AN ABSTRACT OF THE THESIS OF

JUDITH ANNE WITTKOP (Name) for the DOCTOR OF PHILOSOPHY (Degree)

in BIOCHEMISTRY (Major) presented on July 23, 1969 (Date)

Title: THE OXIDATIVE DEMETHYLATION OF CERTAIN N-METHYLHYDRAZINES BY MICROSOMAL LIVER ENZYMES

Redacted for Privacy

Abstract approved: Donald J. Reed

The presence in rat liver microsomes of an enzyme system which demethylates N-methylhydrazines and azo compounds to formaldehyde has been reported. Some of the compounds found to be substrates were monomethylhydrazine, N-isopropyl-α-(2-methylhydrazino)-p-toluamide and the azo derivative of the latter hydrazine. This enzyme can be classified as a mixed function oxidase as defined by Mason (Adv. in Enzymology 19:79, 1957) since the N-demethylase was dependent upon the presence of a NADPH-regenerating system and molecular oxygen. The reaction was not inhibited by SKF 525-A (2-diethylaminoethyl diphenylpropylacetate), a compound that has been found to inhibit many microsomal enzyme systems. The N-demethylase was inducible by both phenobarbital and 3-methylcholanthrene. Addition of carbon monoxide to microsomes and the treatment of microsomes with trypsin led to the conclusion that rat liver microsomes possess a

phenobarbital-inducible N-demethylase dependent upon P-450 and a non-inducible N-demethylase independent of P-450.

A mechanism was proposed for the demethylation of the N-methylhydrazines. Since the azo derivatives of two of the hydrazines were oxidized at a greater rate than their parent compounds, it was postulated that the hydrazines are first oxidized to their azo derivatives which in turn are demethylated to formaldehyde by microsomes. The possible formation of an azoxy intermediate was discussed.

An attempt was made to solubilize and purify the N-demethylase but without success.

The Oxidative Demethylation of Certain N-Methylhydrazines by Microsomal Liver Enzymes

by

Judith Anne Wittkop

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1970

APPROVED:

Redacted for Privacy

Associate Professor of Biochemistry
in charge of major

Redacted for Privacy

Head of Department of Biochemistry

Redacted for Privacy

Dean of Graduate School

Date thesis is presented July 23, 1969

Typed by Illa W. Atwood for Judith Anne Wittkop

DEDICATION

To Father and

in memory of Mother

ACKNOWLEDGMENT

The author wishes to express her sincere appreciation to Dr. D. J. Reed for his helpful guidance and understanding throughout this work.

A special thanks is due the black, white and grey ones for keeping my spirits up and for their encouragement.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
MATERIALS AND METHODS	22
Materials	22
Pre-Treatment of Animals	23
Rat Liver Microsome Preparation	23
Spectral Studies	24
Protein	25
Enzyme Assays	26
Purification of the Pork Liver Demethylase	28
Organic Synthesis	34
Steapsin and Deoxycholate Treatments and Trypsin Digestion of the Microsomes	35
Enzyme Assays Performed Under Various Gaseous Atmospheres	36
PART I. CHARACTERIZATION OF THE N-METHYL-HYDRAZINE DEMETHYLASE	38
RESULTS	38
Cellular Distribution and Substrate Specificity of the N-Methylhydrazine Demethylase	38
Kinetic and Temperature Effects	41
The Effect of Other Reaction Conditions on the N-Demethylase Activity	46
DISCUSSION	50
PART II. INDUCTION OF N-METHYLHYDRAZINE DEMETHYLASE AND THE ROLE OF P-450 IN THE ACTIVITY OF THIS ENZYME(S)	56
RESULTS	56
Spetral Evidence for the Interaction of Hydrazines with Microsomes	56
The Effect of Various Pretreatments on the Microsomal Content of P-450 and on the Demethylation Rates of PCZ, AZO, MMH and DMA	56
The Inhibition of the N-Demethylase by Carbon Monoxide and by Steapsin and Deoxycholate Treatment of the Microsomes	59

Page

The Effect of Trypsin Treatment on the Microsomal Content of P-450, Cytochrome b_5 and N-Demethylase 69

DISCUSSION 73

BIBLIOGRAPHY 81

LIST OF FIGURES

Figure		Page
1	Modified degradation scheme of Procarbazine as proposed by Raaflaub (1965).	5
2	Electron transport in microsomes.	11
3	The effect of PCZ on the spectrum of acetylacetone.	29
4	The effect of PCZ on the spectrum of the Nash reagent.	30
5	The effect of time on formaldehyde formation.	42
6	The effect of protein concentration on formaldehyde formation from PCZ and MMH.	43
7	Lineweaver-Burk Plots.	45
8	The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of AZO by microsomes from rats pretreated with phenobarbital.	64
9	The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of AZO by microsomes from untreated rats.	65
10	Effect of various ratios of oxygen to nitrogen and carbon monoxide to oxygen on the demethylation rate of MMH by microsomes prepared from phenobarbital pretreated rats.	66
11	Effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of MMH by microsomes prepared from untreated rats.	67

Figure		Page
12	The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of DMA by microsomes prepared from untreated rats.	68
13	The effect of trypsin treatment on the content of P-450, cytochrome b_5, and MMH and AZO demethylase activity in microsomes prepared from untreated rats.	70
14	The effect of trypsin treatment on the content of P-450, cytochrome b_5, and MMH and AZO demethylase activity in microsomes prepared from phenobarbital pretreated rats.	71

LIST OF TABLES

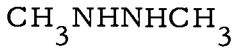
Table		Page
I	Mechanisms in drug metabolism.	2
II	Amount of formaldehyde measured in the presence of N-methylhydrazines.	31
III	Purification of the demethylase.	34
IV	Cellular distribution of demethylase activity.	38
V	Substrate specificity.	39
VI	Temperature dependence of the demethylation of MMH, PCZ, and DMA.	46
VII	Effect of various reaction conditions on the demethylation of MMH, PCZ, AZO and DMA by microsomes prepared from phenobarbital pretreated animals.	47
VIII	Effect of various NADPH concentrations on AZO demethylation.	48
IX	Effect of various compounds in microsomal spectra.	57
X	The effects of various animal pretreatments on P-450 content and on the demethylation of PCZ, MMH, DMA and AZO by liver microsomes.	58
XI	The effect of various treatments on the P-450 content and the demethylase activity of microsomes prepared from phenobarbital pretreated rats.	60
XII	The effect of various treatments on the P-450 content and demethylase activity of microsomes prepared from 3-methylcholanthrene pretreated rats.	61
XIII	The effect of various treatments on the P-450 content and the demethylase activity of microsomes prepared from non-induced rats.	62

Table		Page
XIV	The effect of steapsin treatment on the demethylase activity and cytochrome b_5 content of microsomes prepared from livers of non-induced rats.	72

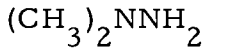
Abbreviations and Symbols

PCZ	- Procarbazine
AZO	- azo derivative of Procarbazine
MMH	- monomethylhydrazine
UDMH	- 1,1-dimethylhydrazine
SDMH	- 1,2-dimethylhydrazine
MBH	- 1-methyl-2-benzylhydrazine
$NADP^+$	- Oxidized nicotinamide-adenine dinucleotide phosphate
NADPH	- reduced nicotinamide-adenine dinucleotide phosphate
TCA	- trichloroacetic acid
SKF 525-A	- 2-diethylaminoethyl diphenylpropylacetate
P-450	- cytochrome P-450
DMA	- N,N-dimethylaniline
NHIP	- non-heme iron protein
mμ	- millimicro
mM	- millimolar
μmoles	- micromoles
Δ	- refers to change in
K_m	- apparent Michaelis constant
V_{max}	- velocity maximum
nm	- nanometers
ml	- milliliter

Structures



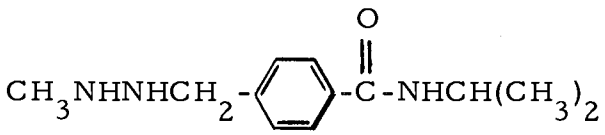
1, 2-dimethylhydrazine
(SDMH)



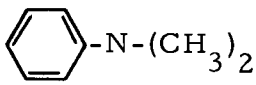
1, 1-dimethylhydrazine
(UDMH)

monomethylhydrazine
(MMH)

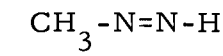
1-methyl-2-benzylhydrazine
(MBH)



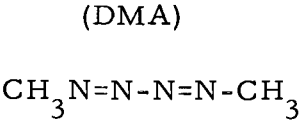
N-isopropyl-α-(2-methylhydrazino)-
p-toluamide (PCZ)



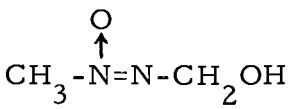
N, N-dimethylaniline
(DMA)



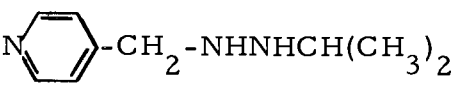
methyldiazene



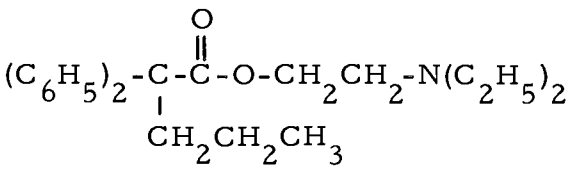
dimethyltetrazene



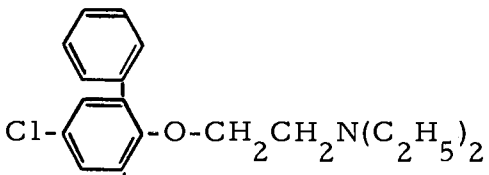
methylazoxymethanol



Iproniazid



SKF 525-A



Cl Lilly 18974

The Oxidative Demethylation of Certain N-Methylhydrazines by Microsomal Liver Enzymes

INTRODUCTION

The biotransformation of compounds foreign to the body plays an extremely important role in drug chemotherapy, without which the effects of many drugs would be too long lasting and/or too toxic to be of any practical value. The therapeutic effect of some drugs actually depends upon their conversion in the body to some "active" metabolite. The body also has mechanisms to detoxify poisonous compounds and in turn can convert compounds, not harmful in themselves, to toxic materials. Many are able to induce drug metabolizing enzymes and thus can increase the rate of their own metabolism. The need for understanding the mechanisms of biotransformation, therefore, becomes obvious.

The metabolism of drugs takes place for the most part in the microsomal fraction of liver cells, the microsomes being that part of the endoplasmic reticulum that sediments after one hour at 105,000 g. Microsomes prepared from lung and kidney tissue have also been shown to possess some drug metabolizing activity but not at the level of liver microsomes. The detoxification of drugs follows relatively few pathways, some of which are listed in Table I. The reaction usually consists of one of the following: deamination,

Table I. Mechanisms in drug metabolism (Taken from J. R. Gillette, Advances in Pharmacology 4, p. 235, 1966).

Hydroxylation of aromatic rings

$$CH_3\text{-}CO\text{-}NH\text{-}C_6H_5 \xrightarrow{(OH)} CH_3\text{-}CO\text{-}NH\text{-}C_6H_5OH$$

Oxidation of side chains

$$R\text{-}CH_2CH_2CH_2CH_3 \xrightarrow{(OH)} R\text{-}CH_2CHOH\text{-}CH_3$$

N-dealkylation

$$R\text{-}NH\text{-}CH_3 \xrightarrow{(OH)} [\,RNHCH_2OH] \longrightarrow R\text{-}NH_2 + HCHO$$

O-dealkylation

$$R\text{-}O\text{-}CH_3 \xrightarrow{(OH)} [\,R\text{-}O\text{-}CH_2OH] \longrightarrow ROH + HCHO$$

S-dealkylation

$$R\text{-}S\text{-}CH_3 \xrightarrow{(OH)} [\,R\text{-}S\text{-}CH_2OH] \longrightarrow RSH + HCHO$$

Deamination

$$RCH_2\text{-}CH(NH_2)\text{-}CH_3 \xrightarrow{(OH)} [\,RCH_2\text{-}COH(\text{-}NH_2)\text{-}CH_3\,]$$

$$\longrightarrow R\text{-}CH_2\text{-}CO\text{-}CH_3 + NH_3$$

Sulfoxide formation

$$R\text{-}S\text{-}CH_3 \xrightarrow{(OH)} [\,R\text{-}S\text{-}CH_2OH] \longrightarrow R\text{-}SO\text{-}CH_3$$

N-dealkylation, O-dealkylation, sulfoxidation, hydroxylation, or S-demethylation. These reactions are related in that they each require the presence of molecular oxygen and NADPH or a NADPH-regeneration system. The enzyme systems which catalyze these reactions have been called "mixed function oxidases" by Mason (1957). There are several other types of reactions that drugs can undergo that do not require molecular oxygen for activity. These include conjugation to form glucuronides, sulfates, mercapturic acids, and carboxylic acids, hydrolysis of esters and amides and dehalogenation. Only the mechanisms of oxidative drug metabolism will be discussed here.

