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Aldehyde oxidase (AO) is a cytosolic molybdenum-containing hydroxylase. It is found in the highest concentrations in the liver and intestines of humans and other mammals but is also expressed in a variety of other organs, such as the kidney, lung and brain. The aim of this research was to investigate AO activity in bovine kidney and to determine if bovine kidney AO contributes to the metabolism of xenobiotics.   Five known substrates for AO were evaluated to quantify AO activity in the kidney and these values were then compared to rabbit liver and bovine liver activities. Experiments with two known inhibitors of AO, menadione and methanol, were performed to help verify that the activity found in the partially purified enzyme preparations were catalyzed by AO.   For all substrates evaluated, rabbit liver showed the highest activity followed by bovine liver and then bovine kidney. No measurable activity for the bovine kidney enzyme was observed with phenanthridine or methotrexate as substrates. However, bovine kidney AO catalyzed the oxidation of benzaldehyde, phenazine methosulfate and N^1-methylnicotinamide. In conclusion, low levels of aldehyde oxidase activity were measured in the bovine kidney using xenobiotic substrates.

MOLYBDENUM HYDROXYLASES FROM BOVINE KIDNEY AND LIVER

by

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APPROVAL PAGE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Aldehyde oxidase (AO; EC 1.2.3.1) and xanthine oxidase (XO; EC 1.2.3.2) are cytosolic enzymes belonging to a class of enzymes termed molybdenum hydroxylases (1). Molybdenum hydroxylases are found in nearly every organism from humans to bacteria and have been determined to be present in a number of various tissues. Xanthine oxidase occurs in high concentration in cow's milk and in the lactating mammary gland, whereas aldehyde oxidase is expressed predominantly in the liver of mammals. However, many other tissues have been shown to contain AO and XO. Xanthine oxidase has been detected in the liver, small intestine, forestomach, oral and nasal cavities, esophagus, tongue, uterus, epidermis and other tissues and organs. Except for the epidermis, aldehyde oxidase has been found to be similarly distributed (2).

Many species have been shown to express aldehyde oxidase and xanthine oxidase activity. Aldehyde oxidase has been found in the livers of bovine and rabbit (as our research also shows), as well as rat, mouse, hog, guinea pig, monkey, hamster, and the livers of fish. No aldehyde oxidase activity has been detected in birds or dogs (2). The highest activity for AO was found in monkeys and humans. This is followed by hamster, rabbit, guinea pig, rat and mouse (2). In addition, aldehyde oxidase has also been found to be prevalent amongst crustaceans, mollusks, and insects (3). Xanthine oxidase has

1

also been found in the livers of a wide variety of species including humans, rodents, and bovines. Four of the best characterized molybdenum hydroxylases include xanthine dehydrogenase (XDH), aldehyde oxidoreductase (AOR), aldehyde oxidase (AO) and xanthine oxidase (XO), the latter two of which this research is focused. Of the four enzymes listed above, all are found in eukaryotes except for AOR, which is a bacterial enzyme referred to as MoP for molybdenum protein (4).

It is an objective of this research to partially purify and measure the activity of aldehyde oxidase and xanthine oxidase in bovine kidney and to compare these values to the activities found in bovine liver and rabbit liver. Past research has shown the presence of the aldehyde oxidase protein in various bovine tissues including that of the kidney as determined by Western blot analysis. In a bovine tissue distribution experiment by Marco Li Calzi et. al. (5) significant amounts of the aldehyde oxidase protein were found in the liver and the lung of the cow, followed by the spleen. Although at much lower levels the eye, kidney, thymus, testis, duodenum, heart, and esophagus also showed detectable amounts of the bovine aldehyde oxidase protein.

The kidney makes a good candidate for this type or research due to the fact that it is a potential site for xenobiotic metabolism. The kidney is a complex organ with numerous biological roles but has received considerably less attention than the liver with respect to xenobiotic metabolism. The kidney is discussed in more detail in Chapter 2 of this thesis. The substrates used to evaluate aldehyde oxidase activity in the bovine kidney and liver include the xenobiotics: phenanthridine, benzaldehyde, and methotrexate

(a drug of pharmacological significance, MTX), the endogenous compound; N^1-methylnicotinamide (NMN) and another well known aldehyde oxidase substrate; phenazine methosulfate (PMS). All activity measurements of this research project were performed on the partially purified enzymes. Phenanthridine is a popular choice substrate for aldehyde oxidase activity measurements and has been used by other researchers for the determination of aldehyde oxidase activity in bovine liver (5). Xanthine, the well known endogenous substrate for xanthine oxidase, is used as the sole substrate for activity measurements of XO for this research project. It is also the predominant substrate used by other researchers for xanthine oxidase activity measurements.

The fact that most metabolic reactions are taking place in the liver makes this organ a good standard for comparison to other tissues, such as the kidney. Valuable comparisons can be made within the same species or across species. This research project shows activity comparison data of the bovine kidney to within the species (bovine liver) and across species (rabbit liver). Due to its availability and high activity levels as determined using a number of substrate types, rabbit liver aldehyde oxidase is the prototypical enzyme and often serves as a standard for aldehyde oxidase activity measurements. Rabbit liver AO is also the most extensively studied of the enzymes (6).

It is well established that liver aldehyde oxidases of many species are able to catalyze the metabolism of various xenobiotics. XO and AO have shown their highest

activities in the liver.   Comparing the activities of AO and XO found in the kidney to the hepatic tissues will aid in understanding if the levels found in the kidney are significant and therefore contribute to the oxidation of xenobiotics. Another key question to be addressed in the research is whether the kidney enzymes (AO and XO) show the same specificity towards substrates as the corresponding liver enzymes.

The ultimate objective of this research is to obtain valuable information as to whether aldehyde oxidase from the extrahepatic tissue bovine kidney contributes to the oxidation of, and therefore, the metabolism of xenobiotics. Xenobiotics are compounds that are foreign to the body, which include drugs such as antibiotics, pollutants such as dioxins, and other substances that are not normally present in the body. Xenobiotic metabolism is the series of metabolic pathways that change the chemical structure of xenobiotics. These reactions often act to detoxify poisonous chemical compounds. Sometimes, however, the product of xenobiotic metabolism can be the cause of toxic effects (7). Such is the case with methotrexate, our primary xenobiotic of interest for this research project. Methotrexate is a clinically important anti-cancer drug and is one of the few drugs that are significantly oxidized by aldehyde oxidase. Its oxidative product, 7-hydroxymethotrexate is cytotoxic. This is discussed in more detail in Chapter 2 of this research paper.

In all known cases, aldehyde oxidase exists exclusively in its oxidase form (1). However, mammalian xanthine oxidase (XO; EC 1.1.3.22) is involved in an inter-conversion from xanthine dehydrogenase (XDH; EC 1.1.1.204) by oxidation of

sulfhydryl residues (that is, cyteines to cystines) (8) by actions of proteases (limited

proteolysis) (8), and upon oxidative stress or other conditions including purification.

When the enzyme is in an oxidized state it exists as the oxidase form, when it is in a

reduced state it exists as the dehydrogenase form. The two forms of this enzyme are

collectively termed xanthine oxidoreductase (XOR) and this term is used throughout this

thesis as it applies. Xanthine oxidoreductase (XOR) is the prototypical molybdenum

hydroxylase and therefore serves as a structural and mechanistic model for the less

studied aldehyde oxidase.   XOR, as purified from bovine milk, is the most frequently

studied enzyme of this family and serves as a standard for the large class of

molybdenum-containing enzymes (9). This is mostly due to the presence of XOR in

cow's milk which is readily obtained and is commercially available on a gram-scale

basis. Xanthine oxidoreductase was first purified over 60 years ago (10) and continues to

be purified or partially purified for a variety of research purposes.

In the non-mammalian species chicken, xanthine dehydrogenase is stable and

does not convert to the oxidase form (8) even though the species is still a eukaryotic

system. As a matter of fact, even within the mammalian tissue, XDH is believed to be

the predominant form of the enzyme (8) under non-pathological (normal) conditions.

The difference between the two forms is largely based on the electron acceptor it utilizes

for its catalytic activity. XO transfers reducing equivalents to O_2, where as XDH transfers

them predominantly to NAD^+ and to a much lesser extent oxygen as well (1).

Aldehyde oxidase and xanthine oxidase are very closely related enzymes (2) in terms of their general chemical structure, biochemical characteristics, and amino acid sequences (11). These two enzymes are complex metalloflavoenzymes that contain one flavin adenine dinucleotide (FAD), two non-identical iron sulfur centers [2Fe-2S], and a molybdenum cofactor as prosthetic groups (8). Though xanthine oxidase and aldehyde oxidase are extremely similar in their protein structure and prosthetic group composition, they are surprisingly different in their substrate specificities (12). In general, these molybdoflavoenzymes, sometimes referred to as MFE's (13), catalyze the oxidation of N-heterocycles via nucleophilic addition reactions (2). Aldehyde oxidase catalyzes the oxidation of aldehydes and nitrogeneous heterocyclic xenobiotics like methotrexate and cyclophosphamide as well as the oxidative metabolism of a variety of endogenous compounds like retinaldehyde, pyridoxal, and N^1-methylnicotinamide (2). The absolute primary physiological function of aldehyde oxidase is yet to be determined. Also the physiological importance of aldehyde oxidase's role in aldehyde oxidation is in question due to the fact that the Michaelis constant (Km) for aldehyde oxidase and xanthine oxidase is higher for aliphatic aldehydes than it is for another mammalian enzyme, aldehyde dehydrogenase (ALDH) [EC; 1.2.1.3]. Aldehyde dehydrogenase, which is not a member of the molybdenum hydroxylase family, is thought to be the main enzyme for aldehyde oxidation in mammals. ALDH, with its wide substrate specificity, removes a variety of aldehydes which are consumed in the diet or formed during xenobiotic metabolism (14). Because of this, aldehyde dehydrogenase is often considered a

6

detoxification enzyme (14). Xanthine oxidase's physiological role is mainly in purine

catabolism in which it catalyzes the oxidation of hypoxanthine to xanthine and then to

uric acid (2). However, it also contributes to oxidative metabolism of certain xenobiotics.

CHAPTER II

BACKGROUND

The Molybdenum Metal

Molybdenum (Mo) is a redox-active transition element that is essential for most

biological systems as it is required by enzymes that catalyze basic metabolic reactions in

the carbon, nitrogen, and sulfur cycles (4). Molybdenum belongs to the second row of

the periodic table and is the only element of that row that is required to grow and sustain

life for a variety of organisms (4). Molybdenum is found in a wide range of

metalloenzymes, however is not an abundant element of the earth or the earth's crust as

would be thought by its demand (15). It is however, very abundant in the oceans in the

form of the dianionic molybdate ion $[MoO_4]^{-2}$ (Fig. 1) at concentrations of about 10

micrograms per liter (16).
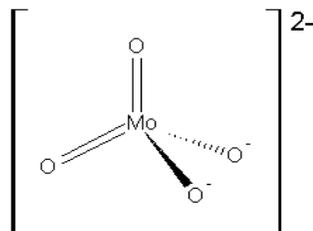
The Molybdate Ion

Figure 1. The Molybdate Ion. A tetrahedral structure containing an oxyanion with
molybdenum in its highest oxidation state of +6.

8

It is in this form by way of complex biological systems that the uptake of molybdenum into the cells takes place. Over the past ten years, the number of molybdenum enzymes identified has grown to a point where a classification system based on active site structure has been developed (17). The enzymes have been grouped into three families; the xanthine oxidase (XO), sulfite oxidase (SO), and dimethylsulfoxide (DMSO) reductase families (9). AO and XO both belong to the xanthine oxidase family along with XDH and AOR (4).

Molybdenum is found in a mononuclear center in all molybdoenzymes except for nitrogenase, where it is part of a multinuclear cluster with seven iron atoms (17). More than 50 different mononuclear molybdenum enzymes in nature have been reported to date, which are mostly from bacterial origin (18). A diverse series of metalloenzymes containing molybdenum occur in plants, animals, fungi, and bacteria (18).

The Cofactor and Active Site (Moco)

Molybdenum itself is biologically inactive unless bound to a tricyclic pterin compound where it then forms the molybdenum cofactor (Moco). Attached to the tricyclic pterin core of Moco is a pyran ring. Moco is located at the active site of all molybdenum enzymes (18). The pterin structure coordinates to the molybdenum metal by way of an enedithiolate side chain (19). The function of the cofactor appears to be the transfer of electrons out of the molybdenum center after the oxidation reaction has taken

place. This results in the reduction of molybdenum from Mo(VI) to Mo(IV) (19). A proposed electron transfer sequence proceeds as shown below (Fig. 2) (20) where Fe/S I and Fe/S II are two distinct iron-sulfur centers. However, this scheme implies a linear sequence of flow and is not necessarily the case for all systems.

Mo → Fe/S I → Fe/S II → FAD

Electron Movement →

Figure 2. Electron Transfer Sequence. Proposed sequence of electron flow out of the molybdenum center.

The cofactor (Fig. 5) is formed by the combination of a molydopterin molecule (Fig. 4) and the active site structure (Fig. 3) through a series of biosynthetic reactions. The molybdenum hydroxylases, aldehye oxidase and xanthine oxidase, contain an active site that is believed to be in a five-coordinate complex with two enedithiolate ligands of the molybdopterin cofactor, one oxo group, one sulfide group, and one hydroxyl or water molecule. This coordination results in a square pyramidal geometric structure and is the site for substrate binding and enzyme inhibition (21).

10
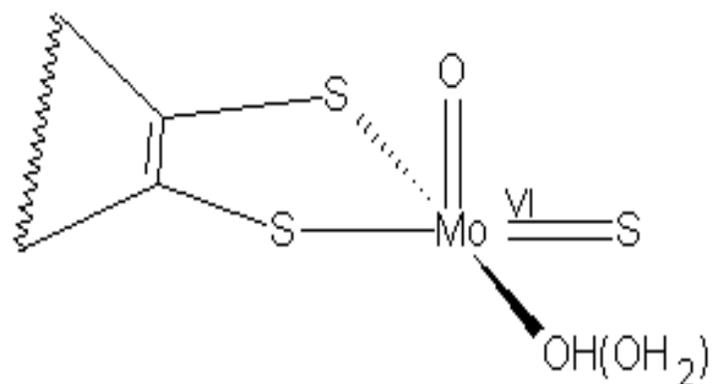
Figure 3. Active Site Structure for the Xanthine Oxidase Family of Enzymes. Square pyramidal structure with Mo in the +6 oxidation state. Identical for xanthine oxidase and aldehyde oxidase enzymes.
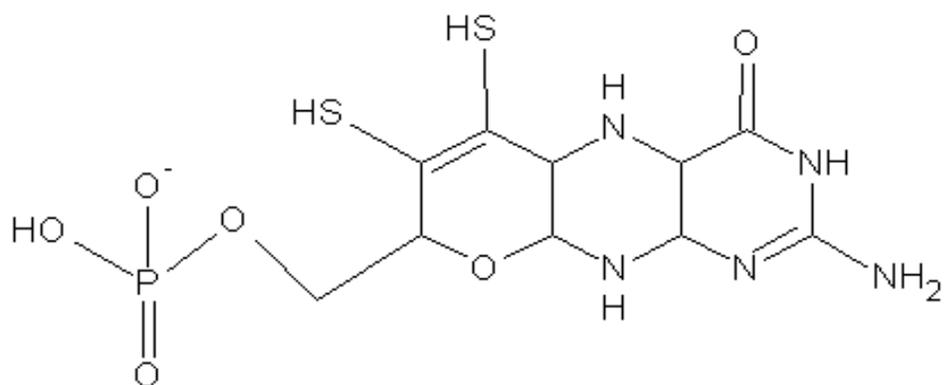
Figure 4. Structural Model for Pyranopterin Molecule (Molybdopterin). The pyranopterin structure for molybdenum hydroxylase enzymes, an organic ligand in the monophosphate form.

11
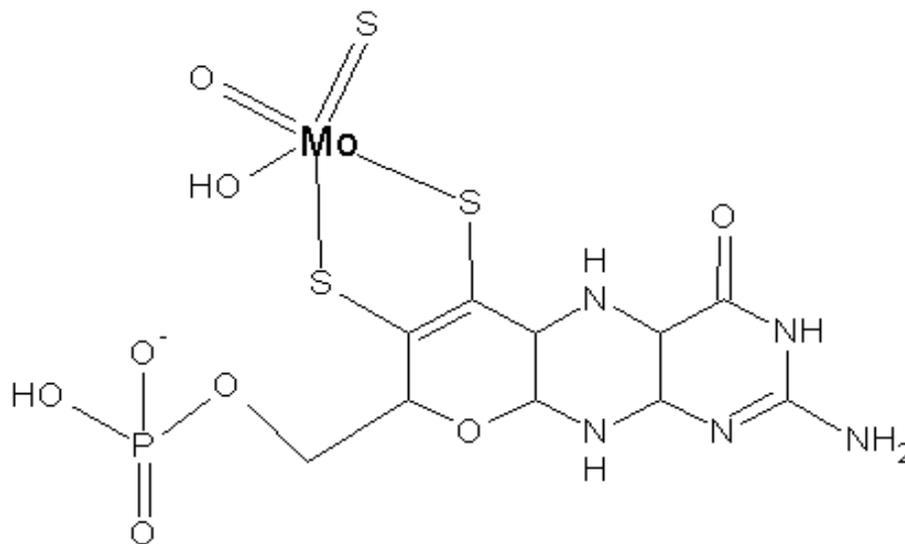
Figure 5. Moco (Molybdenum Cofactor). The active site structure attached to the pyranopterin compound by way of two enedithiolate side chains to produce the molybdenum cofactor (Moco).

AO/XO Enzymes

Aldehyde oxidase and xanthine oxidase are mononuclear molybdenum enzymes belonging to a family termed molybdenum hydroxylases. Molybdenum hydroxylases embody the largest group of mononuclear molybdenum enzymes with more than 20 being identified (17). As of 2005, the crystal structures of three molybdenum hydroxylases have been determined (19). They include xanthine oxidoreductase from *Bos taurus*, xanthine dehydrogenase from *Rhodobacter capsulatus* and aldehyde oxidoreductase from *Desulfovibrio gigas*. In addition, the crystal structure of carbon

12

monooxide dehydrogenase from *Oligotropha carboxidovorans* which catalyzes the

oxidation of a different type of reaction (carbon monoxide (CO) to carbon dioxide (CO_2))

has been determined. CO dehydrogenase bears a strong structural homology to the

molybdenum hydroxyalses (Fig.6) (19). Aldehyde oxidoreductase from *Desulfovibrio*

gigas was the first mononuclear enzyme whose structure at 2.25Å resolution was

determined using x-ray crystallography (17). All of these enzymes bear the same overall

architecture (19).

The crystal structures of the molybdenum hydroxylase enzymes show that the N-

terminus contains two iron-sulfur clusters [2Fe-2S] in different domains and, except for

aldehyde oxidoreductase from *Desulfovibrio gigas*, FAD in a third domain. The binding

portion of the molybdenum atom of the enzyme is at the C-terminus with the

molybdenum center near the intersection of two stretched domains that form about a 90°

angle (19).

Xanthine oxidoreductase from *Bos Taurus*:

[2Fe—2S]	[2Fe—2S]	FAD	Mo domain 1	Mo domain 2

Xanthine dehydrogenase from *Rhodobacter capsulatus*:

[2Fe—2S]	[2Fe—2S]	FAD	Mo domain 1	Mo domain 2
Subunit A	Subunit A	Subunit A	Subunit B	Subunit B

Aldehyde oxidoreductase from *Desulovibrio gigas*:

[2Fe—2S]	[2Fe—2S]		Mo domain 1	Mo domain 2

CO dehydrogenase from Oligotropha carboxidovorans:

[2Fe—2S]	[2Fe—2S]	FAD	Mo domain 1	Mo domain 2
Subunit A	Subunit A	Subunit B	Subunit C	Subunit C

Figure 6. Schematic Representation of the Molybdenum Hydroxylase Homologies. In xanthine dehydrogenase the iron-sulfur centers and the FAD constitute one subunit and the molybdenum binding portion a second subunit. In the CO dehydrogenase , the iron-sulfur centers are together in one subunit, the FAD in a second subunit and the molybdenum center in a third subunit. The FAD domain is absent in the aldehyde oxidoreductase enzyme. The Mo domain 1 and 2 indicate that the molybdenum center is at the interface of two elongated domains that lie across one another.

Hille, R. Molybdenum-containing hydroxylases. *Arch. Biochem. Biophys.* **2005**, *433*, 107-116.

In general, mononuclear molybdenum enzymes catalyze oxygen atom transfer

reactions (21). In the case of molybdenum hydroxylases, this is done through the

hydroxylation of carbon centers where the oxygen atom incorporated into the product

ultimately comes from water and not from molecular oxygen (O_2), which is in contrast to

a monooxygenase reaction (19). These enzymes catalyze the hydroxylation reactions of

substrates containing N-heterocycle or aldehyde functional groups (Fig. 7).

$$R-H \quad + \quad H_2O \quad \longrightarrow \quad R-OH \quad + \quad 2H^+ \quad + \quad 2e^-$$

Substrate   Water Molecule   Oxidized Substrate   Protons   Reducing Equivalents
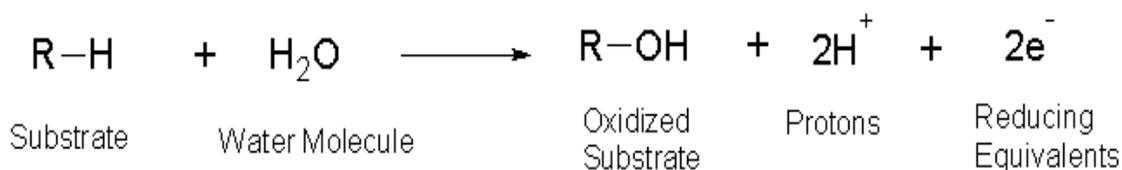
Figure 7. Oxygen Atom Transfer Reaction by Molybdenum Hydroxylases. General
oxidation reaction catalyzed by molybdenum hydroxylases producing an oxidized
substate and two reducing equivalents.

This two-electron redox reaction takes place at the molybdenum center. During

the course of the reaction the molybdenum atom is reduced from Mo(VI) to Mo(IV) after

substrate interaction via the molybdopterin cofactor. The overall reaction generates two

reducing equivalents that are transferred to an external electron acceptor via an electron

transfer system introduced by other cofactors present in the enzyme (21). This is in

contrast to the cytochromes P450 hydroxylation systems that rather consume the reducing

equivalents (9). For the case of an aldehyde substrate the product is a carboxylic acid

(Fig. 8).
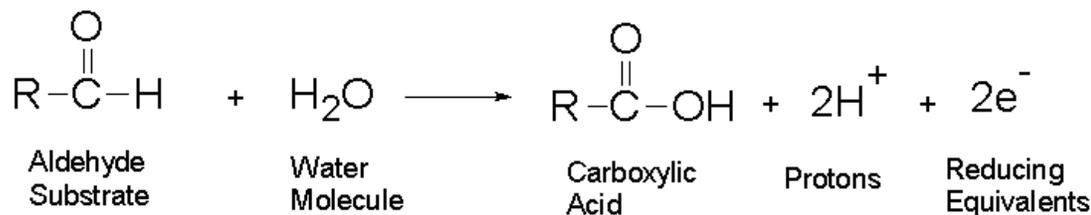
Figure 8. Oxygen Atom Transfer Reaction Involving an Aldehyde. General oxidation reaction catalyzed by molybdenum hydroxylases in which the oxidation of an aldehyde is converted to its corresponding carboxylic acid producing with it two reducing equivalents.

