A standard curve was prepared from a series of K_2HPO_4 solutions covering a range of 0.01 to 0.09 μmoles phosphate. These were carried through the same digestion procedure as the unknown sample.

E. Determination of fatty acid composition of major phospholipids by gas-liquid chromatography:

The method used for the preparation of fatty acid methyl esters was adapted from Kates (173).

Each band (3 to 4 spots) was scraped into tubes provided with Teflon-lined screw caps. Methanolic HCl (2.5%, 4.5 ml) were added to each tube, and the reaction mixture refluxed at 70°C for 1 hour. After adding water (0.5 ml), the fatty acid methyl esters formed were extracted with three 5 ml portions of petroleum ether. The combined petroleum ether extracts were evaporated to dryness at 30°C in a stream of nitrogen. The residue was taken up in $CHCl_3$ and its fatty acid composition determined by gas-liquid chromatography in an F and M Biomedical Model 400 Gas Chromatograph. This instrument is equipped with a hydrogen flame ionization detector system. The glass column contained 10% SP-2330 on 100/120 Chromosorb WAW. The operating conditions used were: column temperature 195°C, N_2 carrier gas flow of 50 ml/min.

Qualitative analysis of fatty acids was done by comparison to known methylated standards obtained from Serdary Research

Laboratories or Supelco, Inc. Fatty acids containing from 14 to 22 carbons were included in this study. Peak areas of fatty acids were obtained by the 'peak height x retention time method' described by Kates (173). Fatty acid composition is expressed in area per cent of total peaks obtained.

XII. Addition of arachidonic acid to mitochondrial fractions:

The method used for the incubations was adapted from Orly and Schramm (239).

The mitochondrial preparation was suspended in 10 mM buffer (either potassium phosphate, pH 7.5, or Tris HCl, pH 8.1) containing 1 mM dithiothreitol (DTT), an antioxidant for sulphydryl groups, and di-t-butyl-hydroxy-toluene (BHT), a reagent which prevents lipid peroxidation. Arachidonic acid in absolute ethanol was added to the membrane suspension to give the final concentration indicated in Table 15. The final ethanol concentration was never allowed to exceed 1% (v/v).

After pre-incubation with the fatty acid (times and temperatures used are specified in Table 15), the substrate was added and the mitochondrial preparations were assayed for monoamine oxidase activity as described in Section VIII.

XIII. Analysis of diet:

The mice used in these experiments received a diet of Purina Rat Chow 5012 pellets. Total lipid was extracted from the pellets using the method of Bligh and Dyer as

described by Kates (173). Methanolysis and analysis of fatty acid methyl esters were performed as discussed in Section XI E. Results of the assay are presented in Table 2.

XIV. Protein determination:

Protein was determined by the method of Lowry et al. (196), using bovine serum albumin as the standard.

XV. Statistical analysis of results:

Statistical analysis of results was done on a Wang Laboratories Model 600 programmable calculator.

Results are expressed as means ± standard error of the mean. Student's t-test was used to determine the significance of differences between mean values.

Table 2

Fatty acid profile of Purina Rat Chow 5012

Fatty Acid	%
14:0	<1
16:0	15.3 ± 1.70
16:1	1.3 ± 0.21
18:0	2.7 ± 0.32
18:1	21.4 ± 2.27
18:2	50.0 ± 3.85
18:3	4.3 ± 0.38
20:1	1.7 ± 0.21
20:4	0
22:1	3.2 ± 0.45
22:6	0

Values are expressed as percent of total fatty acids, and represent the average of 3 pooled samples ± S.E.M.

Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, oleic acid is designated as 18:1.

CHAPTER 3: RESULTS AND DISCUSSION

Results obtained in this study will be presented as follows:

A. Work done on non-treated lean and obese mice,

B. Work done on oxytetracycline-treated mice, and

C. Work done on cold-exposed mice.

I. NON-TREATED LEAN AND OBESE MICE

A. Substrate specificity of mouse liver monoamine oxidase.

Mitochondrial preparations from five organs of lean and obese mice - the brain, heart, liver, pancreas, and white adipose tissue - were assayed for monoamine oxidase activity using tyramine as the substrate. The brain and heart were chosen for this preliminary investigation, because monoamine oxidase has been assigned an important role in the metabolism of endogenous catecholamines in these tissues (62). In the liver, MAO has been assigned a possible role in the metabolism of exogenous monoamines (150). Furthermore, high plasma corticosterone levels have been reported in obese mice (77), and several investigators have observed that an increase in brain and heart MAO activity after adrenalectomy could be antagonized by corticosteroids (10, 245). The pancreas, white adipose tissue, as well as the liver were investigated because of their roles in the development of the obese-hyperglycemic syndrome. Our results (Table 3) indicated that there was no

Table 3

Mitochondrial monamine oxidase activity in lean and obese mice

Organ	Monoamine oxidase activity (units/mg protein)	
	Lean	Obese
Brain	4.49 ± 0.49	4.65 ± 0.39
Heart	2.45 ± 0.17	2.65 ± 0.36
Liver	5.07 ± 0.15	9.39 ± 0.26*
Pancreas	1.08 ± 0.03	1.34 ± 0.02*
White adipose tissue	6.97 ± 0.34	11.89 ± 0.29*

Values are expressed as means ± SEM for three to seven animals per group. Monoamine oxidase was assayed in duplicate, using tyramine as the substrate. Units were defined in Methods (Section VIII).

*$P < 0.001$ when compared to lean animals.

significant difference in the activities of MAO in the brain and heart. Mitochondrial preparations from obese mouse liver, pancreas, and white adipose tissue, on the other hand, showed greater activity towards tyramine than the corresponding lean samples.

Our results for the brain were in agreement with the recently published results of Feldman and Henderson (88). These authors, however, observed no significant difference between homogenates of the liver and pancreas of lean and obese mice. Their experimental protocol differed from ours in two important aspects. They used tryptamine instead of tyramine as their substrate, and whole homogenates of liver and pancreas instead of the mitochondrial fraction. To enable us to draw a valid comparison between their data and ours, enzyme assays were carried out on whole liver homogenates from our animals. Our results (Table 4) showed that the specific activity of MAO in samples from 9 to 12 week old obese mice was about 1.4 times greater than that of the lean controls, regardless of whether tyramine or tryptamine was used as the substrate for the assay.

Feldman and Henderson observed a difference in MAO activity in white adipose tissue and none in heart. Our results are thus similar to theirs even though the group of mice they used for the assays on these tissues were considerably older ($4\frac{1}{2}$ to 7 months) than our animals (9 to 12 weeks).

Table 4

Monoamine oxidase activity in whole liver homogenates from lean and obese mice

	Monoamine oxidase activity (nmol product/min/mg protein)	
Group	Tyramine	Tryptamine
Lean	2.55 ± 0.28	0.51 ± 0.04
Obese	3.51 ± 0.21*	0.69 ± 0.05*

All values are expressed as means ± SEM for five animals from each group.

*$P < 0.001$ when compared with lean groups.

Further studies on monoamine oxidase were subsequently carried out on liver mitochondrial samples. This decision was based on the ease with which mitochondria could be isolated from the liver, the high specific activity of the enzyme and the high yields in this tissue, coupled with the possibility that the altered lipid metabolism in this organ may cause interesting changes in MAO activity.

To determine if the observed higher specific activity of MAO in the liver mitochondria of the obese mouse was due to a generalized effect on all mitochondrial enzymes, the MAO assay was repeated on mitochondria isolated from livers of another group of mice, and an assay of kynurenine hydroxylase, an outer membrane marker (233, 273), was carried out on the same mitochondrial fraction. An assay of cytochrome oxidase was also carried out to determine if the effect was manifested in the inner membrane. Table 5 summarizes the data obtained for all three enzyme assays. No significant difference was observed in the specific activity of kynurenine hydroxylase or cytochrome oxidase. However, the specific activity of MAO in the obese mouse liver mitochondria was again observed to be greater than in the lean controls.

Substrate specificity studies were undertaken in order to better characterize the enzyme in lean and obese mice. The substrates used for the assays were tyramine, serotonin, and benzylamine. This investigation was carried out in parallel with the more extensively studied rat liver

Table 5

Activities of mitochondrial enzymes in the liver of lean and obese mice

Group	Enzyme activity (units/mg protein)		
	Monoamine oxidase	Kynurenine hydroxylase	Cytochrome oxidase
Lean	6.98 ± 0.20	69.7 ± 5.4	1.80 ± 0.20
Obese	10.78 ± 0.30	69.6 ± 5.0	2.10 ± 0.10
P	<0.001	n.s.	n.s.

Units of enzyme activity are defined in the Methods section. Monoamine oxidase was assayed using tyramine as the substrate. Values are means ± SEM for four separate preparations for monoamine oxidase and kynurenine hydroxylase and for two separate preparations for cytochrome oxidase.
Abbreviation: n.s., not significant.

mitochondrial MAO. Our results (Table 6) indicated greater activity towards all three substrates in the obese samples as compared with the lean preparations. As well, it was noted that the substrate specificity in both lean and obese mice differed from that of rat liver mitochondria. In the rat liver, mitochondrial MAO activity ranked thus: tyramine > serotonin > benzylamine. In both lean and obese mice, however, the activities ranked thus: tyramine > benzylamine > serotonin.

It is well documented that two types of functional forms of monoamine oxidase exist in tissues from several species. The two forms, called 'A' and 'B' according to their sensitivity to the inhibitors clorgyline (161) and deprenyl (178) (cf. Review of Literature, section I.A.5), have also been shown to have different substrate specificities (228). For instance, it has been demonstrated in liver and brain from rat and man that serotonin is oxidized mainly by the 'A' form of the enzyme and that benzylamine is a preferred substrate for the 'B' form, while tyramine is a substrate for both forms. The above results would therefore suggest that in the C57BL/6J mice, liver mitochondria contained a much larger proportion of the B form of monoamine oxidase than in rat liver. Furthermore, in the obese mouse, the specific activity of MAO was 74% greater when tyramine was the substrate, 53% with serotonin, and 63% with benzylamine. This

Table 6

Substrate specificity of liver mitochondrial monoamine oxidase in lean mice, obese mice and rats

Animal	Monoamine oxidase activity (units/mg protein)		
	Tyramine	Serotonin	Benzylamine
Lean mouse	5.70 ± 0.30	0.85 ± 0.05	1.55 ± 0.06
Obese mouse	9.89 ± 0.14*	1.30 ± 0.03*	2.51 ± 0.08*
Rat	9.31 ± 0.18	3.19 ± 0.09	1.53 ± 0.05

All values are expressed as means ± SEM for five animals from each of the three groups assayed in duplicate. Units of enzyme activity were defined in Methods (section VIII).

*$P < 0.001$ when compared with lean groups.

may suggest that the factors responsible for the difference in MAO activity in lean and obese mice had a differential action on the two forms of MAO in liver mitochondria.

I.B. Substrate-selective inhibition of liver mitochondrial MAO

The effects of selective inhibition by clorgyline and deprenyl were studied in order to determine the relative proportions of form A and B of monoamine oxidase in lean and obese mouse liver mitochondria. Table 7 gives the results obtained with intermediate concentrations of inhibitors (10^{-6} M and 10^{-7} M). As noted in the Review of Literature, Type A MAO is more sensitive than Type B to inhibition by clorgyline, whereas deprenyl preferentially inhibits the B form of the enzyme. These inhibitors had similar effects on samples from lean and obese mice. However, comparison of the degree of the inhibition effected by each inhibitor on the same substrate revealed interesting differences. For instance, when tyramine was used as the substrate, 10^{-6} M deprenyl almost completely abolished enzyme activity, whereas clorgyline effected only 24% inhibition in both lean and obese mice. Similarly, the activity towards benzylamine was almost completely abolished by 10^{-7} M deprenyl, while clorgyline had hardly any effect on enzyme activity. These observations suggested that the predominant form of MAO in both lean and obese mouse mitochondria was form B.

Results obtained in dose-response studies shown in

Table 7

Substrate-selective inhibition of liver mitochondrial MAO activity

Animal	Substrate	% inhibition by Clorgyline		Deprenyl	
		10^{-7}M	10^{-6}M	10^{-7}M	10^{-6}M
Lean	Tyramine	4	24	83	92
	Serotonin	54	65	33	45
	Benzylamine	0	1	68	96
Obese	Tyramine	1	24	86	94
	Serotonin	50	65	35	47
	Benzylamine	0	3	68	98

Mitochondrial preparations were pre-incubated at 37°C for 10 min with the inhibitor prior to the addition of the substrate. Controls were pre-incubated under the same conditions, but in the absence of the inhibitor. Enzyme activity was then assayed as described in Methods (section VIII). Values given are the averages of two individual experiments assayed in duplicate. The variation between the experiments was less than 5%.

Figures 5 and 6 confirm this observation. These experiments were carried out with concentrations of inhibitor ranging from 10^{-3} to 10^{-9} M, and were run in parallel with the better known rat liver mitochondrial monoamine oxidase. The data obtained for lean and obese mice were essentially the same, suggesting that the factors responsible for the difference in MAO activity in these animals did not exert a discriminative action on the two forms of the enzyme.

The biphasic dose-response curve obtained for the rat suggested that, as previously reported by several investigators (76, 77, 334), about equal amounts of the A and B forms are present in rat liver mitochondria. On the other hand, in the mouse liver, inhibition by clorgyline was observed at a concentration ten-fold as great as that of deprenyl, which was capable of producing nearly complete inhibition of enzyme activity. These data further supported our previous observation of the predominance of the B-form of MAO in lean and obese mouse mitochondria. This finding is in agreement with results published on albino mice (183).

Furthermore, since the dose-response curves indicate that lean and obese-mouse liver mitochondrial MAO contain a negligible amount of Form A of the enzyme, it appears possible that the observed activity towards serotonin may be attributable to Form B of the enzyme. Other investigators (80) have reported that Form B of liver MAO is capable of metabolizing

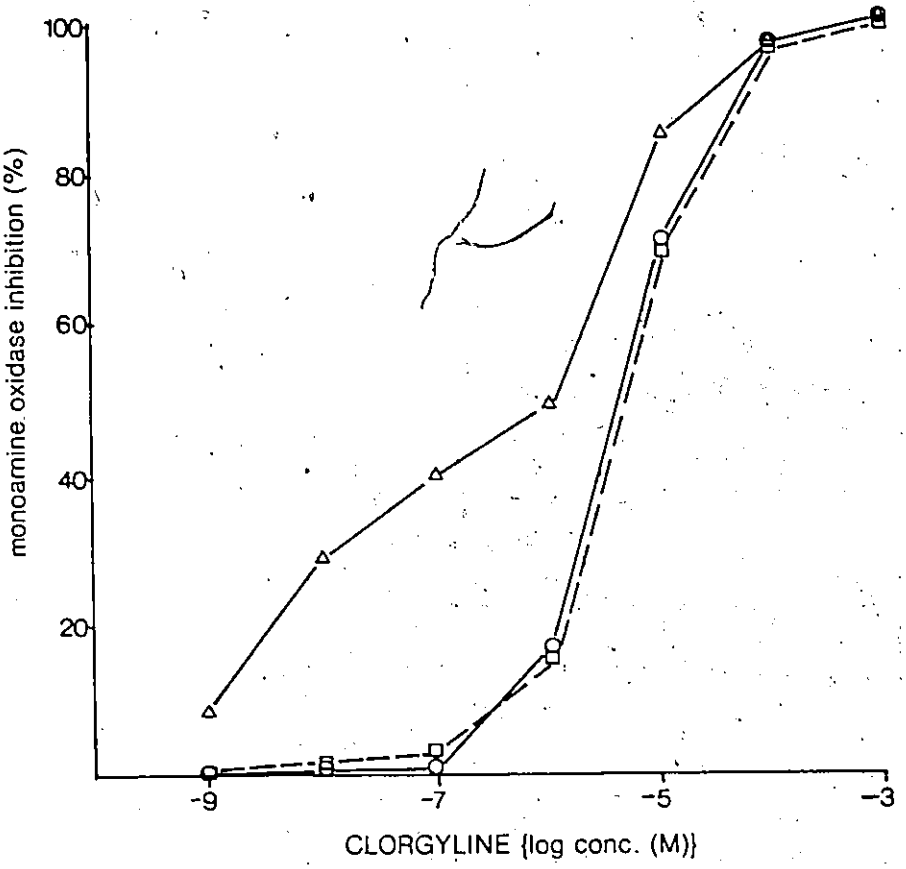

Figure 5. The inhibition in vitro of liver mitochondrial MAO activity. Rat liver mitochondria (Δ — Δ); lean mouse liver mitochondria (□ — □); obese mouse liver mitochondria (O — O). The substrate used was tyramine and the assays were done as described in Methods (VIII).

The results given represent means of two separate experiments done in triplicate for each group.