This study was undertaken to help elucidate the mechanism of action of the carcinogenic, antitumor, cytostatic drug Procarbazine (Zeller, 1963; Rutihauser, 1963; Bollag, 1963a,b; Kelly, 1964). In 1963 Zeller synthesized several N-methylhydrazine derivatives of which Procarbazine had the best therapeutic index. This drug possesses antitumor activity against several types of transplantable tumors in mice and rats (Bollag, 1963a,b) and is presently being tested extensively clinically in the treatment of Hodgkin's disease (Jelliffe, 1965). Kelly (1964) found that when mice are injected with a single or repeated dose of Procarbazine they develop pulmonary tumors and leukemia within 15 weeks. The route of metabolism of this compound has not been firmly established but the degradation

scheme in Figure 1 has been proposed.

The formation of the azo derivative of Procarbazine has been demonstrated to occur in the blood of man, dog and monkeys ten minutes after the administration of Procarbazine (Oliverio, 1964). Raaflaub (1965) found that a major portion of the drug, 25% in 24 hours, is excreted in the urine as N-isopropyl-terephthalamic acid. Methylamine has been suggested as a metabolite since ^{14}C-methylamine was found in the urine of rats treated with $^{14}CH_3$-Procarbazine (Schwartz, 1966). The existence of monomethylhydrazine as a degradation product has not been proven in vivo. Dost et al. (1967) however have reported that both Procarbazine and monomethylhydrazine are metabolized in vivo to carbon dioxide and methane and that the rates of these reactions are consistent with monomethylhydrazine being an intermediate. Several other authors (Dost, 1966, 1967; Baggiolini, 1965; Schwartz, 1966) have reported the formation of $^{14}CO_2$ from ^{14}C-methyl-labelled Procarbazine. Also, through induction studies with phenobarbital, Baggiolini (1965) has determined that the demethylation occurs in the microsomal fraction of the cell. Weitzel (1967a) has found that Procarbazine and monomethylhydrazine split off formaldehyde after mild dehydrogenation in vitro with hexocyanoferrate (III). Further studies by this author (1967b) have shown that Procarbazine is enzymically demethylated to formaldehyde in vitro by microsomes in the presence of

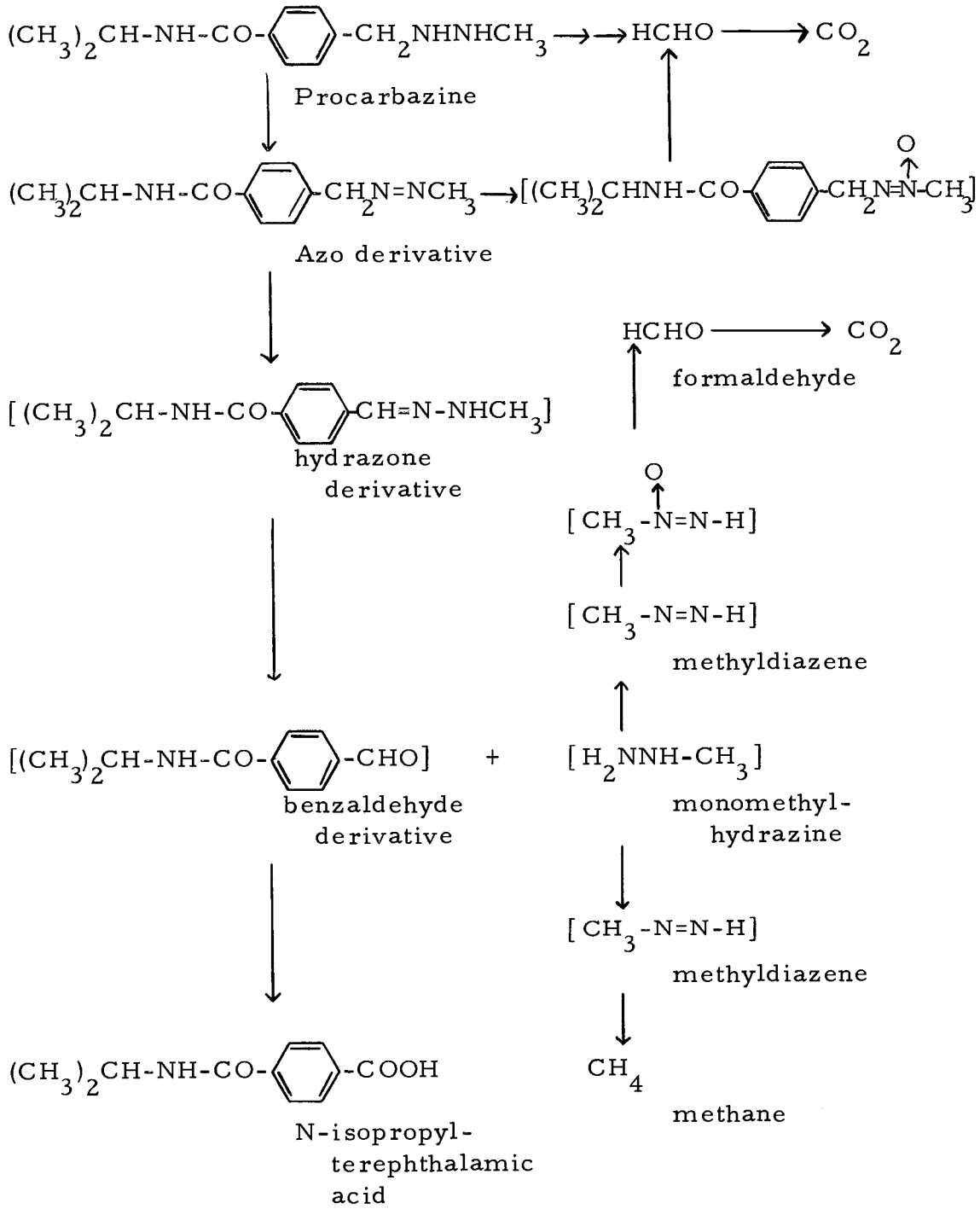


Figure 1. Modified degradation scheme of Procarbazine as proposed by Raaflaub (1965). Brackets indicate compounds which have not been conclusively identified as degradation products of Procarbazine.

molecular oxygen and a NADPH-regenerating system.

At this point of discussion it might be well to review what is known about the reactions involved in microsomal drug metabolism. Mueller and Miller (1953) were the first to note the N-demethylation of 3-methyl-4-methylaminoazobenzene by rat liver homogenates. Since then this reaction has been found to occur in the microsomes along with other reactions as listed in Table I. Lipid solubility is an important factor in determining whether a compound is metabolized in the microsomes. Gaudette (1959) has found that only N-alkyl compounds which are extractable into chloroform are dealkylated. Therefore, sarcosine, creatine and choline which were extracted by an arbitrary procedure established by these authors to an extent of less than 3% into chloroform were not substrates while drugs such as ephedrine and pethidine which are 95% extractable into chloroform are dealkylated by microsomal enzymes.

According to Fouts (1961) the drug metabolizing enzymes appear to be more concentrated in the lighter microsomal fraction, that which is associated with smooth endoplasmic reticulum. It is possible to separate the smooth and rough endoplasmic reticulum by density gradient centrifugation (Rothschild 1961). Thus a slight purification of the drug metabolizing enzymes can be obtained by this method. Conventional means of extracting particulate enzymes such as deoxycholate, lipase or snake venom treatment of the particles

have not been successful in purifying any of the oxidative enzymes which metabolize foreign compounds (Shuster, 1964).

A general mechanism has been proposed for the oxidation of drugs (Gillette, 1966):

$$NADPH + A + H^{+} \longrightarrow AH_2 + NADP^{+}$$

$$AH_2 + NADPH + O_2 \longrightarrow \text{"active oxygen"}$$

$$\text{"active oxygen"} + \text{drug} \longrightarrow \text{oxidized drug} + H_2O + A$$

$$2\ NADPH + O_2 + \text{drug} \longrightarrow \text{Oxidized drug} + H_2O + 2NADP^{+}$$

Conclusive evidence for the stoichiometry of this reaction has not been found. $^{18}O_2$ studies have been attempted but the oxygen of aldehydes exchanges quite rapidly with the oxygen of water and therefore ^{18}O studies on N-, O- or S-dealkylation or deamination must be interpreted with care. Such studies have confirmed that the oxygen incorporated into the drug is derived from atmospheric oxygen (Mason, 1957). Further difficulties in determining the stoichiometry of the reactions are derived from the fact that the microsomes also possess a very active NADPH oxidase and that other enzymes rapidly utilize oxygen even in the absence of drugs. Frequently no change in NADPH uptake is noted in the presence or absence of a drug substrate (Prough, 1969a).

Because of the non-specificity of microsomal enzymes the question has been raised as to the number of oxidative enzymes

present. Several lines of evidence support the conclusion that there are multiple enzyme systems present in microsomes. One argument in support of this postulate is the species difference in the metabolism of drugs. For example, aniline was hydroxylated in the para position in liver microsomes from rabbits and rats but in the ortho position in cats (Posner et al., 1961). Amphetamine was deaminated in rabbits but hydroxylated in dogs (Axelrod, 1954). A sex difference also exists. Male rats appear to metabolize some drugs at a faster rate than females (Holck, 1937). Administration of thyroxine to rats caused an increase in the demethylation of aminopyrine in females, a decrease in males and had no effect on their ability to metabolize aniline and zoxazolamine (Kato, 1968). SKF 525-A competitively inhibits the microsomal oxidation of many drugs (Cooper, 1954); however, the demethylation of methyl- and ethylaniline (Gaudette, 1959) was not inhibited by this compound. Sensitivity to SKF 525-A and sex and species differences therefore indicate that there is a number of different enzymes present in the microsomes to metabolize a variety of drugs.

Iproniazid and Lilly 18947 have also been found to inhibit the same enzyme systems as SKF 525-A (Fouts, 1956). These enzymes are more sensitive to SKF 525-A since concentrations of 10^{-5}M will bring about an inhibition by this compound while concentrations of 10^{-3}M of iproniazid and Lilly 18947 are required (Shuster, 1964).

SKF 525-A or a metabolite appears to be tightly bound to microsomes as the inhibition persists after dialysis (Rogers, 1964). The inhibition by iproniazid can be reversed by dialysis or by washing the microsomes to recover complete activity. When given in vivo, SKF 525-A initially inhibited pentobarbital metabolism and then acted as a stimulant as determined by the sleeping time of rats (Kato, 1964).

Carbon monoxide is a very potent inhibitor of the drug metabolizing enzymes which are dependent upon a P-450 electron transport system. About the same time that the field of microsomal drug metabolism was opening up several laboratories discovered the existence in microsomes of two cytochromes characteristic of this liver cell fraction. The two cytochromes are heme proteins and account for virtually all of the heme present in microsomes (Mason, 1965). One of these, cytochrome b_5 has been purified (Strittmatter, 1968; Ito, 1968) and spectrally characterized but does not appear to play a role in drug metabolism. The other initially seemed to have properties atypical of cytochromes. This latter heme pigment was discovered simultaneously in two laboratories through its spectral properties which could be obtained only when microsomes were reduced and treated with carbon monoxide (Klingenberg, 1958; Garfinkel, 1958). Under these conditions the compound exhibits a strong absorption band at 450 nm. Omura and Sato (1964a, b)

subsequently have called it P-450. These workers have shown that P-450 can be solubilized by treating microsomes with steapsin, deoxycholate or snake venom. Upon solubilization and purification (Omura, 1964b) the absorption maximum of the pigment-CO complex was shifted to 420 nm and P-420, as the solubilized form of P-450 was called, exhibited spectral properties typical of a b-type cytochrome.