AO/XO Mechanism of Action

The mechanism of action (Fig. 9) by which molybdenum enzymes catalyze

oxidation reactions has been a focus of research for many years (22). The reality that

molybdenum hydroxylases use water and not molecular oxygen as the source of oxygen

incorporated into the product has made this mechanism extremely interesting (22). Due

to the increasing availability of crystal structures for some molybdenum hydroxylases,

the chemistry of the mechanism is now better understood (22).

Xanthine oxidase has been studied for more than 40 years and its mechanism of

action has been unclear for most of that time (9). The close relationship between AO and

XO/XOR implies a common mechanism of action for these two enzymes. There have

been two proposed mechanisms for the xanthine oxidoreductase enzyme. The two

mechanisms differ in the source of the labile oxygen atom. The alternate mechanisms

illustrate either the oxygen from the oxo group (Mo=O) or the oxygen from the hydroxyl

group (Mo-OH) as the catalytically labile (least stable) oxygen from the active site of the

enzyme (23). The oxygen used as the labile one defines which oxygen atom is ultimately

transferred to the substrate (17). Until about 1996, most researchers believed it was the

Mo=O group that donated its oxygen to the substrate (17) as an electrophile and the oxo

ligand being revived from a water molecule (24). However, electron spin echo (ESEEM)

and electron-nuclear double resonance (ENDOR) studies of the Mo(V) intermediate of

the reaction have provided evidence that it is the oxygen atom from the Mo-OH moiety

that is incorporated into the substrate (22). Subsequent experiments from other

researchers have agreed with the evidence that supports the Mo-OH group as containing

the labile oxygen. Since this is the case, only the agreed upon mechanism will be shown

in this thesis using xanthine as the example substrate. This oxygen atom transfer

reaction begins with an active site base abstracting the hydrogen from the Mo-OH group.

The group then proceeds to initiate nucleophilic attack at the C-8 position of the xanthine

substrate (22). The C-8 position on xanthine is the site of attack due to it being adjacent

to two ring nitrogens and therefore the more electropositive carbon atom. At the same

time, a hydride transfer is taking place from the C-8 position on the xanthine substrate to

the $Mo^{VI}=S$ group. This then leads to a reduction of the molybdenum atom from +6 to a

+4 oxidation state. Loss of the proton from the sulfur and electron transfer produces

17

transient formation of the Mo(V) intermediate which has a very rapid EPR signal. Water

then enters the reaction, the oxidation product dissociates, and its position on the

molybdenum center is replaced by a water or hydroxyl group. This then results in an

increase in oxidation state of the molybdenum metal from Mo(V) to Mo(VI) (17). The

conversion of aldehydes to the corresponding carboxylic acid proceeds in like manner via

a base-assisted nucleophilic attack of the Mo-OH on the substrate carbonyl with

concomitant hydride transfer to the Mo=S (Fig. 10).
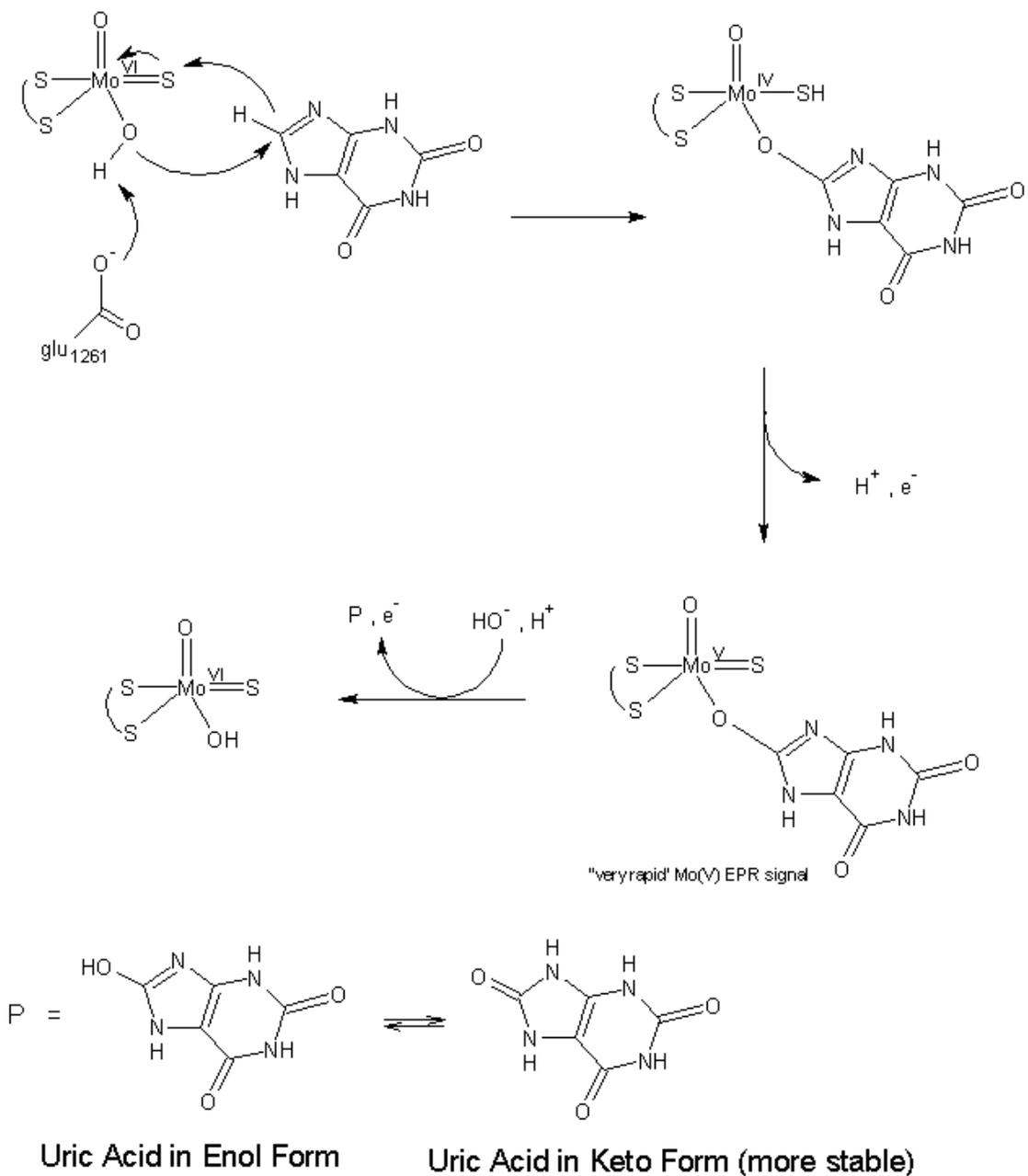
Figure 9. The Reaction Mechanism for Xanthine Oxidase. Active site base abstracts proton form the Mo-OH group which then undertakes nucleophilic attack on the C-8 position of the xanthine substrate with concomitant hydride transfer to Mo=S. Formation of paramagnetic species followed by displacement of product by hydroxide form solvent returns active site to original state.
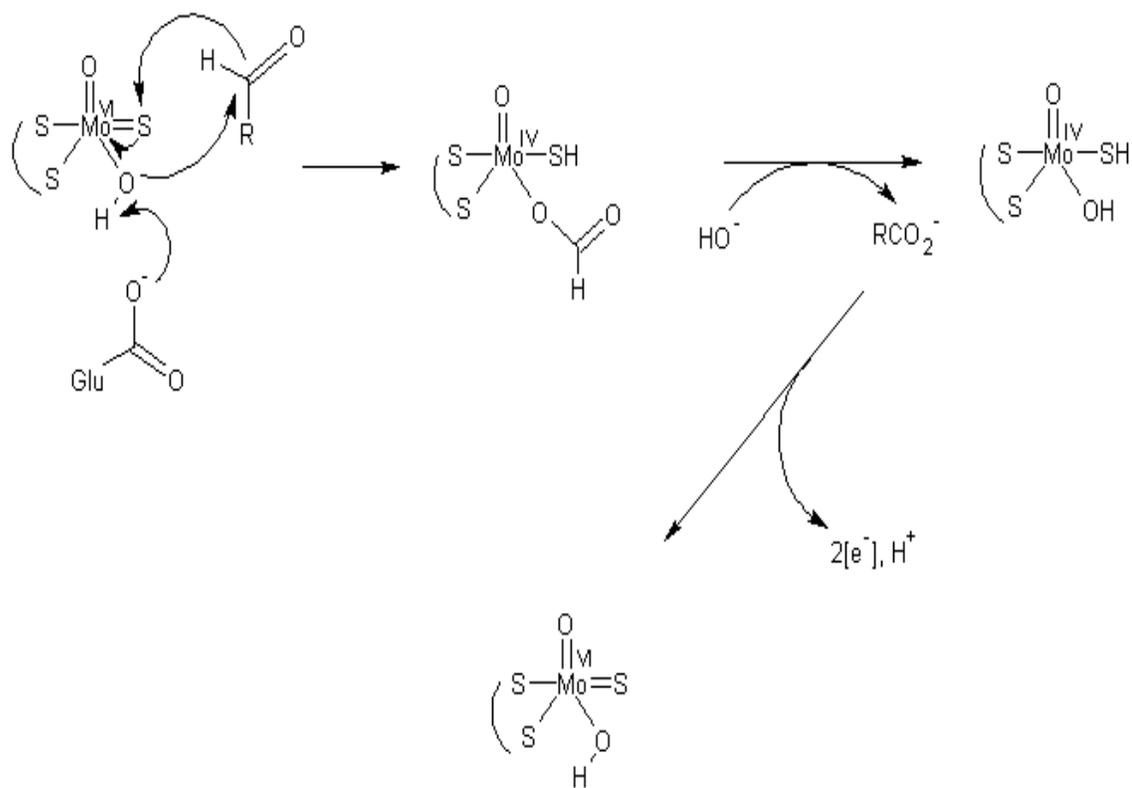
Figure 10. A General Reaction Mechanism for the Aldehyde Oxidase Enzyme. A base-assisted nucleophilic attack of the Mo-OH on the substrate carbonyl with concomitant hydride transfer to the Mo=S which leads to the conversion of the aldehyde to its corresponding carboxylic acid.

Aldehyde oxidase and xanthine oxidase are both complex metalloflavoproteins. They are homodimers due to the existence of two identical subunits. Each subunit has a molecular weight of about 145,000 Daltons (145kDa) for a total of about 290,000 Daltons (290kDa) per dimer (2). However the weight can vary according to species (8). Each monomeric subunit consists of four redox centers which include one molybdenum atom contained in a molybdopterin molecule (85kDa), one FAD molecule (40kDa), and two non-identical iron-sulfur [2Fe-2S] groups (20kDa) identified as Fe/S I and Fe/S II (2). This makes each enzyme contain eight iron atoms and eight sulfur atoms. The geometry and ligation of molybdenum in AO and XO are identical.

In vivo, molecular oxygen serves as the electron acceptor for aldehyde oxidase. Xanthine oxidoreductase, on the other hand, exists primarily in the dehydrogenase form and its electron acceptor is NAD^+. Those enzymes function by being alternately reduced by the substrate and then reoxidized by their respective electron acceptors. In vitro, potassium ferricyanide ($K_3[Fe(CN)_6]$) and 2,6-dichloroindophenol (DCIP) have proven to be effective artificial electron acceptors for these enzymes, replacing molecular oxygen (25).

Within the enzyme structure, flavin adenine dinucleotide (FAD) is the site where electrons are transferred to molecular oxygen where they produce the superoxide anion (O_2·-) in XOR and AO catalysis, or to NAD^+ to form NADH in XDH catalysis (18).

21

However, XDH can utilize both O_2 and NAD^+ as an electron acceptor. XO and AO are known to only use molecular oxygen as an electron acceptor in vivo (18). AO has been known to produce hydrogen peroxide (H_2O_2) as well as the superoxide anion (18). The ability of AO and XOR to produce the reactive oxygen species H_2O_2 and $O_2\cdot^-$ has demonstrated their role in diseases such as ischemia-reperfusion injury and ethanol hepatotoxicity (1). The reduction of oxygen leads to the production of the reactive oxygen species (ROS) O_2^- and H_2O_2. Especially with respect to XOR, this has become a critical issue of interest to researchers due to its apparent role in oxidative stress (2). However, studies have found that the oxidation of aldehydes by AO in the presence of O_2 have also resulted in the production of large amounts of O_2^- and H_2O_2 (11). Oxidative stress is associated with pathological conditions including Alzheimer's disease, diabetes, rheumatoid arthritis, cancer and other such diseases. Oxidative stress is also proposed to be one of the main factors contributing to the biological symptoms of aging (26). Biologically speaking, oxidative stress is an imbalance between the production of reactive oxygen species and a biological systems ability to quickly detoxify the reactive intermediates or easily repair the damage that was caused. Continued oxidative stress causes damages to the entire cell, proteins, lipids and DNA (27). Chemically speaking, oxidative stress is a significant increase in cellular reduction potential (becoming less negative) or a large decrease in the reducing capacity of the cellular redox couples (27).

When molecular oxygen is utilized as the electron acceptor in AO and XOR catalyzed oxidations, the oxygen undergoes a two-electron reduction producing H_2O_2 and

a slower one-electron reduction to produce $O_2\cdot-$ (1, 11, 28). These two pathways are illustrated below in two distinct kinetic phases (Fig. 11). In both phases, the reduction of O_2 occurs at the FAD site (1). The reaction in the case of XDH, where the electron acceptor is NAD^+ is also shown in figure 11. This pathway is a two-electron process (28).

$$FADH_2 \quad + \quad O_2 \quad \longrightarrow \quad FAD \quad + \quad H_2O_2$$

Fully Reduced   Molecular   Flavin Adenine   Hydrogen
Flavin   Oxygen   Dinucleotide   Peroxide
                                                                (a ROS)

$$FADH\cdot \quad + \quad O_2 \quad \longrightarrow \quad FAD \quad + \quad O_2\cdot- \quad + \quad H^+$$

Falvin   Superoxide   Proton
Semiquinone   Oxygen   Flavin Adenine   Anion (a ROS)
                                          Dinucleotide

$$FADH_2 \quad + \quad NAD^+ \quad \longrightarrow \quad NADH \quad + \quad FADH\cdot$$

Fully Reduced   Oxidized   Reduced   Flavin
Flavin   Nicotinamide   Nicotinamide   Semiquinone
                       Adenine   Adenine   (Radical Form)
                       Dinucleotide   Dinucleotide

Figure 11. Reduction Pathways Occuring in XOR/AO and XDH. The three different reduction pathways occurring at the FAD site within the enzyme structure. The O_2 reactions occur within the AO and XO enzyme structure. The NAD^+ reaction occurs within the XDH enzyme structure.

Molybdenum hydroxylases generally catalyze oxidation reactions that largely involve nucleophilic attack of electrophilic carbons including those of aldehyde groups and carbons of N-heterocycles that are para to or adjacent to the ring-nitrogen. The carbons adjacent to the ring-nitrogen in N-heterocycles are normally the most electropositive, eg. in pyrazine, purine, pyrimidine, quinoline, pyridine and pteridine (2).

Aldehyde oxidase and xanthine oxidase catalyze the oxidations of many different N-heterocyclic compounds as well as aliphatic and aromatic aldehydes to their corresponding carboxylic acids (14). As indicated earlier, AO and XO are very closely related enzymes in terms of their structural properties. Despite their ability to oxidize the same class of substrates and their structural similarities, AO and XO vary in their substrate specificities and their response to inhibitors (11, 18). For this reason, it is experimentally possible to differentiate between the enzymes by measuring the activities obtained by reacting them with different substrates or by subjecting the oxidation reactions to different inhibitors. For example, the well known endogeneous substrate xanthine is converted to uric acid by XO but AO is incapable of catalyzing this reaction. On the other hand, AO easily converts N^1-methylnicotinimide to its pyridone derivative but XO does not.

Despite its name, aldehyde oxidase not only oxidizes aldehydes but also catalyzes the hydroxylation of aromatic azaheterocycles possessing a –CH=N- moiety such as phthalazine and purine or aromatic and non-aromatic charged heterocycles with a

24

–CH=N$^+$ group like N^1-methylnicotinamide and N-methylphthalazinium (11). Xanthine

oxidase has a smaller substrate specificity range than AO and is mostly involved in the

oxidation of purines (18). The accepted physiologic role of XOR is catalyzing the

oxidation of hypoxanthine to xanthine and ultimately to uric acid (2). Besides xanthine,

XO also catalyzes the oxidation of a number of xenobiotics, such as the conversion of 6-

deoxyacyclovir to acyclovir (Fig. 12).
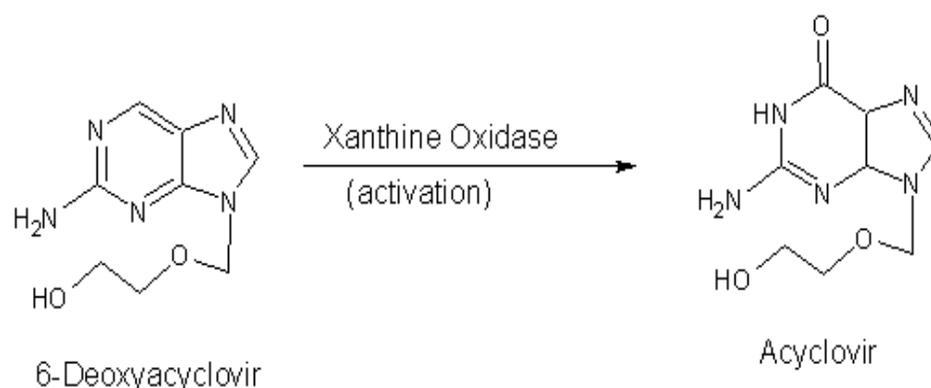
Figure 12. The Activation of 6-Deoxyacyclovir by Xanthine Oxidase. The metabolism of a xenobiotic by xanthine oxidase.

AO and XOR are both important in the metabolism of xenobiotics and

endogeneous compounds (2, 8). Two notable endogenous substrates for AO include

retinaldehyde and pyridoxal (2). Retinaldehyde is the principle component of visual

pigments and for this reason aldehyde oxidase has been suggested to be an important part

of the overall visual process since it catalyzes the biotransformation of this aldehyde to its

corresponding carboxylic acid, retinoic acid, which is the active form of vitamin A (Fig.

13) (5). Because of this it has been suggested that the physiological function of aldehyde

oxidase appears to be the synthesis of retinoic acid from retinal (29). It was once

reported that retinaldehyde is oxidized to retinoic acid by rabbit liver retinal oxidase (EC

1.2.3.11). However, in a report by S. Tomita et. al. (29) retinal oxidase was found to be

identical to aldehyde oxidase.   Pyridoxal is also converted to its corresponding

carboxylic acid by AO to 4-pyridoxic acid (Fig. 14).
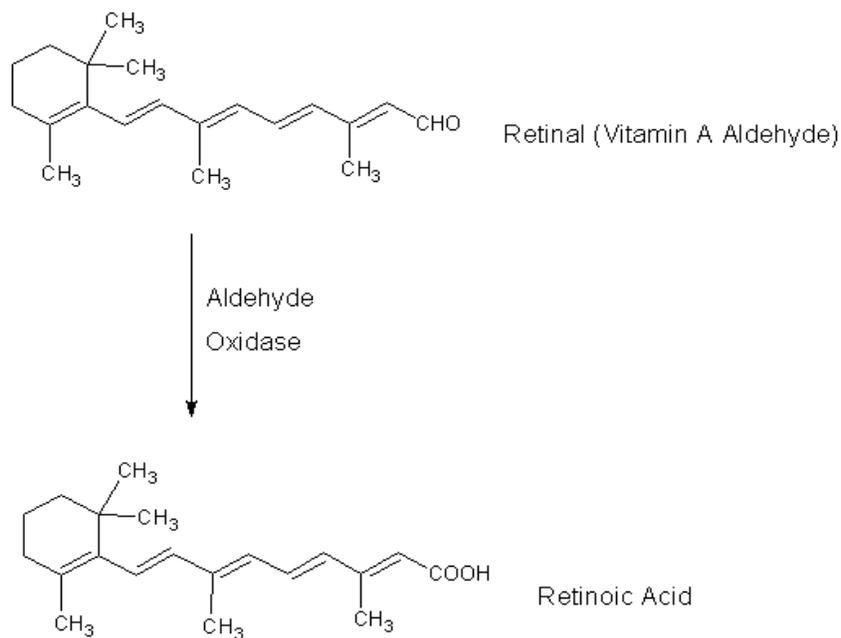
Figure 13. Metabolism of Retinal Catalyzed by Aldehyde Oxidase. Conversion of
retinaldehyde to its corresponding carboxylic acid by aldehyde oxidase. Proposed to be a
primary physiological role for aldehyde oxidase.
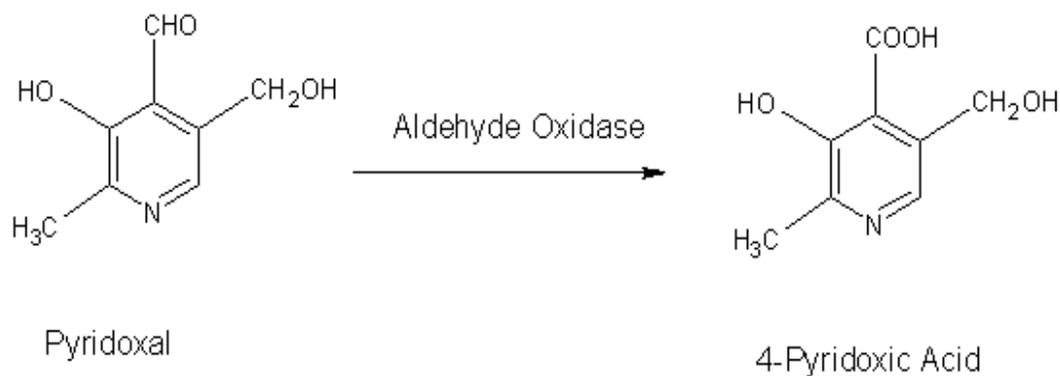
Figure 14. Metabolism of Pyridoxal Catalyzed by Aldehyde Oxidase. Oxidation of pyridoxal to its corresponding carboxylic acid catalyzed by aldehyde oxidase.