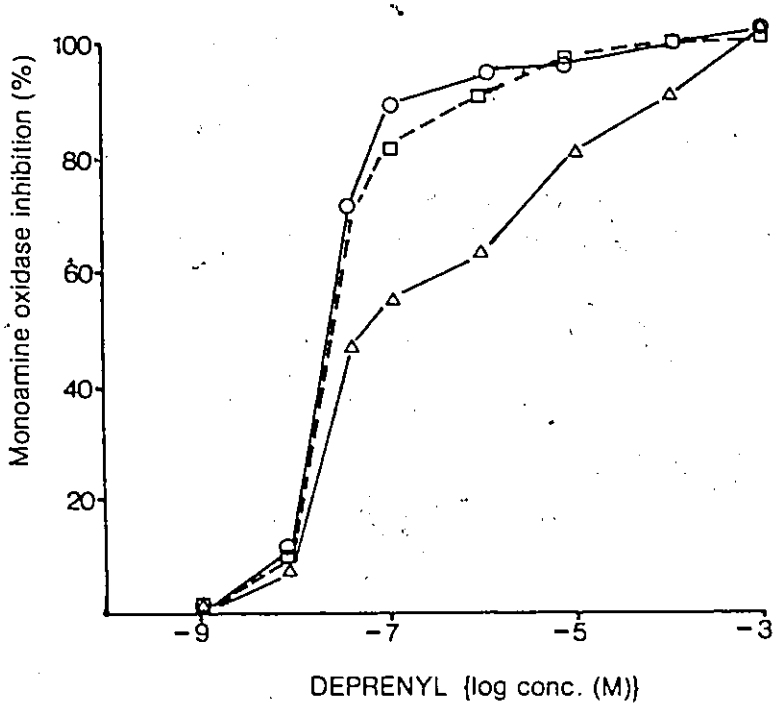

Figure 6. The inhibition in vitro of liver mitochondrial MAO. Rat liver mitochondria (Δ — Δ); lean mouse liver mitochondria (□ --- □); obese mouse liver mitochondria (O — O). Tyramine was used as the substrate and the assays were done as described in Methods (VIII).

The results given represent means of two separate experiments done in triplicate for each group.

serotonin even though this amine is generally considered a substrate for Form A of the enzyme.

I.C. Kinetics of monoamine oxidase in mouse liver mitochondria

Since the higher monoamine oxidase activity observed in the obese mice could be due to an alteration of the kinetic properties of the enzyme, the K_m and V_{max} of the enzyme towards tyramine, serotonin, and benzylamine were determined for both groups of animals. Lineweaver-Burk plots of the data obtained in this experiment are given in Figures 7, 8 and 9. The figures show that for all three substrates used in the assay, there was no difference in apparent K_m between the two groups of animals. On the other hand, the V_{max} value of the enzyme towards all substrates was observed to be higher in the obese animals than in their lean counterparts. This observation would confirm previous results obtained in Section I.A., where saturating concentrations of each of the substrates were used for the assays. The results obtained in this kinetic analysis would also suggest that the greater activity observed in obese mice is not due to an altered affinity of the enzyme for the substrates used in the assays. Since under enzyme-saturating substrate concentrations, the maximum initial velocity V_{max} is proportional to total enzyme concentration (184), the higher V_{max} values obtained for the obese-mouse preparations may suggest a greater amount of enzyme in obese animals than in lean animals. The values for the

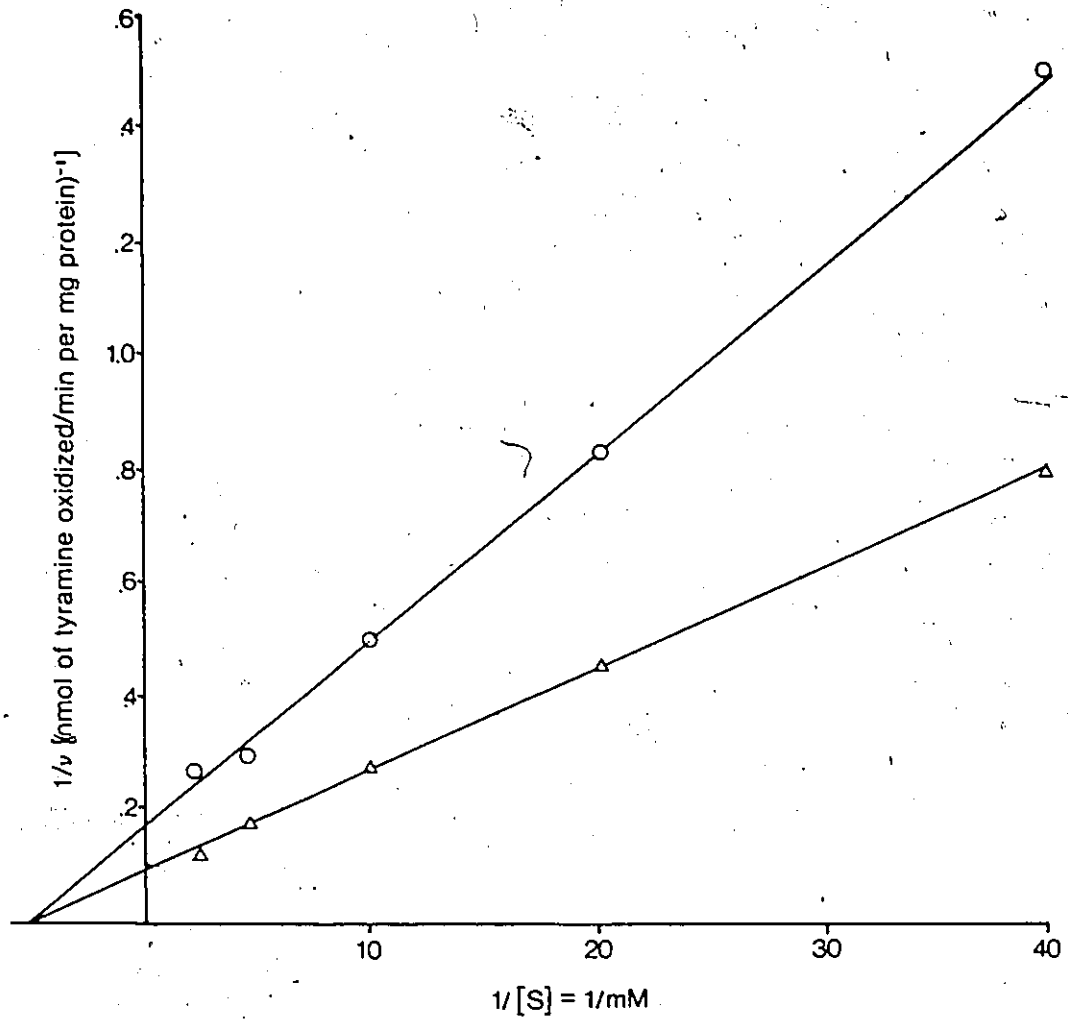

Figure 7. Effect of substrate concentration on the oxidative deamination of tyramine by liver mitochondria. O — O, lean mice; Δ — Δ obese mice. Assays were done as described in Methods (Section VIII).

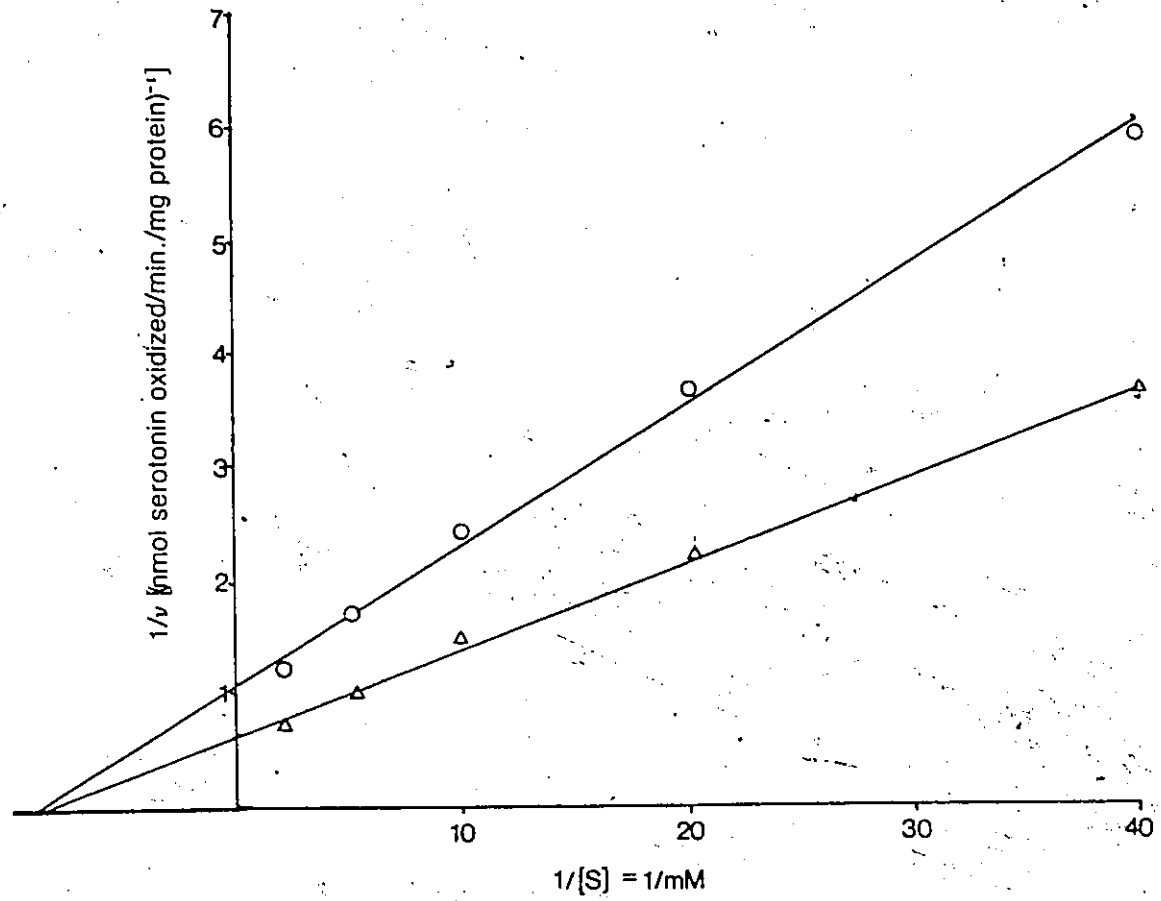

Figure 8. Effect of substrate concentration on the oxidative deamination of serotonin by liver mitochondria. O — O, lean mice; Δ — Δ, obese mice. Assays were done as described in Methods (Section VIII).

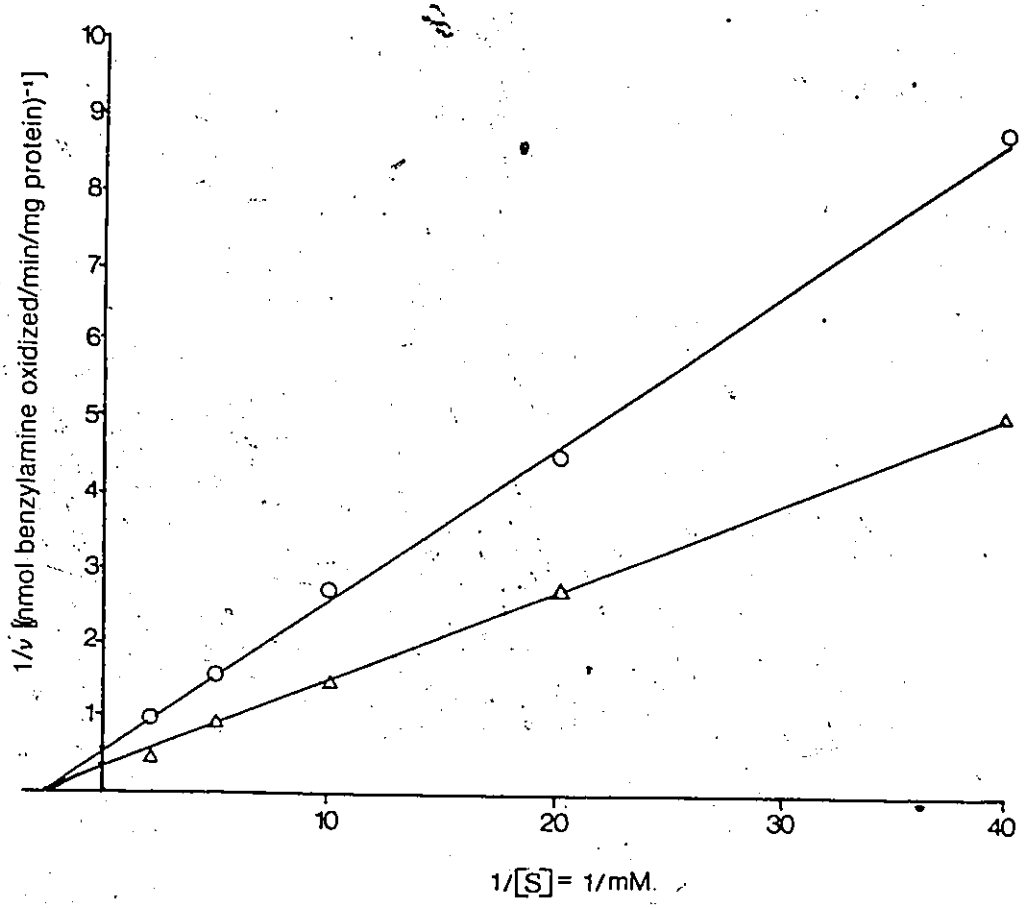

Figure 9. Effect of substrate concentration on the oxidative deamination of benzylamine by liver mitochondria. O — O, lean mice; Δ — Δ, obese mice. Assays were done as described in Methods (Section VIII).

kinetic constants are given in Table 8.

I.D. Effect of lipid environment on enzyme activity

1. Effect of lipid depletion of mouse liver mitochondria:

From the foregoing studies, it appears that the greater specific activity of MAO in obese mouse liver mitochondria was not due to a difference in substrate affinity or proportions of forms A and B of the enzyme, or a greater number of mitochondria in the obese animals.

There are two alternative reasons for the observed difference in activities between lean and obese mice. A possible explanation may be the presence of a greater amount of enzyme protein in obese-mouse mitochondria. The greater V_{max} values obtained for obese-mouse preparations towards all amine substrates studied in the kinetic analysis may be suggestive of this condition. Another basis for the observed difference in enzyme activity may lie in an altered lipid environment of the enzyme in obese-mouse mitochondria.

Although Singer classifies monoamine oxidase as a peripheral protein (cf. Review of Literature, Section I.B.), several investigators (80, 85, 108, 139, 362) have provided evidence indicating that MAO is an integral protein, and as such, the enzyme should have a functional or structural dependence on its lipid environment. Indeed, various groups of workers in the field have demonstrated such a dependence (30, 43, 80, 169, 224, 299, 317). Aithal et al. (1) and

Table 8

Kinetic constants for tyramine, serotonin and benzylamine oxidation by liver mitochondrial MAO in lean and obese mice

Group	K_m (μM)			V_{max} (units/mg protein)		
	Tyramine	Serotonin	Benzylamine	Tyramine	Serotonin	Benzylamine
Lean	199	118	350	6.01	0.93	1.85
Obese	199	118	350	10.81	1.55	2.95

Kinetic constants were obtained from Lineweaver-Burk plots of substrate concentrations vs. initial reaction velocities. Substrate concentrations ranging from 1 to 10^{-3} M were used. Mitochondrial fractions prepared from 6 animals per group were assayed in duplicate for MAO activity as described in Methods (section VIII).

Raison (253), however, are not in agreement with this view, and contend that MAO is not directly dependent on membrane lipids for the expression of its activity.

The role of lipids thus appears to be controversial and needs to be studied further. The obese-hyperglycemic mouse seemed to be a good model for attempting to elucidate the issue, as it has been reported to have a wide range of defective membrane-related processes, such as reduced purine nucleotide binding in brown adipose tissue (364), reduced hormone receptor concentrations (168) and Na^+, K^+-ATPase activity (37), and defective hormonal regulation of adenylate cyclase activity (153). The first step in these studies involved an investigation of the effect of lipid depletion on MAO activity. The results for these experiments are given in Table 9. Extraction of mitochondrial preparations with aqueous acetone (Step II in the table), which removes neutral lipid (94), decreased the activity of the enzyme in both lean and obese mouse samples to the same level. When serotonin was the substrate, the original activity in the lean mice decreased by 54% whereas that of the obese group decreased by 71%. With benzylamine as substrate, the decrease in activity of lean samples amounted to 29%, whereas that of the obese samples decreased by 45%.

Extraction with aqueous acetone and ammonia (Step III) which removes 95% of lipids (94), also left approximately the same residual activity in both groups of animals. With

Table 9

Effect of lipid depletion on liver mitochondrial monoamine oxidase activity

Fraction*	MAO Activity (units/mg protein) Serotonin	Benzylamine
Lean		
I	1.05 ± 0.06	1.90 ± 0.04
II	0.48 ± 0.04	1.35 ± 0.08
III	0.21 ± 0.02	0.75 ± 0.04
Obese		
I	1.70 ± 0.07	2.80 ± 0.11
II	0.50 ± 0.05	1.55 ± 0.07
III	0.25 ± 0.04	0.75 ± 0.03

*I, Mitochondrial fraction; II, mitochondrial fraction extracted with acetone; III, mitochondrial fraction extracted with acetone and ammonia. Details of the extraction procedure are given in Methods (section XI.A.), and units are defined in Methods (section VIII). Values given are the means of two individual experiments ± SEM. Samples were pooled from 5 animals per group.

serotonin as the substrate, the decrease in specific activity in the lean mouse was 80% while that in obese samples was 85%. When benzylamine was the substrate, the observed decrease was 61% in the lean samples, and 73% with the obese. In both procedures, the 'additional' activity of MAO in obese mouse liver mitochondria seems to be readily decreased to the same level as that found in lean mouse mitochondria by this treatment.

Data obtained in our lipid-depletion experiments suggest that MAO in mouse liver mitochondria may be dependent on lipids for expression of its activity. Further, since manipulation of the lipid content of our samples produced a greater change in enzyme activity in mitochondria from the obese animals, it is possible that the greater specific activity observed in obese mice was due to an alteration in the membrane lipid composition of liver mitochondria in these animals.