The work of several laboratories resulted in the formulation of the microsomal electron transport scheme outlined in Figure 2. NADH serves to reduce both cytochrome b_5 and P-450. This reaction is mediated by a FAD containing enzyme in the reduction of Cytochrome b_5 and a FMN containing enzyme in the reduction of P-450. The reduction of P-450 by NADPH through a FAD enzyme appears however to be more active. The further reactions of P-450 in this scheme will be discussed later.

In 1965 Cooper found that the inhibition of C-21 hydroxylation of 17-hydroxyprogesterone by carbon monoxide could be reversed by monochromatic light at a wavelength of 450 nm. He further found that formation of the P-450-CO complex was reversible by light of the same wavelength. Steroids have been suggested as the natural substrates for the drug metabolizing enzymes (Kuntzman, 1964), as they undergo reactions similar to drugs and also act as competitive inhibitors of drug metabolism (Wada, 1968; Tephly, 1968). The involvement of P-450 in drug and steroid metabolism was therefore

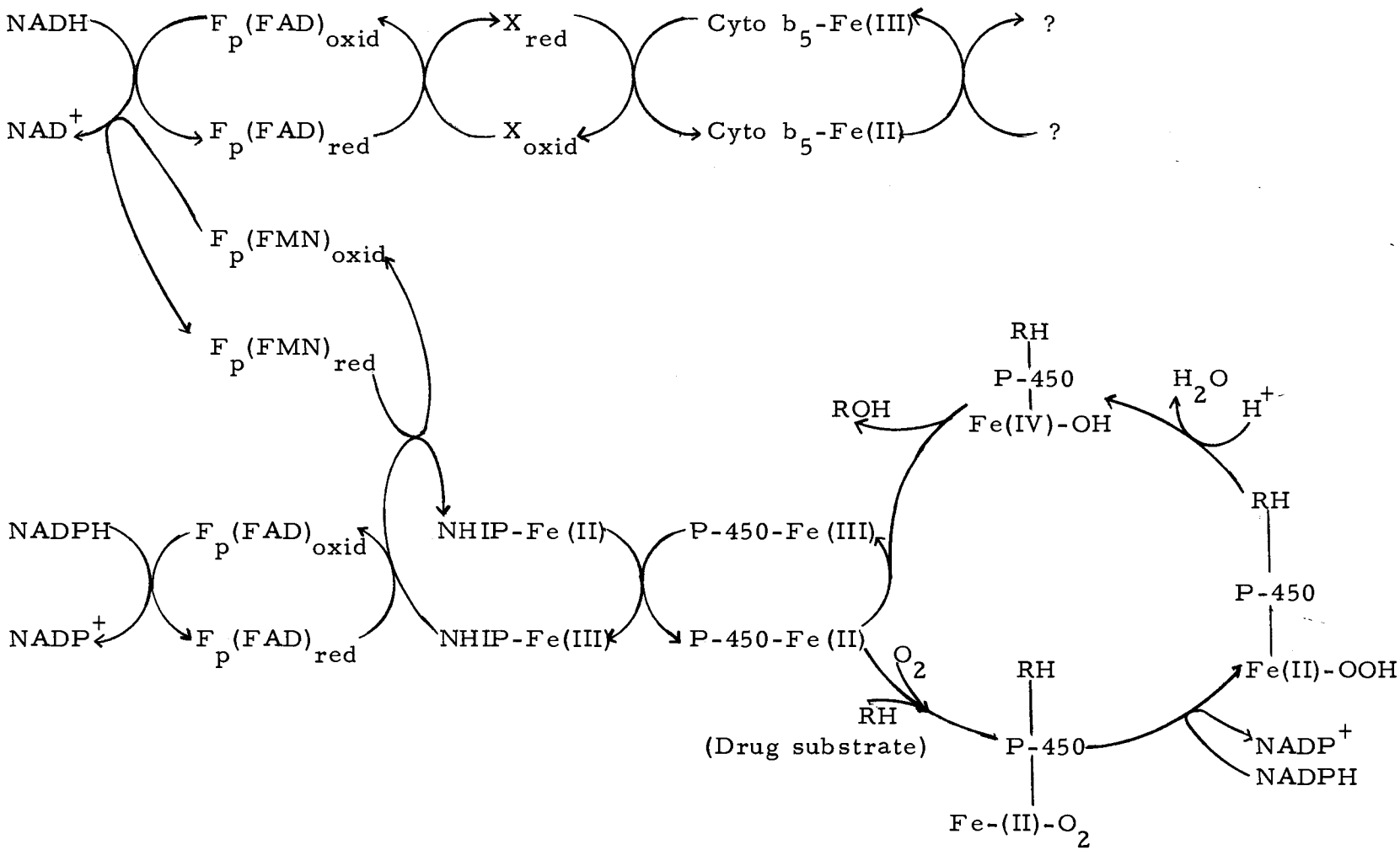


Figure 2. Electron transport in microsomes.

postulated. Since then several other lines of evidence have been produced to support this hypothesis. Treatment of microsomes with steapsin or deoxycholate not only caused solubilization of P-450 to P-420 (Omura, 1964a), but also inactivated several drug metabolizing enzymes (Gillette, 1957). Stitzel (1963) has found that drugs metabolized by microsomal enzymes competitively inhibited the metabolism of other drugs, suggesting the existence of a microsomal component common to the metabolism of each of these drugs, possibly P-450. Schenkman (1967b) has observed that substrates for microsomal demethylations and hydroxylations caused two types of changes in microsomes which can be observed spectrally. He classified the substrates for microsomal enzymes as exhibiting either a class I or class II-type interaction. The spectral changes caused by Type I substrates are characterized by an absorption maximum at 385 nm and minimum at 415 nm while Type II are characterized by a maximum at 430 nm and a minimum at 390 nm. These spectrally observed changes in microsomes were attributed to an interaction of the compound with P-450 since the spectrum of purified cytochrome b_5 was not affected. In 1960 Conney showed that several foreign compounds such as phenylbutazone, 3-methylcholanthrene, and phenobarbital stimulated in vivo drug metabolism in rats. A parallel rise in P-450 content and drug metabolism was found by Remmer (1965) in animals pretreated with phenobarbital. Orrenius

(1967) observed in rats that the quantity of ^{14}C-aniline bound to microsomes was stoichiometrically related to the P-450 content. Phenobarbital pretreatment of the rats increased the binding of ^{14}C-aniline to the same level it increased the P-450 content. Omura (1965) has found by reconstitution studies on the hydroxylation of steroids in the 11β position that P-450 is the terminal electron acceptor in the NADPH electron transport chain of the microsomes. He purified the non-heme iron protein indicated in Figure 2 from adrenal cortex microsomes and suggested such a protein was probably functional in liver microsomes also. P-450 then appears to act as a terminal oxidase by reacting with oxygen and subsequently transferring an "active oxygen" to the drug or steroid substrate. The order of binding of oxygen and the drug substrate is not known. The presence of the various iron complexes of P-450 shown in Figure 2 has not been demonstrated but their role in drug metabolism has been postulated by several authors (Kato, 1966; Sih, 1969). In this scheme NADPH serves a dual role. First,to keep P-450 in its reduced ferrous state and second, to provide the reducing equivalents necessary to cause the splitting of the oxygen-oxygen bond of molecular oxygen. Evidence for the latter role of NADPH has been provided by Sih (1968) who noted through kinetic studies that NADPH and a steroid substrate were combining with the same enzyme which would not be expected to occur if the only role of NADPH were at the

beginning of the electron transport chain.

The direct involvement of P-450 in microsomal oxidations could explain why attempts to purify these enzymes have proved unsuccessful. The solubilization techniques employed also cause the conversion of P-450 to its inactive form P-420. Enzymes which have been solubilized such as aniline hydroxylase (Omai, 1960) and DMA demethylase (Ziegler, 1968) may still possess enzymic activity due to the presence of a small amount of P-450 which is solubilized to the extent that it is bound to a fragment of the original microsomal membrane which does not sediment at 105,000 g.

One very important characteristic of drug metabolism already mentioned in the previous section is induction, which is caused by both administration of foreign compounds and starvation. The induction of liver microsomal enzymes is significant because it alters both the nature and rate of biotransformation of drugs and thereby shortens their duration of action. Induction has also been used as an important tool in learning more about the mechanisms of drug metabolism. As already pointed out, induction studies have lead to the conclusion that P-450 plays a central role in drug and steroid oxidation (Remmer, 1965). They have also led to the now popular belief that there are two functional P-450's present in the microsomes, differing in that one is preferentially induced by the carcinogen 3-methylcholanthrene (Alvares, 1969; Kuntzman, 1968). The

phenomenon of microsomal enzyme induction was first noted by Brown (1954) who found that dietary changes caused an increase in the demethylation rates of drugs in rats. In 1960 Conney reported a number of drugs, carcinogens and pesticides that were inducers of microsomal enzymes. Over two hundred compounds have been found to possess this property. There is no structural relationship between many of these compounds, nor is there any apparent relationship between these compounds and their biological or pharmacological actions. Some appear to be more specific inducers of one enzyme than another and their apparent mechanisms of induction differ. Phenobarbital but not 3-methylcholanthrene stimulated the dealkylation of ethylmorphine while phenobarbital and 3-methylcholanthrene both stimulated 3-methyl-4-methylaminoazobenzene dealkylation and when given together their effects were additive (Sladek, 1969a,b). Microsomes from rats given 3,4-benzpyrene and phenobarbital stimulated the metabolism of acetanilide, zoxazolamine and phenacetin more so than if they were administered alone (Gillette, 1963a,b). However, the effects of administration of this hydrocarbon plus phenobarbital were not always additive. Treatment with two polycyclic hydrocarbons did not appear to produce additive or greater stimulatory effects than when either of the hydrocarbons was administered alone. One characteristic all of these compounds have in common though is that they are lipid soluble at

at physiological pH (Conney, 1967).

Several explanations for enzyme induction have been offered by Sladek (1969a). He suggested the inducer could (1) stabilize the enzyme or decrease enzyme degradation rate, (2) cause the synthesis of a more active enzyme, (3) increase enzyme synthesis or (4) directly or indirectly activate the existing enzyme. The latter was ruled out since inducers do not produce their effect in vitro and experiments to determine if they effected some enzyme activator or inhibitor were negative (Conney, 1963).

Alvares (1967) has found that ethionine, an inhibitor of protein synthesis (Villa-Trevino, 1963), blocks 3-methylcholanthrene induction of the hydroxylation of benzpyrene. Ethionine and puromycin, which inhibits translation (Yarmolinsky, 1959), and actinomycin D, which inhibits transcription (Reich, 1961), have been found to block induction brought about by either 3-methylcholanthrene or phenobarbital. These results seem to support the third hypothesis. Sladek (1969a) raised the question of whether or not apparent blocking of protein synthesis is a result of a decrease of enzyme levels through enhanced catabolism of the enzyme and not really blocking induction. In the case of 3-methylcholanthrene induction of benzpyrene hydroxylation this possibility does not appear to be so since untreated animals and those treated with ethionine possessed the same enzyme activity (Alvares, 1967). Furthermore, Gelboin (1967) has found that

phenobarbital and 3-methylcholanthrene caused an increase in the activity of RNA polymerase in rat liver nuclei and therefore caused an increase in the rate of amino acid incorporation into protein.