With respect to the rate of aldehyde oxidation, aldehyde oxidase has generally been shown to achieve faster rates of conversion than xanthine oxidase. A good example is the aromatic aldehyde vanillin which has been shown to be rapidly converted to its metabolite vanillic acid by AO but only slowly converted to its metabolite by XO (Fig. 15) (14). In addition, the molybdenum site in AO may be more accessible to solvent than the molybdenum site in XO as determined by the fact that cyanide, a compound that renders both enzymes inactive by covalent modification, works faster in AO than XO.

O=\
   \H   O=\
                                  \OH

4-hydroxy-3-methoxybenzaldehyde   4-hydroxy-3-methoxybenzoic acid

(Vanillin)   (Vanillic Acid)
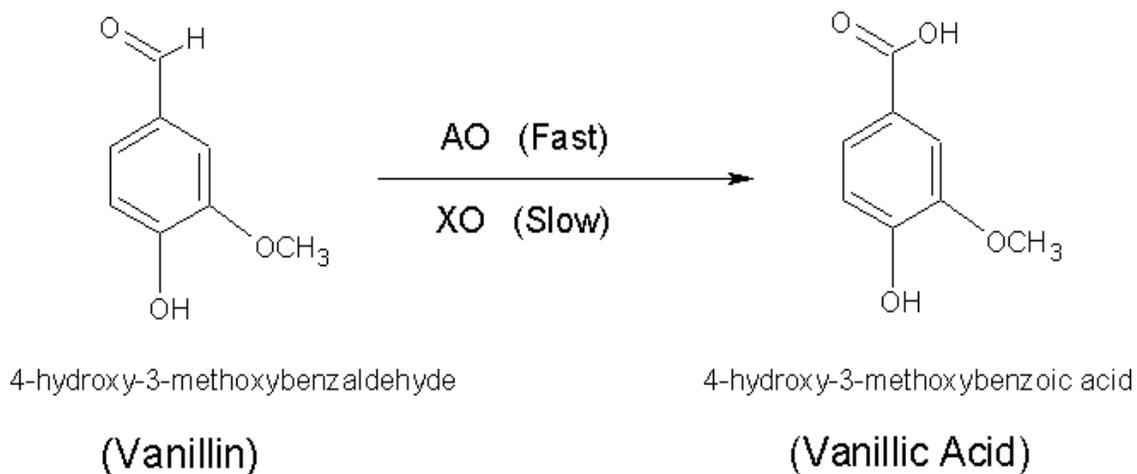
Figure 15. Metabolism of Vanillin to Vanillic Acid. Conversion of vanillin to its corresponding carboxylic acid catalyzed by either aldehyde oxidase or xanthine oxidase. Aldehyde oxidase results in a faster conversion.

AO and XO have many common substrates as well. AO, like XO, can also catalyze the oxidation of purines and in some cases with higher activity than by XO (28). For example, 6-mercaptopurine is oxidized by AO to 6-mercaptopurin-8-one and by XO to 6-mercaptopurin-2-one (2). XO ultimately converts this to 6-thiouric acid (Fig. 16).
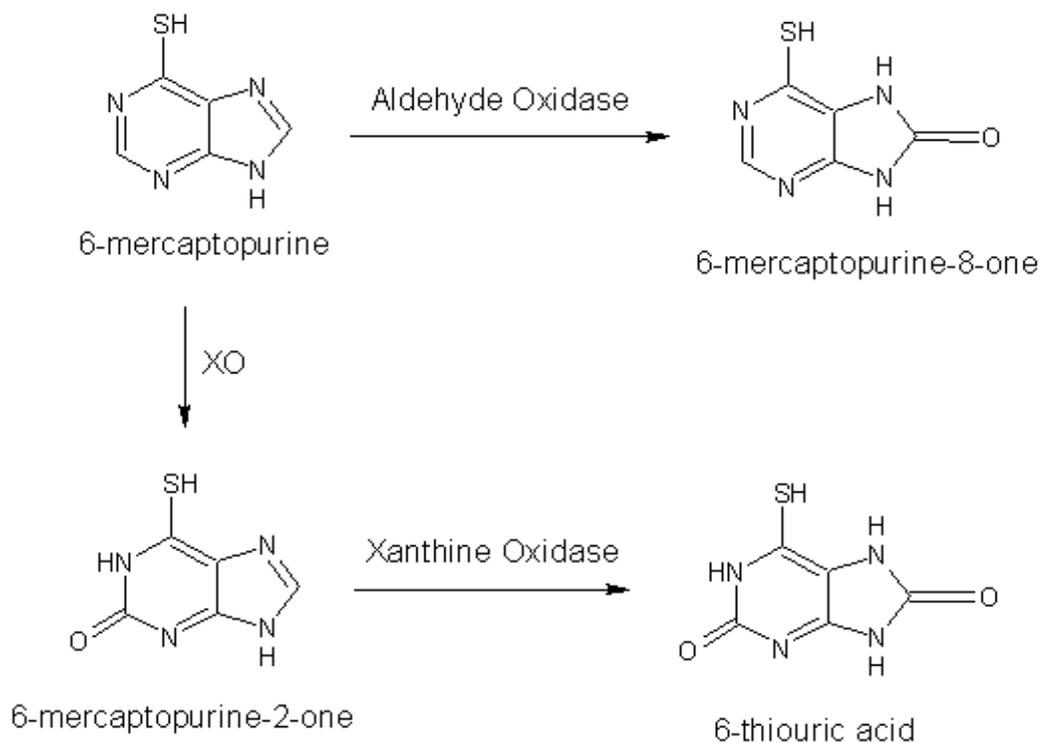
Figure 16. The AO and XO Conversion of 6-Mercaptopurine. The oxidation of 6-mercaptopurine, a common substate for AO and XO, to different oxidative products.

AO/XO Inhibitors

AO and XO are often expressed together in mammalian tissues. In order to increase the level of certainty that aldehyde oxidase is the enzyme responsible for the activity measured with its substrates, documented inhibitors of AO are useful in studies with crude tissue homogenate or partially purified enzyme fractions. Measurements of activity in the presence and absence of inhibitors aid in confirming that the activity measured is truly from the enzyme of interest, that is in our case, aldehyde oxidase.

These studies also help to gain a better understanding of structural determinants that are critical for effective aldehyde oxidase inhibition and help determine the role inhibition plays in drug interactions (30).

Menadione (1×10^{-5} M), methanol (0.3 M), and potassium cyanide (1×10^{-4} M) have all been shown to inhibit the conversion of benzaldehyde to benzoic acid by human liver aldehyde oxidase by 55%, 67%, and 66% respectively (31). It has been demonstrated that methanol inhibits the enzyme by attacking at or near the substrate-binding site. Menadione, on the other hand, inhibits by interfering with the reduction and reoxidation of the internal electron transport chain components and does not bind to the substrate binding site (31). Cyanide and methanol have been determined to be just as effective at the inhibition of phenazine methosulfate (PMS) oxidation as they are for benzoic acid by human liver aldehyde oxidase but not menadione (31). However, menadione is very effective at inhibiting PMS oxidation of rabbit liver aldehyde oxidase (31). Menadione is a well established AO inhibitor and is often used as a standard for inhibition studies of AO catalyzed reactions. Cyanide has been shown to be a potent irreversible xanthine oxidase family inhibitor. Some classifications of Moco containing enzymes have been based on cyanide inhibition studies (4).

N^1 - methylnicotinamide (NMN) is a charged aromatic heterocycle containing a $(-C=N^+<)$ and is a stable iminium ion (28). NMN is formed from nicotinamide by nicotinamide methyltransferase. It is widely dispersed throughout the animal kingdom and has been discovered to be toxic to nerve tissue, however its oxidative products are not. Aldehyde oxidase has been found to catalyze the oxidation of NMN to the non-toxic compounds N^1-methyl-2-pyridone-5-carboxamide (2-PY) and N^1-methyl-4-pyridone-3-carboxamide (4-PY) (Fig. 17) with varying amounts according to species analyzed (32). NMN has been proven to be a good substrate for AO and is inactive as a substrate for XO. Therefore NMN provides a specific probe for the presence of the aldehyde oxidase enzyme.
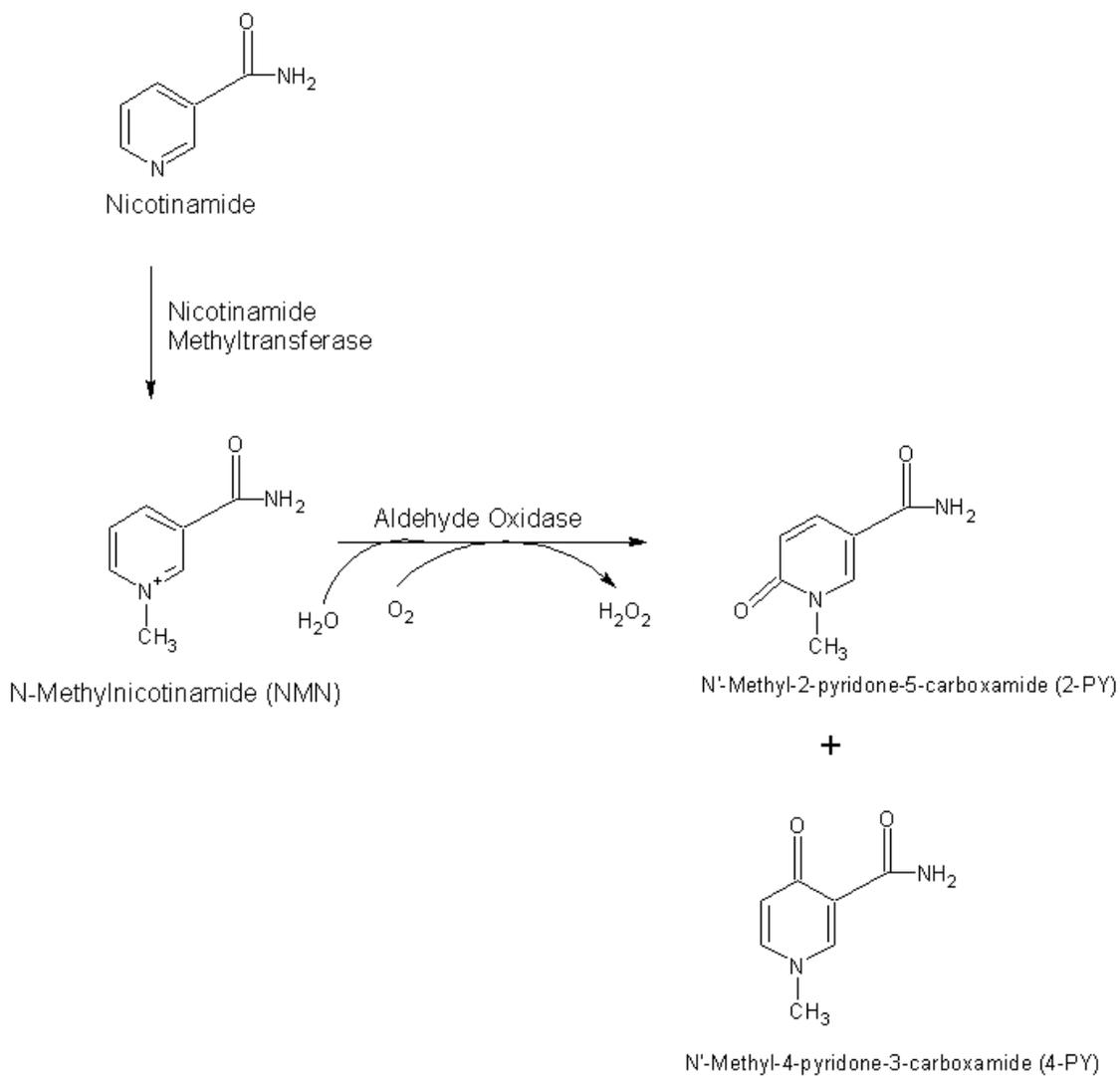
Figure 17. Nicotinamide to Pyridone Derivatives of NMN. Methylation of nicotinamide by nicotinamide methyltransferase followed by oxidation of N^1-Methylnicotinamide by aldehyde oxidase to N^1-methyl-2-pyridone-5-carboxamide (2-PY) and N^1-methyl-4-pyridone-3-carboxamide (4-PY).

Phenanthridine is an azaphenanthrene and has been detected in cigarette smoke, urban air particles, petroleum, shale oil, and coal products (33). For this reason, phenanthridine is a xenobiotic that enters the body as a pollutant. Phenanthridine and other azaphenanthrenes were mutagenic in *Salmonella typhimurium* in the presence of rat liver homogenate. Phenanthridone is an oxidative product and minor oxidative metabolite of phenanthridine which was also found to be mutagenic in *S. typhimurium*. Phenanthridine is a good example of an uncharged xenobiotic substrate of aldehyde oxidase. The oxidative product, phenanthridone, is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Fig. 18) (34).
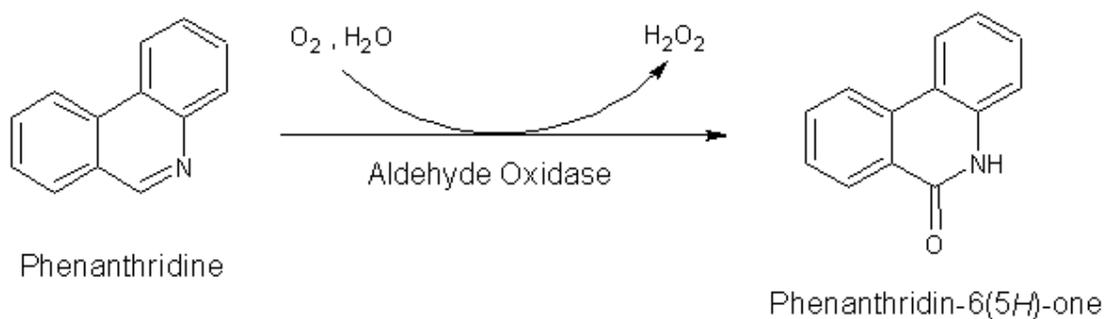
Figure 18. Oxidation of Phenanthridine to Phenanthridone by Aldehyde Oxidase. Phenanthridine is a common substrate for aldehyde oxidase activity studies.

Methotrexate (MTX, 4-amino-N^{10}-methylpteroyl-L-glutamic acid) formally known as amethopterin, is a pteridine compound. A pteridine is an aromatic heterocycle containing –C=N< moiety (28). Methotrexate has been for a long time a common drug used in the treatment of leukemia in children and other adult and childhood cancers. More recently it has been a standard drug used for the treatment of psoriatic and rheumatoid arthritis (35). It has been reported that this nitrogeneous heterocyclic xenobiotic is oxidized to its major metabolite 7-hydroxymethotrexate (7-OH-MTX) by rat liver aldehyde oxidase (36) and rabbit liver aldehyde oxidase (Fig. 19) (37). When MTX is used for therapeutic purposes, the amount is extremely important since 7-OH-MTX has been determined to be cytotoxic (36). The low solubility of 7-OH-MTX in aqueous solutions is the cause of its potentially high cytotoxicity following either intermediate or high dose methotrexate therapy (38). It was shown in monkey kidney following high dose MTX therapy that crystalline deposits of 7-OH-MTX had accumulated within the tubules thereby causing impairment of renal function (39). Since it is the case that 7-OH-MTX is cytotoxic and has significant pharmacological effects, the hydroxylating activity of methotrexate to 7-hydroxymethotrexate becomes extremely important for the clinical administration of MTX. This then makes methotrexate a good candidate for AO activity assay studies by kidney aldehyde oxidase for the purpose of determining if AO may be contributing to the presence of 7-OH-MTX, therefore contributing to cytotoxicity of kidney cells.
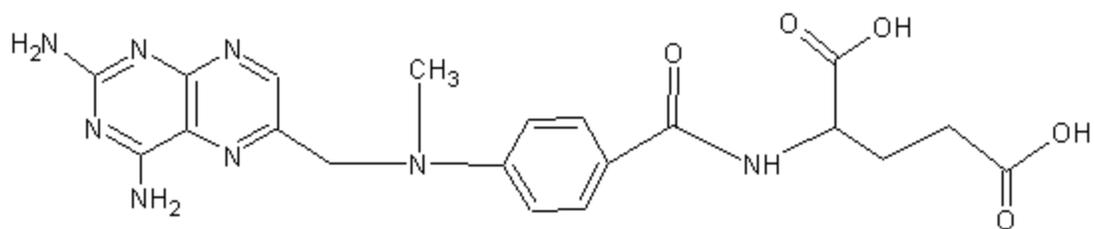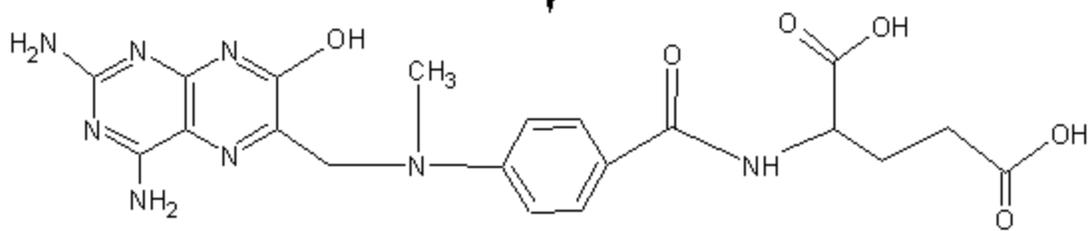
Figure 19. Oxidation of Methotrexate (MTX) to 7-Hydroxymethotrexate (7-OHMTX) by Aldehyde Oxidase.

Benzaldehyde is the simplest of aromatic aldehydes and is a well known

xenobiotic substrate for aldehyde oxidase. It is an important component of the scent of

almonds and the primary component of bitter almond oil extract. It can also be extracted

from other natural sources such as cherry and apricot (40). Therefore, benzaldehyde is a

xenobiotic in that it is injested through various food stuffs. On oxidation, benzaldehyde

is converted to the odorless benzoic acid. Benzaldehyde often serves as a standard for

comparison to other substrates for the measurement of aldehyde oxidase activity.

Benzaldehyde assays are very sensitive to the inhibitor menadione, which can be useful

when xanthine oxidase is also present in partially purified enzyme preparations.

Partially purified human liver aldehyde oxidase has been shown to have a high affinity

toward the benzaldehyde substrate (41). The aldehyde oxidase enzyme catalyzes the

conversion of benzaldehyde to its corresponding carboxylic acid, benzoic acid (Fig. 20).
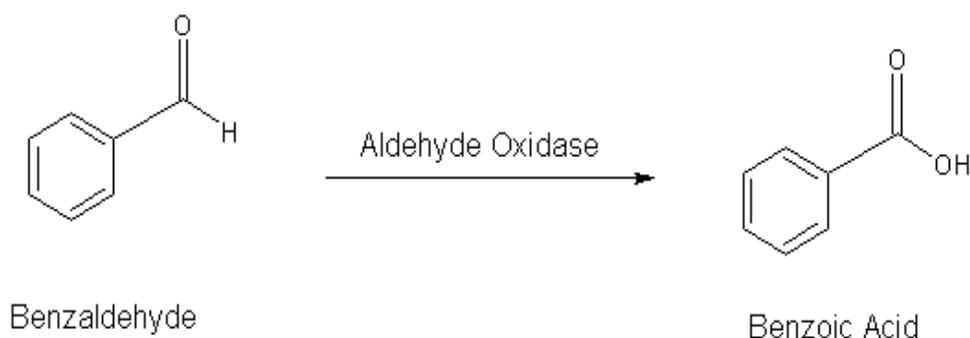
Figure 20. Oxidation of Benzaldehyde to Benzoic Acid. Conversion of benzaldehyde to its corresponding carboxylic acid, benzoic acid, catalyzed by aldehyde oxidase.

Xanthine is an endogenous compound that is derived from guanine in a reaction catalyzed by guanase and is also formed when hypoxanthine is oxidized by either xanthine oxidase or xanthine dehydrogenase. It is ultimately converted to uric acid by XO or XDH (Fig. 21) (23). Xanthine is a purine base that is found in most body tissues and body fluids and is a product on the pathway of purine degradation (42). Xanthine is, therefore, the most common substrate used for measurement of xanthine oxidase activity.
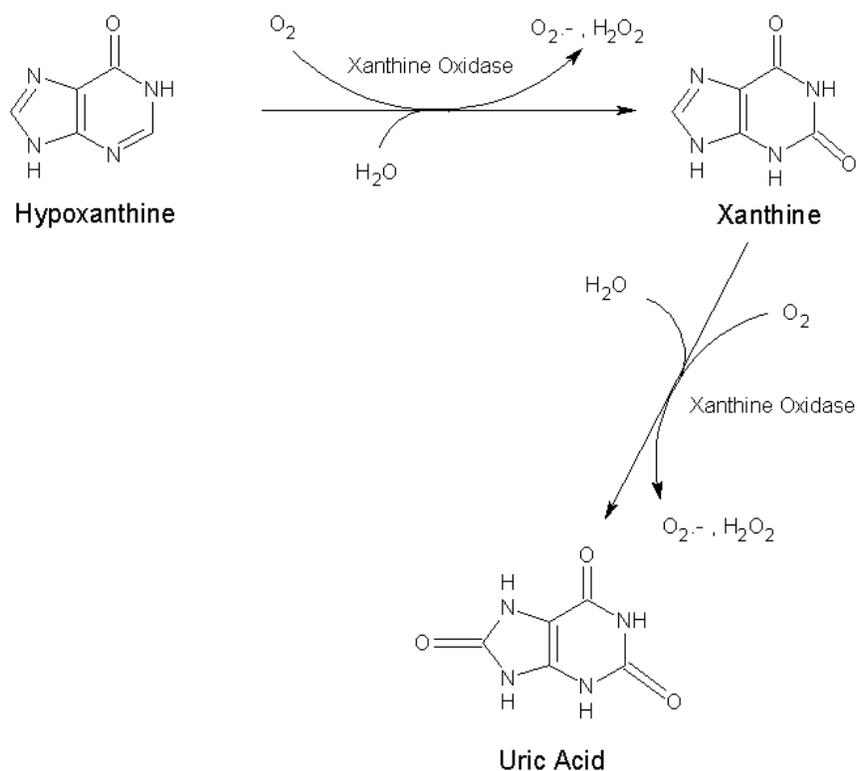
Figure 21. Hypoxanthine to Uric Acid by Xanthine Oxidase. Oxidation of hypoxanthine to xanthine and then to uric acid, catalyzed by xanthine oxidase, the primary physiological role of xanthine oxidase.