I.D. 2. Phospholipid composition of lean and obese mouse liver mitochondria

Membrane-associated enzymes often require specific phospholipids for the expression of maximal activity. This requirement has been demonstrated in the case of the sugar specific phosphotransferases of *E. coli* membranes, guinea-pig kidney Na^+,K^+-ATPase, and mitochondrial D(-)β-hydroxybutyric acid dehydrogenase.

Ekstedt *et al.* (81) have observed that when monoamine

oxidase solubilized from pig liver mitochondria by extraction of phospholipids was incubated with lipid-depleted mitochondrial residues, binding of the enzyme to the residues occurred in the presence of cardiolipin, and mixtures of cardiolipin and lecithin, but not of lecithin alone. Olivecrona and Oreland (236) have also shown that phosphatidylinositol and phosphatidylserine can cause binding of MAO to lipid-depleted membrane residues.

In view of these published results, and since phospholipids account for three-quarters of the total lipid content of mitochondria, it seemed appropriate to determine if there was a difference in the phospholipid composition between the obese and lean mouse mitochondria, and if such a difference existed, to determine which of the phospholipids is or are necessary for the expression of enhanced monoamine oxidase activity in the obese mouse.

The results obtained in this study are summarized in Table 10. These results show that PC and PE were present in largest quantity in both groups of mice, while PI + PS and CL + PA were present in smaller amounts. The numbers in parentheses indicate the proportions of these phospholipids, taking sphingomyelin and lysophosphatidylcholine as 1.00.

Application of Student's t-test to these results, however, did not reveal any significant differences between any of the phospholipid groups in lean and obese mice.

Table 10

Phospholipid composition of lean and obese mouse liver mitochondria

Phospholipid Class	Composition (%) Lean	Obese
Sphingomyelin + lysophosphatidylcholine	12.7 ± 3.7 (1.00)	13.5 ± 3.2 (1.00)
Phosphatidylcholine	29.8 ± 1.2 (2.55)	31.8 ± 1.8 (2.36)
Phosphatidylinositol + phosphatidylserine	12.9 ± 1.6 (1.02)	11.4 ± 2.1 (0.84)
Phosphatidylethanolamine	22.1 ± 2.4 (1.74)	21.7 ± 1.8 (1.61)
Phosphatidylglycerol	7.7 ± 1.1 (0.61)	8.1 ± 1.9 (0.60)
Cardiolipin + phosphatidic acid	13.7 ± 2.1 (1.08)	12.8 ± 2.1 (0.95)

The results represent means ± SEM for four experiments each done in triplicate and are expressed as percentage of total phospholipid fraction.

It is interesting in this respect that in a recent study on membrane fluidity done on adipocyte membranes, Hyslop and York (153) found that the microviscosity of membranes from ob/ob mice was significantly reduced at physiological temperature. However, an analysis of the gross phospholipid composition did not reveal any significant differences between ob/ob and lean-mouse membrane preparations. These authors have postulated that the decrease in the microviscosity of ob/ob samples may reflect an increased occurrence of unsaturated fatty acids in the membrane phospholipids.

I.D. 3. Fatty acid composition of phospholipids in lean and obese mouse liver mitochondria

It has been reported by Winand (346) that the total liver phospholipid fraction in the ob/ob mouse liver contains different proportions of fatty acids from the same fraction of the lean mouse liver. At the time that our study was undertaken, it was not known whether there were significant differences in the fatty acid composition of liver mitochondrial phospholipids in these mice. The degree of unsaturation of the fatty acyl moiety in mitochondrial phospholipids has been reported to affect the degree of activation of phospholipid-dependent enzymes such as mitochondrial D(-)β-hydroxybutyric acid dehydrogenase and the *E. coli* membrane enzyme which transfers galactose from UDP-galactose to galactose-deficient lipopolysaccharide (cf. Review of

Literature, Section I.B.).

In view of the possibility that the greater activity of MAO in the obese mouse liver is due to a higher content of unsaturated fatty acids, the fatty acid composition of liver mitochondrial phospholipids of lean and obese mice was determined. Thus, preparations of whole mitochondria, purified on a discontinuous Ficoll gradient, were used for the studies on fatty acid composition. The phospholipid fraction from the lipid extract of these mitochondrial samples was subjected to methanolysis, and the resulting fatty acid methyl esters assayed by gas-liquid chromatography. The fatty acid percentage composition of the phospholipids studied are presented in Tables 11, 12, 13, and 14.

Arachidonic acid (20:4) was found to be significantly higher in three of the phospholipid fractions in the obese: the arachidonate content in the PC fraction of preparations (16.4%) was 1.8 times as great as that of the lean (9.2%). In the PI + PS fraction, the arachidonate in the obese (29.8%) was 2.1 as high as in the lean (14.1%). In the PE + PG fraction, there was also a 2.1 fold difference between samples from the obese (22.7%) and the lean mice (10.8%).

Of all the phospholipid fractions studied, it was only in cardiolipin that a difference in 20:4 was not observed between lean- and obese-mouse preparations. Our study showed that oleic acid (18:1) in this fraction of the obese (28.6%)

Table 11

Fatty acid composition of phosphatidylcholine in liver mitochondria of lean and obese mice

Fatty Acid	Group	
	Lean	Obese
14:0	<1	<1
16:0	27.3 ± 1.59	26.6 ± 1.34
16:1	<1	<1
18:0	22.9 ± 1.39	23.8 ± 0.94
18:1	13.7 ± 1.08	12.7 ± 1.12
18:2	12.7 ± 1.41	10.4 ± 0.27
18:3	0	0
20:4	9.2 ± 1.64	16.4 ± 0.76*
22:6	9.6 ± 0.95	9.2 ± 0.40

Values given are means ± SEM of five individual experiments. Results are expressed as percentage of total fatty acids in phosphatidylcholine.

Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, while linoleic acid is designated as 18:2.

*Significantly different from lean, $P < 0.001$.

Table 12

Fatty acid composition of phosphatidylinositol + phosphatidylserine in liver mitochondria of lean and obese mice

Fatty Acid	Group	
	Lean	Obese
14:0	<1	<1
16:0	15.0 ± 1.46	12.5 ± 1.54
16:1	<1	<1
18:0	48.1 ± 2.44	45.0 ± 2.32
18:1	7.2 ± 0.25	6.8 ± 0.67
18:2	3.4 ± 0.49	2.8 ± 0.29
18:3	0	0
20:4	14.1 ± 1.85	29.8 ± 2.90*
22:6	7.4 ± 2.20	5.2 ± 1.25

Values given are means ± SEM of five individual experiments. Results are expressed as percentage of total fatty acids in the phosphatidylinositol + phosphatidylserine fraction.

Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, while linoleic acid is designated as 18:2.

*Significantly different from lean, $P < 0.025$.

Table 13

Fatty acid composition of phosphatidylethanolamine + phosphatidylglycerol in liver mitochondria of lean and obese mice

Fatty Acid	Group	
	Lean	Obese
14:0	<1	<1
16:0	19.7 ±1.95	16.9 ±1.24
16:1	<1	<1
18:0	40.8 ±2.83	37.5 ±2.11
18:1	10.9 ±1.65	10.7 ±0.94
18:2	6.2 ±0.49	4.9 ±0.51
18:3	0	0
20:4	10.8 ±1.53	22.7 ±2.24*
22:6	9.6 ±0.90	9.6 ±1.80

Values given are means ± SEM of five individual experiments. Results are expressed as percentage of total fatty acids in the phosphatidyethanolamine + phosphatidylgycerol fraction.

Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, while linoleic acid is designated as 18:2.

*Significantly different from lean, $P < 0.05$.

Table 14

Fatty acid composition of cardiolipin + phosphatidic acid in liver mitochondria of lean and obese mice

Fatty Acid	Group	
	Lean	Obese
14:0	<1	<1
16:0	18.4 ± 2.37	15.2 ± 2.24
16:1	2.2 ± 0.22	2.9 ± 0.84
18:0	14.9 ± 3.02	11.0 ± 1.11
18:1	17.1 ± 0.98	28.6 ± 2.95*
18:2	29.3 ± 4.25	31.0 ± 3.13
18:3	0	0
20:4	5.1 ± 0.34	5.7 ± 0.98
22.6	5.9 ± 1.60	4.9 ± 0.80

Values given are means ± SEM of five individual experiments. Results are expressed as percentage of total fatty acids in the cardiolipin + phosphatidic acid fraction.

Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0; while linoleic acid is designated as 18:2.

*Significantly different from lean, $P < 0.01$.

was 1.7 times as great as that of the preparation from the lean (17.1%). Since cardiolipin is believed to be present predominantly in the inner mitochondrial membrane (54), and MAO is generally believed to be on the outer mitochondrial membrane (275), the difference in oleic acid content may not be associated with the observed difference in enzyme activity between lean and obese animals.

In rat liver mitochondria, the major phospholipids of the outer mitochondrial membrane have been found to be phosphatidylcholine and phosphatidylethanolamine. The outer mitochondrial membrane in rat liver has also been reported to be markedly richer in phosphatidylinositol than the inner membrane. The analysis of gross phospholipid composition has demonstrated that lean and obese-mouse liver mitochondria contained about 31% PC, 22% PE, 8% PG, and 12% PI + PS. It does not appear inconceivable, therefore, that the higher arachidonic acid content in obese mouse liver mitochondrial phospholipids may indeed have a role in the expression of greater MAO activity, either through an increase in membrane fluidity, or through direct interaction of the more highly unsaturated fatty acid with the enzyme.

One may be criticized for performing this study on whole mitochondria instead of the outer mitochondrial membrane. However, Chaffee et al. (44) have reported that in golden hamster the fatty acid composition of whole mitochondria did not differ significantly from the fatty acid profile of

mitochondrial membranes. Patton and Platner (246) have confirmed this observation in rat liver mitochondria. Further, Parkes and Thompson (243) found that in guinea pig liver, the fatty acid composition of inner and outer mitochondrial membrane phospholipids are basically similar. This observation confirms the findings of Stoffel and Schieffer for rat liver (302). It was, therefore, not considered necessary to utilize the outer mitochondrial membrane in this investigation.

I.D. 4. Effect of arachidonic acid on the oxidative deamination of tyramine by lean mouse liver mitochondria

An attempt was made to obtain direct evidence as to whether arachidonic acid is responsible for the greater specific activity of mitochondrial MAO in obese mouse liver. It has been found possible to insert free fatty acids artificially into biological membranes so as to study their effect on membrane properties (239). Thus, certain fatty acids acted as crenators of erythrocytes, and protected these against hemolysis in hypotonic media (256, 279). Addition of sodium laurate to secretory granules of rat parotid gland sealed the granule membrane against leakage at low temperatures (274). Manipulation of membrane functions by insertion of free fatty acids has the following advantages. Experiments are not dependent on the successful esterification of the fatty acid, since the free fatty acid can serve as such to modify the physical properties of the membrane. Moreover,

any effect, can be directly related to the specific chemical structure of the fatty acid. The protocol used in the experiments is presented in Table 15.

The first two experiments were carried out to compare the degree of activation by arachidonic acid at physiological temperature and a temperature well below normal body temperature, i.e. 22°C. Raison (253) has previously observed that 23°C is the critical temperature below which the kinetics of mitochondrial enzymes of homeotherms changed, and that these changes could be associated with a phase change in membrane lipids. It was hoped that if arachidonic acid was increasing MAO activity by enhancing membrane fluidity in the obese mouse, its effect on enzyme activity would be more pronounced at this lower temperature than at physiological temperature. No enzyme activation was observed at either temperature used.

Since the amount of lipid added was reported to be critical in these experiments (239), it was decreased from 1 mM to 0.11 mM as shown in the third experiment. No significant increase in activity was observed at this concentration or intermediate concentrations of arachidonic acid.

It has been previously reported by Ekstedt et al. (81) and Olivecrona and Oreland (236) that anionic phospholipids bind more readily than zwitterionic phospholipids to lipid-depleted mitochondrial residues. It was therefore, decided

Table 15

Conditions used in studies on the effect of arachidonic acid on the oxidative deamination of tyramine by lean mouse liver mitochondria

Expt. No.	Buffer	pH	Conc. of 20:4ω6 (mM)	Temp. (°C) Pre-incubation	Assay	Pre-incubation time (min)
1	potassium phosphate	7.5	1.00	0-4°	37°	10
2	potassium phosphate	7.5	1.00	0-4	22	10
3	potassium phosphate	7.5	0.11	0-4	37	10
4	Tris-HCl	8.1	0.11	0-4	37	15
5	Tris-HCl	8.1	0.11	37	37	15

The specific activity in the control tubes was 5.40 ± 0.40, and no change was observed under the condition outlined above.

Abbreviation: 20:40 ω6 - arachidonic acid.

to perform the experiment using a higher pH, 8.1, which was successfully used by Orly and Schramm (239) in a similar experiment on a different system. It was hoped that at this pH the arachidonate ion would attach itself more readily to the mitochondrial membrane. However, no physiologically significant increase in activity was observed under these conditions. Improvement was not realized by increasing pre-incubation time or temperature.

In experiments with lymphocyte membrane preparations, Klausner et al. (177) observed that after 1 hour, most of the fatty acids (78%) incorporated into the membranes were still in the unesterified form. The above results may suggest that arachidonic acid enhances MAO activity only when incorporated into phospholipid molecules. In this respect, Sandermann has cited the importance of the interaction between lipid head groups and polar amino acid side chain in the formation of enzymatically active lipoprotein complexes. It has been demonstrated, for instance, that lipids with mobile fatty acid chains will only activate lipid-dependent C_{55}-isoprenoid alcohol kinase when they have sufficiently hydrated polar head groups (269).

II. OXYTETRACYCLINE-TREATED LEAN AND OBESE MICE

(i) RESULTS:

A. Substrate specificity studies

1. MAO activity in liver homogenates from OTC-treated mice and their controls:

Lean and obese mice were treated as described in Methods (section III. A.) Since the purpose of this experiment was to determine total MAO activity, the assay was performed on the 600 x g supernatant rather than the mitochondrial fraction used in the previous experiments.

Table 16 shows that oxytetracycline treatment decreased total MAO activity of the obese mice to the same level as the non-treated lean animals. This decrease in enzyme activity was accompanied by a dramatic reduction in the weight and protein content of the liver of the obese mice. Although a slight decrease in the liver weights of food restricted animals was noted, changes observed in the OTC-treated obese mice did not appear to be attributable to decreased food intake since values in the food-restricted group were not different from those found for the control obese mice.

The specific activity of the enzyme in treated obese mice was reduced to 69% of that in the non-treated obese group. Treatment of the lean mice decreased specific activity to 74% of that in the non-treated lean animals. The decrease

Table 16

Monoamine oxidase activity in oxytetracycline-treated animals and their controls

Treatment	Liver wt. (g)	Protein/ Liver (mg)	MAO Activity Units/Liver	Units/mg protein
Lean non-treated	1.61±0.01	120.2±2.35	148.3±1.30	1.24±0.03
Lean OTC-treated	1.13±0.01	101.2±1.80	93.2±3.65	0.92±0.02
Obese non-treated	4.05±0.07	363.9±5.40	869.6±1.65	2.39±0.04
Obese OTC-treated	1.61±0.03	104.3±1.95	175.1±4.35	1.28±0.04
Obese food-restricted	3.10±0.01	336.4±5.30	770.9±9.40	2.33±0.02

Values given are means ± SEM for two separate experiments.

Four animals per group were sacrificed by decapitation. Livers were removed, weighed, and homogenized with 9 volumes of isolation medium, pH 7.5. The homogenate was centrifuged for 10 minutes at 600 x g. The pellet was discarded and the supernatant containing most of the MAO activity was assayed using tyramine as the substrate.

Abbreviation: OTC - oxytetracycline.

in weight and protein content of treated livers in the lean mice was very much less than that observed in the livers of treated obese mice.

II. (i) A. 2. Subcellular fractionation studies:

It has been reported that MAO is synthesized on cytoribosomes and then later incorporated into mitochondria (109), so that the microsomal MAO may have a precursor-pool relationship to the mitochondrial MAO.

The tetracycline antibiotics have been reported to inhibit protein synthesis in cell-free preparations of rat liver by preventing the transfer of amino acid to the ribosomal protein (97). In addition, oxytetracycline has been reported to inhibit mitochondrial protein synthesis in rapidly proliferating mammalian tissue (73, 136). It was of interest, therefore, to determine and compare the effect of oxytetracycline on MAO activity in the different subcellular fractions.

The results obtained in this study are summarized in Table 17. Although a significant percentage of the activity is found in the microsomal fraction, the major part of the activity (77-85%) sedimented with the mitochondria in both treated and non-treated mice. This is in agreement with published results on adult rat liver homogenates (21). The data obtained showed that the effect of OTC-treatment previously observed in mitochondrial preparations was also

Table 17

Subcellular distribution of monoamine oxidase activity in livers of oxytetracyline-treated lean and obese mice

Group	Specific activity (units/mg protein)			
	Homogenate	Mitochondrial fraction	Microsomal fraction	Soluble fraction
lean, non-treated	1.32	5.37	1.59	no activity
lean, OTC-treated	0.80	3.37	0.75	no activity
obese, non-treated	2.95	10.48	2.32	no activity
obese, OTC treated	1.90	7.44	1.32	no activity

Values represent averages of assays done in duplicate on samples pooled from livers of 4 mice per group. Assays were done as described in the Methods section (VIII), using tyramine as the substrate.