Since phenobarbital but not 3-methylcholanthrene caused an increase in microsomal protein the first and third hypotheses could explain the mechanism of induction by this compound. Phenobarbital also has been found by Rubin (1964) to cause an increase in the V_{max} but not the K_m values of ethylmorphine, chlorpromazine or hexobarbital metabolism indicating more enzyme activity but no new enzyme was present. Guarino (1969) has further reported that both the V_{max} and K_m for aniline hydroxylation are increased with phenobarbital pre-treatment of animals, indicating that a more active enzyme was produced. The different results found by these two authors were explained by Guarino (1969). He concluded that phenobarbital has an effect on enzymes which metabolize substrates such as aniline by increasing their apparent K_m values. Aniline causes a type II spectral change in microsomes as classified by Schenkman (1967). Phenobarbital, however, has no such effect on substrates such as chlorpromazine, hexobarbital, and ethylmorphine. These latter substrates cause type I spectral changes in microsomes.

Sladek (1969a) has postulated a mechanism of microsomal induction by phenobarbital and 3-methylcholanthrene that appears to explain most of the aforementioned findings. He found that

thioacetamide which supresses the release of RNA from the nucleus, inhibited phenobarbital induction but not 3-methylcholanthrene induction. He further determined that phenobarbital stimulated a 3-methyl-4-methylaminoazobenzyne demethylase which had the same kinetic properties and sensitivity to SKF 525-A as the enzyme from untreated microsomes and that 3-methylcholanthrene stimulated a 3-methyl-4-methylaminoazobenzene demethylase that showed a different sensitivity to SKF 525-A and different kinetics (Sladek, 1969b). From these findings Sladek concluded that the m-RNA involved in phenobarbital induction is templated in the nucleus while that involved in 3-methylcholanthrene induction is templated in the cytoplasm through a relatively stable m-RNA and therefore would not require immediate RNA synthesis in the nucleus. Consequently only phenobarbital induction would be blocked by thioacetamide and both phenobarbital and 3-methylcholanthrene induction would be blocked by ethionine, puromycin and actinomycin D.

Returning to the metabolism of the drug Procarbazine, the biochemical mechanism(s) by which it produces its carcinogenic and antitumor properties has not been firmly established. The formation of hydrogen peroxide from the oxidation of Procarbazine has been pointed to as the cause of DNA degradation (Berneis, 1963a,b) but recently Gale (1967) has determined that this effect is not due to only hydrogen peroxide but to some other metabolite of

Procarbazine. Weitzel (1968) has shown that hydrogen peroxide and formaldehyde inhibited DNA polymerase and DNA dependent RNA polymerase. Procarbazine, its azo derivative, p-hydroxy-methylisopropyltoluamine and p-carbonylisopropyltoluamide inhibited uridine-^{14}C incorporation (Weitzel, 1967). The latter two compounds also inhibited the intracellular concentration of the nucleosides suggesting a blockage of nucleotide transport into cells (Weitzel, 1967). Sartorelli (1966) has proposed that there is a secondary antitumor effect of Procarbazine and/or its metabolites greater than its role in inhibiting DNA and RNA synthesis since most cell death occurred after both of these processes had recuperated. Koblet (1968) and Weitzel (1967a) have found that Procarbazine also inhibited the incorporation of labelled amino acids into protein possibly due to an effect of this compound on polysomes. Procarbazine treated polysomes have been shown to inhibit amino acid incorporation in vitro (Koblet, 1968). This could, however, be an indirect result of its effect on DNA and RNA synthesis.

Kreis (1965, 1966) has found that the ^{14}C of ^{14}C-methyl labelled Procarbazine when administered to mice appeared in DNA in the seven position of guanine. This appears to be where most carcinogenic or antitumor alkylating agents have their most observable effect on DNA. Procarbazine also caused breaks in DNA but did not interfere with its helical structure (Berneis, 1963a, b).

Prough (1969a, b) has reported that monomethylhydrazine and the azo derivative of Procarbazine are oxidized to methane by the microsomes in the presence of a NADPH-regenerating system. A free radical or ionic mechanism was proposed as the mechanism of methane formation from both of these compounds. Therefore, DNA alkylation by Procarbazine could result from the formation of a methyl carbonium ion (CH_3^+) or a methyl radical ($CH_3\cdot$) from the azo derivative of Procarbazine or monomethylhydrazine.

The intermediate formation of diazomethane cannot be ruled out as a possibility in explaining the carcinogenic properties of Procarbazine in rats and mice although so far it has not been detected as a metabolite. Diazomethane has been implied as the source of carcinogenic activity of trialkyltriazenes (Preussmann, 1969) and is generally accepted as the carcinogenic metabolite of N, N-dimethylnitrosoamine (Magee, 1967). Diazomethane has been found to alkylate DNA in the seven position of guanine (Kriek, 1964) as does Procarbazine (Kreis, 1966, 1965) and to cause breaks in DNA which are due to the release of methylated bases (Kriek, 1964). In aqueous solutions diazomethane forms methylcarbonium ions (Kriek, 1963) which have been implicated as the alkylating agents which alter DNA.

Similarities between the properties of diazomethane and methylazoxymethanol, the carcinogenic component of the cycad nut, have been noted (Shank, 1967). The $CH_3\overset{\overset{O}{\uparrow}}{N}=N-$ group in

methylazoxymethanol poses some interesting implications which could apply to Procarbazine. Fish (1956) has found that the N-oxides of several biological amines are demethylated to formaldehyde in the presence of ferric iron at a pH of seven. Zeigler (1964) has observed the presence in microsomes of an N-oxide dealkylase which demethylates the N-oxide of N,N-dimethylaniline at a rate greater than that for formaldehyde formation from N,N-dimethylaniline. He concluded that the N-oxide is an intermediate in the microsomal demethylation of N,N-dimethylaniline. Since the azo derivative of Procarbazine is rapidly formed from the parent compound in vivo it might actually be the substrate for the microsomal N-demethylation reported by Weitzel (1967b) for Procarbazine. If the N-oxide of the azo derivative is formed, it might be responsible for the carcinogenic and antitumor properties of Procarbazine.

The research presented in this thesis was undertaken to further characterize the microsomal enzyme which metabolizes Procarbazine and to determine the specificity of this enzyme toward other methylhydrazines and methylazo compounds. The properties of this enzyme were compared to another microsomal demethylase, that which demethylates N,N-dimethylaniline. The properties of this latter enzyme have been well studied (Ziegler, 1964, 1966; Machinist, 1966; Dehner, 1968).

MATERIALS AND METHODS

Materials

Sprague-Dawley male rats were obtained from Pacord Research, Inc., Portland, Oregon. Rats used in experiments weighed 250-300 g. Fresh pork livers were generously provided by Steen Brothers Meat Packing Company, Albany, Oregon. Monomethylhydrazine 1,1-dimethylhydrazine, and N,N-dimethylaniline were purchased from Matheson-Coleman-Bell. 1,2-dimethylhydrazine was obtained from Aldrich Chemical Co. Isocitrate, isocitrate dehydrogenase (type IV), NADPH, $NADP^+$, nicotinamide, steapsin, deoxycholate, trypsin (2x crystallized), and trypsin inhibitor (egg ovalbumin) were purchased from Sigma Chemical Co. Protamine sulfate (grade I), guanidine, glycine, Triton X-100 and X-45, Sephadex G 100-120, DEAE-cellulose, and 3-methylcholanthrene were also obtained from Sigma. SKF 525-A was a gift of Smith Kline and French Laboratories. Procarbazine and 1-methyl-2-benzylhydrazine were generously provided by Hoffmann La-Roche Laboratories. Phenobarbital and sucrose were obtained from Mallinckrodt Chemical Works. Ammonium sulfate, special enzyme grade, and the acetate of methylazoxymethanol were purchased from Mann Research Laboratories, Inc. Tanks of prepurified nitrogen, oxygen and air were obtained from the Salem Steel Co. Tanks of carbon monoxide

(99.95%) and nitrogen (108 ppm oxygen) were purchased from and analyzed for percent composition by the Matheson Co. Sodium dithionite was obtained from J. J. Baker Chemical Company. Other chemicals used came from laboratory stores and were analyzed reagent grade. All solutions were made in deionized glass-distilled water.

Pre-Treatment of Animals

Rats were starved for 24 hours prior to sacrifice but were allowed water ad libitum. Phenobarbital was administered i. p. daily for three days prior to sacrifice in doses of 100 mg/kg. Three-methylcholanthrene (30 mg/kg) and Procarbazine (200 mg/kg) were administered i.p. daily for two days prior to sacrifice. SKF 525-A (50 mg/kg) was given in a single i.p. dose one hour prior to sacrifice.

Rat Liver Microsome Preparation

The rat livers were sliced and washed twice in equal volumes of 0.25 M sucrose, and homogenized in five volumes of the same. The homogenate was centrifuged at 15,000 g for 30 minutes to remove the cell debris and mitochondria. The supernatant was centrifuged at 140,000 g for four hours to sediment the microsomes. The microsomal pellet was resuspended with a teflon-glass

homogenizer in 0.1 M phosphate buffer pH 7.4 (1 ml per gram of wet liver weight).

Microsomes used in the spectral studies were prepared by Mason's procedure (private communication, 1968). The rat livers were sliced, washed in 0.25 M sucrose, homogenized in 0.88 M sucrose (3 ml/g of liver, wet weight) and centrifuged at 9000 g for 20 minutes. The lipid layer which was on the surface of the supernatant was removed and the remainder of the supernatant was diluted with five volumes of 1.15% KCl and centrifuged for one hour at 140,000 g. The microsomal pellet was resuspended by homogenization in 10 volumes of 0.1 M phosphate buffer pH 7.0 and recentrifuged at 140,000 g for one hour. The pellet was resuspended in 1 M phosphate buffer/g of liver weight and stored under an atmosphere of molecular hydrogen in test tubes equipped with serum stoppers. Preparation of microsomes in this manner caused negligible formation of P-420 as determined spectrally. A Beckman Model L-2 ultracentrifuge was used to sediment microsomes.

Spectral Studies

The microsomal content of P-450 and cytochrome b_5 was determined spectrally according to methods described by Smuckler (1967). For P-450 determinations 2.5 mg of microsomal protein was diluted to 6 ml with 0.1 M phosphate buffer pH 7.4. A few mg

of sodium dithionite was added, and the solution was divided between two cuvettes. A baseline was then recorded on a Shimadzu MPS-50L spectrophotometer which was used throughout for spectral studies. The sample in one cuvette was bubbled for 20 seconds with carbon monoxide and the difference spectrum was taken. The $\Delta OD_{450} - \Delta OD_{500}$ was taken as the relative amount of P-450.

For cytochrome b_5 determinations 2.5 mg of microsomal protein and 0.3 ml of 10% deoxycholate were diluted to six ml with 0.1 M phosphate buffer pH 7.4 and the base line was recorded. To one cuvette was added a few mg of sodium dithionite and the difference spectrum was taken. The $\Delta OD_{427} - \Delta OD_{410}$ was taken as the relative amount of cytochrome b_5.

The effects of various hydrazines on the spectra of microsomes were determined by the methods of Schenkman (1967b). Microsomal protein was diluted to 2 mg/ml, divided between two cuvettes and the base line was recorded. The compound to be tested was then added to one cuvette and the difference spectrum was taken.

Protein

Protein was determined by the method of Lowry or by the OD_{260}/OD_{280} method as described by Chaykin (1966). It was found that the ratio method gave approximately two times higher protein concentration than the Lowry method for microsomal protein. Since

the Lowry method is a more accepted procedure for protein determination in microsomal preparations, all protein concentrations were corrected to that which would be determined by this method, e.g. the amount of protein determined by the ratio method was divided by two.