Phenazine methosulfate (PMS) serves as an electron acceptor for both the xanthine oxidase and aldehyde oxidase enzymes. It is a dye that produces a red or yellow color when oxidized under aerobic incubation but a clear color in its reduced state (43, 44). PMS can also serve as a substrate for oxidation by aldehyde oxidase (Fig. 22) (43). The red oxidative product produces a significant rise in absorbance at a wavelength of 520 nm, which provides a sensitive and reliable spectrophotometric assay for aldehyde oxidase activity (43). PMS is an exception to the rule that oxidation normally takes place at the carbon atom adjacent to the ring nitrogen of an N-heterocycle structure, and this reaction is one of the few examples of oxidation by a molybdenum hydroxylase occurring in a benzenoid ring system.
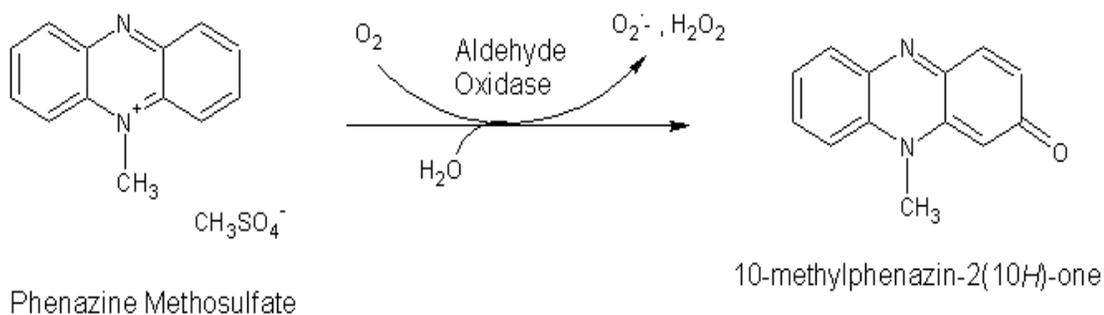
Figure 22. Oxidation of Phenazine Methosulfate to 10-Methylphenazinone by Aldehyde Oxidase.

Renal and Hepatic Drug Metabolism

The principle site of drug metabolism is the liver. However, the kidneys, lungs, and gastrointestinal tracts are also important metabolic sites (45). This makes the enzymatic reactions that take place in those extrahepatic organs also an important area of research. The kidney tissue, though only representing about 0.5% of the body weight, receives between 20-30% of the cardiac output (46). The kidney also accounts for approximately 10% of the oxygen consumption for the whole body. The kidneys have many important physiological functions including filtering out urea, which is waste product carried in the blood, and concentrating it for excretion and maintaining and adjusting the pH of the blood (47). It is also the main route for molybdenum excretion (48). Other important physiological functions of the kidneys include maintenance of water and electrolyte balance, synthesis, metabolism and secretion of hormones. In addition, the kidneys also play a major role in the excretion of drugs, hormones, and xenobiotics (49). The role of the kidney in the metabolism of endogenous and exogenous compounds has not been given due research or attention. Most of the research and acquired knowledge concerning xenobiotic metabolism is based on liver studies. However, it is becoming increasingly apparent that the kidney is actively involved in the metabolism of many drugs and xenobiotics. As a matter of fact, it has even been shown that in some cases certain biotransformations occur faster in the kidney than in the liver; e.g., the glycination of benzoic acid (Fig. 23) (50).
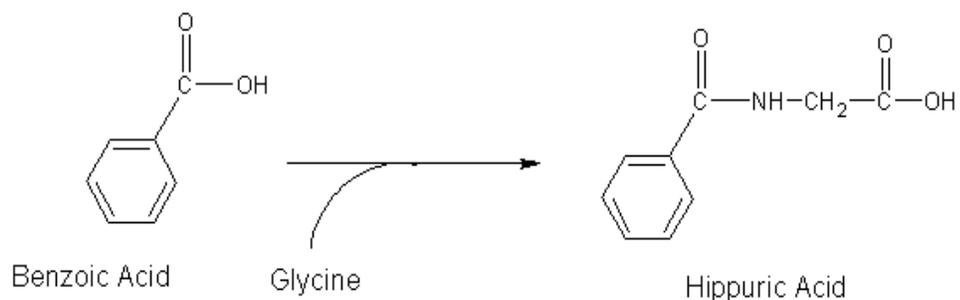
Figure 23. Glycination of Benzoic Acid. A biotransformation reaction that occurs faster in the kidney than in the liver.

Experimental data accumulated over the last 20 years has shown a large capacity for the metabolism in the kidney that leads to activation or inactivation of many different compounds (49). Additionally, the metabolites produced by the kidney may exert significant toxic effects. Such is the case with the oxidation of methotrexate, which was discussed earlier. The kidney has the ability to metabolize a large variety of substrates depending on their availability in the flow of blood. The fact that the blood flows through the kidney carrying with it a variety of metabolites illustrates the importance of what enzymatic reactions may occur within that organ. The oxidative metabolism in the kidney occurs mostly in the cortical segments of the nephron.

Hepatic drug metabolism is already well established. Enzymatic activity in the liver is extremely important since the liver is considered the body's central metabolic clearing house. The liver is responsible for maintaining the correct levels of nutrients in the blood for use by the brain, muscles, and other tissues. All the nutrients absorbed by

the intestines except fatty acids drain directly into the liver. The liver, being the body's major metabolic processing unit, has a great many specialized biochemical functions which includes the detoxification of biologically active substances such as drugs, poisons, and hormones by a number of oxidation, reduction, hydrolysis, conjugation, and methylation reactions (47).

Molybdenum Hydroxylases vs. Monooxygenases

Cytochromes P450 are a very large family of heme-containing monooxygenase enzymes that are better known for metabolizing most of the xenobiotics and foreign chemicals in the body. Like AO and XO, the P450 enzymes that are responsible for metabolizing xenobiotics show their highest activities in the liver, while they are also present in extrahepatic tissue including the lung and the kidney (26).

Cytochromes P450 generally catalyze electrophilic oxidation reactions whereas molybdenum hydroxylases catalyze nucleophilic oxidation reactions. This then results in different oxidative products (2). For example, caffeine is oxidized to 1,7-dimethyl-xanthine by a P450 enzyme and to 1,3,7-trimethyluric acid by the AO enzyme (2). In contrasting the reaction mechanisms for the two different systems, molybdenum hydroxylases use water as the source of the oxygen atom incorporated into the product whereas the monooxygenase system uses molecular oxygen (O_2). Another important difference lays in the fact that molybdenum hydroxylases produce reducing equivalents

during the course of the oxidation reactions whereas P450 systems consume the reducing

equivalents. The two distinct reaction stoichiometries for the monooxygenases and

molybdenum hydroxylases are illustrated below (Fig. 24) (19).

Figure 24. Cytochrome P450 vs. Molybdenum Hydroxylases. Reactions showing the differences in these two systems. The source of oxygen is the primary difference in the reactions.

There are some partnerships between the cytochrome P450's and molybdenum

hydroxylases. For example, nicotine is oxidized to the nicotine iminium ion by the

cytochrome P450 system and then on to cotinine by aldehyde oxidase (Fig. 25) (2).
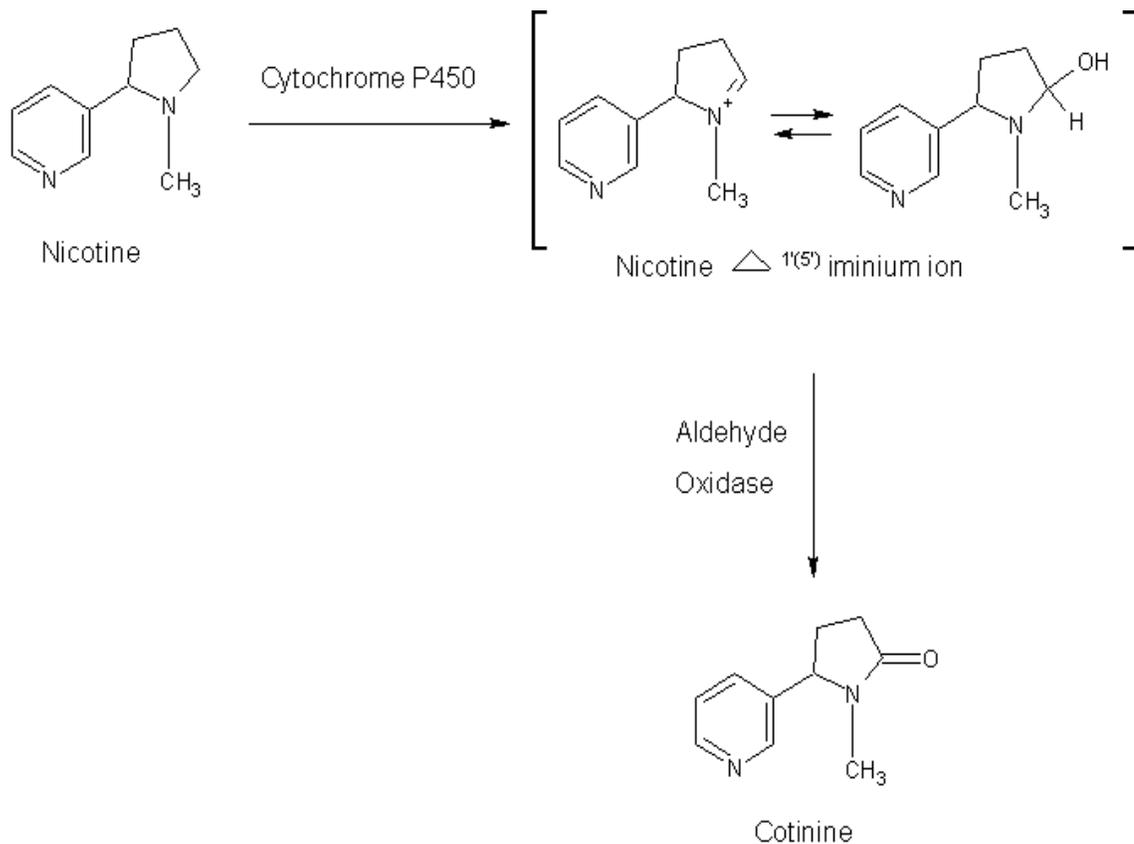
Figure 25. Reaction Showing Partnership Between Cytochrome P450 and Aldehyde
Oxidase. First oxidation reaction catalyzed by cytochrome P450 enzyme followed by
second oxidation reaction catalyzed by aldehyde oxidase.

CHAPTER III

EXPERIMENTAL

Experimental Approaches

There were two main approaches used to quantify aldehyde oxidase and xanthine oxidase activity in the partially purified enzymes; direct continuous and direct discontinuous assay methods. Both methods utilized UV-Visible spectrophotometry to determine the absorbance change during substrate reactions. The direct continuous assays, hereafter only referred to as continuous assays, allow for the production of a progress curve (Fig. 26) (51).

[PRODUCT]
OR
[SUBSTRATE]

REACTION PROGRESS CURVE
SHOWING DECLINATION OF
PRODUCT FORMATION
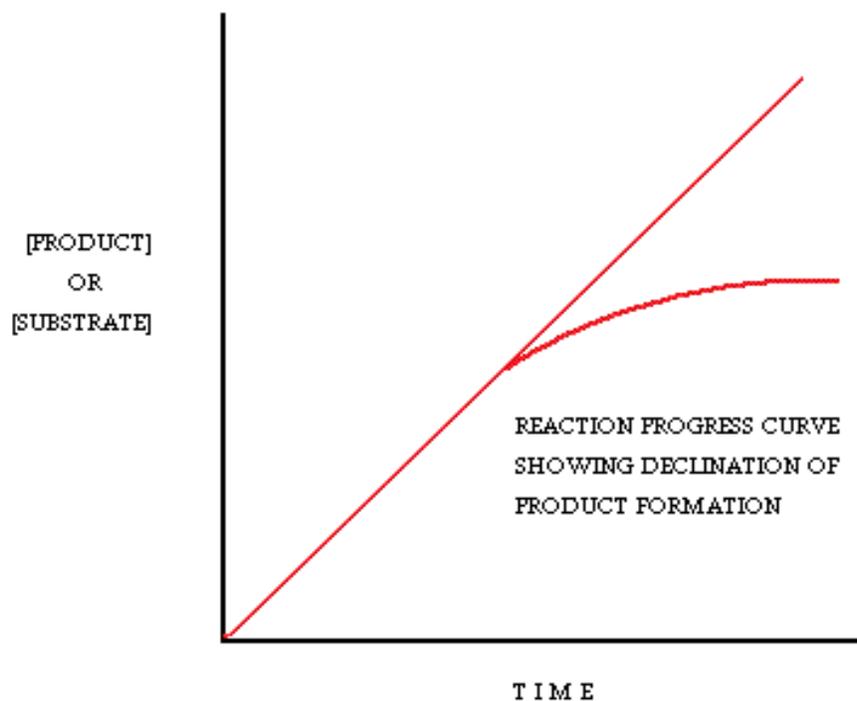
T I M E

Figure 26. Illustration of an Enzyme-Catalyzed Reaction Curve. The enzyme-substrate reaction deviates from a straight line after an extended period of time. Not an actual curve from this research project.

The use of progress curves allows for easy determination of initial rates (51). For this research project, continuous assays were employed to evaluate the following six enzyme-substrate oxidation reactions: AO – phenazine methosulfate, AO – N[1]-methylnicotinamide, AO – phenanthridine, AO – benzaldehyde, AO – methotrexate, and XO – xanthine. These assays were each performed using partially purified bovine liver,

45

bovine kidney, and rabbit liver enzymes. Direct discontinuous assays, hereafter only

referred to as discontinuous assays, give the extent of a reaction after a predetermined

fixed time. This involves stopping the reaction at the chosen time and directly reading

the absorbances. The discontinuous assay method was employed to evaluate the

following four enzyme-substrate oxidation reactions: AO – phenanthridine, XO –

xanthine, AO – N^1-methylnicotinamide, and AO – phenazine methosulfate. These assays

were performed using the partially purified bovine kidney enzyme only.

Specific Activity Formulas

The specific activity formulas that are used to express the activity for enzyme

assays for this research project uses the basis of the Beer's Law (Fig. 27) and

incorporates into it other factors that are necessary in determining the specific activity of

an enzyme in micromoles per minute per milligram of protein. These formulas (Fig. 28

and Fig. 29) (52) are applicable to the two-minute initial rate continuous enzyme assays.

Discontinuous assays use the straight forward Beer's Law as seen in figure 27. The

specific activity formula for the initial rate continuous assays that utilize the natural

electron acceptor, molecular oxygen, differ by a factor of 0.5 to the specific activity

formula for the reactions that utilize ferricyanide as an artificial electron acceptor. This is

due to the fact that one mole of substrate is oxidized per two moles of ferricyanide that

are reduced. This is illustrated in figures 28 and 29.

$$A = \varepsilon bc$$

Figure 27. Beer's Law. Where A is the absorbance, ε is the molar absorptivity, b is the pathlength, and c is the concentration of the sample.

$$[(\Delta A / min) / \varepsilon] \ \times \ mL_{FCV} \ \times \ 1{,}000 \ \times$$

$$(mL_{OLPA} \ / \ mL_{ESI})$$

Figure 28. Specific Activity Formula (O_2 as Electron Acceptor). $(\Delta A / min)$ = absorbance change per minute (raw data generated by UV-Vis software); mL_{FCV} = final cuvette volume in mLs; mL_{OLPA} = dilution factor in mLs if original Lowry Protein Assay solution was diluted prior to transferring to cuvette. If no dilution was done to the original LPA solution then the value equals 1. A value of 1 was used for all assays in this research project; mL_{ESI} = mLs of enzyme solution transferred into the cuvette; **1000** = factor that arises because the units of activity are in µmols/min and because the molar absorptivity gives the concentration in M^{-1} and the cuvette volume is in mL.

$$[(\Delta A / min) / \varepsilon] \; x \; mL_{FCV} \; x \; 1{,}000 \; x$$

$$(mL_{OLPA} \; / \; mL_{ESI}) \; x \; (1 \; mol \; / \; 2 \; mol)$$

Figure 29. Specific Activity Formula (Ferricyanide as Electron Acceptor). **(ΔA / min)** = absorbance change per minute (raw data generated by UV-Vis software); **mL_{FCV}** = final cuvette volume in mLs; **mL_{OLPA}** = dilution factor in mLs if original Lowry Protein Assay solution was diluted prior to transferring to cuvette. If no dilution was done to the original LPA solution, then the value equals 1.   A value of 1 was used for all assays in this research project; **mL_{ESI}** = mLs of enzyme solution transferred into the cuvette; **1000** = factor that arises because the units of activity are in μmols/min and because the molar absorptivity gives the concentration in M^{-1} and the cuvette volume is in mL; **(1 mol / 2 mol)** = factor arises due to the conversion for two mols of potassium ferricyanide that are reduced for each mole of substrate being oxidized.

Specific activities cannot be calculated from the raw data obtained from the thirty-minute discontinuous assays since this assay is not measuring the initial reaction rates of the enzyme-substrate complex. This type of assay only allows for the molarity of the oxidized product or total amount of product produced to be calculated. This is accomplished by using the straight forward Beer's Law equation that was discussed earlier. In the case of the potassium ferricyanide method for the discontinuous assays, the Beer's Law equation is divided by 2 because two moles of potassium ferricyanide are reduced for each mole of substrate being oxidized.

The Artificial Electron Acceptor Method

The use of artificial electron acceptors such as dichloroindophenol (DCIP) and potassium ferricyanide have proven to be effective replacements for the natural electron acceptor dioxygen for the monitoring of oxidation reactions that are catalyzed by aldehyde oxidase, xanthine oxidase and many other enzymes. These electron acceptor compounds normally exhibit a maximum absorbance at high wavelengths that are normally within the visible range (380 nm – 780 nm). The maximum absorbances are 600 nm and 420 nm for DCIP and potassium ferricyanide, respectively.

One of the biggest benefits coming from the artificial electron acceptor method is that an enzyme activity experiment can be performed even when the max absorbance (λ_{max}) is not known for a particular substrate. This is in contrast to activity assays using oxygen as electron acceptor where the max absorbance must be known prior to performing the assay. For the sake of the cost of research, time and money on experiments can be saved when using the artificial electron acceptor method since λ max and, therefore, molar absorptivities will not have to be experimentally determined if they are not already known. There have been many substrate oxidations correlated to the reduction of these electron acceptor compounds. These include aromatic and non-aromatic aldehydes, heterocycles, purines, azaphenanthrenes, pteridines and others. For this research project, the potassium ferricyanide assay was utilized to evaluate the following enzyme-substrate oxidation reactions: AO – N^1-methylnicotinamide, AO –

methotrexate, and XO – xanthine. The following reaction illustrates the overall

stoichiometry for the ferricyanide method (Fig. 30). The reaction uses a general aldehyde

as an example substrate though the stoichiometry is the same for all substrates.

$$R-\overset{\overset{\displaystyle O}{\|}}{C}-H \quad + \quad 2[Fe(CN)_6]^{-3} \quad + \quad H_2O$$

$$\text{Aldehyde} \qquad\qquad \text{Ferricyanide } (Fe^{3+})$$

$$\downarrow \text{AO or XO}$$

$$R-\overset{\overset{\displaystyle O}{\|}}{C}-OH \quad + \quad 2[Fe(CN)_6]^{-4} \quad + \quad 2H^+$$

$$\text{Carboxylic Acid} \qquad \text{Ferrocyanide } (Fe^{2+})$$
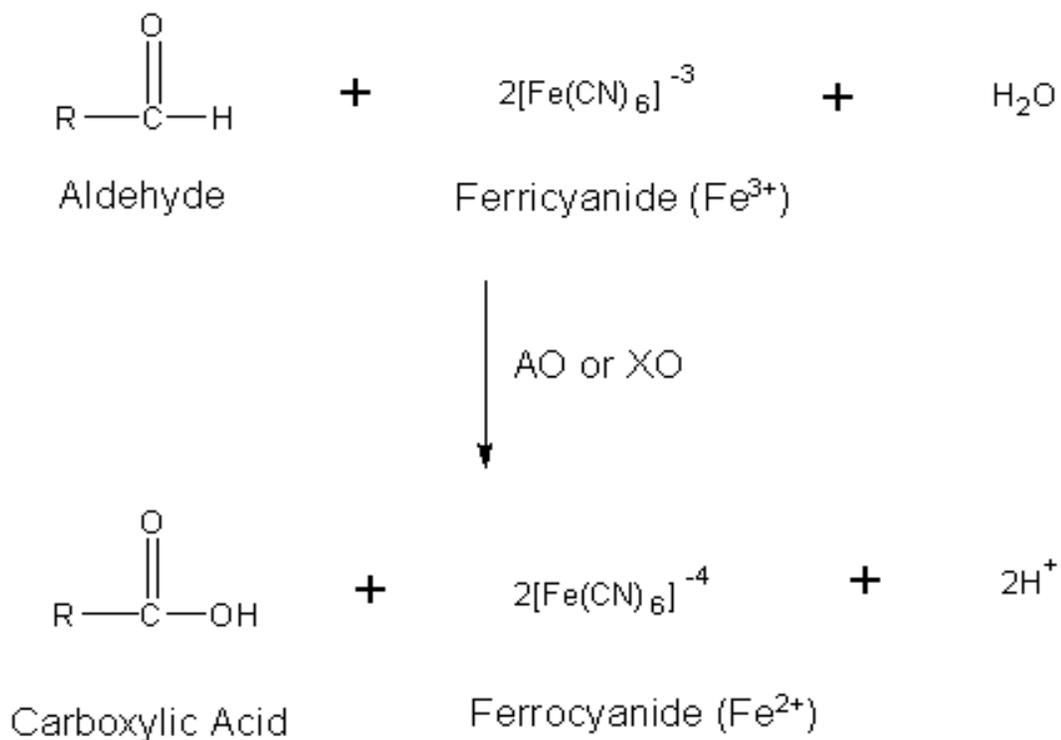
Figure 30. The Ferricyanide Reaction Using an Aldehyde Substrate. Ferricyanide is reduced to ferrocyanide during the course of the reaction.

As can be seen from the reaction above, the aldehyde or substrate, undergoes a

two-electron oxidation which requires two successive one-electron reductions of

ferricyanide to produce ferrocyanide. This spectrophotometric method monitors the coupled reduction of ferricyanide $[Fe^{+3}(CN)_6]$ to ferrocyanide $[Fe^{+2}(CN)_6]$ at 420 nm wavelength. Many assays can be performed this way since ferricyanide shows reduction at the same wavelength regardless of the nature of the oxidative product and that the reduction of ferricyanide parallels the oxidation of the product. This method worked well for our research because aldehyde oxidase and xanthine oxidase are both able to transfer electrons from oxidized substrates to electron acceptors other than oxygen.

Enzyme Purification Procedure

Aldehyde oxidase and xanthine oxidase were partially purified to the ammonium sulfate fraction from the commercial tissues: bovine kidney, bovine liver, and rabbit liver, using a modification of the method of K.V. Rajagopalan, I. Fridovich, and P. Handler, *J. Biol. Chem.*, 237 (1962) 922-928.

Rabbit livers were purchased from Pel-Freez Biologicals of Rogers, Arkansas. Golden Forest® brand beef livers and beef kidneys were purchased from Food Lion LLC of Salisbury, North Carolina.   Rumba® brand beef livers and beef kidneys were purchased from Wal-Mart Stores Inc. of Bentonville, Arkansas. If frozen, the tissue samples were thawed in a 0.05 M potassium phosphate buffer solution containing 0.1 mM EDTA and had a pH of 6.8.