Abbreviation: OTC - oxytetracycline.

manifested in the microsomal fraction.

II (i) A. 3. Mitochondrial monoamine oxidase activity in the liver and brain of OTC-treated mice and their controls:

It was of interest to make a comparative study of the effect of oxytetracycline treatment on the liver and a tissue in which there is no difference in MAO activity between non-treated lean and obese mice. The brain was chosen for this study, and the assays were performed on the mitochondrial fraction. A decrease in the specific activity of MAO was observed in the mitochondrial fractions of livers from OTC-treated mice, whereas the brain mitochondrial preparations did not show any significant change with treatment in either lean or obese animals (Table 18).

This difference in the effect of oxytetracycline in the two tissues may reflect the distribution of the drug in the body. The tetracyclines are removed from the blood by the liver (338), and then excreted into the intestine by way of the bile. The tetracyclines diffuse into the brain but their concentration in this tissue appears to depend on their lipid solubility, whereas in the liver there does not seem to be a distinct relation between lipid solubility and concentration. Thus, Barza et al. (16) have shown that oxytetracycline, the least lipid-soluble of the four tetracyclines they studied (minocycline, doxycycline, tetracycline and oxytetracycline), accumulates in the smallest

Table 18

Mitochondrial monoamine oxidase activity in the liver and brain of oxytetracycline-treated mice and their controls

Group	Monoamine Oxidase Activity units/mg protein	
	Liver	Brain
Lean		
Control	5.85 ± 0.50	5.27 ± 0.09
OTC-treated	3.83 ± 0.12	5.23 ± 0.13
Obese		
Control	10.25 ± 0.81	5.64 ± 0.14
OTC-treated	6.88 ± 0.45	5.92 ± 0.21

Results shown are the means ± SEM of 2 individual experiments on organs pooled from four animals per group. Enzyme activity was assayed using tyramine as the substrate. Units are defined in Section VIII of Methods.

amount in the brain, but does reach the highest concentration in the liver.

II. (i) A. 4. Activities of mitochondrial marker enzymes in the liver of oxytetracycline-treated lean and obese mice and their controls:

In order to establish whether or not the effect of oxytetracycline was also manifested on other mitochondrial enzymes, the activity of cytochrome c oxidase, a mitochondrial marker enzyme, was determined in OTC-treated mice and their controls. The results of this experiment are summarized in Table 19. Whereas the specific activity of MAO was again observed to be lower in the oxytetracycline-treated animals, no significant differences in the specific activity of cytochrome oxidase were observed between the treated animals and their controls. Thus, it appears that not all liver mitochondrial enzymes are affected in the same manner by OTC treatment.

II. (i) A. 5. Substrate specificity:

Some drugs, e.g. thyroxine, have been reported to exert a discriminative effect on monoamine oxidase activity (323), which varied with the substrate used in the enzyme assay. Lyles and Callingham (199) observed that cardiac MAO activity was increased by 47% when tyramine was used as the substrate, but only by 19% with benzylamine as the substrate. In young thyroxine-treated rats, Tong and D'Iorio (323) noted that the

Table 19

Activities of mitochondrial marker enzymes in the liver of oxytracycline-treated lean and obese mice and their controls

Group	Enzyme Activity (units/mg protein)		Mitochondrial Protein (mg/g liver)
	Monoamine oxidase	Cytochrome oxidase	
Lean, non-treated	5.40±0.44	1.80±0.02	5.74±0.26
Lean, OTC-treated	3.55±0.11*	1.85±0.15	5.89±0.27
Obese, non-treated	9.89±0.14	2.10±0.10	5.84±0.15
Obese, OTC-treated	6.90±0.19*	2.01±0.19	4.53±0.16**

Units of enzyme activity were defined in the Methods section. Monoamine oxidase was assayed with tyramine as the substrate. Values given are means ± SEM of individual experiments each assayed in duplicate. Mitochondrial fractions were prepared from tissue pooled from 4 to 8 animals per group.

* $P < 0.05$, when compared with non-treated animals.

** $P < 0.001$, when compared with non-treated obese mice.

decrease in liver MAO activity was 20% with serotonin and 44% with benzylamine. In order to determine if this differential effect was also observable in the case of oxytetracycline, the enzyme activity of liver mitochondrial preparations was assayed using three substrates: tyramine, serotonin, and benzylamine. The results obtained are presented in Table 20. As in previous experiments, when tyramine was used as the substrate, the decrease in specific activity was 32% in OTC-treated lean mice ($P < .005$) and 29% in treated obese animals ($P < .001$). With benzylamine, a 22% decrease was observed with obese mice ($P < .001$) and 27% with the lean group ($P < .005$). However, with serotonin, no significant difference was observed between treated and non-treated animals. These results would suggest that oxytetracycline exerts a differential effect on liver mitochondrial monoamine oxidase activity towards the substrates utilized in this experiment.

II. (i) B. Studies on the mechanism of action of oxytetracycline on monoamine oxidase.

1. Inhibitor studies:

Inhibitor dose-response studies were carried out with concentrations of clorgyline ranging from 10^{-9} to 10^{-3}M and enzyme activity was assayed using tyramine as the substrate. The results obtained (Figs. 10 and 11) showed no significant difference in inhibitor sensitivity between non-treated and

Table 20

In vivo effect of oxytetracycline on lean and obese mouse liver mitochondrial monoamine oxidase

Group	Specific Activity nmol/min/mg of protein		
	Tyramine	Serotonin	Benzylamine
Lean, non-treated	5.72 ± 0.41	0.85 ± 0.05	1.55 ± 0.06
Lean, OTC-treated	3.87 ± 0.08*	0.81 ± 0.02	1.13 ± 0.05*
Obese, non-treated	9.82 ± 0.14	1.30 ± 0.03	2.51 ± 0.08
Obese, OTC-treated	6.92 ± 0.41*	1.26 ± 0.05	1.97 ± 0.08*

Values given are means ± SEM for five animals from each of the groups studied. All assays were done in duplicate.

*$P < 0.005$, when compared with controls.

Abbreviation: OTC - oxytetracycline.

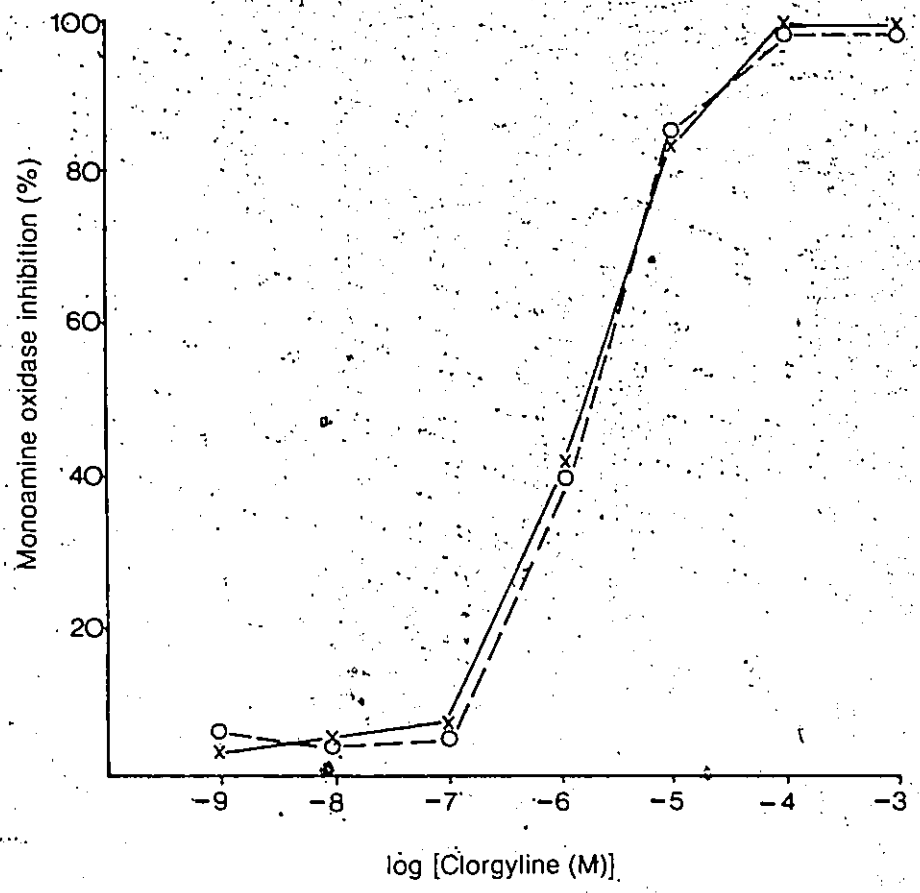

Figure 10. Inhibition in vitro of lean mouse liver mitochondrial monoamine oxidase activity by clorgyline. 0 — 0, non-treated; X — X, oxytetracycline-treated.

Mitochondrial preparations were pre-incubated at 37°C for 10 minutes in the presence of different concentrations of clorgyline before adding the substrate. Controls were pre-incubated under the same conditions in the absence of inhibitor. Tyramine was used as the substrate for the enzyme assays which were carried out as described in the Methods section (VIII).

The results given represent means of two separate experiments done in triplicate for each group.

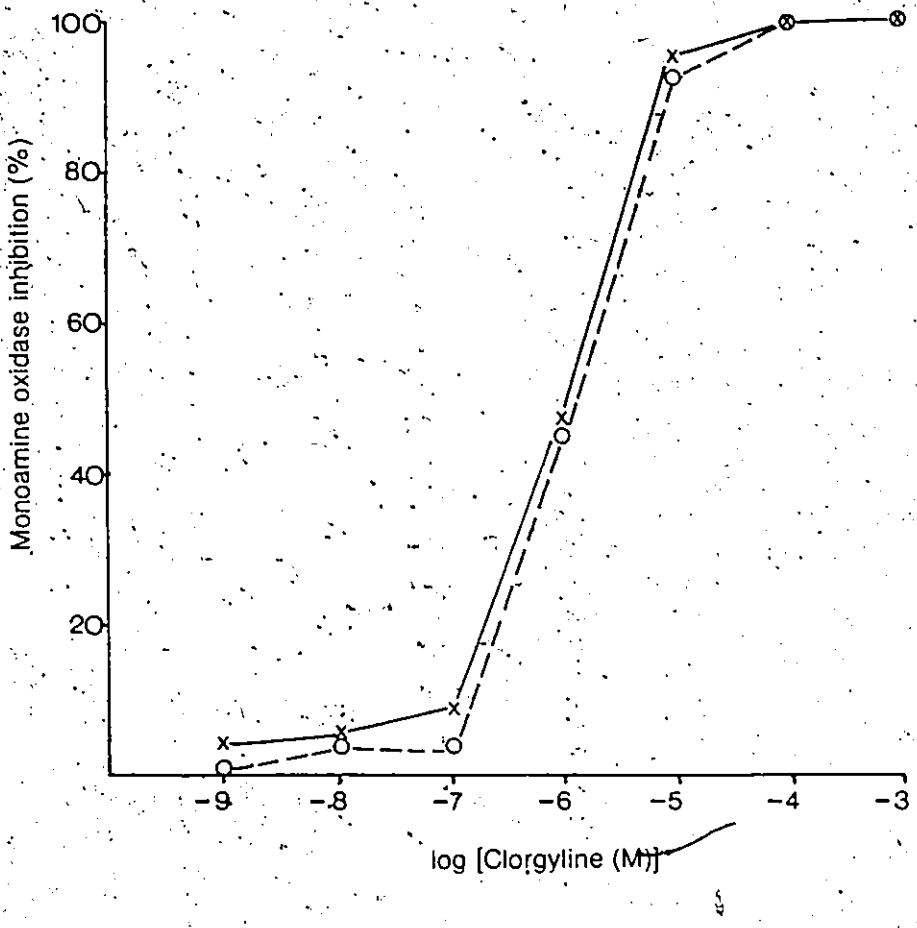

Figure 11. Inhibition in vitro of obese mouse liver mitochondrial monoamine oxidase activity by clorgyline. O — O, non-treated; X — X, oxytetracycline-treated.

Mitochondrial preparations were pre-incubated at 37°C for 10 minutes in the presence of different concentrations of clorgyline before adding the substrate. Controls were pre-incubated under the same conditions in the absence of inhibitor. Tyramine was used as the substrate for the enzyme assays which were carried out as described in the Methods section (VIII).

The results given represent means of two separate experiments done in triplicate for each group.

OTC-treated lean and obese mice. The observations in this study also support our previous findings (cf. Results and Discussion, section I.B.) that liver mitochondrial monoamine oxidase in this strain of mice is predominantly of the B-form.

II. (i) B. 2. Kinetic studies:

Since the observed decrease in MAO activity in OTC-treated mice may be due to an alteration of the kinetic properties of the enzyme, the V_{max} and apparent K_m of the enzyme towards the substrates affected by the treatment, i.e., tyramine and benzylamine, were determined for both groups of mice.

It appeared from the Lineweaver-Burk plots of the data from this study (Figs. 12, 13, 14 and 15) that treatment with oxytetracycline did not alter the affinity of MAO towards tyramine or benzylamine, since the K_m values for the enzyme from treated mice were identical to those of the non-treated groups. However, the V_{max} values for both substrates were decreased by OTC treatment (Table 21).

II. (i) B. 3. Direct inhibition of MAO activity:

The observed decrease in liver mitochondrial MAO activity after the administration of OTC may be the result of direct enzyme inhibition by the drug or an endogenous inhibitor produced by OTC treatment of the intact animal.

a. Mixing experiments:

The latter possibility was tested by mixing mitochondrial

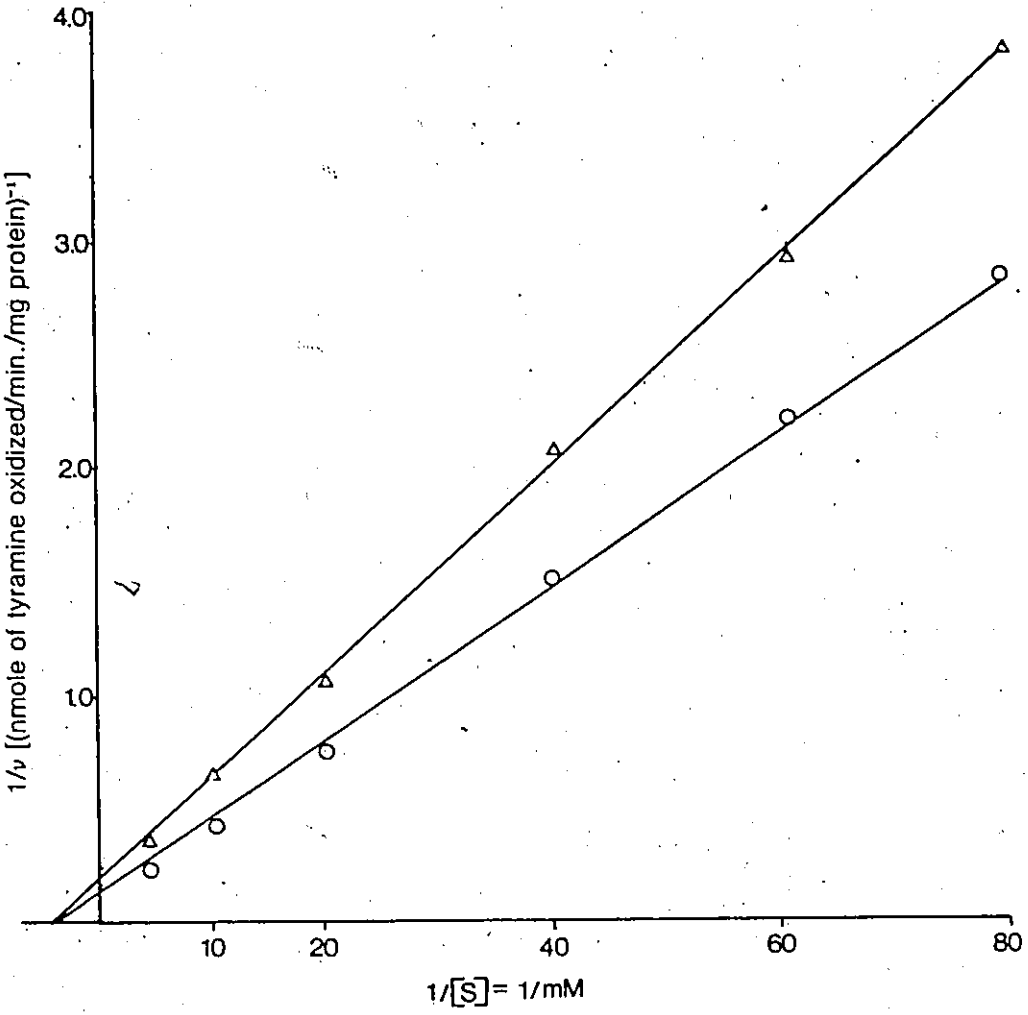

Figure 12. Effect of substrate concentration on the oxidative deamination of tyramine by liver mitochondria from nontreated lean mice (O — O) and OTC-treated lean mice (Δ — Δ). Assays were done as described in Methods (Section VIII).