Enzyme Assays

Twelve ml conical centrifuge tubes were used for enzyme assays. A typical reaction mixture consisted of substrate, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 0.01 mM $MnCl_2$, 5 mM isocitrate, 0.32 units of isocitrate dehydrogenase, microsomes and 0.1 M phosphate buffer pH 7.4 to a final volume of two ml. The time of incubation was 40 minutes at 37^o unless otherwise noted. The reaction was stopped by the addition of one ml of 20% trichloroacetic acid. When AZO was used as the substrate, the reaction was stopped by precipitation of the protein on addition of one ml of methylene chloride to permit the extraction of AZO prior to the formaldehyde determination. More than 80% of the AZO was extracted into the methylene chloride layer as determined by its extinction coefficient at 232 nm ($1.46 \times 10^4 M^{-1}$). The organic layer was then re-extracted with phosphate buffer to remove any formaldehyde. This was added back to the original aqueous layer. Such a procedure was used since it was found that treatment of the AZO with 20% TCA caused its cleavage to

formaldehyde to a slight extent (approximately 0.6%). This same procedure was used when the azo of 1-methyl-2-benzylhydrazine was used as substrate, as it also undergoes the same cleavage reaction when treated with 20% TCA. Formaldehyde was measured by the method of Nash (1953). After addition of the TCA, the reaction mixture was centrifuged to sediment the protein. A two ml aliquot of the reaction medium was then pipetted into 0.6 ml of the Nash reagent and incubated at 37^{o} for 60 minutes. The optical density was then measured at 412 nm. The Nash reagent consists of 1.5 g ammonium acetate, 0.02 ml acetylacetone and 0.03 ml glacial acetic acid diluted to 10 ml with glass distilled water. Boiled microsomal protein was used in the control for enzyme assays. Unless otherwise stated microsomes prepared from phenobarbital pretreated rats were used in the enzyme assays. Assays were performed in duplicate and on liver microsomes prepared from at least two animals.

Nash (1953) observed that certain amines will substitute for ammonia in forming diacetyldihydrolutidine; methylamine (0.1 M) caused a 4% loss in formaldehyde and ethylenediamine (0.1 M) caused a 20% loss. At first it was thought that the hydrazines were reacting with the formaldehyde formed in the assay medium to give the corresponding hydrazone but spectral studies indicated that PCZ, MMH, UDMH and SDMH interact in some way with the Nash reagent causing a considerable decrease in the absorption of the reagent at 295 nm.

The hydrazines appear to be reacting with acetyl acetone, since addition of a hydrazine also caused a decrease in its absorption at 276 nm. An example of the spectra obtained can be found in Figures 3 and 4. When a known amount of formaldehyde was added to a typical assay mixture of one of these hydrazines (20 μmoles), TCA, protein, and the Nash reagent, only a small percentage of the added formaldehyde formed diacetyldihydrolutidine which absorbs at 412 nm ($E = 8 \times 10^{3} M^{-1}$) (Table 2). When the same amount of formaldehyde was added to the Nash reagent alone, 100% of the added formaldehyde was measured at 412 nm by using the above extinction coefficient. It was concluded that the N-methylhydrazines were reacting in some way with a component of the Nash reagent, possibly in a manner similar to that of methylamine and ethylenediamine. A correction factor was therefore applied to the amount of formaldehyde formed from these hydrazines (Table 2). A Beckman DU was used to measure the absorption of diacetyldihydrolutidine at 412 nm.

Purification of the Pork Liver Demethylase

A method to purify DMA demethylase from pork liver was provided by Ziegler (private communication, 1968) and was used in an attempt to purify the N-methylhydrazine demethylase. A pork liver (approximately 1100 g) was obtained fresh, sliced immediately in 1 cm strips and placed on ice. The liver slices were washed in an

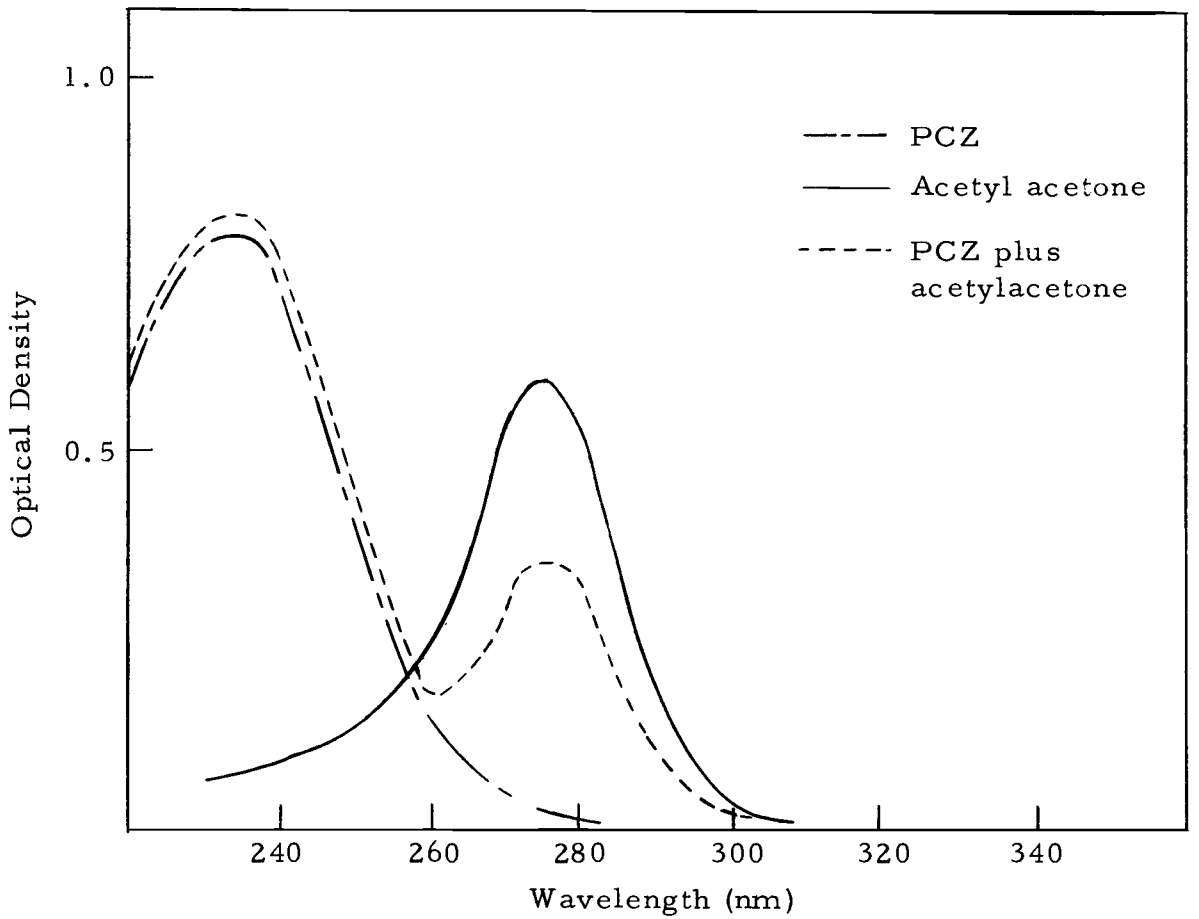


Figure 3. The effect of PCZ on the spectrum of acetylacetone. The same concentrations of PCZ and acetylacetone were used as would be in a typical enzyme assay.

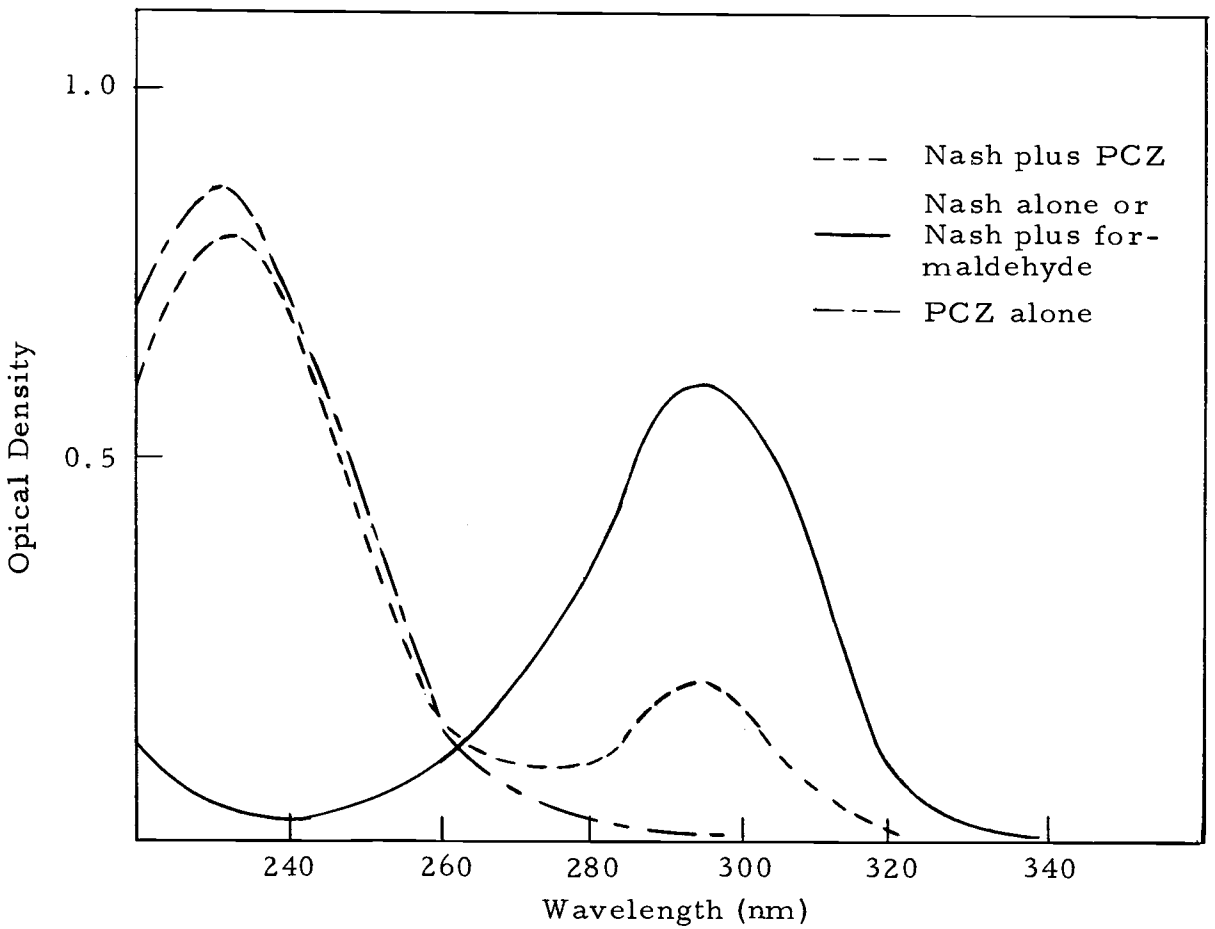


Figure 4. The effect of PCZ on the spectrum of the Nash reagent. The same concentrations of PCZ and Nash reagent were used as would be in a typical enzyme assay.

Table II. Amount of formaldehyde measured in the presence of N-methylhydrazines.