The tissue samples were homogenized using a Waring® laboratory blender for 3 minutes in a 1:5 mixture of tissue sample and 0.05 M potassium phosphate buffer which contained 0.1 mM EDTA, pH of 6.8. The mixture was then rapidly warmed to a temperature of 50^oC in a preheated 75^oC water bath and the temperature of the solution was maintained between 50^oC and 55^oC for ten minutes. The mixture was then placed in an ice-bath and allowed to cool to between 10^oC and 15^oC with frequent stirring. The mixture was then centrifuged for 30 minutes at 4000 rpm and 4^oC in a refrigerated type IEC Centra-8R centrifuge manufactured by International Equipment Company. The supernatant obtained was filtered through glass wool into a graduated cylinder in order to directly determine the volume of supernatant collected. The supernatant was then transferred to a suitable Erlenmeyer flask and placed in an ice-bath. While stirring in the ice-cold water bath, ammonium sulfate was added during a time-duration of five minutes in the amount of 313 grams $(NH_4)_2SO_4$ per liter of original supernatant collected. The solution was allowed to equilibrate for 45 minutes longer while stirring in the ice-bath and then centrifuged for 10 minutes at 4^oC and 4000 rpm. The supernatant collected was discarded and the precipitate was dissolved in 0.05 M potassium phosphate buffer which contained 0.1 mM EDTA, pH of 7.8 at an amount of (80 milliliters buffer per liter of original supernatant). All solutions from the centrifuge tubes were then collected and transferred to a single vessel in which the pH was adjusted to 7.8 with concentrated sodium hydroxide solutions using a Fisher Scientific Accumet® Model 10 pH meter. The resulting solution was then re-centrifuged to remove any protein that precipitated

during the latter stages of the purification procedure or pH adjustment. The purification was stopped here, at the ammonium sulfate fractionation, and the solution was stored in small plastic vials which were stored in a freezer at -20°C for future enzymatic assay tests.

Lowry Protein Assay

Protein concentrations of the partially purified enzyme samples were determined spectrophotometrically according to the often-cited general use protein assay first described in 1951 by Lowry et al., *J. Biol. Chem.*, (1951) 265-275. This Lowry Protein Assay (LPA) utilizes albumin from bovine serum as the primary standard. This protein standard was purchased from the Sigma Chemical Company. Five concentrations of the standard were prepared in triplicate and their absorbances measured at 750 nm on a Cary 100 Bio UV-Vis Spectrophotometer. A calibration curve was then constructed using the fifteen data points. Two dilutions of the sample enzyme solutions were prepared in triplicate. These solutions were treated in like manner to the standard solutions and their absorbances also read at 750 nm. Calculations were performed using the calibration curve to determine the protein concentrations in the dilute sample solutions. These values were then used to determine protein concentrations in the original enzyme solutions and the values averaged. This average value was then used to calculate the volume of enzyme solution needed for the desired final protein concentration in the

cuvettes during enzyme assays.   In most cases, a 1 mg/mL final protein concentration

was used for this research.

Enzyme Assays

All assays were determined by measuring the absorbances of a mixture that

contained the enzyme and substrate in a solution that contained 33 mM potassium

phosphate buffer and 0.07 mM ethylenediaminetetraacetic acid (EDTA) and had a pH of

7.8.   All concentrations noted in this research paper are the final concentrations that are

present in the cuvette. Refer to Table 1 for the concentrations and parameters used in the

assays. An upward arrow (↑) or downward arrow (↓) in front of a wavelength value

within the experimental conditions table signifies whether there was an increase or

decrease in the absorbance being measured for that particular assay. In order to produce

positive absorbance changes, the sample cuvettes and blank cuvettes were switched in the

holders where a decrease in absorbance occurred. All spectrophotometric assays were

run in the presence of a reference cuvette that contained all ingredients except the

substrate. The ingredients in the cuvettes consisted of 2000 μL of 0.05 M potassium

phosphate buffer - 0.1 mM EDTA – pH 7.8, 100 μL of 0.03 M potassium ferricyanide

solution if using the ferricyanide method, appropriate enzyme preparation solution to give

a 1mg/ml final protein concentration (normally about 200 μL), appropriate amount of

substrate solution to give the desired final substrate concentration as shown in Table 1

(normally about 100 µL), and sufficient de-ionized water to give a final assay volume of 3000 µL. In the reference cuvette, the substrate solution was replaced by the solvent used to dissolve the substrate. Phenazine methosulfate, benzaldehyde, and N^1-methylynicotinamide substrates used de-ionized water as a solvent. Methotrexate used 0.01 M NaOH, xanthine used 0.02 M NaOH, and phenanthridine used acetone as solvents. The raw data obtained from the initial rate continuous assay method is in terms of absorbance change per minute. This allows calculation of the specific activity of the enzyme. The procedural steps for this assay are as follows: all prescribed ingredients except for the substrate solution were transferred to the blank and sample cuvettes. The cuvettes were then placed in the UV-Vis spectrophotometer cell holder which had been adjusted to a temperature of 37.0°C. The solutions were allowed to incubate for 5 minutes. At that point, the prescribed volume of substrate solution was transferred to the sample cuvette and the cuvette was inverted 3 to 4 times to mix which was then immediately placed back in the instrument holder. The instrument was programmed to monitor the enzyme-substrate reaction for two minutes at the wavelength specific for the oxidized product. In the case of the enzyme assays that used the artificial electron acceptor, potassium ferricyanide, the instrument monitored the coupled reduction of ferricyanide [$Fe^{+3}(CN)_6$] to ferrocyanide [$Fe^{+2}(CN)_6$] at a wavelength of 420 nm. After the data had been collected, the absorbance change per minute was calculated by the Cary Win UV software. The raw data so obtained was then be used to calculate the specific activity of the enzymes.

55

In contrast to the initial rate continuous assay method where the UV solution ingredients were mixed in the cuvette, the discontinuous assay (long-term incubation) solution ingredients are transferred to and mixed in a 25 mL Erlenmeyer flask. See Table 1 for the concentrations and parameters used in the assays. The procedural steps were as follows: all ingredients minus the substrate are placed in the 25 mL Erlenmeyer flasks. The same principle applies for ingredient volumes for the long-term incubation discontinuous assays as did for the two-minute initial rate continuous assays discussed earlier. A blank solution was also prepared. The 25 mL flasks were then placed in a Fisher Shaking Water Bath Model 127$_®$ where the bath temperature had been adjusted to 37.0^{o}C. While gently shaking, the solutions were allowed to stay in the shaker bath for 5 minutes to assure that they are at the temperature of the bath (37°C). At this point, the prescribed volume of substrate solution is added to the sample solution flask. The solutions are then allowed to incubate while gently shaking for exactly 30 minutes at 37°C. The reaction is then stopped by adding 1.0 mL of ice-cold 95% ethanol to the reaction mixtures. This should also precipitate out the protein. The contents of the flask are then immediately transferred to test tubes which are placed on ice and allowed to cool for 15 minutes. The tubes are then centrifuged in an IEC Clinical Centrifuge for 10 minutes to spin down the protein and to clarify the solution for UV-Vis analysis. The solutions were then transferred to a cuvette and placed in the holder. The wavelength specific for the oxidative product was entered into the software and the absorbances were read at a temperature of 25°C. For the case of potassium ferricyanide assays, the

wavelength was set at 420 nm. The blank solution was placed in the appropriate holder to subtract out the absorbance of this solution that did not contain substrate.

The two minute initial rate continuous assay method was used to evaluate the menadione inhibition of phenazine methosulfate oxidation by rabbit liver and bovine kidney aldehyde oxidase. The prescribed amount of inhibitor solution was added to both the sample and blank solution and the absorbance change per minute was obtained as described earlier for the two-minute continuous assays. Two concentrations of menadione, 3.2×10^{-5} M and 9.1×10^{-5} M, were evaluated for their effect on the oxidation reactions for the two enzymes. The results are discussed in the Results section.

The initial rate two-minute continuous assay method was also used to evaluate the inhibition of 0.3 M methanol on phenazine methosulfate oxidation by bovine kidney, bovine liver and rabbit liver aldehyde oxidase. The prescribed amount of methanol was added to both the blank and sample solutions contained in the 1cm UV cuvettes. The absorbance change per minute was obtained as described earlier for the menadione assay. The results are discussed in the Results section.

A scanning experiment was performed on the phenazine methosulfate oxidation reaction by bovine kidney aldehyde oxidase and rabbit liver aldehyde oxidase. This scanning experiment was performed in order to help verify that the oxidative product of phenazine methosulfate was truly the cause of the increase in absorbance at 520 nm due to the fact that phenazine methosulfate is known to be involved in non-enzymatic

57

oxidation. The solutions were scanned every five minutes for 55 minutes (12 total scans) from 350 nm to 600 nm. Within this range, the peak arising from phenazine methosulfate as well as the peak arising from its red oxidized product can be observed. Phenazine methosulfate has a maximum absorbance at about 380 nm while its red oxidized product has a maximum absorbance at 520 nm. Initially, the PMS concentration used for the scans was the same as used for the specific activity assays, which was 0.2 mM. However, this concentration produced a very large PMS peak with an absorbance of about 3.2. The concentration was reduced to 0.1 mM but still produced a high absorbance of about 2.5. The concentration was then reduced five-fold to 0.04 mM. This produced a reasonable absorbance value between 1.0 and 1.2 for the PMS peak. After the best concentration was determined to produce a reasonable level of absorbance, the procedure was carried out the same as the two-minute assay procedure with respect to solution ingredients, except for the difference in substrate concentration just discussed.

Evaluation of the purification procedure was accomplished by use of the 30 minute discontinuous assay method as described earlier. This evaluation utilized the PMS oxidation reaction and the xanthine oxidation reaction, both by the bovine kidney enzyme.

Substrate Name	Substrate Conc.	Wavelength (λ) (Increase or Decrease)	Molar Absorptivity ($M^{-1}cm^{-1}$)	Potassium Ferricyanide Conc.
Phenazine Methosulfate	0.2 mM	↑ 520 nm	11,700	N/A
N'-Methylnicotinamide	1.0 mM	↓ 420 nm	1,020	1.0 mM
Benzaldehyde	0.05 mM	↓ 249 nm	17,540	N/A
Methotrexate	0.167 mM	↓ 420 nm	1,020	1.0 mM
Methotrexate	0.333 mM	↓ 420 nm	1,020	1.0 mM
Phenanthridine	1.0 mM	↑ 322 nm	9,000	N/A
Xanthine (2'Assay)	0.03 mM	↑ 295 nm	9,500	N/A
Xanthine (30'Assay)	0.17mM	↓ 420 nm	1,020	1.0mM

Table 1. Experimental Conditions for Enzyme Assays. Concentrations are what are present in 3 mL of enzyme solution. Up/down arrows indicate an increase or decrease in absorbance being measured. Same conditions used for 2 minute continuous assays and 30 minute discontinuous assays unless otherwise indicated.

CHAPTER IV

RESULTS

Bovine Kidney Enzyme Specific Activities

The specific activity of the bovine kidney enzyme as determined by the two-minute initial rate continuous assay method showed a large amount of xanthine oxidase activity (0.0327 µmols/min/mg) relative to the aldehyde oxidase activities measured. The highest aldehyde oxidase activity measured was from the N^1-methylnicotinamide substrate (an endogenous substrate for AO) and this activity was only 38% of the xanthine oxidase activity found using the xanthine substrate (the primary endogenous substrate for XO). The order of activity for the aldehyde oxidase substrates from highest to lowest is N^1-methylnicotinamide with 0.0124 µmols/min/mg followed by phenazine methosulfate at 0.0052 µmols/min/mg and finally benzaldehyde at 0.0027 µmols/min/mg. There was no detectable activity observed for the aldehyde oxidase substrates, methotrexate and phenanthridine for the bovine kidney enzyme (Fig. 31, Table 2). However, our research shows that bovine kidney aldehyde oxidase catalyzed the oxidation of the endogenous substrate N^1-methylynicotinamide and the xenobiotic benzaldehyde as well as the well-known aldehyde oxidase substrate phenazine methosulfate.

Figure 31. Beef Kidney Enzyme Specific Activities. Substrate comparisons as determined by the two-minute initial rate continuous enzyme assays of the bovine kidney enzyme.

Substrate	μmols/min/mg
Xanthine	0.0327 +/- 0.0060 (N=3)
N'-Methylnicotinamide	0.0124 +/- 0.0029 (N=2)
Phenazine Methosulfate	0.0052 +/- 0.0017 (N=5)
Benzaldehyde	0.0027 +/- 0.0005 (N=3)
Methotrexate	None Detected (N=2)
Phenanthridine	None Detected (N=2)

Table 2. Activity Values for Beef Kidney (Corresponding to Fig. 31). +/- Values are standard deviations. N is number of sample preparations.

Bovine Liver Enzyme Specific Activities

Like that of the bovine kidney enzyme, the specific activity of the bovine liver enzyme as determined by the two-minute initial rate continuous assay method showed a higher activity for xanthine oxidase than for aldehyde oxidase. The highest aldehyde oxidase activity measured was from the oxidation of the N^1-Methylnicotinamide substrate and this activity was only 76% of the xanthine oxidase activity found using the xanthine substrate. The order of activity for the five aldehyde oxidase substrates from highest to lowest is N^1-Methylnicotinamide at 0.0600 μmols/min/mg followed by benzaldehyde at 0.0384 μmols/min/mg, phenazine methosulfate at 0.0363 μmols/min/mg, phenanthridine at 0.0262 μmols/min/mg and lastly methotrexate at 0.0234 μmols/min/mg. Xanthine oxidase specific activity for the bovine liver enzyme was measured to be 0.0793 μmols/min/mg (Fig. 32, Table 3). Our research showed that bovine liver aldehyde oxidase catalyzed the oxidation of all aldehyde oxidase substates evaluated.
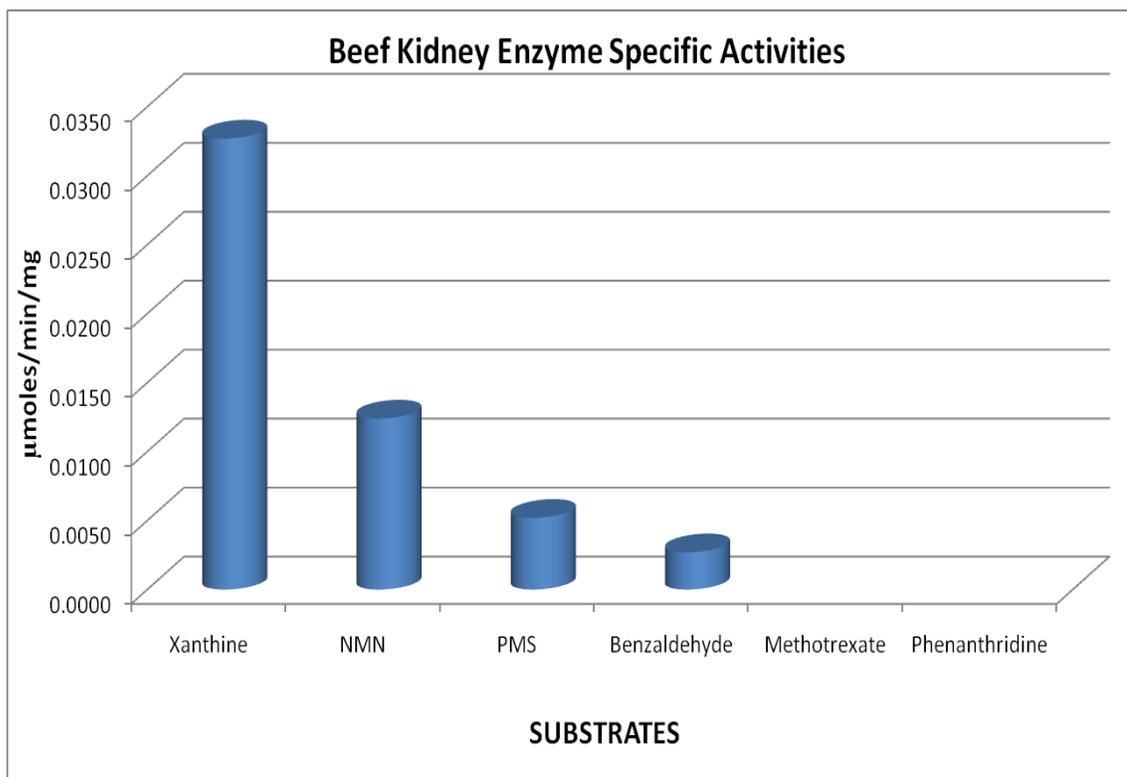
Figure 32. Beef Liver Enzyme Specific Activities. Substrate comparisons as determined by the two-minute continuous initial rate enzyme assays of the bovine liver enzyme.

Substrate	μmols/min/mg
Xanthine	0.0793 +/- 0.0063 (N=3)
N'-Methylnicotinamide	0.0600 +/- 0.0039 (N=3)
Benzaldehyde	0.0384 +/- 0.0026 (N=3)
Phenazine Methosulfate	0.0363 +/- 0.0033 (N=3)
Phenanthridine	0.0262 +/- 0.0028 (N=3)
Methotrexate	0.0234 +/- 0.0002 (N=2)

Table 3. Activity Values for Beef Liver (Corresponding to Fig. 32). +/- Values are standard deviations. N is number of sample preparations.

Rabbit Liver Enzyme Specific Activities

Unlike that of the bovine kidney and bovine liver enzymes, the rabbit liver enzyme as determined by the two-minute initial rate continuous assay method showed a lower xanthine oxidase activity than aldehyde oxidase activity. The xanthine oxidase activity was 50% lower than the lowest aldehyde oxidase activity measured and 87% lower than the highest aldehyde oxidase activity measured. The order of activity for the five aldehyde oxidase substrates evaluated from highest to lowest is N^1-Methylnicotinamide at 0.8243 μmols/min/mg followed by methotrexate at 0.5385 μmols/min/mg, phenazine methosulfate at 0.3658 μmols/min/mg, phenanthridine at 0.3649 μmols/min/mg, and lastly benzaldehyde at 0.2069 μmols/min/mg. The specific activity for xanthine oxidase was measured to be 0.1033 μmols/min/mg (Fig. 33, Table 4). Our research showed that rabbit liver aldehyde oxidase significantly catalyzed the oxidation of all five aldehyde oxidase substrates evaluated.
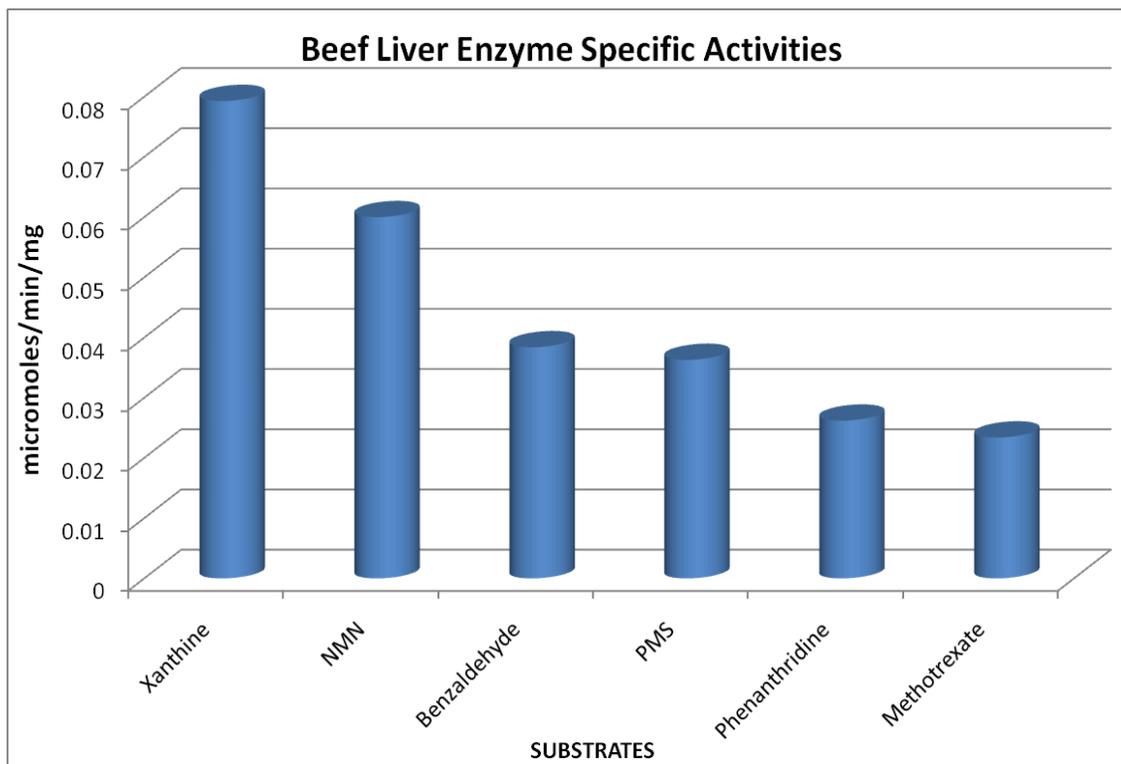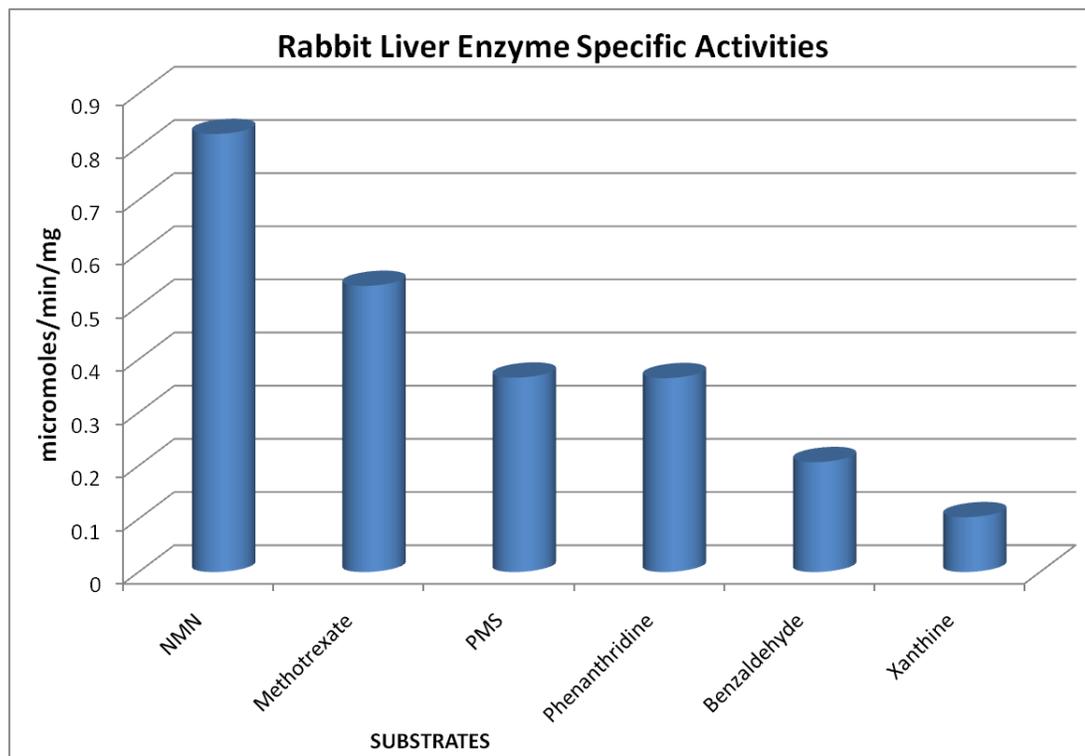
Figure 33. Rabbit Liver Enzyme Specific Activities. Substrate comparisons as determined by the two-minute initial rate continuous enzyme assays of the rabbit liver enzyme.