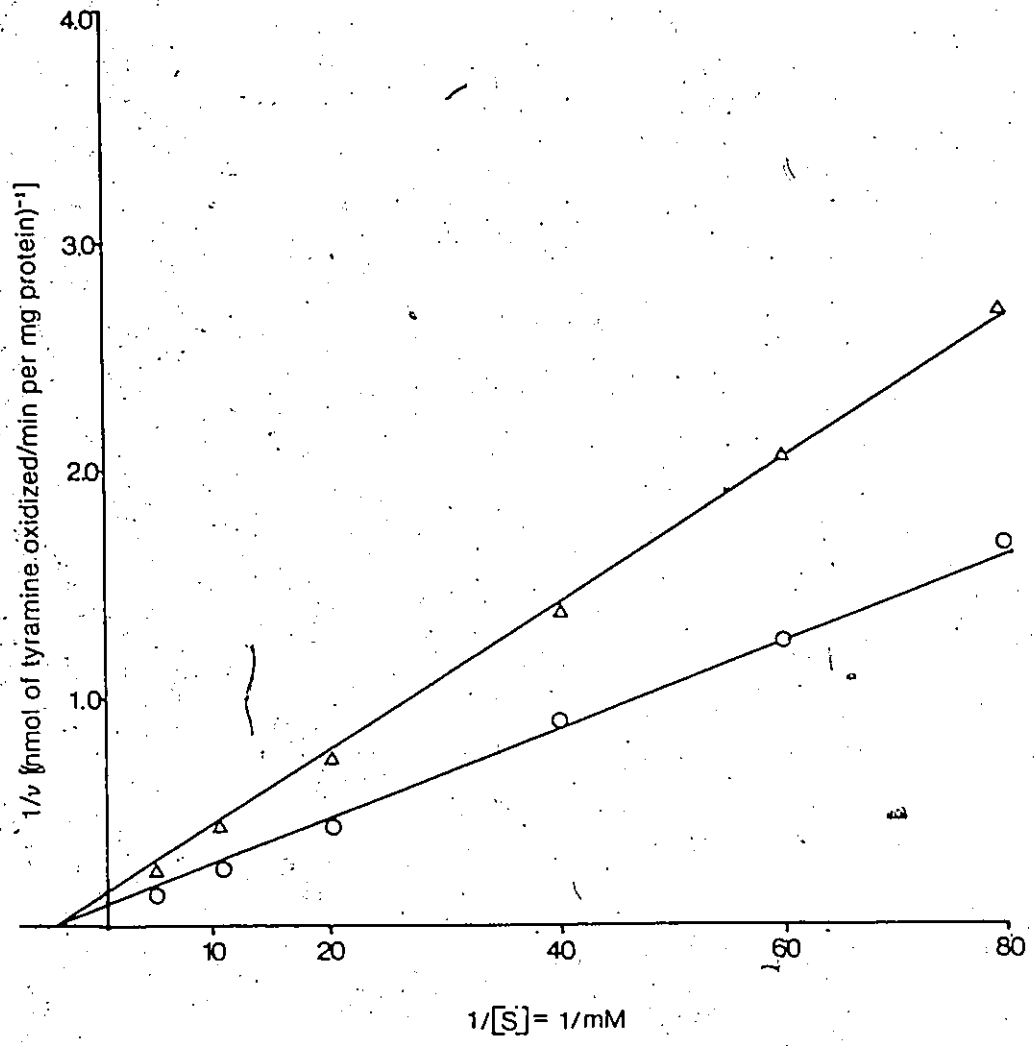

Figure 13. Effect of substrate concentration on the oxidative deamination of tyramine by obese mouse liver mitochondria. O — O, nontreated; Δ — Δ, OTC-treated. Assays were done as described in Methods (Section VIII).

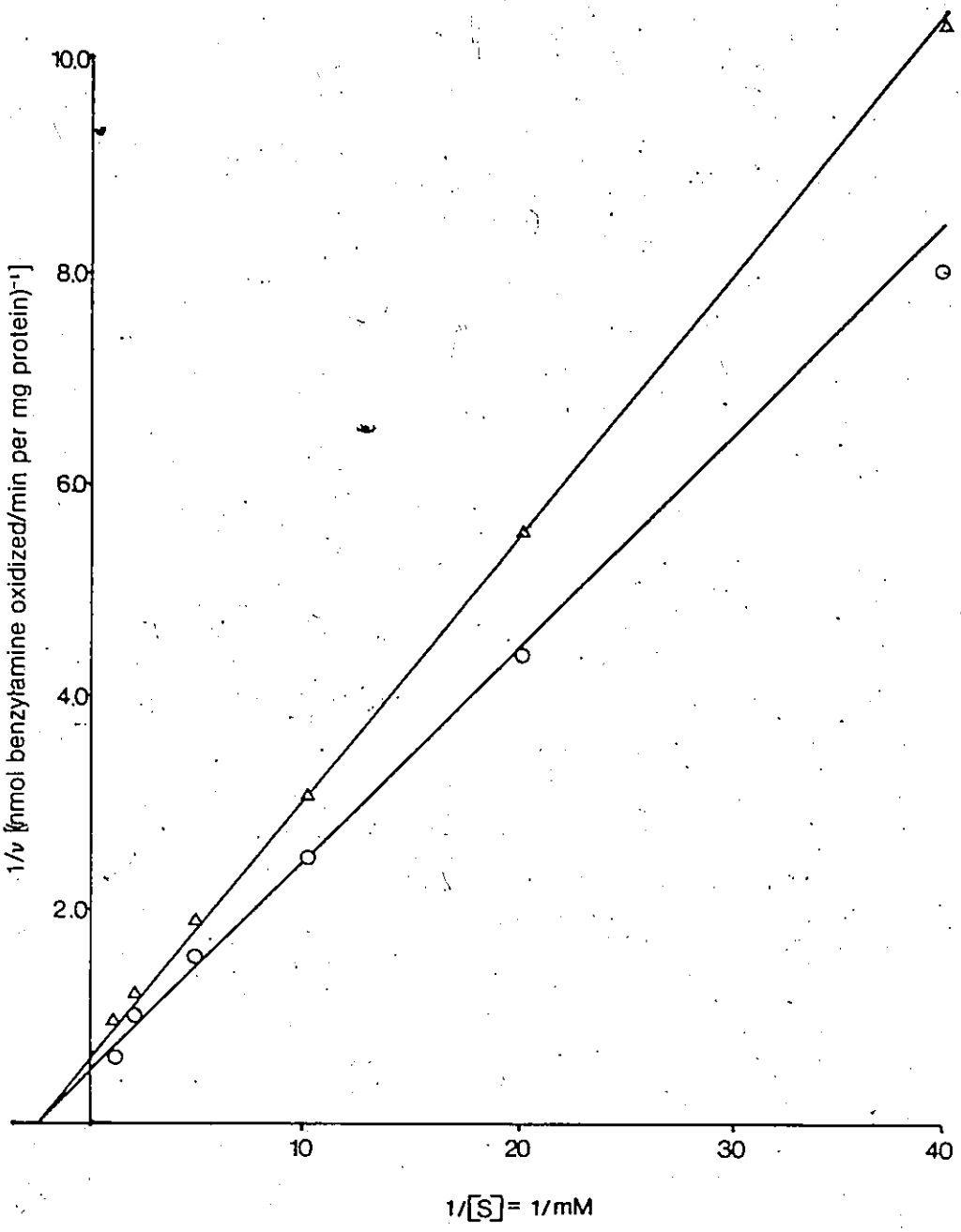

Figure 14. Effect of substrate concentration on oxidative deamination of benzylamine by lean mouse liver mitochondria. O — O, nontreated; Δ — Δ, OTC-treated. Assays were done as described in Methods (Section VIII).

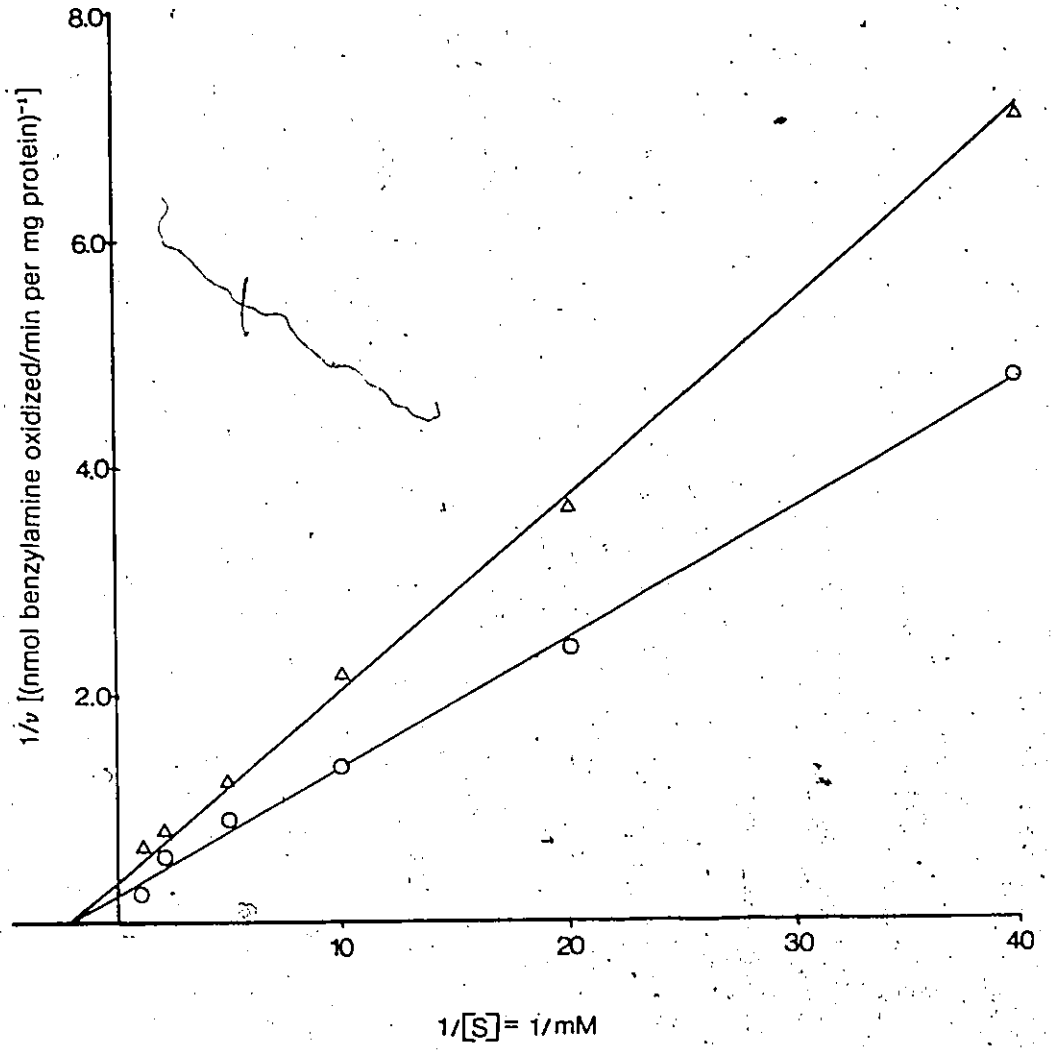

Figure 15. Effect of substrate concentration on the oxidative deamination of benzylamine by obese mouse liver mitochondria. O — O, non-treated; Δ — Δ, OTC-treated. Assays were done as described in Methods (Section VIII).

Table 21

Kinetic constants for tyramine and benzylamine oxidation by liver mitochondrial MAO in oxytetracycline-treated mice and their controls

Group	K_m (μM)		V_{max} (units/mg protein)	
	Tyramine	Benzylamine	Tyramine	Benzylamine
Lean, control	208	335	6.14	1.80
Lean, OTC-treated	208	335	4.52	1.44
Obese, control	210	333	10.29	3.05
Obese OTC-treated	210	335	6.54	2.03

Kinetic constants were obtained from Lineweaver-Burk plots of substrate concentrations vs. initial reaction velocities. Substrate concentrations ranging from 1 to 10^{-3} M were used. Units of enzyme activity were defined in Methods (section VIII).

Abbreviations: OTC - oxytetracycline

MAO - monoamine oxidase

preparations from the livers of non-treated animals with the cytosol from the lean-treated or obese-treated group, prior to assay for enzyme activity. Our results (Table 22) showed that the mitochondrial MAO activities of control animals were not affected by the soluble fraction from the OTC-treated animals. This observation would rule out direct inhibition of the enzyme by a soluble inhibitor in the livers of OTC-treated mice.

b. In vitro inhibition of monoamine oxidase by oxytetracycline:

The purpose of this study was to determine whether or not oxytetracycline can exert direct inhibitory action on monoamine oxidase that has already been incorporated into the mitochondrial membrane. The antibiotic (0.5 mmoles/mg protein) was added to mitochondrial preparations from non-treated animals, and the resulting mixture was pre-incubated at 37°C for 30 minutes, prior to assay of MAO activity.

Whereas a small decrease in enzyme activity towards serotonin was observed in vitro, no significant decrease in activity was observed towards tyramine and benzylamine in both lean and obese mice (Table 23), in contrast to the results obtained earlier in the in vivo experiments.

The concentration of oxytetracycline used in this study represents the maximum amount that would go into aqueous solution at pH 7.4 (1 mg/ml). Other investigators (73, 136), using twice as large a drug dose as that used by us in the

Table 22

Effect of the cytosol from oxytetracycline-treated animals on liver mitochondrial MAO activity in non-treated mice

Fraction	Specific Activity (units/mg. of protein) Lean	Obese
Mitochondrial fraction	5.56	8.18
Mitochondrial fraction + lean OTC cytosol	5.70	8.12
Mitochondrial fraction + obese OTC cytosol	5.35	7.86

The experiment consisted in mixing 1 part of mitochondrial fraction from non-treated animals with 7 parts cytosol from either the lean-treated or obese-treated group. Enzyme assays were done as described in the Methods section (VIII), using tyramine as the substrate.

Abbreviation: OTC - oxytetracycline.

Table 23

In vitro effect of oxytetracycline on lean and obese mouse liver mitochondrial monoamine oxidase

Group	Additions	Specific activity ($\frac{\text{nmol product/min}}{\text{mg of protein}}$) Tyramine	Serotonin	Benzylamine
Lean	none	5.22 ± 0.26	1.32 ± 0.01	1.39 ± 0.67
Lean	OTC (1mg/ml)	4.37 ± 0.17	1.02 ± 0.05[1]	1.73 ± 0.13
Obese	none	10.05 ± 0.06	1.58 ± 0.05	2.47 ± 0.06
Obese	OTC (1mg/ml)	9.16 ± 0.93	1.42 ± 0.08[2]	2.07 ± 0.17

Values given are means ± SEM for three samples pooled from 4 to 6 animals for each group.

[1]Significantly different from lean control group, $P < 0.025$.

[2]Significantly different from obese control group, $P < 0.02$.

Prior to enzyme assay, oxytetracycline was added to the incubation mixture to achieve a concentration of 1 mg OTC/ml. The resulting mixture was pre-incubated at 37°C for 30 minutes, then assayed for MAO activity as described in Methods (Section VIII).

Abbreviation: OTC - oxytetracycline.

in vivo treatment of mice with OTC, report a drug concentration of 6 µg OTC per ml of serum in treated animals. It appears, therefore, that the above observations in vitro would argue against the direct inhibition by OTC of the tyramine- and benzylamine-specific MAO activity in the intact animal.

II. (I) B. 4. Fatty acid composition of obese mouse liver mitochondrial phosholipids:

Oxytetracycline has been previously reported to alter lipid metabolism in obese-mouse liver (22, 23), and since another antibiotic, chloramphenicol, has been shown to inhibit and alter the acylation of phospholipid precursors in BHK-21 cultured cells (189), it was thought that OTC treatment might produce significant changes in the fatty acid composition of liver mitochondrial phospholipids in the obese mice.

These studies were done on OTC-treated mice and two groups of controls - the non-treated mice and the food-restricted controls. The results which had been obtained with non-treated mice (cf. Tables 24, 25, 26 and 27) suggested that arachidonic acid may be responsible for the enhanced MAO activity in obese-mouse liver mitochondria. Arachidonate is synthesized from linoleic acid, an essential fatty acid. Since previous investigators (23) have observed that OTC-treated mice consume approximately 25% less food than non-treated mice, it was considered important to

Table 24

Fatty acid composition of phosphatidylcholine in liver mitochondria of oxytetracycline-treated obese mice and their controls

Fatty Acid	Group		
	Obese, non-treated	Obese, food-restricted	Obese, OTC-treated
14:0	<1	<1	<1
16:0	23.6 ± 1.34	23.0 ± 2.40	21.0 ± 2.04
16:1	<1	<1	<1
18:0	23.8 ± 0.94	21.6 ± 1.70	22.4 ± 2.06
18:1	12.7 ± 1.12	11.2 ± 0.60	13.0 ± 0.58
18:2	9.4 ± 0.27	10.8 ± 0.40	14.3 ± 2.54*
18:3	0	0	0
20:4	16.4 ± 0.76	17.8 ± 1.40	15.8 ± 1.73
22:6	9.2 ± 0.40	11.4 ± 2.30	11.0 ± 2.12

Results given represent means ± SEM of three to five different samples, and are expressed as percentage of the total fatty acids in the phosphatidylcholine fraction.

Abbreviations: OTC - oxytetracycline. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, while linoleic acid is designated as 18:2.

*Significantly different from non-treated group, $P < 0.05$.