Substrate	Formaldehyde Added in mμmoles					Correction Factor*
	100	300	500	700	1000	
	mμmoles Formaldehyde Measured					
MMH	16	31	56	74	158	6.2
PCZ	17	24	31	51	72	6.2
UDMH	50	175	258	371	494	2.0
SDMH	31	90	155	222	273	3.3

* Correction factor applied to the amount of formaldehyde measured at 412 nm in the presence of the various hydrazines in order to more closely approximate the actual amound of formaldehyde present.

$$\text{Correction factor} = \frac{\text{formaldehyde added (}\mu\text{moles)}}{\text{formaldehyde observed (}\mu\text{moles)}}$$

Assay medium as described in materials and methods. The amount of MMH, PCZ, UDMH or SDMH added to the medium was 20 μmoles. One ml of 20% TCA was added to the medium before addition of microsomal protein to permit measurement of only the formaldehyde added and not formaldehyde formed enzymically from the substrate used.

equal volume of 0.25 M sucrose, ground in a meat grinder, and suspended in 1 1/2 volumes of sucrose. The suspension was then run through a continuous flow homogenizer similar to that described by Zeigler (1966) and the homogenate was centrifuged at 1,200 g for 20 minutes. The supernatant was centrifuged at 105,000 g for one hour to sediment the microsomes which were then resuspended in 0.1 ml 0.25 M sucrose/one g of original liver weight and frozen in a trichloroethylene-dry ice bath overnight. The following day the microsomes were thawed and diluted with two volumes of 0.25 M sucrose and centrifuged for one hour at 105,000 g. The microsomes were resuspended in 0.25 M sucrose-0.1 M guanidine pH 8.0 and sedimented at 105,000 g for 20 minutes. The particles were resuspended in water to a final concentration of 20 mg/ml protein. To extract the demethylase, 0.25 mg Triton x-100 and 0.25 mg Triton X-45 per mg protein were added and the suspension was stirred in the cold for 60 minutes. One mg of protamine sulfate/ml suspension was then added and the suspension was centrifuged for one hour at 105,000 g. The supernatant was immediately fractionated by a 35% saturation with ammonium sulfate. This precipitate was removed by centrifugation and the supernatant was taken to 50% ammonium sulfate saturation. The 35-50% precipitate was suspended in a minimum volume of 0.05 M glycine pH 6.0 and immediately frozen. This fraction contained all the demethylase activity as measured by the amount

of enzymic formaldehyde formation. The following day the 35-50% fraction was thawed and then fractionated with ammonium sulfate. That which precipitated between 40-50% ammonium sulfate was suspended in a minimal volume of glycine buffer and dialyzed against the same. It was then placed on a DEAE cellulose column (1 cm by 8 cm). The column retained most of the protein and that which was eluted was concentrated about 3-fold in a Diaflo pressure cell of 10 ml volume with a XM-50 filter. The concentrate was then passed through a Sephadex G100-120 column (3 cm by 30 cm) equilibrated with 0.05 M glycine pH 6.3. The purified demethylase came out with the void volume. An approximately 700 fold purification was thus obtained only once for the enzyme or enzymes which demethylate MMH and DMA although this entire purification procedure was attempted several times. Frequently the activity would precipitate in the 35-40% second ammonium sulfate fractionation and could not be extracted from this fraction by either further ammonium sulfate fractionation or by placing the fraction on the DEAE-cellulose column. The data obtained at each step of the purification can be found in Table III. The final yield of protein was very low and the total activity obtained was not sufficient to do studies on the purified enzyme.

Table III. Purification of the demethylase.

	Protein		Specific Activity		Fold Purification	
	mg/ml	Total	MMH	DMA	MMH	DMA
Microsomes	108.6	14,120	2.0	5.8		
Guanidine treatment	102.8	13,400	2.0	5.7		
Triton X-100 and X-45, protamine, and $(NH_4)_2SO_4$ 35-50%	2.5	133	3.9	10.0	2	2
2nd $(NH_4)_2SO_4$ 40-50%	5.0	25.0	67.0	97.4	32	17
DEAE-cellulose column	1.6	3.1	147	530	73	90
Sephadex G 100-120 column	0.07	0.06	1540	3750	771	650

Protein was determined by the method of Lowry. Enzyme assays as described in Materials and Methods. Specific Activity = mμmole formaldehyde/mg protein/40 minutes.

Organic Synthesis

PCZ and MDH were dehydrogenated to the corresponding azo derivatives by a method adapted from a procedure provided by Hoffman La-Roche Laboratories. One gram of the hydrazine hydrochloride was mixed with 2.5 ml anhydrous ether, and 2.5ml 100% ethanol. Six grams of HgO was added to start the reaction. The reaction vessel was shaken for 40 minutes and then filtered through a sintered

glass filter to remove the mercury, and unreacted HgO and hydrazine hydrochloride which was only slightly soluble in organic solvents. This entire procedure was carried out under a nitrogen atmosphere. The filtrate was then evaporated to dryness in a vacuum with a rotary evaporator and extracted with 40 ml of anhydrous ether. The ether was removed by a rotary evaporator and the residue was taken as the product and identified by its spectral properties.

The N-oxide of AZO was synthesized in our laboratory by Mr. Russell A. Prough by a procedure adapted from that of Bollag (1964).

Steapsin and Deoxycholate Treatments and Trypsin Digestion of the Microsomes

Microsomes were incubated with 0.14% steapsin or 1% deoxycholate at 37° for one hour. They were then removed, placed on ice and assayed for demethylase activity as already described. Untreated microsomes served as the control.

For the trypsin digestion, 30 mg of trypsin was incubated with 10 ml of microsomes (approximately 200 mg of protein) for various time intervals at 25°. At the end of each time interval a two ml aliquot was removed and added to 8 mg of trypsin inhibitor (egg ovalbumin) which was dissolved in 0.1 M phosphate buffer pH 7.4. Each sample was centrifuged for one hour at 140,000 g to sediment

the intact microsomes. The solubilized protein in the supernatant was discarded. Two types of controls were utilized in these experiments. One type consisted of diluting two ml of untreated microsomes to the same final volume as the treated microsomes with 0.1 M phosphate buffer pH 7.4 and centrifuging the suspension for one hour at 140,000 g. The other control consisted of taking a two ml aliquot of the trypsin-digested microsomes at zero time, adding it to the trypsin inhibitor and centrifuging it. The microsomal pellets were resuspended in two ml of 0.1 M phosphate buffer pH 7.4 and assayed for demethylase activities as already described.

Enzyme Assays Performed Under Various Gaseous Atmospheres

The entire assay media except microsomes and isocitric dehydrogenase were placed in conical centrifuge tubes equipped with serum stoppers and were bubbled for five minutes with the gases. Various CO/O_2 ratios were obtained by first bubbling each tube with 99.95% CO and then adding appropriate volumes of oxygen by a syringe after the same volume of added CO had been similarly removed from each tube in order to maintain atmospheric pressure within the tube. The same procedure was used to obtain the various N_2/O_2 ratios, except that nitrogen containing 108 ppm oxygen was used to obtain an atmosphere of 0.01% O_2, 99.99% N_2. Nitrogen was purified of oxygen by passing it over a hot activated copper

column. Nitrogen purified in this manner has less than 4×10^{-7} atm. of oxygen at 1 atm. pressure (Meyer and Ronge, 1939).

Both microsomes and isocitric dehydrogenase were each placed in conical centrifuge tubes equipped with serum stoppers and equilibrated with O_2 free nitrogen. They were added to the sealed reaction tubes with one ml syringes equipped with 26 guage one half inch needles.

For the experiments in which the assay medium was bubbled with air or CO plus air, the assay was bubbled for five minutes with compressed air or for five minutes with a 50-50 mixture of CO and air. DMA demethylation served as the control to determine if all the P-450 was complexed with CO since this reaction was found to be almost completely inhibited when only CO complexed P-450 was present. In this case the microsomes were also bubbled for five minutes with the same gases as the assay mixtures prior to addition of the microsomes. Air bubbled microsomes and assay medium were run as controls to determine whether such agitation caused loss in enzyme activity. All assays run in an atmosphere containing CO were run in the dark since the complex formed between P-450 and CO is light reversible (Cooper, 1965).

PART I. CHARACTERIZATION OF THE N-METHYLHYDRAZINE DEMETHYLASE

RESULTS

Cellular Distribution and Substrate Specificity of the N-Methylhydrazine Demethylase

The microsomal fraction contained essentially all of the enzymic activity for formaldehyde formation; the nuclear, mitochondrial, and soluble fractions produced less than 1.0 mμmole of formaldehyde/mg of protein/40 minutes (Table IV). DMA was

Table IV. Cellular distribution of demethylase activity.

Cellular Fraction	mμmoles Formaldehyde/mg protein/40 minutes		
	MMH	PCZ	DMA
Nuclear	< 1.0	< 1.0	< 1.0
Mitochondrial	< 1.0	< 1.0	< 1.0
Microsomal	75	12	54
Soluble	< 1.0	< 1.0	< 1.0

Assay medium: 20 μmoles MMH, 20 μmoles PCZ or 10 μmoles DMA, and 5 mg of protein, 0.1 M phosphate buffer pH 7.4 to a final volume of 2 ml. Assay of the microsomal fraction also contained the NADPH-regenerating system.

included as a substrate throughout this study as a measure of microsomal activity from preparation to preparation and as a tool for comparison since its microsomal oxidative demethylation has been extensively studied (Ziegler, 1964, 1966; Machinist, 1966; Dehner, 1968).

The observed substrate specificity of the demethylase system for various methylhydrazines is shown in Table V. UDMH, MMH,

Table V. Substrate specificity.

Substrate	mμmoles formaldehyde/mg protein/40 minutes
1,1-dimethylhydrazine	81
Monomethylhydrazine	75
1,2-dimethylhydrazine	29
Procarbazine	12
AZO	22
N-oxide of AZO	<1
1-methyl-2-benzylhydrazine	<1
AZO of 1-methyl-2-benzylhydrazine	19
Methylazoxymethanol	75

Assay medium: 20 μmoles substrate, 0.5 mM $NADP^+$, 5 M $MgCl_2$, 0.01 mM $MnCl_2$, 5 mM isocitrate, 0.32 units isocitrate dehydrogenase, 2.5 mg microsomal protein, and 0.1 M phosphate buffer pH 7.4 to a final volume of 2 ml. Incubation time was 40 minutes at 37°. Microsomes were prepared from animals pretreated with phenobarbital.

SDMH, and PCZ are demethylated at rates of 81, 75, 29 and 12 mμmoles of formaldehyde per mg of protein per 40 minutes respectively. Since PCZ is converted to AZO by microsomes (Prough, 1969b) and nonenzymically in aqueous solutions in the presence of oxygen (Zeller, 1963), both PCZ and AZO may be serving as substrates in the experiments with PCZ. This possibility is supported by the fact that AZO was found to be demethylated at a rate twice that of PCZ. Therefore, one could be measuring the sum of formaldehyde formed from PCZ and AZO, when PCZ is used as substrate. Due to the acid lability of AZO the addition of 20% TCA to stop the assay reactions could result in a small portion of any AZO formed from PCZ to cleave and yield formaldehyde. This was eliminated by comparing the amount of formaldehyde formed when 20% TCA and methylene chloride were used to stop the reaction. The same amount of formaldehyde was formed under both conditions, indicating that 20% TCA caused an insignificant quantity of formaldehyde to be formed nonenzymically when PCZ was used as substrate.

If the azoxy derivative of PCZ were an intermediate in the demethylation of PCZ and if it has a suitable lipid solubility it should be demethylated at a faster rate than the parent compound. However, it did not appear to be a substrate (Table V). It should be noted that approximately 58 mμmoles of formaldehyde/mg of protein/40 minutes were formed non-enzymically in an assay medium

containing this compound and either boiled or unboiled microsomes. When boiled microsomes were used with AZO as substrate an average of 20 mμmoles of formaldehyde/mg of protein/40 minutes was formed.

The acetate of methylazoxymethanol was one of the better substrates tested since it yielded 75 mμmoles of formaldehyde/mg of protein/40 minutes.

1-Methyl-2-benzylhydrazine (MBH) did not appear to be demethylated to formaldehyde by microsomal preparations. If any formaldehyde is being enzymically formed from MBH the amount is less than 1 mμmole. This value (1mμmole) is the lower limit of formaldehyde detection by this reagent (Nash, 1953). The azo derivative of MBH was a good substrate for the N-demethylase; 19 mμmoles of formaldehyde being formed per mg of protein per 40 minutes.