Substrate	μmols/min/mg
N'-Methylnicotinamide	0.8243 +/- 0.1515 (N=3)
Methotrexate	0.5385 +/- 0.0657 (N=3)
Phenazine Methosulfate	0.3658 +/- 0.0580 (N=6)
Phenanthridine	0.3649 +/- 0.0542 (N=3)
Benzaldehyde	0.2069 +/- 0.0171 (N=3)
Xanthine	0.1033 +/- 0.0139 (N=3)

Table 4. Activity Values for Rabbit Liver (Corresponding to Fig. 33). +/- Values are standard deviations. N is number of sample preparations.

Bovine Kidney 30' Discontinuous Enzyme Assays
Long-Term (Time-Course) Incubation Method

Due to very low activities found for bovine kidney aldehyde oxidase following the two-minute initial rate continuous assay method, discontinuous thirty-minute incubation assays were used to evaluate the activity of the enzyme using aldehyde oxidase substrates phenanthridine, N^1-methylnicotinamide, and phenazine methosulfate (PMS). The activity of bovine kidney xanthine oxidase was also evaluated using xanthine as the sole substrate. As previously discussed, the discontinuous long-term incubation assays allow for the calculation of the amount of oxidative product formed in terms of molarity or micromoles of product produced. For this research project, the amount shown is in micromoles of oxidized product formed in 3 mLs of solution since this is the total cuvette volume used in the two-minute initial rate continuous assays. For the aldehyde oxidase substrates, phenanthridine produced the largest amount of product at 0.3108 μmols/3 ml followed by N^1-methylnicotinamide at 0.0755 μmols/3ml and finally phenazine methosulfate at 0.0348 μmols/3ml. The amount of product produced by the xanthine oxidase-xanthine substrate complex was 0.1534 μmols/3ml which falls between that of phenanthridine and N^1-methylnicotinamide substrates (Fig. 34, Table 5). There was approximately one order of magnitude difference between the highest amount of product produced (oxidized phenanthridine) to the smallest amount of product produced (oxidized PMS).
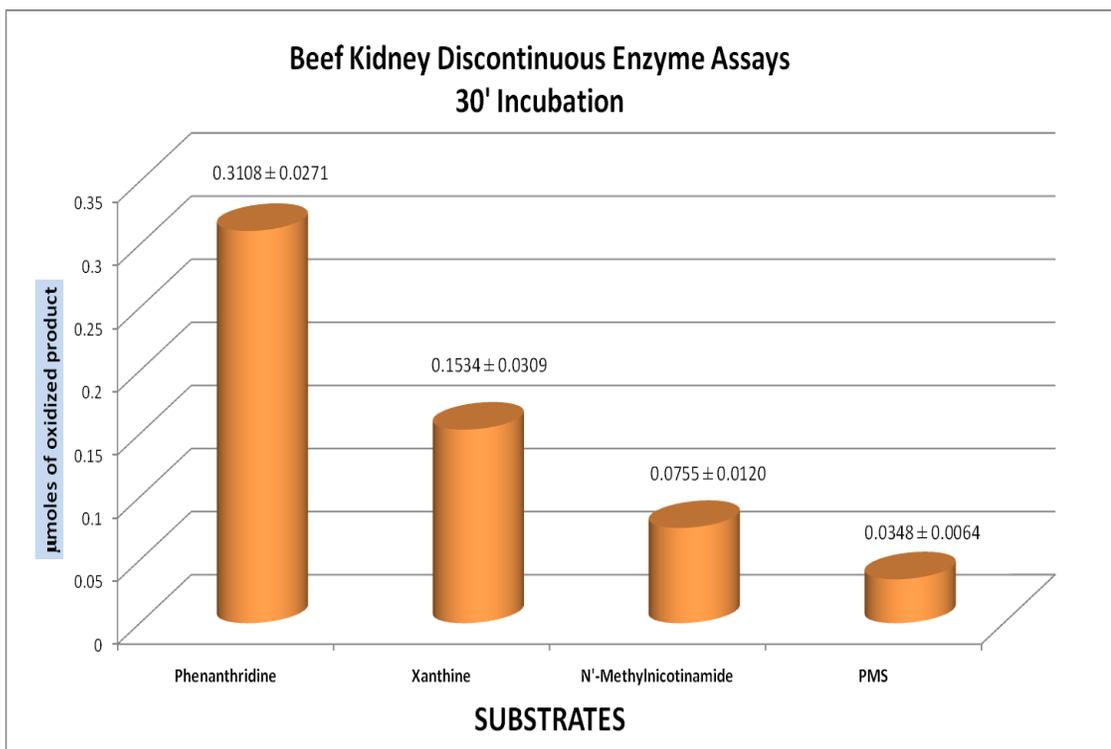
Figure 34. Beef Kidney Discontinuous Enzyme Assays 30' Incubation. Substrate comparisons as determined by the thirty-minute discontinuous long-term incubation enzyme assays of the bovine kidney enzyme.

Substrate	Results
Phenanthridine	0.3108 +/- 0.0271 (N=3)
Xanthine	0.1534 +/- 0.0309 (N=6)
N'-Methylnicotinamide	0.0755 +/- 0.0120 (N=4)
Phenazine Methosulfate	0.0348 +/- 0.0064 (N=13)

Table 5. Beef Kidney Assay Values (Corresponding to Fig. 34).   +/- Values are standard deviations. N is number of sample preparations.

The benefit of doing repetitive scans of the phenazine methosulfate oxidation reaction over the wavelength from 350 nm to 600 nm is the ability to observe the decrease in absorbance of the substrate and the increase in absorbance of the oxidized product. If a decrease in the absorbance of the substrate is observed concurrently with an increase in absorbance of oxidized product this provides good evidence that the actual oxidation reaction is taking place. The substrate in this case is phenazine methosulfate and the oxidized product is 10-methylphenazine-2(10H)-one which is a red product that has a maximum absorbance at about 520 nm. A repetitive scanning experiment was performed on a blank solution to determine if any non-enzymatic reactions were taking place during the 55 minute run that would cause a rise in absorbance at 520 nm (Fig. 36). The blank cuvette did not contain any enzyme solution. Three other repetitive scanning experiments were performed which included phenazine methosulfate oxidation by bovine kidney aldehyde oxidase at two different concentrations of enzyme (1 mg/mL and 4 mg/mL) (Fig. 37 and Fig. 38) and by rabbit liver aldehyde oxidase at 1 mg/ml protein concentration (Fig. 35). The results of the repetitive scanning experiments are as follows: A slight rise at 520 nm was seen in the blank repetitive spectrum. The difference in the initial scan absorbance (0 minutes) at 520 nm and the final scan absorbance (55 minutes) at 520 nm for the blank solution was equal to 0.0321. The difference in initial scan absorbance (0 minutes) at 520 nm and final scan absorbance (55 minutes) at 520 nm for

the 1 mg/mL bovine kidney enzyme was 0.0750 and the difference for the 4 mg/mL

bovine kidney enzyme was 0.0902. A much larger difference of 0.4942 was seen in the 1

mg/mL rabbit liver enzyme. For all repetitive scanning experiments performed, a

correlation between the drop in absorbance of the phenazine methosulfate substrate peak

to the oxidized phenazine methosulfate peak was observed. The bovine kidney enzyme

repetitive spectrums indicate that there is a definite rise in absorbance at 520 nm thus

revealing the production of oxidized PMS. At the 4 mg/mL bovine kidney enzyme

concentration the scans begin to take on a similar appearance to that of the rabbit liver

enzyme. As mentioned earlier, the blank spectrum also shows an increase in absorbance

at 520 nm. Regarding this as some form of non-enzymatic oxidation it would make sense

to subtract that value from the results of the enzyme preparations. Being that the case, a

positive value is still obtained for all three enzyme preparations indicating that the

oxidation of phenazine methosulfate by aldehyde oxidase has occurred. It is important to

point out though, that the spectrums for rabbit liver and bovine kidney are not identical.

When comparing the rabbit liver spectrum (Fig. 35) to the 4 mg/mL bovine kidney

spectrum (Fig. 38) the isosbestic points differ. This could indicate that different chemical

species and therefore different oxidation products are forming by the rabbit liver enzyme

and the bovine kidney enzyme.

Figure 35. Repetitive Scanning Spectrum of the Oxidation of PMS by Rabbit Liver Aldehyde Oxidase. 1 mg/ml enzyme concentration and 0.04 mM PMS concentration. Scans ran at 0 minutes and every 5 minutes thereafter up to 55 minutes (12 scans total).
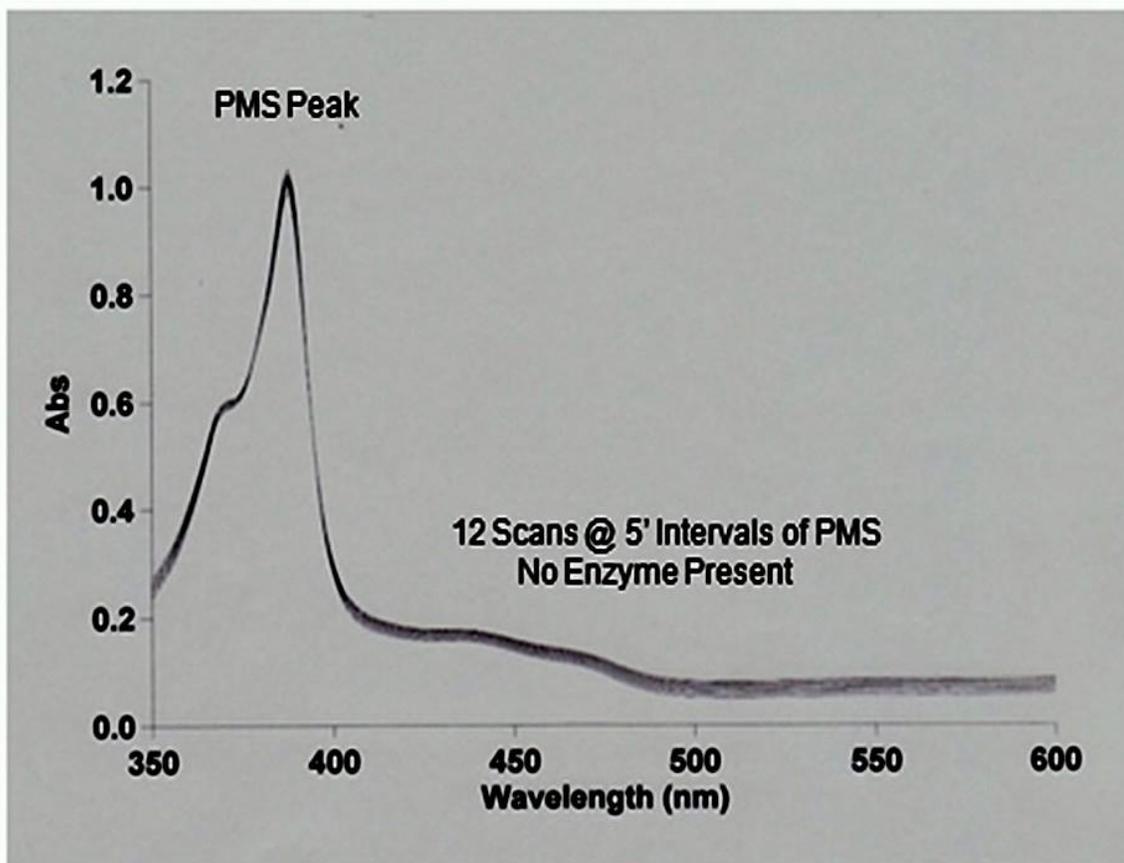
Figure 36. Repetitive Scanning Spectrum of 0.04 mM PMS Solution. 0 mg/mL of enzyme solution. Scans ran at 0 minutes and every 5 minutes thereafter up to 55 minutes (12 scans total).

Figure 37. Repetitive Scanning Spectrum of the Oxidation of PMS by Bovine Kidney Aldehyde Oxidase (1 mg/mL). 1 mg/ml enzyme concentration and 0.04 mM PMS concentration. Scans ran at 0 minutes and every 5 minutes thereafter up to 55 minutes (12 scans total).
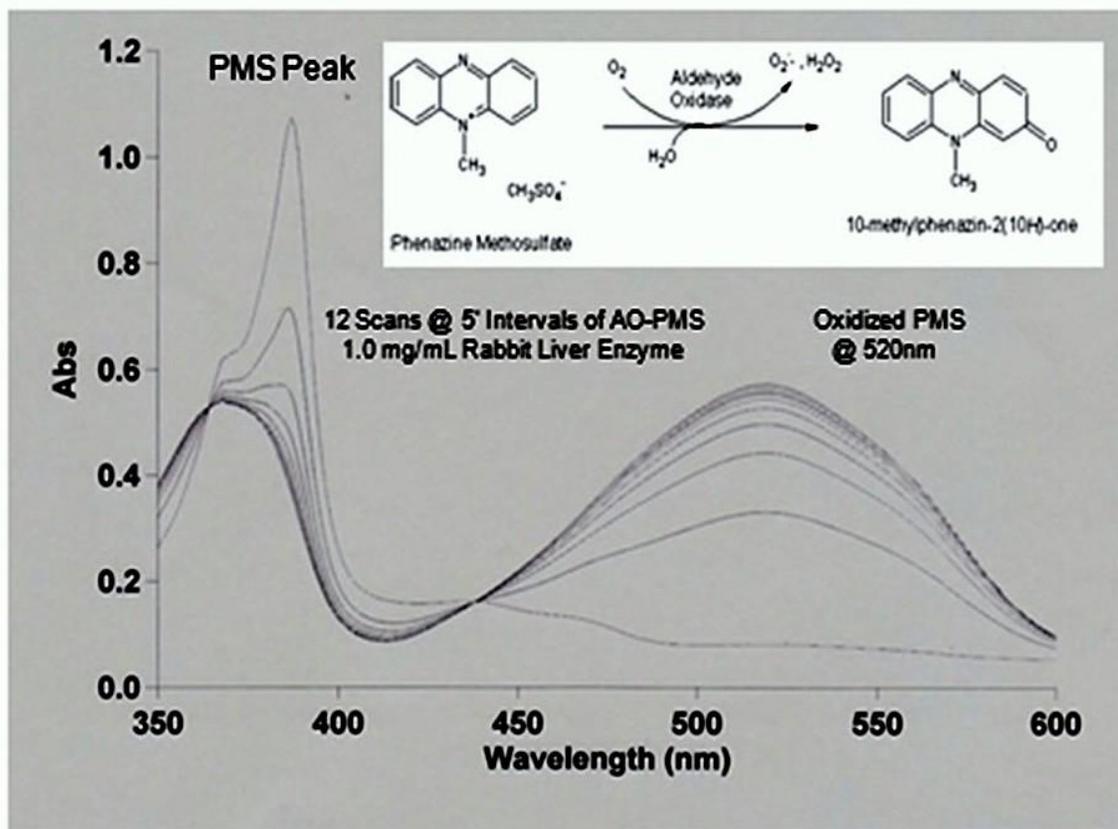
Figure 38. Repetitive Scanning Spectrum of the Oxidation of PMS by Bovine Kidney Aldehyde Oxidase (4 mg/mL). 4 mg/ml enzyme concentration and 0.04 mM PMS concentration. Scans ran at 0 minutes and every 5 minutes thereafter up to 55 minutes (12 scans total).
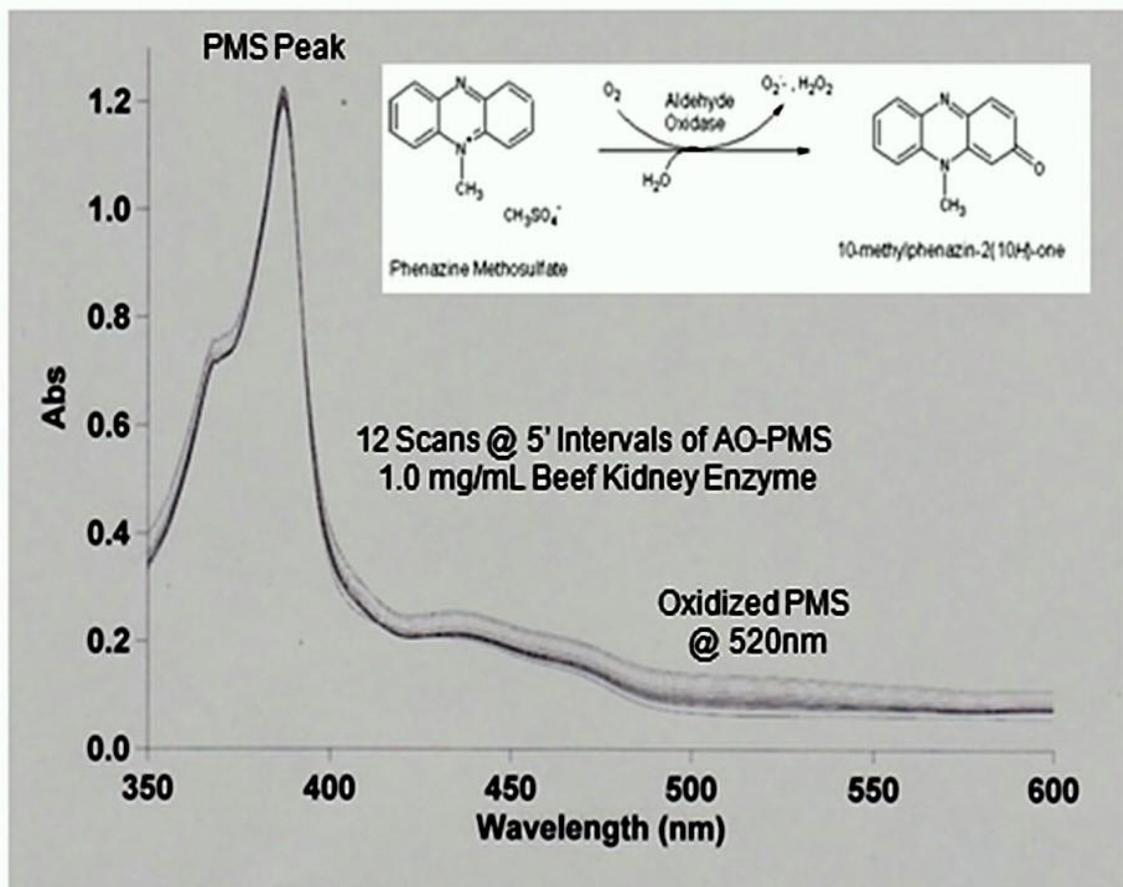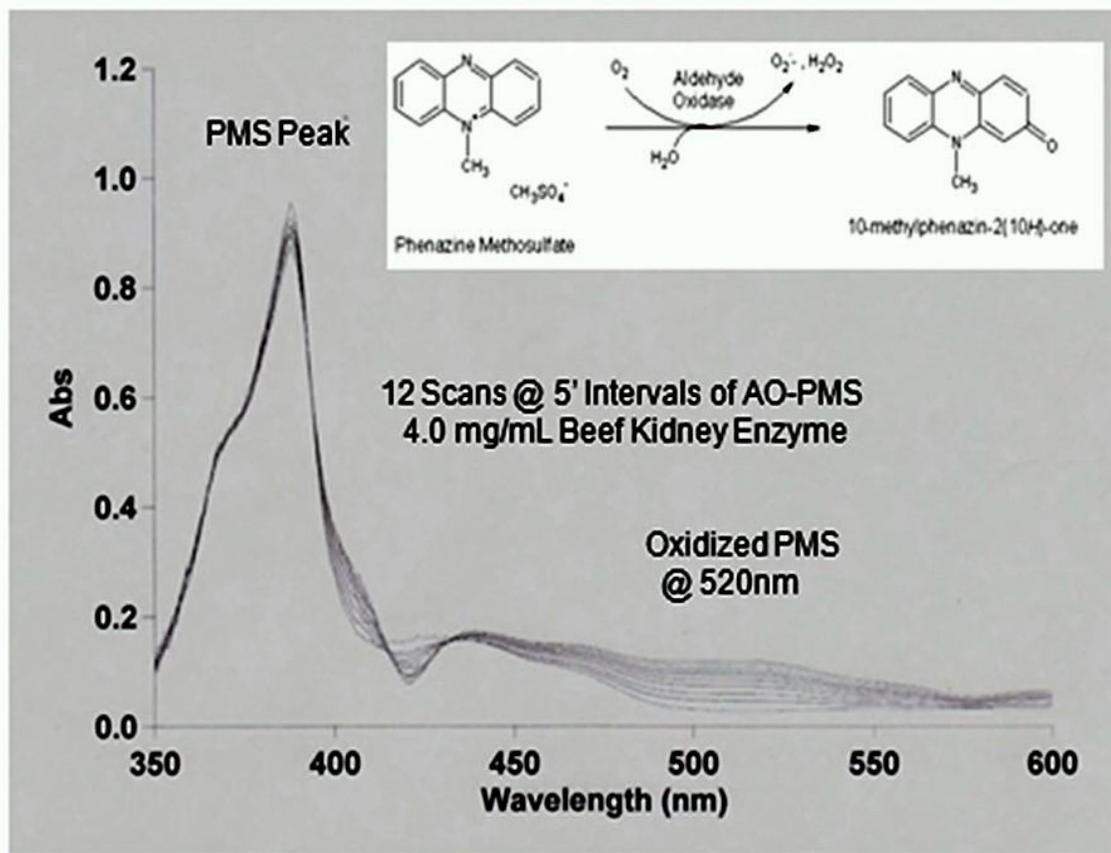
Inhibition of Phenazine Methosulfate Oxidation by Menadione and Methanol

The inhibition of the oxidation reaction of phenazine methosulfate by rabbit liver and bovine kidney aldehyde oxidase as determined by the two-minute initial rate continuous assay method showed two very different results when studying the well known menadione inhibitor against the two different enzymes. At a menadione concentration of 3.2×10^{-5} M the oxidation reaction catalyzed by the rabbit liver enzyme was inhibited by 36.3%. Increasing the concentration of menadione to 9.1×10^{-5} M, a 3-fold increase, the oxidation of phenazine methosulfate by rabbit liver aldehyde oxidase was inhibited by 53.6% (Fig. 39). On the contrary, the oxidation of phenazine methosulfate by bovine kidney aldehyde oxidase showed no inhibition at either of the concentrations of menadione used in the rabbit liver experiments. It is important to note however, that the activity as measured by the phenazine methosulfate oxidation reaction by bovine kidney aldehyde oxidase was at low levels and the inhibition studies were performed and calculated on these values.