Table 25

Fatty acid composition of phosphatidylinositol + phosphatidylserine in liver mitochondria of oxytetracycline-treated obese mice and their controls

Fatty Acid	Group		
	Obese, non-treated	Obese, food-restricted	Obese, OTC-treated
14:0	<1	<1	<1
16:0	10.5 ± 1.74	8.6 ± 1.05	8.2 ± 1.79
16:1	<1	<1	<1
18:0	45.0 ± 2.82	45.2 ± 2.00	50.0 ± 1.67
18:1	6.8 ± 0.76	8.3 ± 1.80	8.9 ± 1.66
18:2	2.8 ± 0.49	3.6 ± 0.20	3.2 ± 0.98
18:3	0	0	0
20:4	28.8 ± 2.90	25.4 ± 2.95	24.5 ± 4.85
22:6	4.2 ± 0.40	3.5 ± 0.54	3.8 ± 0.50

Values given are means ± SEM of three to five different samples, and are expressed as percentage of the total fatty acids in the phosphatidylinositol + phosphatidylserine fraction.

Abbreviations: OTC - oxytetracycline. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, and oleic acid as 18:1.

Table 26

Fatty acid composition of phosphatidylethanolamine + phosphatidylglycerol in liver mitochondria of oxytetracycline-treated obese mice and their controls

Fatty Acid	Group		
	Obese, non-treated	Obese, food-restricted	Obese, OTC-treated
14:0	<1	<1	<1
16:0	13.9 ± 2.24	11.8 ± 1.25	14.1 ± 0.68
16:1	<1	<1	<1
18:0	34.5 ± 4.11	32.4 ± 2.40	31.0 ± 2.54
18:1	10.7 ± 0.94	9.5 ± 0.46	9.6 ± 0.60
18:2	4.5 ± 0.61	3.3 ± 0.50	5.2 ± 0.62
18:3	0	0	0
20:4	22.7 ± 2.24	26.6 ± 1.10	24.2 ± 0.69
22:6	16.0 ± 2.80	14.4 ± 1.40	13.4 ± 1.62

Values given are means ± SEM of three to five different samples, and are expressed as percentage of the total fatty acids in the phosphatidylethanolamine + phosphatidylglycerol fraction.

Abbreviations: OTC - oxytetracycline. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, and oleic acid as 18:1.

Table 27

Fatty acid composition of cardiolipin + phosphatidic acid in liver mitochondria of oxytetracycline-treated obese mice and their controls

Fatty Acid	Group		
	Obese, non-treated	Obese, food-restricted	Obese, OTC-treated
14:0	<1	<1	<1
16:0	12.2 ± 2.24	10.6 ± 1.60	11.9 ± 1.04
16:1	4.9 ± 1.34	3.1 ± 0.55	2.1 ± 0.17
18:0	7.0 ± 1.11	6.7 ± 0.65	9.7 ± 0.29
			P < 0.02
18:1	28.6 ± 2.95	20.4 ± 0.80	19.9 ± 1.39
	P < 0.05		
18:2	35.0 ± 5.13	42.3 ± 4.25	37.7 ± 2.54
18:3	0	0	0
20:4	7.7 ± 0.98	4.8 ± 0.40	6.3 ± 0.81
	P < 0.05		
22:6	4.6 ± 0.80	4.8 ± 1.00	5.4 ± 1.10

Values given are means ± SEM of three to five different samples, and are expressed as percentage of the total fatty acids in the cardiolipin + phosphatidic acid fraction.

Abbreviation: OTC - oxytetracycline. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, and oleic acid as 18:1.

include a group of food-restricted controls in the study. These animals were allowed to consume only 4.5 g of Purina Chow per day, the reported average daily consumption of OTC-treated mice (23).

The data from this series of experiments are presented in Tables 24, 25, 26 and 27. Treatment produced an increase in linoleic acid in PC, whereas food restriction resulted in a decrease in arachidonic acid and oleic acid in the CL + PA fraction. A greater amount of stearic acid in the CL + PA fraction was observed in the OTC-treated mice as compared to the food-restricted animals. No changes were observed in the PI + PS and PE + PG fractions. Oxytetracycline treatment did not result in a significant decrease in arachidonic acid in any of the fractions studied.

Cardiolipin has been reported to be localized predominantly on the inner mitochondrial membrane (54), and it is generally believed that monoamine oxidase is bound to the outer membrane (273). Therefore, it may not be possible to relate the changes in the CL + PA fraction to the decrease in MAO activity brought about by OTC treatment.

Linoleic acid is an essential fatty acid, and cannot, therefore, be synthesized endogenously by the obese mouse. Purina Chow has a high linoleate content (cf. Table 2); however, the observed increase in linoleic acid cannot be attributed to an enhanced food intake, since as cited earlier, OTC-treated obese mice have been found to ingest less food

than their non-treated counterparts. The rise in linoleic acid content in the PC fraction may reflect an enhanced acylation of phosphatidylcholine by linoleic acid and/or a block in the biosynthetic pathways that use linoleic acid as a precursor.

Arachidonic acid is the fatty acid that was found to be present in significantly greater quantity in the PC, PE + PG, and PI + PS fractions of non-treated obese-mouse liver mitochondria when compared to lean-mouse samples. Since there was no significant decrease in arachidonate, it appears that OTC treatment produced the observed decrease in MAO activity by a mechanism that does not seem to involve an alteration of mitochondrial fatty acid composition.

II. (ii). Discussion:

The effect of OTC treatment on mitochondrial enzymes appeared to be selective. Whereas the specific activity of MAO was observed to be lower in the oxytetracycline-treated animals, no significant difference was noted in the specific activity of cytochrome oxidase activity. Oxytetracycline has been reported to inhibit mitochondrial protein synthesis in cultured rat heart cells at concentrations (20 µg/ml) approximately three times that achieved by *in vivo* administration in rats of 200 mg OTC/kg/day I.M. (73). In the intact animal, a decrease in mitochondrial protein has been observed in rapidly proliferating tissue such as the regenerating

liver of partially hepatectomized rats (73), or brown adipose tissue of rats during cold acclimation (136). In the liver of treated obese mice, a 22% decrease in mitochondrial protein on a per-gram-liver basis was observed, whereas no decrease was observed in preparations from treated lean animals. In this respect, Himms-Hagen (136) observed that in cold-acclimated rats, oxytetracycline treatment decreased the activity of cytochrome oxidase in brown adipose tissue, but not in liver or muscle, and has attributed this response to a preferential action of the drug for rapidly dividing tissue. Thus, the difference in response between lean and obese mice may be associated with the hyperplasia that has been observed in obese-mouse liver (84).

It has been previously claimed that the mitochondrial protein-synthesizing system is involved in the formation of enzymatically active or spectrally recognizable cytochrome c oxidase (73). Although one could reasonably predict a decrease in total activity of cytochrome oxidase in treated obese animals due to the decrease in mitochondrial protein, a change in specific activity would result only if factors other than the decrease in mitochondrial protein also affect enzyme activity subsequent to oxytetracycline treatment. It has been reported that the apoprotein of cytochrome oxidase is synthesized on the cytoribosomes (73). Since there was no decrease in enzyme specific activity in treated obese animals, it appears that the <u>in vivo</u> OTC treatment did not

affect the synthesis of the enzyme on the cytoribosomes.

Treatment also had a differential effect on MAO activity which varied with the substrate used. A decrease in specific activity was observed towards benzylamine and tyramine, but not towards serotonin. As cited in Section I.B. of Results and Discussion, it appears that lean- and obese-mouse liver MAO is predominantly, or even exclusively, Form B. It was also noted that the observed activity towards serotonin which is generally regarded as a substrate for Form A, could be due, to Form B of MAO. The difference in response between serotonin- and the other amine-metabolizing activities to in vivo treatment may suggest a difference in the mechanism of drug action towards these substrates. It is probably relevant to recall the difference in structure between serotonin and the other amines used in these experiments. Serotonin possesses an indole nucleus in contrast to tyramine and benzylamine which have a benzene nucleus. Since the data obtained with the non-treated mice suggests that the same enzyme species is involved in the deamination of serotonin, and there is no apparent reason to conclude that different active principles were produced by treatment, it is not inconceivable that the discriminative action observed in vivo is due to the difference in structures of the amines used in the study.

The decrease in activity towards benzylamine and

tyramine did not appear to be attributable to a change in the affinity of MAO towards these substrates, since the apparent K_m values were not altered in the treated animals. Furthermore, the decreased enzyme activity towards these amines in the intact treated animals did not appear to result from a direct inhibition by OTC or from an endogenous inhibitor resulting from chronic treatment with the drug. The differences in mitochondrial MAO activity between treated and non-treated mice cannot be explained on the basis of a different intracellular distribution of the enzyme, since the pattern of distribution of activity was similar in all subcellular fractions tested from treated and non-treated animals.

The antibiotic chloramphenicol has been reported to inhibit rat liver mitochondrial monoamine oxidase in vitro and in vivo (15). It has also been reported by Lipton and McMurray (189) that BHK-21 cells cultured in chloramphenicol, and in particular, the mitochondria of these cells show reduced labelling of phospholipids with $^{32}P_i$, [^{3}H] glycerol, [^{14}C] acetate, [^{14}C] linoleate or [^{14}C] palmitate. This observation was general to all phospholipids of all subcellular fractions, and was attributed to the inhibition of mitochondrial and to a lesser extent, microsomal capacity, to acylate sn-glycerol 3-phosphate and dihydroxyacetone phosphate. Mukherjee and Mukherjee (219) observed that the incorporation of injected ^{14}C-labelled palmitate in liver

phospholipids decreased in the intact tetracycline-treated rat. In view of these published results, and the observation made by Bégin-Heick et al. (22) on the effects of oxytetracycline treatment on lipid metabolism in the obese-hyperglycemic mouse, it was thought that oxytetracycline treatment might also alter the lipid environment of MAO in liver mitochondria in the obese mouse.

In our work with the non-treated mice, it was observed that arachidonic acid was present in much larger quantity in obese-mouse liver mitochondrial phospholipids than in the corresponding lean mouse samples (cf. Tables 11, 12, 13 and 14), and it appeared distinctly possible that the lipid dependency suggested by the results obtained with acetone-extracted mitochondria (cf. Results and Discussion, Section I.D.1.) might be due to this difference. This study was undertaken to find out if treatment would result in changes in the fatty acid composition of liver mitochondrial phospholipids, and if these changes could be related to changes in MAO activity. OTC treatment, however, did not affect the arachidonate content of any of the phospholipid fractions studied. The changes observed were small and did not show a trend which would lead us to believe that they were physiologically significant. As such, they do not appear to be associated with the large decrease in total activity of MAO in the obese mouse to the level of that in the non-treated lean mouse.

III. COLD - EXPOSED MICE

As stated in section I.B. of the Review of Literature, biological membranes can be considered as fluid mosaics of various proteins imbedded in, or on the surface of a predominantly phospholipid bilayer matrix. This matrix must be sufficiently fluid to allow free movement of various enzymes and transport proteins in the bilayer, but must not be so free-flowing that the structural integrity of the membrane is threatened (217). The degree of fluidity of the matrix is determined partly by the nature of fatty acids of the membrane phospholipids (217, 299, 336).

An inverse relationship between temperature and desaturation has been observed repeatedly in organisms ranging from bacteria, protozoa, fungi and algae to higher plants and animals. This inverse correlation has been noted in a wide variety of poikilotherms (128). In warm-blooded animals, an adjustment has also been reported to take place. Fox (96) observed that there is a temperature gradient in the legs of the reindeer: the highest temperature being near the body, and the lowest near the hooves. To compensate for this gradient, the cells near the hooves have membranes whose phospholipids are enriched in unsaturated fatty acids. There seems little doubt that these phenomena represent temperature adaptations on the part of the organisms to maintain the integrity and function of cell membranes (217).

As mentioned earlier, the activity of MAO appears to be regulated by its lipid environment in the mitochondrial membrane (30, 43, 80, 169, 224, 310). Studies by Trayhurn and James (cf. Review of Literature section I.C.7) demonstrated that at 4°C ob/ob mice die rapidly of hypothermia, because of a reduced capacity for heat production. The same investigators have shown, however, that survival at 12°C was longer, although the body temperature of the mice dropped to 31°C. In this part of the project, studies were done on cold-exposed mice that were housed in a cold room maintained at 14 ± 1°C for two weeks, while the controls were maintained at 24 ± 1°C during the same period. Obese mice have been reported to be hypothermic at 24°C (153, 325). Since an increase in the unsaturation of mitochondrial phospholipids and enhanced MAO activity were observed in obese mice at 24 ± 1°C, it was decided to determine if a further reduction of their core temperature by chronic exposure to cold would result in a further change in fatty acid unsaturation and liver MAO activity.

III (i). Results:

A. Hepatic mitochondrial monoamine oxidase activity in cold-exposed lean and obese mice and their controls:

Monoamine oxidase activity of mitochondrial preparations from livers of cold-exposed lean and obese mice and their controls was assayed with the substrates tyramine, serotonin

and benzylamine, as described in Methods (section VIII). Table 28 summarizes the results obtained in this study. In both lean and obese samples, no significant changes in MAO activity were observed towards any of the amine substrates used.

III (i) B. Fatty acid composition of liver mitochondrial phospholipids in cold-exposed obese mice and their controls:

Inasmuch as chronic exposure of obese mice to the cold has been reported to result in a lowering of their core temperature, it was decided to check the effect of exposure to 14 ± 1°C for two weeks on the fatty acid composition of the liver mitochondrial phospholipids of these animals. The percentages of the different fatty acids contained in the phospholipid fractions studied are presented in Table 29, 30, 31 and 32.

The percentage of each fatty acid in the PI + PS and CL + PA fractions was similar in preparations of warm- and cold-exposed animals. However, the proportion of oleic acid (18:1) in PC and docosahexaenoic acid in PE + PG (22:6) was smaller in samples from cold-exposed animals than in the controls.

III (i) C. Arrhenius plots of the activity of liver mitochondrial MAO from non-treated lean and obese mice:

As stated previously, Trayhurn and James (325) have reported that chronic cold exposure of obese mice at 12°C had lowered their core temperature to 31°C. Such a lowering

Table 28

Effect of cold-exposure on liver mitochondrial mononamine oxidase activity in lean and obese mice

Group	Monoamine oxidase activity units/mg protein					
	Tyramine		Serotonin		Benzylamine	
	W	C	W	C	W	C
Lean	4.88±0.01	3.99±0.21	1.58±0.09	1.38±0.07	1.70±0.11	1.54±0.07
Obese	11.00±1.61	9.66±1.61	2.07±0.12	1.84±0.02	2.34±0.45	2.37±0.44

Values are means ± SEM of two individual experiments on samples pooled from 4 to 6 animals per group. Assays were done in triplicate. Units were defined in Methods (section VII).

Abbreviations: C, cold-exposed at 14 ± 1°C;

W, controls maintained at 24 ± 1°C.

Table 29

Fatty acid composition of phosphatidylcholine in liver mitochondria of cold-exposed obese mice and their controls

Fatty Acid	Group W	Group C
14:0	<1	<1
16:0	30.3 ± 3.84	28.0 ± 2.68
16:1	0.7 ± 0.23	1.2 ± 0.31
18:0	29.8 ± 2.04	34.0 ± 3.16
18:1	15.0 ± 1.31	9.8 ± 1.10*
18:2	9.1 ± 1.73	11.7 ± 1.73
18:3	0	0
20:4	10.1 ± 1.71	8.9 ± 0.93
22:6	5.0 ± 0.51	5.4 ± 0.02

Results are means ± SEM of two individual experiments on 4 to 6 animals per group, and are expressed as percentage of the total fatty acids in the phosphatidylcholine fraction.

Abbreviations: C, cold-exposed. W, controls. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, palmitic acid is designated as 16:0, and oleic acid as 18:1.

*Significantly different from controls, $P < 0.05$.

Table 30

Fatty acid composition of phosphatidylinositol + phosphatidylserine in liver mitochondria of cold-exposed obese mice and their controls

Fatty Acid	Group W	Group C
14:0	<1	<1
16:0	10.7 ± 2.80	9.5 ± 2.50
16:1	<1	<1
18:0	73.6 ± 1.70	75.6 ± 1.53
18:1	3.6 ± 0.39	3.8 ± 0.25
18:2	1.4 ± 0.22	1.2 ± 0.13
18:3	0	0
20:4	9.3 ± 1.74	9.4 ± 1.33
22:6	1.2 ± 0.35	0.8 ± 0.35

Results are means ± SEM of two individual experiments on 4 to 6 animals per group, and are expressed as percentage of the total fatty acids in the phosphatidylinositol + phosphatidylserine fraction.

Abbreviations: C, cold-exposed, W, controls. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, stearic acid is designated as 18:0 and linoleic acid as 18:2.

Table 31

Fatty acid composition of phosphatidylethanolamine + phosphatidylglycerol in liver mitochondria of cold-exposed obese mice and their controls

Fatty Acid	Group W	Group C
14:0	<1	<1
16:0	13.4 ± 0.43	15.9 ± 0.43
16:1	<1	<1
18:0	30.2 ± 3.03	35.5 ± 4.58
18:1	17.1 ± 2.82	16.4 ± 0.89
18:2	0.8 ± 0.01	1.0 ± 0.08
18:3	0	0
20:4	26.7 ± 4.50	24.5 ± 2.58
22:6	11.5 ± 1.05	6.4 ± 1.00*

Results are means ± SEM of two individual experiments on 4 to 6 animals per group, and are expressed as percentage of the total fatty acids in the phosphatidylethanolamine + phosphatidylglycerol fraction.

Abbreviations: C, cold-exposed, W, controls. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, stearic acid is designated as 18:0, and linoleic acid as 18:2.

*$P < 0.01$, when compared with controls.