Kinetic and Temperature Effects

To determine reaction conditions which would give maximum enzyme activity, a study was made on the effects of protein concentration and incubation time upon microsomal N-demethylase activity. The rate of formaldehyde formation from MMH and DMA was linear with time up to 40 minutes and up to 120 minutes for PCZ (Figure 5). The reaction with MMH and PCZ as substrates was approximately linear up to 2.5 mg of protein (Figure 6). An incubation time of

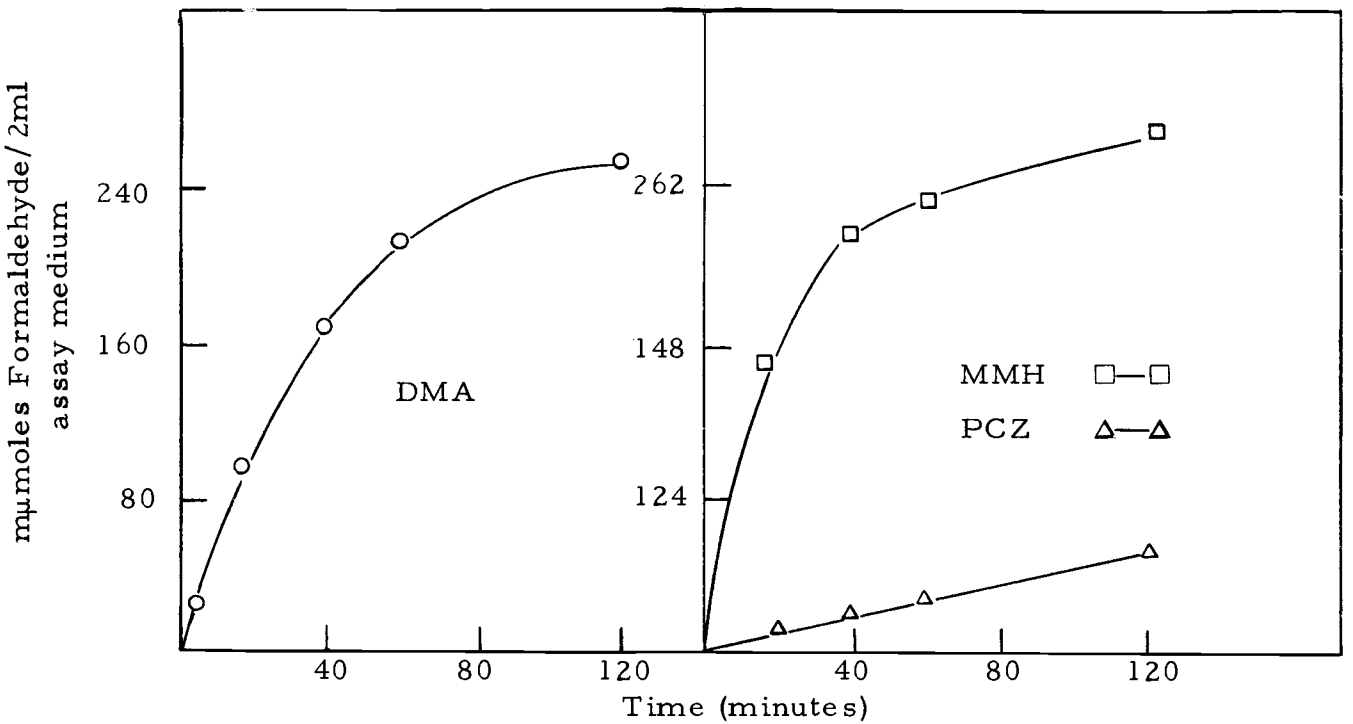


Figure 5. The effect of time on formaldehyde formation. Enzyme assays were carried out as described in Materials and Methods except that the time of incubation was varied.

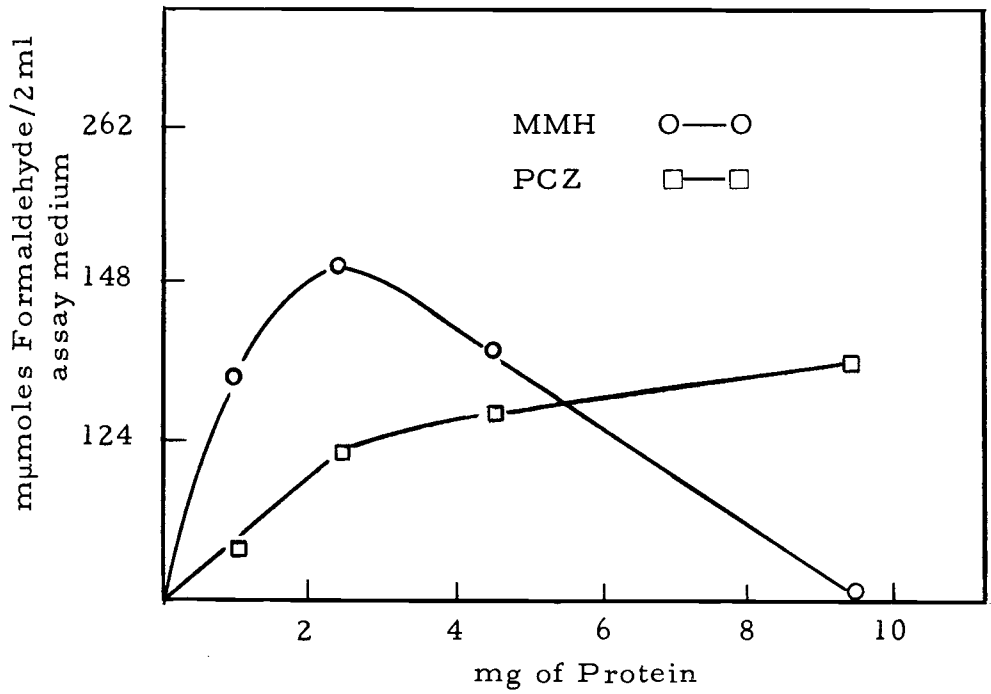


Figure 6. The effect of protein concentration on formaldehyde formation from PCZ and MMH. Enzyme assays were carried out as described in Materials and Methods except that protein concentration was varied.

40 minutes was therefore chosen for all assays and 2.5 mg of microsomal protein was added to the reaction mixture for routine assays. Even though the formation of formaldehyde from PCZ was linear with time up to 120 minutes, an incubation time of 40 minutes was chosen for assays with this compound. Since PCZ is autooxidized in aqueous solutions with time to AZO (Zeller, 1963) and since the AZO is more lipid soluble than PCZ, a shorter incubation time would be more representative of formaldehyde formation from PCZ itself.

DMA, MMH, and AZO gave hyperbolic velocity versus substrate concentration curves. Lineweaver-Burk plots of this data (Figure 7) gave K_m values for MMH, AZO and DMA of 0.8×10^{-3}M, 1.6×10^{-3}M and 2.4×10^{-3}M, respectively. The data for MMH were not corrected for the amount of formaldehyde measured in the presence of this compound and therefore represent minimum values. The K_m value as calculated from the Lineweaver-Burk plot for MMH is then at best an approximation.

The effect of temperature on the demethylase reaction of PCZ, MMH and DMA was examined (Table VI). An incubation temperature of 37^{o} gave maximum demethylase activity. Treatment of the microsomes for 2, 4, or 7 minutes at 60^{o} caused a decrease in the demethylation rate of PCZ, MMH and DMA. DMA and PCZ demethylases appeared to be the most heat labile.

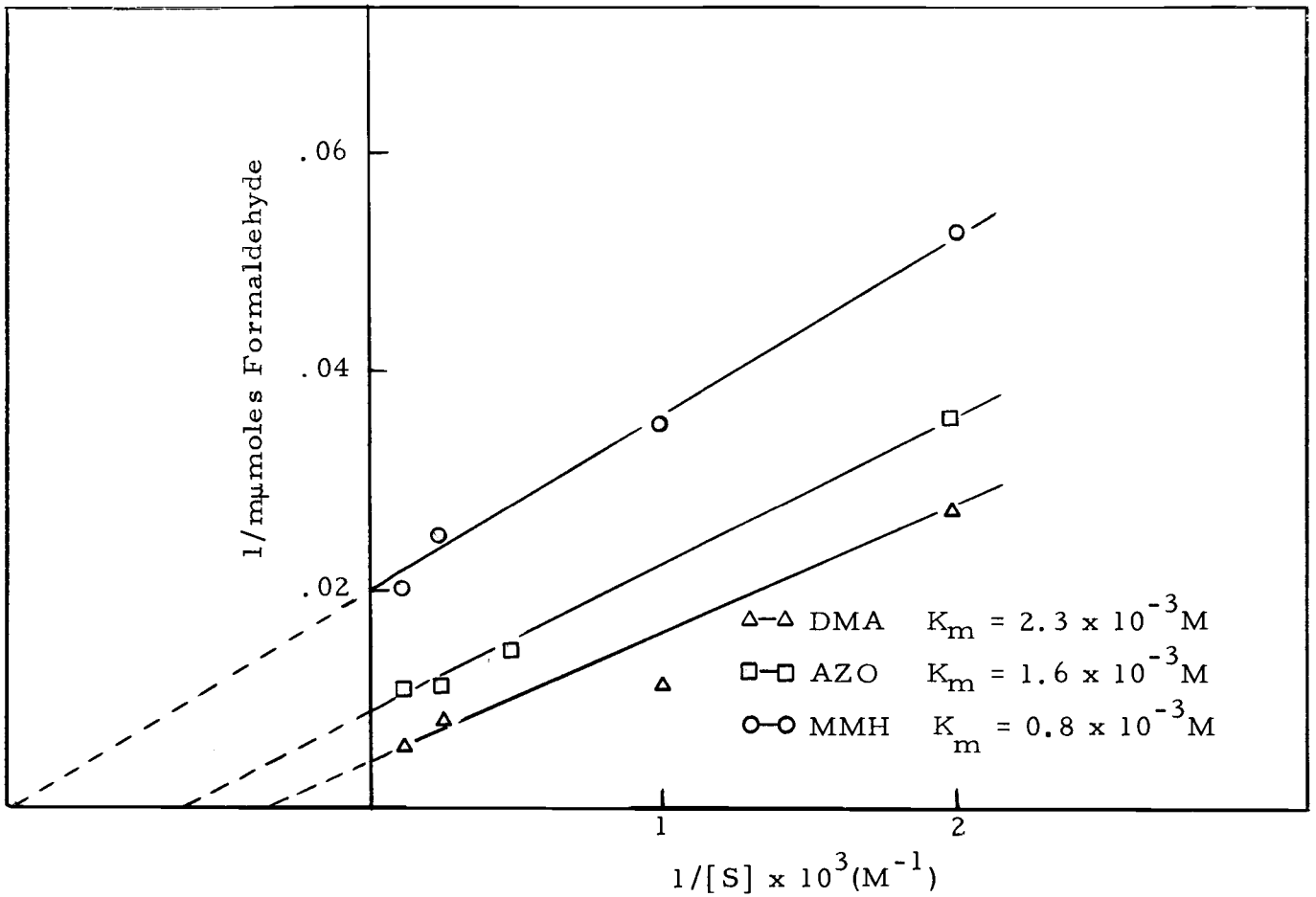


Figure 7. Lineweaver-Burk Plots. Determination of the K_m values for AZO, DMA, and MMH demethylation. Enzyme assays were carried out as described in Materials and Methods. The data for the MMH K_m determination are not corrected for the amounts of formaldehyde measured in the presence of this compound and therefore represent minimum values.

Table VI. Temperature dependence of the demethylation of MMH, PCZ, and DMA.

Substrate	mμmoles Formaldehyde Formed						
	Incubation Temp. °C				Pretreatment Time* of Microsomes at 60° Minutes		
	0	15	25	37	2	4	7
DMA	7	36	55	135	7	3	2
PCZ	0	14	21	40	14	0	0
MMH	0	82	113	188	50	21	14

* After the microsomes were treated at 60°, they were assayed for activity at 37° C.

Assay medium: 10μmoles DMA, 20μmoles MMH, or 20μmoles PCZ. Other assay conditions as described in materials and methods unless otherwise stated.

The Effect of Other Reaction Conditions on the N-Demethylase Activity

The data in Table VII indicate that the demethylation of PCZ and MMH is dependent upon the presence of either a NADPH-regenerating system or NADPH. The concentration of isocitric dehydrogenase was doubled (0.64 units) to determine if the rate of NADPH regeneration was limiting. No increase in formaldehyde production was noted in these experiments. Addition of a limiting amount of NADPH (0.5mM) caused a three to four fold increase in the demethylation of MMH and PCZ. The demethylation rate of AZO did not

Table VII. Effect of various reaction conditions on the demethylation of MMH, PCZ, AZO and DMA by microsomes prepared from phenobarbital pretreated animals.