The inhibition of the oxidation reaction of phenazine methosulfate by rabbit liver, bovine liver, and bovine kidney AO as determined by the two-minute initial rate continuous assay method showed similar results for the bovine tissues but a different result for the rabbit liver tissue when comparing them to methanol inhibition (Fig. 40, Table 6). At a methanol concentration of 0.3M, both bovine aldehyde oxidases were inhibited by 30%. Rabbit liver AO, however was only inhibited by 6%.

74
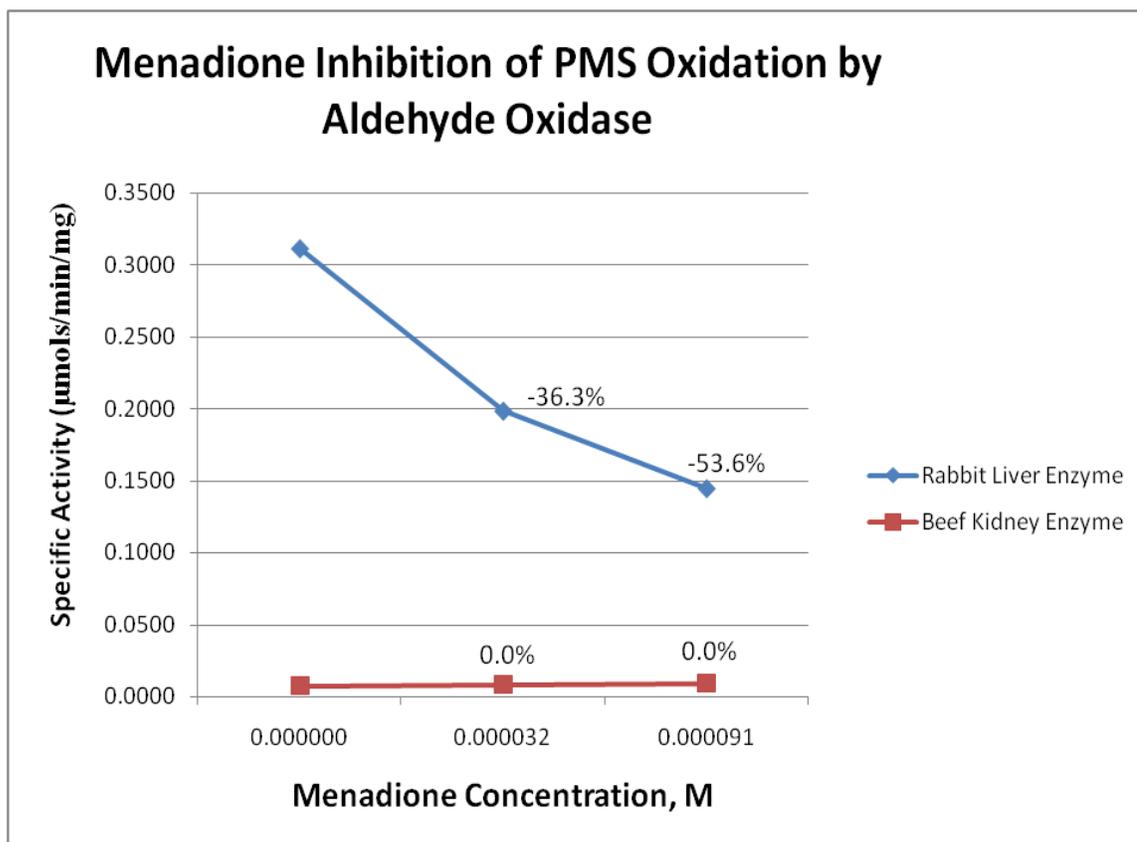
Figure 39. Menadione Inhibition of PMS Oxidation by Aldehyde Oxidase. Inhibition of rabbit liver and bovine kidney aldehyde oxidase by 3.2×10^{-5}M and 9.1×10^{-5}M menadione. Determined on the oxidation of phenazine methosulfate reaction by the two-minute initial rate continuous enzyme assay.
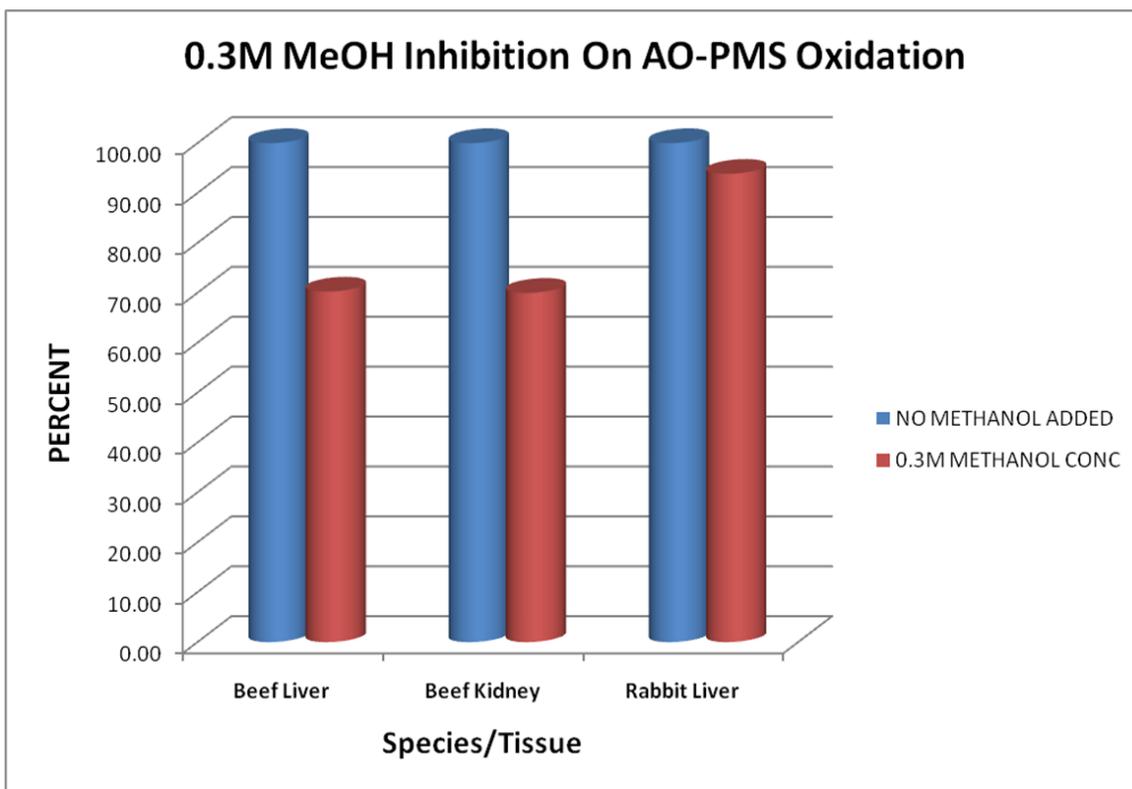
Figure 40. 0.3M MeOH Inhibition on AO-PMS Oxidation. Inhibition of bovine kidney, bovine liver, and rabbit liver aldehyde oxidase by 0.3M methanol. Determined on the oxidation of phenazine methosulfate reaction by the two-minute initial rate continuous enzyme assay. Bars on the right are with 0.3M methanol. Bars on the left are without the methanol inhibitor.

Species/Tissue	% Inhibited by 0.3M MeOH
Beef Liver	29.75
Beef Kidney	30.00
Rabbit Liver	6.15

Table 6. Percent Inhibition by Methanol of AO-PMS (Corresponding to Fig. 40)

76

Evaluation of the Enzyme Purification Procedure

The primary purpose of performing the following experiments was to verify that the purification procedure was producing expected and typical results though at the same time valuable enzymatic activity data was obtained. Expected and typical results for an enzyme purification procedure are an increase in enzyme activity measurements as the steps in the purification procedure progress. Our experiments show this to be the case as described below.

Recalling from the enzyme purification procedure previously discussed; the procedure begins by homogenizing the tissue in 0.05 M potassium phosphate buffer, heat treating the homogenized solution between 50-55°C, and obtaining a supernatant by centrifuging at 4000 rpm. The supernatant was then reacted with ammonium sulfate at 313 g/L of original supernatant, and the precipitate obtained and collected by centrifuging at 4000 rpm. The precipitate was then dissolved in 0.05 M potassium sulfate buffer at 80 mL/L of original supernatant, and the solution adjusted to a pH of 7.8. The solution was re-centrifuged to remove any additional precipitated protein. Using the 30 minute long term incubation assay method to measure the production of uric acid catalyzed by xanthine oxidase, three different points of the purification procedure were evaluated. The results were as expected, with an increase in the micromoles of uric acid produced as the steps in the purification procedure progressed. There was a 2.2-fold increase (120.1%) in micromoles of uric acid formed when testing the supernatant obtained after the ten-

minute 50-55°C heat treatment over the crude homogenate.   There was a 4.7-fold

increase (368.2%) in uric acid formation when testing the ammonium sulfate fraction,

which is the final step carried out for this research project, compared to the crude

homogenate from the Waring Laboratory blender (Fig. 41, Table 7).

Figure 41. Beef Kidney XO-Xanthine Oxidation 30' Incubation Assay. 30-minute discontinuous, long-term incubation enzyme assays for purpose of evaluating the purification procedure. Results are determined on the xanthine oxidation reaction by bovine kidney xanthine oxidase. 1 mg/mL protein concentrations for each step.

Purification Point	Results
Crude Homogenate from Blender	0.0274 +/- 0.0042 (N=3)
Supernatant Following Heat Treatment	0.0603 +/- 0.0134 (N=3)
Ammonium Sulfate Fraction	0.1283 +/- 0.0160 (N=3)

Table 7. Beef Kidney XO Assay Results (Corresponding to Fig. 41). +/- Values are standard deviations. N is number of sample preparations.

An additional 30 minute long term incubation assay evaluation was performed on the supernatant following heat treatment and the ammonium sulfate fraction by measuring the micromoles formed of oxidized phenazine methosulfate catalyzed by aldehyde oxidase. There was a 1.3-fold increase (26.9%) in micromoles of oxidized phenazine methsulfate formed when testing the ammonium sulfate fraction over the supernatant following heat treatment (Fig. 42, Table 8). Both experiments described above show an increase in product formation when testing the latter step of the enzyme purification as the procedure progresses. This is the expected outcome of such an experiment.
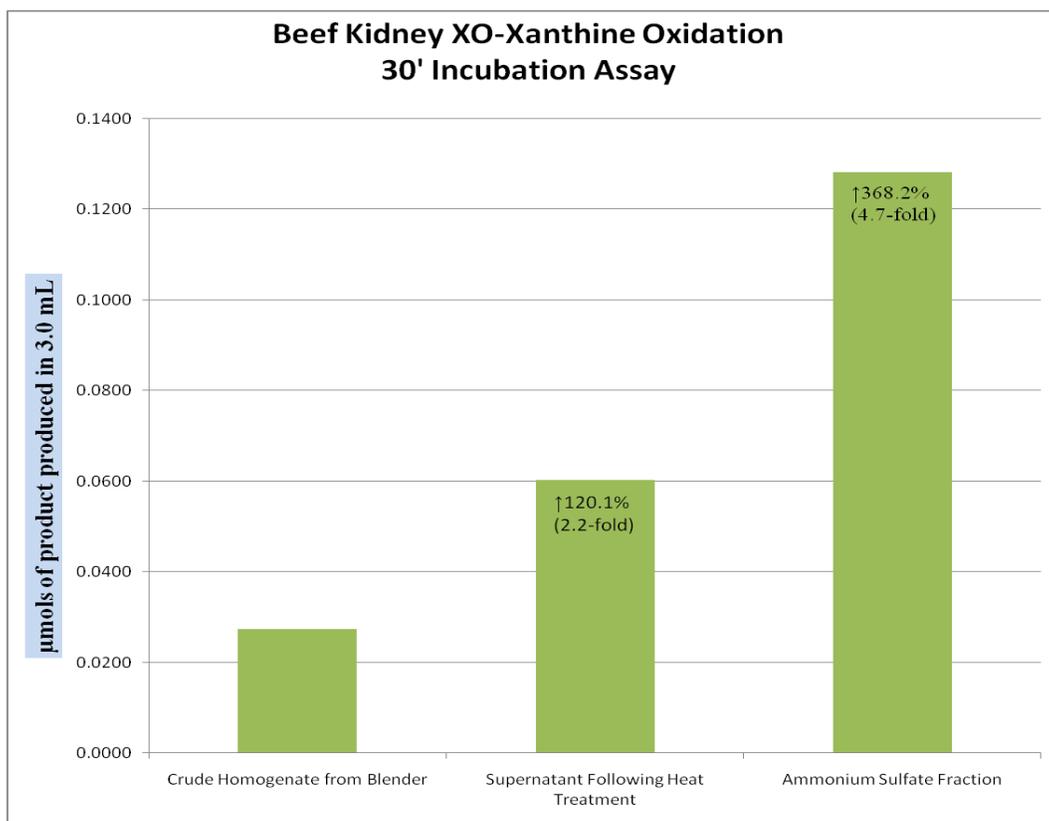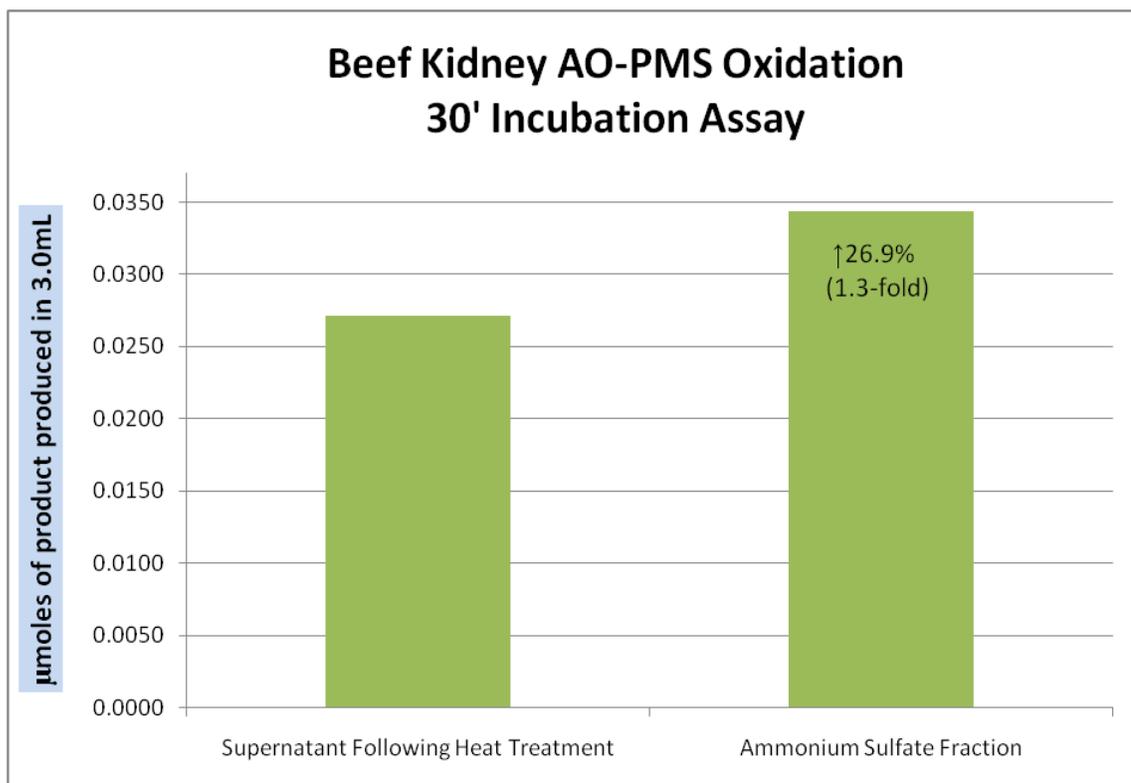
Figure 42. Beef Kidney AO-PMS Oxidation 30' Incubation Assay. 30-minute discontinuous, long-term incubation enzyme assays for purpose of evaluating purification procedure. Determined on the oxidation of phenazine methosulfate reaction by bovine kidney aldehyde oxidase. 1 mg/mL protein concentration for each step.

Purification Point	Results
Supernatant Following Heat Treatment	0.0271 +/- 0.0005 (N=3)
Ammonium Sulfate Fraction	0.0344 +/- 0.0020 (N=3)

Table 8. Beef Kidney AO Assay Results (Corresponding to Fig. 42). +/- Values are standard deviations. N is number of sample preparations.

CHAPTER V

DISCUSSION

Results from our research have agreed with previous work by other scientists that the enzyme aldehyde oxidase does exist within the bovine kidney tissue and contributes to the metabolism of xenobiotics as shown by our limited number of substrate oxidation experiments, repetitive scanning experiments, and inhibition studies on the partially purified enzyme. When comparing the bovine kidney aldehyde oxidase activity to that of bovine liver and rabbit liver the results are approximately one order of magnitude and two orders of magnitude lower, respectively. Of the three tissues evaluated, rabbit liver showed the highest aldehyde oxidase activity for all five AO substrate oxidations followed by bovine liver and then bovine kidney, as the following figures and tables illustrate (Figs. 43-47, Tables 9-13).

Figure 43. Phenanthridine-Enzyme Activity. Tissue comparisons for the oxidation of phenanthridine by aldehyde oxidase as determined by the 2-minute initial rate continuous enzyme assay.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.3649 +/- 0.0542 (N=3)
Beef Liver	0.0262 +/- 0.0028 (N=3)
Beef Kidney	None Detected (N=2)

Table 9. Assay Results for Phenanthridine (Corresponding to Fig. 43). +/- Values are standard deviations. N is number of sample preparations.
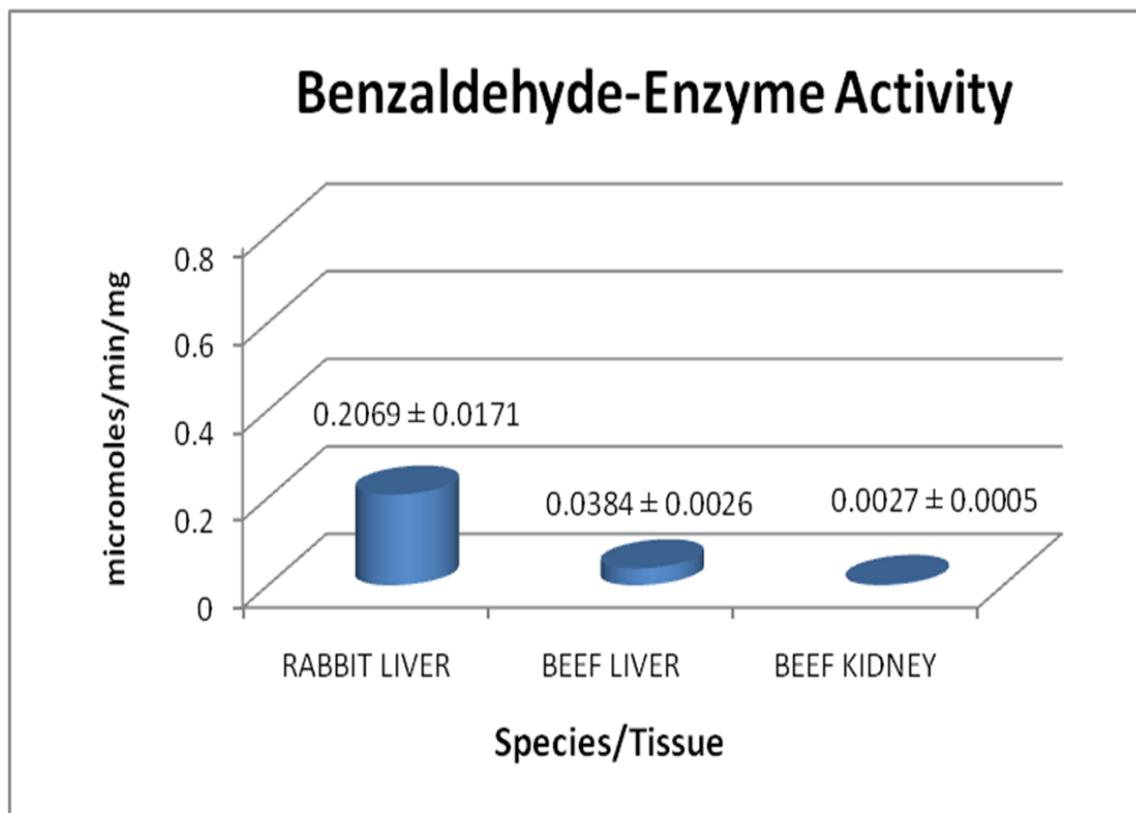
Figure 44. Benzaldehyde-Enzyme Activity. Tissue comparisons for the oxidation of benzaldehyde by aldehyde oxidase as determined by the 2-minute initial rate continuous enzyme assay.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.2069 +/- 0.0171 (N=3)
Beef Liver	0.0384 +/- 0.0026 (N=3)
Beef Kidney	0.0027 +/- 0.0005 (N=3)

Table 10. Assay Results for Benzaldehyde (Corresponding to Fig. 44). +/- Values are standard deviations. N is number of sample preparations.
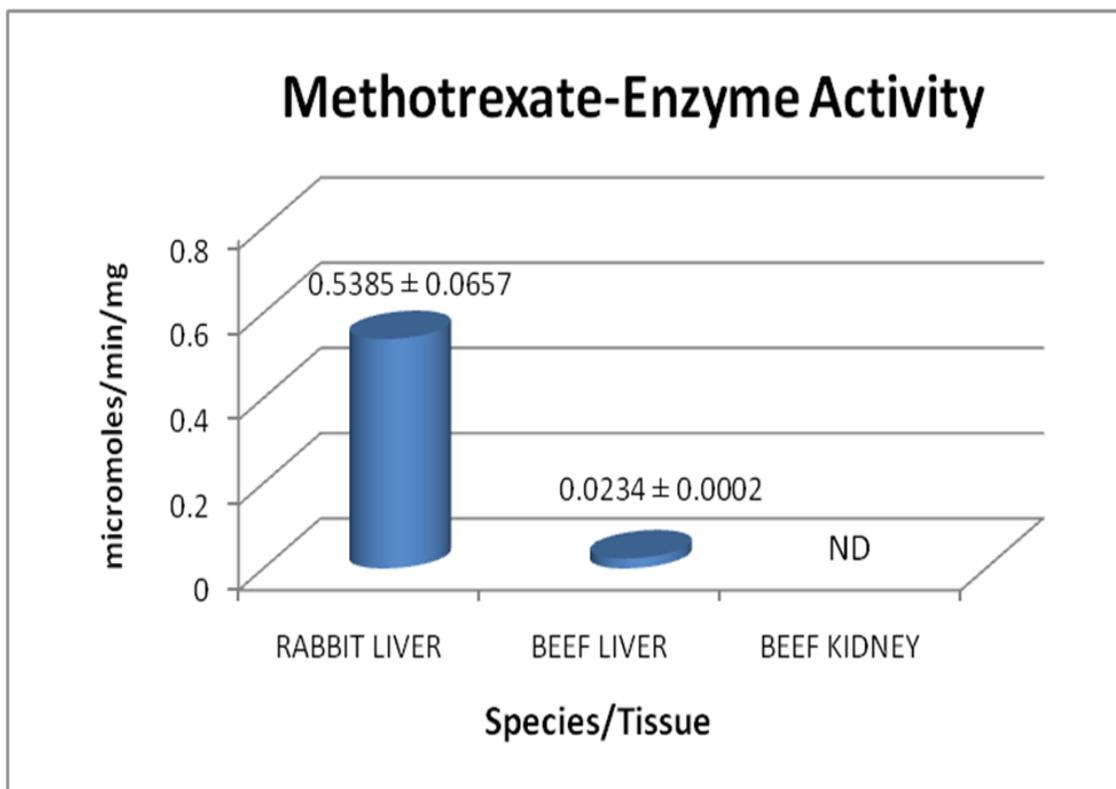
Figure 45. Methotrexate-Enzyme Activity. Tissue comparisons for the oxidation of methotrexate by aldehyde oxidase as determined by the two-minute initial rate continuous enzyme assay.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.5385 +/- 0.0657 (N=3)
Beef Liver	0.0234 +/- 0.0002 (N=2)
Beef Kidney	None Detected (N=2)