Table 32

Fatty acid composition of cardiolipin + phosphatidic acid in liver mitochondria of cold-exposed obese mice and their controls

Fatty Acid	Group W	Group C
14:0	<1	<1
16:0	9.8 ± 1.94	12.0 ± 1.72
16:1	1.8 ± 0.21	2.0 ± 0.13
18:0	8.3 ± 0.64	9.6 ± 2.55
18:1	22.4 ± 0.47	19.9 ± 2.81
18:2	48.9 ± 3.43	45.8 ± 3.19
18:3	0.4 ± 0.30	1.4 ± 0.68
20:4	2.9 ± 0.27	3.1 ± 0.39
22:6	4.1 ± 0.77	5.3 ± 0.85

Results are means ± SEM of two individual experiments on 4 to 6 animals per group, and are expressed as percentage of the total fatty acids in the cardiolipin + phosphatidic acid fraction.

Abbreviations: C, cold-exposed; W, controls. Fatty acids are designated by the number of carbon atoms and double bonds in the chain. Thus, stearic acid is designated as 18:0 and linoleic acid as 18:2.

of body temperature would normally be expected to result in an adjustment of the fatty acid composition of membranes. Since the changes observed in the fatty acid composition of the liver mitochondrial phospholipids were minor, and were not accompanied by physiologically significant changes in MAO activity, it was decided to check the effect of temperature on enzyme activity in samples from non-treated obese mice. Enzyme activity was determined at different temperatures between 7° and 37°C. This study was run in parallel with samples from non-treated lean mice.

Arrhenius plots for several membrane-bound enzymes are biphasic or multiphasic, and the slopes either intersect or are discontinuous at unique transition temperatures. These 'break' points, detected either by enzymatic or physical methods are closely associated with lipid-phase separations within the membrane (143, 308), and the temperatures at which these occur are related to membrane composition (284). The data obtained in this study were analyzed by the method of least-squares, and were best fitted by two intersecting straight lines (Fig. 16). The transition temperature appeared at 22.9°C for both lean- and obese mouse preparations, and the values for Arrhenius activation energy, E_a, did not differ significantly between the two groups of animals, above and below this temperature (Table 33).

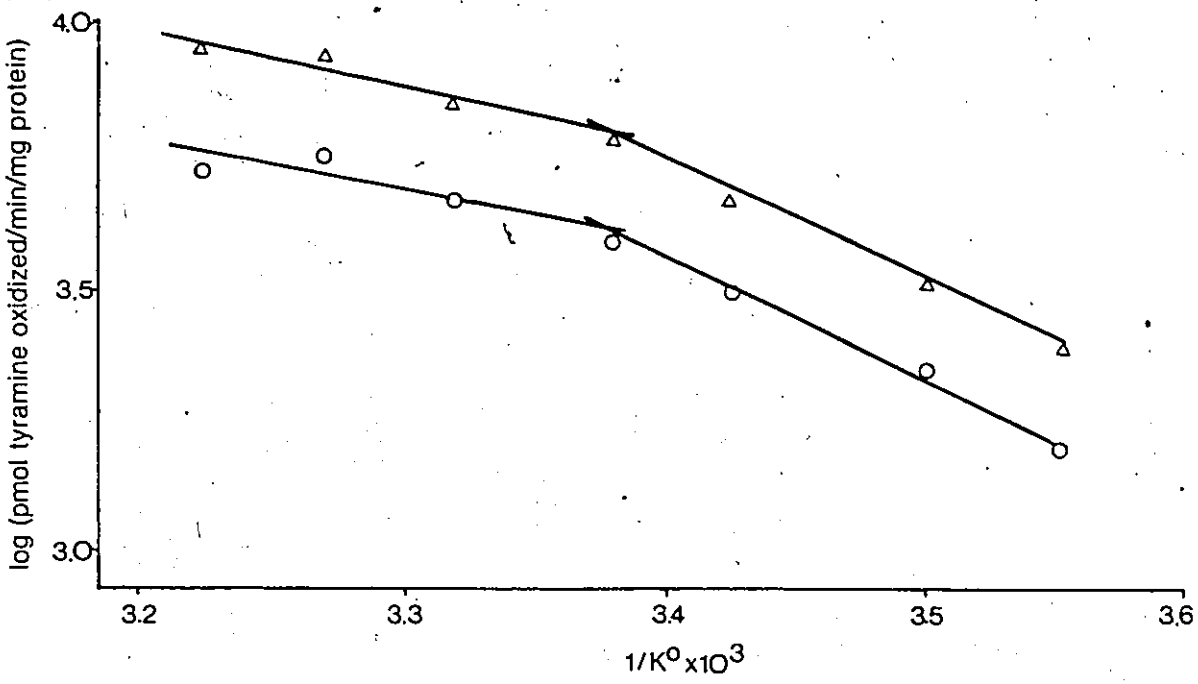

Figure 16. Arrhenius plots of monoamine oxidase activity in lean- and obese-mouse liver mitochondria.

Monoamine oxidase was assayed using tyramine as substrate as described in Methods (section VIII). All points on the plot are the average of two determinations which varied by less than 5% from each other.

Table 33

Transition temperatures and energies of activation for liver mitochondrial monoamine oxidase in lean and obese mice

	Group	
	Lean	Obese
Transition temperature (°C)	22.9°	22.9°
Activation energy below transition temperature (kcal/mol)	11.4	10.6
Activation energy above transition temperature (kcal/mol)	5.3	4.9

III (ii). Discussion:

Chronic cold exposure did not alter MAO activity in the lean mice (Table 28). The lean animals have no difficulty maintaining their body temperature within the normal range (36.1 - 36.7°C) during cold exposure (325). On this basis, there is no requirement for an adjustment of the fatty acid composition of cell membranes in the lean mouse, and if the expression of MAO activity is dependent on the nature of fatty acids in its microenvironment, then there should indeed be no change in MAO activity. On the other hand, ob/ob mice have been reported to experience a reduction in core temperature to 31°C, on chronic exposure to 12°C (325). In the light of results obtained in other experimental models (96, 127), such a drop in body temperature should be accompanied by an increase in unsaturated fatty acid content of membrane lipids. Such a change was not observed (Tables 29 to 32), for mitochondrial phospholipids nor was there any physiologically significant alteration of MAO activity in these animals (Table 28).

It appears, from the Arrhenius plots (Fig. 16) that the transition temperature for liver mitochondrial lipids in both groups of animals is far below their physiological temperatures. It seems possible, therefore, that after treatment, monoamine oxidase was still functioning *in vivo* in a largely fluid lipid environment. This factor may

explain why in the obese mice, there was no physiologically significant change in MAO activity or increase in unsaturated fatty acid content of mitochondrial phospholipids, in spite of the lower body temperature of these animals.

There is no obvious reason as to why exposure to cold produced a decrease in 18:1 in PC and 22:6 in PE + PG but similar changes have been reported for other systems. Patton and Platner (241) have reported a decrease in 22:6 of liver mitochondria in cold-acclimated rats, and a decrease in total unsaturation of mitochondrial fatty acids in the same animals. Williams and Platner (345) have previously observed an increase in unsaturation of white fat in cold-adapted rats and hamsters; and have postulated that one of the possible reasons is the preferential assimilation from plasma of unsaturated fatty acids by white adipose tissue, and that a possible source for these fatty acids may be the liver. Cold-acclimation, however, has been demonstrated to decrease hepatic lipogenesis in rats (208, 209) and hamsters (72). Chronic cold exposure may have had the same effect on hepatic lipogenesis in ob/ob mice and caused the observed decrease in 18:1 and 22:6. A decrease in liver weights on a mg liver/g body weight basis was indeed observed in cold-exposed obese mice (Table 34) despite an increase in food intake (Table 35), which may be indicative of a mobilization of energy reserves from the liver. The decrease in 18:1 and 22:6 may simply reflect a reduced

Table 34

Effect of cold-exposure on body weights and liver weights of lean and obese mice

Group	Body Weights (g)		Liver Weight (g)		Liver Wt./Body Wt. (mg/g)	
	W	C	W	C	W	C
Lean	28.3±0.46 (12)	29.3±0.92 (12)	1.61±0.06 (12)	1.92±0.06 (12)	57.1±2.58	65.9±2.01
	NS		P < 0.001		P < 0.01	
Obese	54.0±0.40 (8)	50.1±1.13 (8)	4.70±0.14 (8)	3.23±0.16 (8)	75.9±5.23*	64.3±2.47
	P < 0.001		P < 0.005		P < 0.005	

*P < 0.001, when compared to warm-acclimated lean group.

**NS, when compared to cold-exposed lean group.

The numbers in parentheses represent the number of animals in each group, and the data given are means ± SEM for the numbers specified.

Abbreviations: C, cold-exposed animals; W, controls; Wt., weight; NS, not significant.

Table 35

Daily food consumption of cold-exposed lean and obese mice and their controls

Group	W g/day/mouse	C g/day/mouse
Lean (12)	4.19 ± 0.19	7.53 ± 1.34
	$P < 0.05$	
Obese (8)	7.41 ± 0.30*	9.18 ± 0.42**
	$P < 0.01$	

The numbers in parentheses represent the number of animals in each group, and the data given are means ± SEM for the numbers specified.

*$P < 0.001$, when compared to warm-acclimated lean mice.

**NS, when compared to cold-exposed lean mice.

Abbreviations: C, cold-exposed; W, controls; NS, not significant.

availability of unsaturated fatty acids for phospholipid synthesis.

The fact that there was no significant change in MAO activity despite the decrease in 18:1 and 22:6 in two of the major phospholipids found in mouse liver mitochondria may suggest that these two fatty acids are not found in the immediate lipid environment of monoamine oxidase, or if present therein, they do not function in the modulation of MAO activity in the obese mice. As mentioned earlier (cf. Review of Literature, section I.C. 7), the obese mouse has a reduced capacity for thermogenesis and its body temperature, though normal at 34°C, is depressed at ambient temperatures of 20 - 25°C. The importance of making an adjustment to this lower setting for body temperature is highlighted by the reported higher content of unsaturated fatty acids in the mitochondrial lipids of poikilotherms (257). This observation has been correlated to the ability of poikilotherms to adapt to the lowering of their body temperature following exposure to lower environmental temperatures. The higher content of unsaturated acids in the liver mitochondria of non-treated obese mice as compared to their lean controls may be part of a general mechanism in obese animals to adjust to the lower set-point of body temperature. It appears that in the case of monoamine oxidase, despite the decrease in 18:1 and 22:6 in the cold-exposed obese mouse, this adaptive mechanism was able to maintain the enzyme in a sufficiently compatible environment, so that no significant change in MAO activity was observed.

CHAPTER 4: GENERAL DISCUSSION

Preliminary studies having revealed that the liver, pancreas and white adipose tissue of the ob/ob mouse had greater specific activities for monoamine oxidase than the corresponding tissues in the lean controls, we undertook to characterize the liver enzyme further and to examine whether differences in lipid environment might have an effect on the expression of enzyme activity.

The higher specific activity for MAO was found with all substrates tested and with homogenates as well as crude and purified mitochondrial preparations. While these studies were in progress, Feldman and Henderson (88) reported no significant difference in the activity of monoamine oxidase toward tryptamine in ~~total~~ liver homogenates of lean and obese mice. The protocol used by these authors differed in several respects from that which was used in our studies, including the age of experimental animals, the method used for assaying enzyme activity, and the removal of food from animal cages 12 hours before sacrifice; and the discrepancy could probably be explained on that basis. There is no doubt, however, that in our hands there was a significant and reproducible difference in the specific activity of the enzyme in the liver of lean and obese mice.

Subcellular fractionation studies demonstrated that

the major portion of MAO activity (80%) sedimented with the mitochondrial fraction, whereas about 20% sedimented with the microsomal fraction. This finding is in general agreement with studies done on rat liver (21) where it has been reported that mitochondria contained approximately 70% and the microsomal fraction, 24% of the total activity. The subcellular distribution was essentially the same in lean and obese mouse liver, and it must be concluded that the greater specific activity observed in mitochondrial preparations from obese mouse liver was not due to a difference in subcellular compartmentation. It is unlikely that the enhanced MAO activity in the obese mouse liver would be attributable to an increase in the number of mitochondria per unit of liver protein, since the activity of two marker enzymes - kynurenine hydroxylase (outer membrane) and cytochrome oxidase (inner membrane) are not different in the liver mitochondrial fractions of lean and obese mice.

The pattern of substrate specificity was similar in lean and obese mice but differed from the pattern observed in rat liver (296). The pattern of specificity in the mice suggested a predominance of form B. This was confirmed by inhibitor studies with the substrate-selective inhibitors, clorgyline and deprenyl. These data are in accord with reports in the literature concerning the substrate specificity of liver mitochondrial MAO in albino mice (183) as well as inhibitor response experiments in mice of the NMRI strain

(295). The data cited above rules out the possibility that the increased specific activity of the ob/ob mouse liver enzyme may be due to differences in subcellular localization of the enzyme activity or to the presence of different forms of the enzymes.

The Michaelis constant of liver MAO for tyramine, serotonin, and benzylamine were similar in lean and obese mice indicating that the increased MAO activity is not due to an altered affinity of the enzyme for the substrates used. An increased V_{max} of obese mouse liver MAO was observed towards all three amines used, confirming our data in substrate specificity studies where enzyme-saturating concentrations of the substrates were used for assays. Since V_{max} is proportional to total enzyme concentration (184), this suggests the presence of a greater amount of enzyme protein per unit of mitochondrial protein, or alternatively, that due to a difference in environment, the enzyme is more active in the liver mitochondria from the ob/ob mice.

Studies were undertaken to investigate the latter possibility. Several investigators have provided evidence that MAO is a membrane-bound enzyme (85, 108, 139). This, however, is by no means universally accepted and some authors (253, 281) contend that MAO is a protein peripheral to the membrane and as such would not have a structural or functional dependence on membrane lipids. On the other hand, the work of Oreland and Olivecrona (238), Ekstedt (80), and

Naoi and Yagi (224) indicate a certain degree of interdependence between MAO activity and membrane lipids.

The experiments on lipid depletion provided a first indication that lipids may play a role in the modulation of the activity of the enzyme. Sequential removal of phospholipids from the mitochondria reduced the activity of the enzyme such that the residual specific activity was equal in samples from lean and obese mice. While it could be argued that the solvent extraction simply inactivated the enzyme, it is difficult to envisage how a non-specific inactivation would reproducibly reduce the higher activity found in the untreated obese mouse mitochondria to precisely the same value as in the lean mouse mitochondria. The activity of many membrane-bound enzymes are known to be affected by the type of phospholipid which surrounds them and/or by the fatty acid composition of these phospholipids.

An investigation into the phospholipid profile of mitochondria of lean and obese mice revealed no difference between the two groups. Upon analysis of the fatty acid composition of these individual phospholipids, several differences were noted between the mitochondria of the lean and obese mice. Most striking was the increased content of arachidonic acid (20:4) in several phospholipid fractions (PC, PI + PS, & PE + PG). Notable also was the increase in oleic acid in the CL + PA fraction. The fact that

phospholipid profiles were essentially similar in lean and obese mouse mitochondria cannot be taken as a proof that specific phospholipids do not affect monoamine oxidase activity, since it gives no information about specific lipid domains which might be associated with the enzyme.

The differences in fatty acid composition, on the other hand, provided a basis for further investigation. As mentioned earlier, Hyslop and York (153) noted reduced microviscosity in adipose tissue membranes from obese as compared to lean mice, and not having observed any difference in phospholipid percentage composition, proposed that the decrease in microviscosity observed in the obese animals might be attributable to an increased occurrence of unsaturated fatty acids in the membrane phospholipids. Further, various investigators (26, 43, 169) have reported a correlation between a lack or absence of dietary unsaturated fatty acids and a decrease in MAO activity in rat liver and brain.

It should be noted that the greater proportion of oleic acid in certain fractions in the obese mouse is in harmony with the observations of Lemmonier et al., (187) and Winand (347) that monoene species are synthesized more rapidly in the liver of the obese mouse than in controls. Microsomal 9, 10-dehydrogenase which has been reported to be insulin-dependent by Gellhorn and Benjamin (104), was observed by Enser (84) to have a higher activity per cell in

the livers of ob/ob mice as compared to lean mice. Although no reports have been made on the activity of the enzymes responsible for the synthesis of arachidonic acid from linoleic acid, it is not inconceivable that the desaturases involved in such a transformation are also more active in obese-mouse liver. Enser (84) has postulated that the enhanced activity of stearic acid desaturase is due to induction by high plasma insulin concentrations in obese mice. This hypothesis may be open to question, since a decrease in the number of insulin receptors (168) and reduced insulin binding (22, 291) by obese-mouse liver has been reported by other investigators. However, there appears to be a lack of a simple relationship betwen insulin binding and the effect of insulin on lipid metabolism. In this respect, Le Marchand et al., (186) observed that while insulin binding was increased and lipogenesis curtailed in livers from fasted and streptozotocin-treated obese mice, in vitro addition of insulin to these livers failed to stimulate lipogenesis. Thus, these authors proposed that alterations in events subsequent to insulin-receptor binding may affect liver responsiveness to the hormone independently of the state of insulin receptors.

While the higher arachidonic acid content of obese mouse liver mitochondria could result from the enhanced activity of liver desaturases, it could also be a reflection of lipid nutrition, particularly since arachidonic acid is

synthesized from an essential fatty acid. Linoleic acid constitutes 50% of total fatty acids in the laboratory chow used in our studies. When allowed to eat ad libitum, the obese mouse consumes approximately twice as much food as its lean counterpart, thus the amount of linoleic acid consumed is twice as great in the obese as in the lean mouse. In view of the fact that partial food restriction in the obese mouse did not decrease appreciably the arachidonic acid content of the major phospholipids, it is unlikely that the increased food intake alone could account for the differences observed between lean and obese mice.

One of the in vivo treatments utilized in an attempt to establish that arachidonic acid has a role in the enhancement of MAO activity in the obese mouse consisted of chronic treatment with the antibiotic, oxytetracycline. Bégin-Heick et al., (22, 23) had reported previously that OTC altered lipid metabolism in the liver of treated obese mice. In particular, their results suggested that OTC treatment might affect de novo synthesis of fatty acids. Mukherjee and Mukherjee (219) observed that the incorporation of injected palmitate into liver phospholipids decreased in the intact tetracycline-treated rat. Another antibiotic, chloramphenicol, which has been shown to interfere with the acylation of phospholipid precursors in BHK-21 cells (189), has been reported to inhibit rat liver mitochondrial MAO in vitro and in vivo (15).

OTC treatment decreased the specific activity of liver mitochondrial MAO towards tyramine and benzylamine, but not

towards serotonin. Total activity in obese-mouse liver, which was observed to be several times as great as that in preparations from lean mice, was reduced to the level of the non-treated lean-mouse. Treatment, however, did not affect the arachidonate content of any of the phospholipid fractions studied, and the changes observed in some fatty acids were small and did not show a trend which would suggest that they were physiologically significant. It appears then that OTC treatment produced the observed decrease in MAO activity by a mechanism that does not seem to involve an alteration of mitochondrial fatty acid composition.

Kinetic analysis showed that whereas there was no change in the affinity of the enzyme towards tyramine and benzylamine, treatment had reduced V_{max} values for both amine substrates. Since on the assumption that all other properties of the lean and obese-mouse liver enzyme are similar, V_{max} may be considered proportional to the amount of enzyme protein (184), it is not inconceivable that the reduced V_{max} for both tyramine and benzylamine may be attributable to a decreased amount of enzyme protein following OTC treatment. The large decrease in protein content of obese-mouse liver accompanying the large decrease in total activity, and the decrease of enzyme activity in the microsomal fraction, which is the reported site of MAO synthesis (86, 227), appear to favor this view. Bégin-Heick et al., (23) have also noted previously that the decrease in lipid content of obese-mouse liver following

treatment was accompanied by a reduction in total liver protein. It is possible that the decrease in MAO activity in our studies is secondary to the general decrease in liver protein content effected by antibiotic treatment. It should be pointed out, however, that no decrease was observed in the specific activity of another mitochondrial marker enzyme, cytochrome oxidase, in treated animals. The apoprotein of cytochrome oxidase, like MAO, is synthesized on the cytoribosomes (73). A decrease in the specific activity of this enzyme should also have been observed if such a generalized action as decrease in total protein content were involved.

It is likewise relevant to recall that the inhibitor studies with the non-treated mice indicated that the mouse liver enzyme is predominantly, or even entirely of the 'B' form, and that Ekstedt (80) has shown that Form B of the enzyme can metabolize serotonin, which is generally considered a substrate for the A form. If the oxidative deamination of tyramine, benzylamine and serotonin are due to the same enzyme form and the effect of OTC treatment is attributable to a decrease in the amount of enzyme protein, then a question can be raised as to why the serotonin-metabolizing activity is not affected by the treatment in vivo. In view of these considerations, it appears that a decrease in enzyme protein may not be an adequate explanation for the observed reduced activity in OTC-treated animals.

The increased arachidonic acid content of liver mitochondrial phospholipids in the obese mouse may affect MAO activity in various ways. It may facilitate the binding of the enzyme to the mitochondrial membrane. Alternatively, it may enhance enzyme activity by altering the conformation of the enzyme or the fluidity of the mitochondrial membrane. The work of Steim (6) has indicated that a high proportion of the phospholipids in some natural membranes are in a fluid state, and as noted earlier (cf. Review of Literature, section I.B.), fatty acyl chain length and unsaturation are among the important factors in determining the fluidity of membrane lipids. The fluidity of the fatty acyl chains presumably provide the required motional freedom allowing enzymes within membranes to undergo conformational changes and movements associated with their activity.

A correlation between the activity of membrane-bound enzymes and motional parameters of the surrounding lipid phase has frequently been sought by a comparison of their temperature dependencies. Our study of the temperature dependency of liver MAO activity yielded bi-phasic Arrhenius plots for both lean- and obese-mouse mitochondrial preparations which were reminiscent of those reported by Kimelberg and Papahadjopoulos (176) in their study of the effect of phospholipid acyl chain fluidity on the reactivation of rabbit kidney Na^+, K^+-ATPase. In their

study, these authors observed that the discontinuities in the Arrhenius plots bore a qualitative, and in some cases, quantitative correspondence with the phase transition of the phospholipids as determined by differential scanning calorimetry.

The Arrhenius plots shown above are in contrast with those reported by Raison (253) and Aithal (1), who obtained straight-line functions for rat liver mitochondrial MAO. While it is not possible to comment on the reason for the discrepancy between Raison's findings and ours in the absence of a report on the details of the experimental protocol used by these investigators, it is not unlikely that Aithal's results could be attributed to the treatment of membrane preparations with the detergent Triton X-100. Detergents like Tritons disrupt membrane lipid-protein interactions (330), and have been observed to abolish the 'break' or discontinuity in the Arrhenius plots of various oxidative mitochondrial enzymes (252).

Whereas most soluble enzymes do not exhibit diphasic Arrhenius kinetics, membrane-bound enzymes generally yield bi- or tri-phasic curves (270). Although the molecular mechanisms underlying multiphasic Arrhenius plots have not as yet been definitively elucidated, it has been shown that an explanation in terms of protein solubility in the lipid phase may be possible (351). Discontinuities in Arrhenius plots of microsomal and mitochondrial enzyme activities have

indeed been attributed to lipid thermal phase transitions by several investigators (175, 151). Noteworthy is the decrease in activation energy for both lean- and obese-mouse MAO above the discontinuity to half of its value below this temperature. This would suggest that the enhancement of enzyme activity is favoured by the transition from an ordered quasi-crystalline to a more fluid, disordered, liquid-crystalline state. In this context, the biphasic Arrhenius kinetics obtained in our study imply that mouse liver mitochondrial MAO may indeed depend on its lipid environment for the modulation of its activity, and that contrary to the contention of Singer (281) and Aithal (1) appears to be an integral rather than a peripheral membrane protein.

In Arrhenius plots of enzyme activity, fatty acid effects are generally manifested as a change in slope at a temperature which is uniquely determined by the fatty acid composition of the membrane. In our studies, a single break was obtained in the Arrhenius plots for the lean and obese-mouse MAO, and this occurred at precisely the same temperature for both lean and obese animals. There is evidence that certain enzymes can segregate specific lipids out of the bulk lipid pool to constitute their immediate lipid environment or 'lipid annulus' (151, 337). The physical properties of such a lipid annulus may then determine to some extent the activity of the enzyme, and

such properties may or may not reflect the bulk lipid pool.

Enzyme activity, according to Steim (299), would be responsive to phase transitions in the immediate lipid environment of the functional protein. Hesketh et al., (133) however, have shown that enzyme protein can sense changes in the physical state of the bulk lipid phase, as well as that of its immediate lipid environment. In their study of Ca^{2+}, Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, these authors obtained two inflections (27 - 37°C and 37.5 - 38.5°C) at high lipid to protein ratios. As the proportion of lipid was decreased, the upper 'break' remained in the same temperature range, but eventually broadened into a continuous curve below a ratio of 30 dipalmitoyllecithin molecules per ATPase. At the same time, the lower 'break' became more pronounced, while remaining in the same temperature range. Experiments with spin-labeled phospholipids indicated that approximately 30 lecithin molecules interact directly with the enzyme. These authors proposed that each ATPase molecule prevents a normal phase transition from occurring in an annulus of about 30 dipalmitoyllecithin molecules, and that the lipid molecules immediately outside this annulus are able to undergo a normal dipalmitoyllecithin transition at close to 41°C, the transition temperature for the pure lipid.

Hesketh's study demonstrates that perturbations of a

lipid bilayer by interdigitated protein are essentially restricted to the phospholipid annulus, and that the physical properties of the bulk lipid phase would be unaffected by protein insertion above a certain lipid to protein ratio. Thus, a protein with a lipid annulus not reflecting the composition of the bulk lipid pool may still sense lipid phase separations occurring in the bulk lipid, but would have superimposed upon this any changes in the physical state of its annular lipid.

If MAO, like other proteins (260, 272, 337) seeks regions of compatible lipid composition, then it is not inconceivable that the immediate environment of obese-mouse MAO has a higher content of C 20:4 - rich phospholipids than that of the lean-mouse enzyme. Using this model, the single 'break' in the Arrhenius plots cannot be rationalized by stating that the composition, and therefore, the phase transition of the annular lipids reflected that of the bulk lipid pool. However, it is possible for the changes in state of the annular lipids to have occurred outside the temperature range used in our study, and if there were differences in physical properties resulting from differences in composition between annular and bulk lipids, that no changes other than those due to phase separations in the bulk lipid pool would lead to the appearance of breaks in the Arrhenius plots of enzyme activity. Indeed, Houslay and Palmer (144) have shown that a number of very different

proteins associated with hamster liver plasma membranes exhibit remarkably similar breaks in Arrhenius plots of their activities, and have attributed these common breaks to phase separations occurring in the bulk lipid phase. Similarly, the Arrhenius plots of the respiratory enzymes and oligomycin-sensitive Mg^{+2}-activated ATPase of the inner membrane of rat liver mitochondria all exhibited a discontinuity at approximately 23°C, which coincides with the phase transition temperature for rat-liver mitochondrial lipids as determined by e.s.r. specroscopy (252). Also relevant to this discussion are the observations made by Kimelberg and Papahadjopoulos (176) in studies on the reactivation of kidney Na^+, K^+-ATPase. Whereas 'breaks' in Arrhenius plots were observed by these authors when dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylglycerol were used, a straight-line function was obtained with dioleylphosphatidylglycerol. Differential scanning calorimetry showed that dioleyl-phosphatidylglycerol was fluid throughout the temperature range (0 - 60°C) used for the study. Chapman et al., (46) have shown that the temperature at which a particular phase can exist for a given phospholipid will depend on the melting temperature of the hydrocarbon chains. It should be noted that the melting point for oleic acid is 5 - 7°C while that for arachidonic acid is -49.5°C. Thus it is not inconceivable that the melting point of arachidonic acid-rich phospholipids may be well below the temperature

range used in our studies, and would be inacessible in our experimental analyses.

Alternatively, the phase transition temperature for annular lipid may have been broadened or shifted as a result of Van der Waal's interactions between lipid and protein. In this respect, Marcelja (204, 205) using theoretical calculations, has demonstrated that these interactions can be so great that the transition for annular lipids is broadened and shifted, or so broadened as to be experimentally undetectable. In this context, the discontinuity observed in the Arrhenius plots could represent a phase separation in the bulk lipid pool rather than in the lipid annulus, and may explain why there was no detectable difference in the transition temperature of lean and obese-mouse preparations. Further, the similarity of the energies of activation above and below the transition temperature would indicate that the change in the physical state of the bulk membrane lipids elicited the same effect on the kinetic properties of the enzyme from both groups of animals.

On the basis of the results obtained in the experiments involving the incorporation of arachidonic acid into lean mouse mitochondria, it appears unlikely that any effect arachidonic acid may have on the activity of liver MAO is being exerted by altering the fluidity of mitochondrial lipids. In this respect, Klausner et al., (177) have found that free fatty acids readily intercalate into the plasma

membranes of splenic lymphocytes and baby hamster kidney cells, and over short periods of time, remain there as unesterified fatty acids. These investigators postulate the presence of discreet relatively gel-like and fluid regions in the membrane, and have observed that cis-unsaturated free fatty acids preferentially partition into fluid domains and disorder the membrane interior. Such an increase in the degree of disorder within the membrane would conceivably lead to enhanced fluidity (336). Based on this model, the observation that MAO activity was not altered in the experiments involving the incorporation of C 20:4 into lean-mouse mitochondria might imply that the increased enzyme activity in obese mice is not due to the enhancement of the fluidity of membrane lipids. This conclusion appears reasonable regardless of whether the arachidonic acid had intercalated into the bulk lipid phase or the 'lipid annulus'.

Noteworthy is the fact the 'break' in the Arrhenius plots occurred at a temperature (23°C) well below the physiological temperature of both lean and obese mice, which suggests that the bulk phase is fluid, and that <u>in vivo</u>, the enzyme is in a largely fluid lipid environment in both groups of animals. In the case of the obese mice, the absence of an adaptive increase in unsaturated fatty acid content of mitochondrial phospholipids in response to cold exposure further suggests that the enzyme is in a fluid environment even at the lower core temperature (31°C) of

these animals following chronic exposure to cold. The fact that the slopes, and therefore, the energies of activation above the 'break' in the Arrhenius plots are similar for lean and obese mice, may suggest that in vivo the enzyme in the lean animals may exist in an equally fluid lipid environment. Although our data does not permit us to firmly exclude the possibility of greater fluidity in the annular lipids of obese-mouse MAO, it must be concluded from the similarity of the Arrhenius break temperatures and the activation energies above and below these temperatures, that the fluidity of bulk membrane lipids does not appear to be the principal determinant for the enhanced enzyme activity in obese-mouse liver.

Other possible roles, however, may be postulated for the presence of C 20:4-rich phospholipids in obese-mouse liver mitochondria. In studies done on the livers of essential fatty acid-deficient rats, Collins (55) reported that the presence of arachidonic acid in the 2-position of a phosphoglyceride, lowers the ^{32}P turnover rate of the phosphoglyceride and proposed that arachidonic acid acts as a modulator of phospholipid metabolism. This author further suggests that the symptoms of essential fatty acid deficiency may be attributable to an increased phospholipid turnover followed by partial disorganization of the enzymes on lipo-protein membranes.

Arachidonic acid-rich phospholipids may also

facilitate the binding of monoamine oxidase to the mitochondrial membrane. In this respect, Oreland and Olivecrona (238) have proposed that the good correlation between the extent of phospholipid depletion of pig liver mitochondria and the decrease in the activity of mitochondrial MAO would indicate that specific phospholipids may have a role in the binding of the enzyme to the mitochondrial membrane.

Alternatively, the configuration of a long-chain fatty acid such as arachidonic acid with its multiple cis-double bonds may alter the conformation of the enzyme in such a manner as to facilitate interaction between the active site and substrate, or expose more active sites to the incoming substrate. Increased amounts of arachidonic acid-rich phospholipids may also favor a configuration of obese-mouse MAO that facilitates the breakdown of the enzyme-substrate complex into enzyme and product, thus increasing the rate constant for this process and V_{max}, for the same amount of enzyme protein.

The importance of the structure of fatty acyl chains, and the possibility that enzyme activity can be enhanced through means other than an increase in membrane fluidity is implied in a study of ox heart mitochondrial β-hydroxybutyrate dehydrogenase by Houslay *et al.* (151). In this study, the authors demonstrated that dioleyllecithin and dilauroyllecithin activated the enzyme as compared with

endogenous mitochondrial lipids. On the other hand, dicaproyllecithin was found to actually inhibit the enzyme. As stated earlier (cf. Review of Literature, section I.B.), a higher degree of unsaturation and a shorter chain length both favour mobility of fatty acyl chains, and consequently, the fluidity of the membrane. The fact that dilauroyl-lecithin (12:0 chains) activated β-hydroxybutyrate dehydrogenase, whereas dicaproyllecithin (10:0 chains) reduced enzyme activity was interpreted by the authors to mean that a minimum chain length is essential to maintain an active enzyme conformation similar to that in vivo.

Although we cannot exclude the possibility of a greater amount of MAO protein in obese-mouse liver mitochondria as a basis for the enhanced monoamine oxidase activity observed in these animals, and the decrease in total liver protein accompanying the reduction in MAO activity in OTC-treated animals seems to support this view, the data obtained in the lipid-depletion experiments with non-treated animals suggest a greater dependence of the obese-mouse enzyme on its lipid environment. The presence of a higher proportion of arachidonic acid in obese-mouse liver mitochondrial phospholipids may indeed imply a role for this polyunsaturated fatty acid in the lipid-enzyme interaction. The results of our studies suggest areas for further investigation that may provide more definitive evidence regarding the precise function of 20:4 or 20:4-rich phospholipids. These

would include:

- A comparative study of the fluidity of lean and obese mouse liver mitochondrial membrane by means of ESR spectra obtained with spin-labelled fatty acids and phospholipids. Ideally, probing the immediate environment of MAO may involve the use of a spin-labelled fatty acid attached to a molecule having a high affinity for the enzyme, such as a specific inhibitor.
- An analysis of endogenous or boundary lipid to establish any preference or requirement for polar head-group and/or fatty acyl chain.
- Once a requirement or preference for a specific phospholipid and/or fatty acid chain is established, direct lipid binding studies can be carried out to establish the amount required to attain the V_{max} observed in non-treated animals. Data on the conformation of enzyme protein in lean and obese mice at these binding levels can then be obtained by a number of different techniques such as circular dichroism, nuclear magnetic resonance, or low angle X-ray scattering.
- Finally, in order to determine if there is an increased amount of MAO protein in obese-mouse liver mitochondria, outer mitochondrial membranes may be isolated and their protein composition checked by SDS-polyacrylamide gel electrophoresis.

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