Table 11. Assay Results for Methotrexate (Corresponding to Fig. 45). +/- Values are standard deviations. N is number of sample preparations.
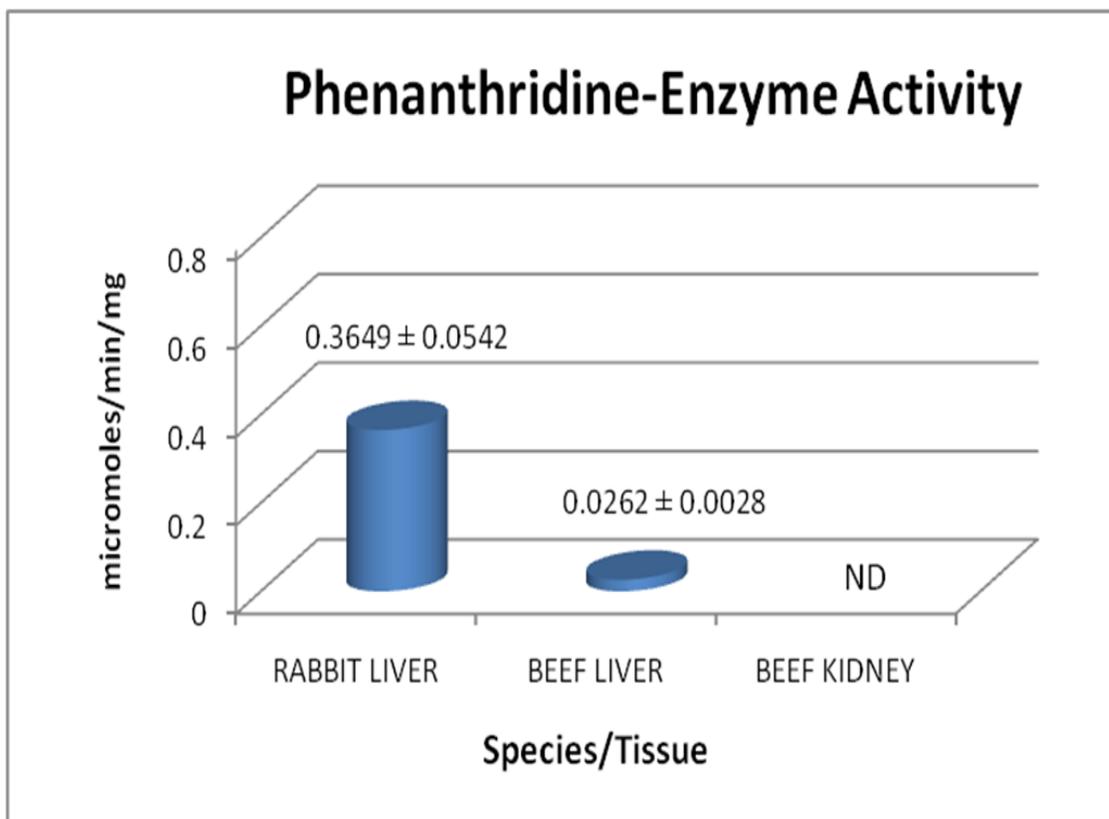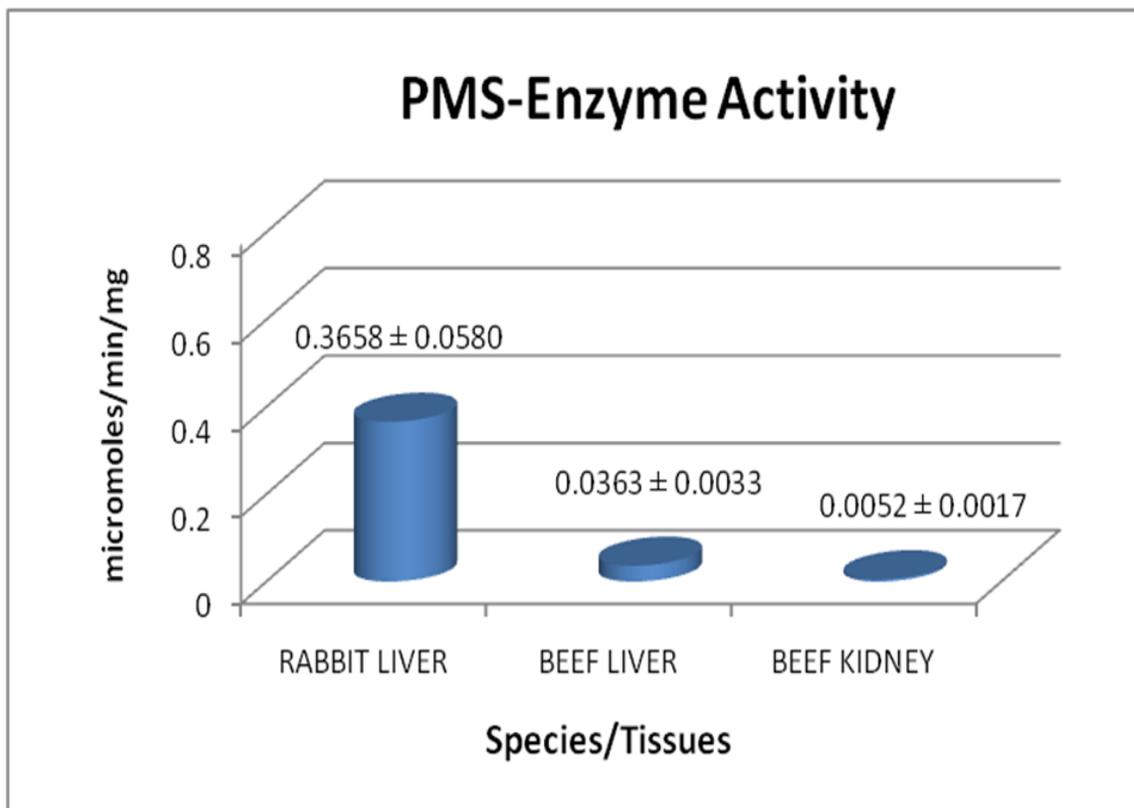
Figure 46. PMS-Enzyme Activity. Tissue comparisons for the oxidation of phenazine methosulfate by aldehyde oxidase as determined by the 2-minute initial rate continuous enzyme assay.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.3658 +/- 0.0580 (N=6)
Beef Liver	0.0363 +/- 0.0033 (N=3)
Beef Kidney	0.0052 +/- 0.0017 (N=5)

Table 12. Assay Results for Phenazine Methosulfate (Corresponding to Fig. 46). +/- Values are standard deviations. N is number of sample preparations.

Figure 47. NMN-AO Activity. Tissue comparisons for the oxidation of N^1-Methylnicotinamide by aldehyde oxidase as determined by the two-minute initial rate continuous enzyme assay.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.8243 +/- 0.1515 (N=3)
Beef Liver	0.0600 +/- 0.0039 (N=3)
Beef Kidney	0.0124 +/- 0.0029 (N=2)

Table 13. Assay Results for NMN (Corresponding to Fig. 47). +/- Values are standard deviations. N is number of sample preparations.

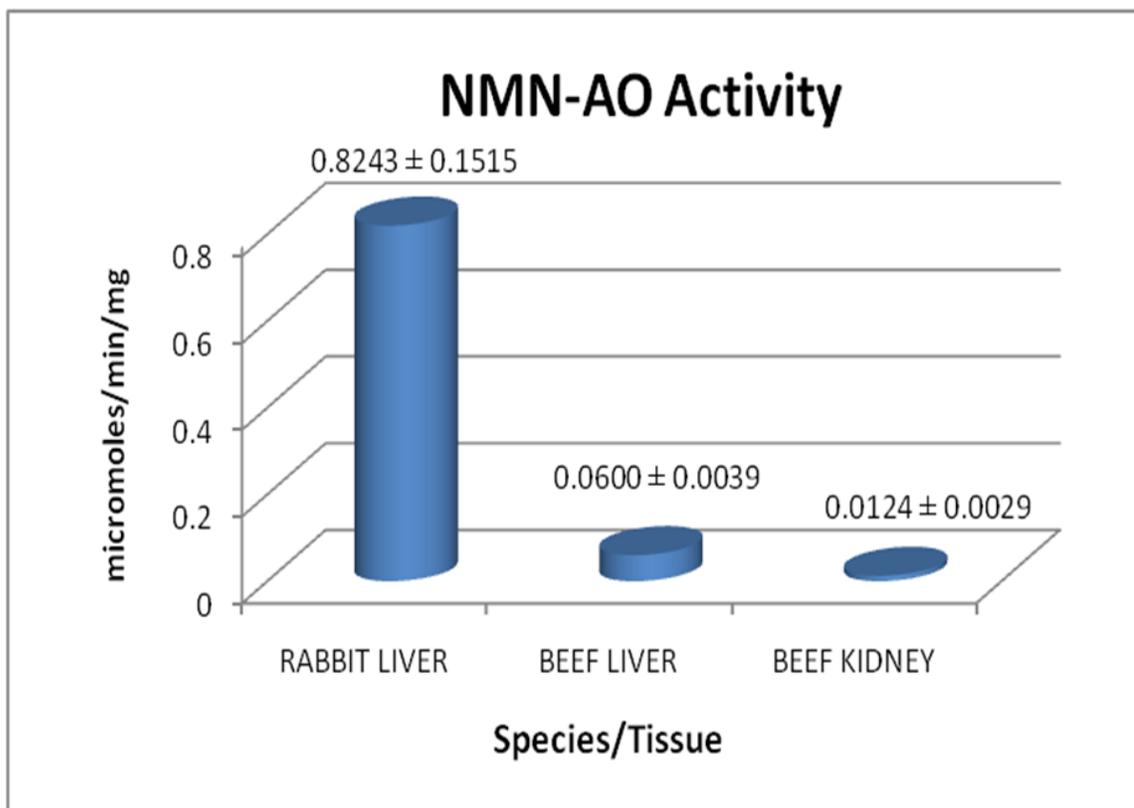This rank order of activities for the tissues are what was expected due to the fact that the liver is the main organ responsible for metabolic reactions and that the rabbit liver enzyme has shown to be one of the more potent sources of AO for many years and is often used as a standard. Of the five AO substrates evaluated, the endogenous compound N^1-methylnicotinamide produced the highest activity results for all three tissues. The values for this endogenous substrate were higher than any of the xenobiotic substrates evaluated. The research also indicates that the levels of AO activity found in the tissues are dependent upon the substrate used. In a study by A. David Rodrigues (53) on the human liver aldehyde oxidase enzyme, it was also determined that the charged substrate, NMN, produced a much higher aldehyde oxidase activity than did uncharged substrates like benzaldehyde or 6-methylpurine. Our study also showed that the aldehyde oxidase activity was higher for the charged compound N^1-methylnicotinamide than for the uncharged compound benzladehyde for all three tissues evaluated. The reason for this substrate variability could be in the existence of and levels of isoenzymes. Isoenzymes often display different kinetic properties and therefore can have different affinities for different substrates. Isoenzymes have been discovered in rodents such as mice but not in humans. Two separate isoenzymes for mouse aldehyde oxidase have been identified as AOX1 and AOH1 (54). Currently, only a single isoenzymatic form of aldehyde oxidase has been detected for the human liver enzyme (54) though more research is necessary to confirm the lack of other forms. For this project, no research was conducted to indicate either the lack of or existence of isoenzymes for the bovine tissues.

88

As with many experimental research projects, unusual, unexpected, and aberrant results can be produced. Such is the case when comparing the thirty-minute long term incubation enzyme assay results to the two-minute initial rate continuous assay results for the oxidation of phenanthridine by bovine kidney aldehyde oxidase. The activity rank order for the 30 minute discontinuous assay for the following substrates is phenanthridine > xanthine > NMN > PMS. The activity rank order for the 2 minute continuous assay for the same substrates is xanthine > NMN > PMS > phenanthridine (not detectable). The phenanthridine substrate showed the highest oxidase activity for the 30 minute long term incubation assay but was not detected by the 2 minute initial rate assay. Was there some form of non-enzymatic oxidation taking place during the 30 minute incubation that was not seen in the 2 minute assay? An explanation for these unexpected aberrant results is unknown at this time. Further research is warranted to confirm the product produced in the 30 minute incubation assay method is valid.

Our research also showed that there is a significant amount of xanthine oxidase activity within the two bovine tissues. As a matter of fact, xanthine oxidase activity exceeded aldehyde oxidase activity for the bovine kidney and the bovine liver tissues. The reverse was seen in the rabbit liver tissue. Though the rank order of activities from highest to lowest for the tissues remained the same for xanthine oxidase activity (rabbit liver followed by bovine liver and then bovine kidney), the results were much closer as compared to the aldehyde oxidase values as the following figure and table shows (Fig. 48, Table 14).
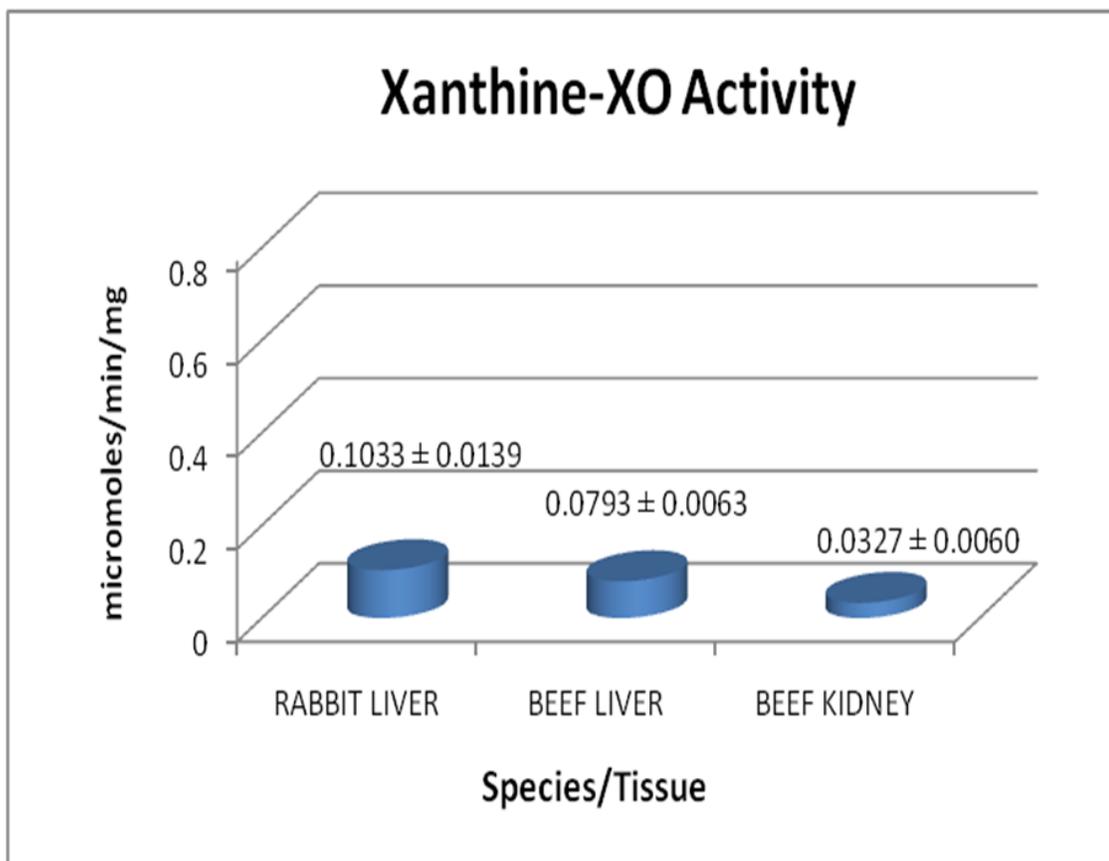
Figure 48. Xanthine-XO Activity. Tissue comparisons for the oxidation of xanthine by xanthine oxidase as determined by the 2-minute initial rate continuous enzyme assay. The bars are much closer is size than those of the aldehyde oxidase charts.

Species/Tissue	μmols/min/mg
Rabbit Liver	0.1033 +/- 0.0139 (N=3)
Beef Liver	0.0793 +/- 0.0063 (N=3)
Beef Kidney	0.0327 +/- 0.0060 (N=3)

Table 14. Assay Results for Xanthine (Corresponding to Fig. 48). +/- Values are standard deviations. N is number of sample preparations.

Instead of one and two orders of magnitude higher, rabbit liver xanthine oxidase activity was only 1.3 and 3.2 times greater than bovine liver and bovine kidney respectively. In terms of the ratio of xanthine oxidase activity over aldehyde oxidase activity, bovine kidney is at 8.0, bovine liver is at 2.1 and rabbit liver is at 0.2.

It is important to note that the purification procedure utilized for the partial purifications was the same for aldehyde oxidase and xanthine oxidase. This could be resulting in co-purification of these two enzymes. It may not be known in all cases how much contribution each enzyme has for catalyzing these oxidation reactions. However, it is known that xanthine is not oxidized by aldehyde oxidase and that NMN is not oxidized by xanthine oxidase. Benzaldehyde, on the other hand, is not known to only be oxidized by either one of the partially purified enzymes, though it is more commonly used as an aldehyde oxidase substrate. In this case, one or both of the enzymes may have contributed to oxidation of benzaldehyde.

Methotrexate, the aldehyde oxidase substrate of clinical significance and of important relevance to humans, was not oxidized to its metabolite 7-hydroxymethotrexate by partially purified bovine kidney aldehyde oxidase based on our limited experimental work. This then indicates that the aldehyde oxidase enzyme from bovine kidney does not contribute to the oxidation of this xenobiotic thereby not contributing to cytotoxicity of the bovine kidney cells by 7-hydroxymethotrexate. However, this work does show that bovine liver and rabbit liver aldehyde oxidase do contribute to the cytotoxicity of liver

91

cells by catalyzing the oxidation of methotrexate to 7-hydroxymethotrexate. In earlier

work by Aya Moriyasu et. al. (36) and S. Kitamura et. al. (55) it was shown that

methotrexate is converted to 7-OH-MTX by rat liver aldehyde oxidase also.

The inhibition studies performed showed that the oxidation of phenazine

methosulfate by bovine kidney aldehyde oxidase was inhibited by methanol but not by

menadione. However, the oxidation of phenazine methosulfate by rabbit liver aldehyde

oxidase was inhibited by both methanol and menadione. In a similar experiment by

David G.Johns (31) it was found that menadione was ineffective at inhibiting the

phenazine methosulfate oxidation reaction by human liver aldehyde oxidase although it

was effective at inhibiting rabbit liver aldehyde oxidase by about 58% at a menadione

concentration of 2.5×10^{-5}M for the same reaction (31). Our study showed a 54%

inhibition of the phenazine methosulfate oxidation by rabbit liver aldehyde oxidase at a

menadione concentration of 9.1×10^{-5}M. Those results were not what are expected due to

the fact that menadione has proven to be a potent AO inhibitor and in most cases usually

eliminating all or nearly all AO activity for that particular substrate. In an experiment

conducted by Yoshihara and Tatsumi (56), a 0.5 μM and 1.0 μM concentration of

menadione inhibited the oxidation of benzaldehyde by mouse liver aldehyde oxidase to

91% and 96% respectively. In another experiment by S. Kitamuro et al. (55) an 84%

inhibition of the oxidation of methotrexate by the purified rat liver aldehyde oxidase was

shown. Though the methanol inhibitor was able to inhibit the phenazine methosulfate

oxidation reaction by all three tissues it did not do so very effectively. In a work by

David G. Johns (31) where they used a different oxidation reaction, it was shown that a 0.3M concentration of methanol inhibited the oxidation of benzaldehyde to benzoic acid by human liver aldehyde oxidase by 67%. This research only showed a 30% and 6% inhibition of the oxidation of phenazine methosulfate by the bovine aldehyde oxidases and rabbit liver aldehyde oxidase, respectively. The inhibition data above indicates that the oxidation of phenazine methosulfate is not as sensitive to menadione and methanol inhibition than to other oxidation reactions. However, the inhibition data was consistent with other similar experimental work which helps verify that aldehyde oxidase was the enzyme responsible for the activity values measured. Inhibition studies using other substrate oxidations such as N^1-methylnicotinamide, would be valuable experiments and help to better characterize the bovine tissue enzymes.

The evaluation of the purification procedure showed typical and expected results. In normal cases, as an enzyme purification procedure progresses, the enzyme activity also increases since the enzyme becomes more pure. The percent yield however, decreases mainly due to enzyme loss at different points in the procedure. Our evaluation showed a 2-fold increase in bovine kidney xanthine oxidase activity when comparing the heat treatment step to the crude homogenate and a 5-fold increase when comparing the 50% ammonium sulfate fraction to the crude homogenate. In a paper by J.G.P. Stell, A.J. Warne, and C. Lee-Woolley (57), they also stated that there is an approximate 6-fold gain in specific activity at the ammonium sulfate precipitation step. They also state that at this step there is little loss in enzyme activity (57). In another similar experiment by Maia

and Mira (1), a 2-fold increase in rat liver xanthine oxidase was observed when comparing the heat treatment step to the crude homogenate and a 9-fold increase when comparing a 60% ammonium sulfate fraction to the crude homogenate. For aldehyde oxidase activity, Maia and Mira (1) found a 3.2-fold increase in the rat liver enzyme when comparing a 60% ammonium sulfate fraction to the heat treated sample. Our results showed a 1.3-fold increase when comparing the 50% ammonium sulfate fraction to the heat treated sample for the bovine kidney enzyme. These results and comparisons indicate that our technique for the purification procedure was effective, efficient and comparable to the work of other researchers.

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