Reaction Condition	% of Control Formaldehyde			
	DMA	MMH	PCZ	AZO
Control	100	100	100	100
Deletion of Regenerating System	6	10	0	120
Deletion of Regenerating System; addition of NADPH[1]		30	40	
Addition of 10^{-4}M SKF 525-A	28	145	160	150
Gassed for 2 min. with N_2	8	22	20	25
Addition of 100 μmoles Nicotinamide	112	25	28	100

[1] NADPH concentration was 0.5 mM.

Assay medium: 10 μmoles DMA, 20 μmoles MMH, 20 μmoles PCZ, or 20 μmoles AZO. 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 0.01 mM $MnCl_2$, 5 mM isocitrate, 0.32 units isocitrate dehydrogenase, 2.5 mg of protein, and 0.1 M phosphate buffer pH 7.4--unless otherwise stated. Incubation time was 40 minutes at 37° C.

require the presence of a NADPH-regenerating system for maximum activity but instead seemed to be slightly reduced (20%) by its presence. Further study indicated that high concentrations of NADPH inhibited the reaction while low concentrations actually stimulated the reaction (Table VIII). Therefore, the presence of 0.05 μmole to 1 μmole NADPH increased formaldehyde production from AZO 20%. The nature of the inhibition caused by NADPH on AZO demethylation is not known.

Table VIII. Effect of various NADPH concentrations on AZO demethylation.

NADPH Concentration μmoles	mμmoles Formaldehyde/ mg protein/40 min.
0	9
0.05	11
0.1	11
0.25	5
0.5	6
1.0	5

Reaction mixture consisted of: 20 μmoles substrate, NADPH, 2.5 mg microsomal protein (microsomes from phenobarbital pretreated animals) and 0.1 M phosphate buffer pH 7.4 to two ml. Incubation time was 40 minutes at 37° C.

The demethylation reaction is also dependent on molecular oxygen since gassing the assay medium for two minutes with nitrogen caused a 74-80% inhibition of formaldehyde formation (Table VII). Additional details of oxygen effects are described later in this thesis. Schenkman (1967a), has recently reported that nicotinamide, which is often added to the assay medium to inhibit pyridine nucleotidase, inhibits aminopyrine demethylation and aniline hydroxylation. Addition of 100 μmoles of nicotinamide caused a 75% inhibition of the formaldehyde formed from PCZ and MMH, it had no effect on AZO demethylation, and increased by 12% the formaldehyde formed from DMA. Nicotinamide was not used due to these results and those observed by Schenkman (1967a).

SKF 525-A has been reported to inhibit the metabolism of many drugs (Cooper, 1954). However, a few exceptions can be found in the literature. Gaudette and Brodie (1959) have observed that the demethylation of methyl- and ethyl-aniline is not inhibited by SKF 525-A. Addition of SKF 525-A (10^{-4}M) to the assay medium (Table VII) did not inhibit the demethylation of PCZ, MMH, or AZO but actually stimulated it approximately 50%. The demethylation of DMA was inhibited 72%.

DISCUSSION

The microsomal fraction of rat liver cells possesses an enzyme system which demethylates several compounds that contain the $N\text{-}N\text{-}CH_3$ group. Substrates for the demethylation reaction include MMH, UDMH, SDMH, PCZ, AZO, the azo derivative of MBH and methylazoxymethanol. Several authors (Dost, 1966, 1967; Baggiolini, 1965; Schwartz, 1966) have reported the in vivo formation of carbon dioxide from methylhydrazines but only for PCZ has there been reported any enzymic formation of formaldehyde in vitro (Weitzel, 1967b). The enzyme described here is heat labile and possesses typical enzyme kinetics. The reaction rate is linear with time and protein concentration and gives linear Lineweaver-Burk plots. The reaction is dependent upon the presence of molecular oxygen and a NADPH-regenerating system with the exception of AZO whose demethylation is inhibited slightly in the presence of high concentrations of NADPH. NADPH is rapidly oxidized by microsomes even in the absence of substrate (Prough, 1969a) and therefore the addition of one μmole of NADPH while not expected to give the maximum activity of the NADPH-regenerating system did enhance the reaction rate 30 to 40%. This enzyme has properties similar to the enzyme system that demethylates DMA (Zeigler, 1966) and can be classified as a mixed function oxidase as defined by Mason (1957).

The effect of SKF 525-A on the demethylation rate of MMH, PCZ and AZO poses an interesting problem. Most oxidative drug metabolizing enzymes are inhibited by this compound (Cooper, 1954). Rats pretreated with SKF 525-A accumulate the drug imipramine in liver (Bickel, 1968) causing an 84% inhibition of the demethylation of imipramine. In vitro SKF 525-A actually stimulated the demethylation of MMH, AZO and PCZ 1.5 fold. DMA demethylation was inhibited 72%. Ziegler (1966) has found that in pork liver microsomes SKF 525-A inhibited formaldehyde formation from DMA 94%. Microsomes pretreated with SKF 525-A metabolized MMH at 85%, AZO at 77% and DMA at 90% of the rate of untreated microsomes (Table X). Baggiolini (1966) has found that SKF 525-A (5×10^{-5}M) when added to isolated perfused rat liver inhibited CO_2 formation from MMH 15% and PCZ 50%. Although a direct comparison cannot be made since the systems and products being studied are different, Baggiolini's results appear to support the in vivo data obtained with SKF 525-A and contradict the in vitro data presented here. The reason for the stimulatory action of SKF 525-A on the demethylation rate of MMH, PCZ and AZO is not known. The differences seen between the addition of SKF 525-A in vitro and treatment of rats with SKF 525-A could be explained if the inhibition due to the latter was attributable to some metabolite of SKF 525-A. When this compound is added in vitro possibly enough time does not elapse for this

metabolite to accumulate in the system to an inhibitory concentration and then SKF 525-A by some unknown mechanism stimulates the reaction.

Recently Prough (1969b) has reported the formation of hydrocarbon from the parent alkylhydrazine by rat liver microsomes. This reaction was also dependent on the presence of molecular oxygen and a NADPH-regenerating system. The following mechanism was proposed: an oxidation occurs in which the alkylhydrazine is converted to the corresponding diazene (R-N=N-). This compound then collapses to form hydrocarbon and nitrogen via either a free radical or ionic mechanism. If the alkyldiazene is formed and has a sufficient half life it could be the substrate for the oxidative demethylation of the N-methylhydrazines described. This author (1969a) has further found that AZO is enzymically formed from PCZ by microsomes. Data have been reported here that support this mechanism. AZO was demethylated at a rate two times that of PCZ. The azo of MBH but not MBH itself was demethylated by microsomes. The more rapid rate of N-methyl-azo demethylation could be due to the increased lipid solubility of the azo compound and/or to the fact that the azo is the substrate for the N-demethylase and the formation of the azo from the parent hydrazine is the rate-limiting step.

A different mechanism must be proposed for the demethylation of UDMH than MMH for two reasons. First, no methane is formed

from UDMH and therefore MMH cannot be a metabolite of this compound. Second, UDMH is not oxidized to a corresponding diazene but to a diazonium ion and then forms its tetrazene (Smith, 1966). The tetrazene of UDMH could then be the actual substrate for the N-demethylase. Structurally related compounds, the carcinogenic aryldialkyltriazenes, have been reported by Preussmann (1969) to be demethylated by microsomes to formaldehyde.

Data presented here indicated that the acetate of methylazoxymethanol was an excellent substrate for microsomal N-demethylation. This compound was isolated from the cycad nut as its glycoside and has been shown to be the potent carcinogenic component of the nut (Laquer, 1966, 1967). PCZ has also been shown to possess carcinogenic activity (Kelly, 1964) and both compounds alkylate DNA in the seven position of guanine (Shank, 1967; Kreis, 1966). N-oxides of tertiary amines have been proposed by Fish (1956) and Ziegler (1964) to be the intermediates in N-demethylation reactions by microsmal preparations. The strongest evidence against such an intermediate was reported by McMahon (1964) who determined through isotope dilution experiments that the N-oxide of l-propoxyphene was not an intermediate in the demethylation of this compound by rat liver microsomes. Considering the facts that methylazoxymethanol is demethylated by microsomal preparations and that it possesses biological properties similar to PCZ and, furthermore, that

N-oxides have been proposed as intermediates in oxidative demethylation by microsomes, it is tempting to postulate the formation of the N-oxide or azoxy derivative of AZO as the intermediate in its oxidative demethylation. Therefore, the N-oxides of methyldiazene, azomethane and the tetrazene of UDMH can be postulated as intermediates in the demethylation of MMH, SDMH and UDMH respectively. The sequence of reactions shown below is a summation of the foregoing postulations.

$$CH_3\text{-}NH\text{-}NH\text{-} \longrightarrow CH_3\text{-}N{=}N\text{-} \longrightarrow CH_3\text{-}\overset{\overset{O}{\uparrow}}{N}{=}N\text{-}$$

No net amount of formaldehyde was observed from the azoxy-derivative of AZO after comparing the yield of formaldehyde when boiled microsomes were present to unboiled microsomes. However, the amount of product formed under both of these conditions was approximately three times that formed from AZO in the presence of boiled microsomes. It is believed therefore that the azoxy cannot be ruled out as a possible intermediate in the demethylation of AZO since this compound is probably unstable in aqueous solution and slowly decomposes to formaldehyde. Thus the azoxy once it is formed from AZO in microsomes may be an unstable intermediate that spontaneously decomposes to product. Further study must be done to determine the lipid solubility of this compound and its stability in aqueous solutions before a definite statement can be made

as to whether it is an intermediate or not. The N-oxide of DMA as described by Ziegler (1964) appeared to be demethylated by microsomes but the K_m value of the N-oxide dealkylase was very high (0.193 M) for an enzymic reaction. However, lipid solubility and consequently availability of polar substrates to the demethylase enzyme system is an important factor in comparing the demethylation rates of the lipid soluble amine and its relatively lipid insoluble N-oxide.

Returning to the degradation scheme of PCZ in Figure 2, it seems that PCZ is first autooxidized to AZO which in turn can act as substrate for a microsomal N-demethylase to form formaldehyde and consequently CO_2. Or the AZO can be further degraded to its benzaldehyde derivative and MMH. MMH can then either be metabolized in the microsomes to methane or formaldehyde and CO_2.

The exact metabolic process that is affected by PCZ to cause it to be carcinogenic or cytotoxic has not been pinpointed. Many of the degradation products of PCZ have been found to inhibit DNA and protein synthesis in the cell. PCZ also methylated DNA in the seven position of guanine possibly via intermediate formation of diazomethane and carbonium ions. Any one or all of these effects of PCZ and its metabolites on cell metabolism could be responsible for the cytostatic and carcinogenic properties of this drug.

PART II. INDUCTION OF N-METHYLHYDRAZINE DEMETHYLASE AND THE ROLE OF P-450 IN THE ACTIVITY OF THIS ENZYME(S)

RESULTS

Spectral Evidence for the Interaction of Hydrazines with Microsomes

Substrates of microsomal enzymes cause two types of changes in microsomes as determined spectrally (Schenkman, 1967b). Since the spectrum of purified cytochrome b_5 is not affected by these substrates and since the total heme in microsomes can be attributed to cytochrome b_5 and P-450, Schenkman (1967b) concluded that these changes in microsomes were due to an interaction of the substrate with P-450. Addition of 3.4 mM PCZ and 7 mM MMH caused spectral changes which are included in the group designated as class II along with aniline (Table IX). Class II changes are characterized by an absorption maximum at 430 nm and a minimum at 390 nm. The interaction by DMA was characterized by an absorption maximum at 385 nm and a minimum at 415 nm: a class I interaction.

The Effect of Various Pretreatments on the Microsomal Content of P-450 and on the Demethylation Rates of PCZ, AZO, MMH and DMA

As previously mentioned induction studies with phenobarbital led to the conclusion that P-450 was involved in oxidative demethylation in microsomes. Pretreatment of rats with phenobarbital caused

Table IX. Effect of various compounds on microsomal spectra.

Compound	Concentration mM	Absorption Peaks Max mμ	Min mμ	Class
MMH	7.0	428	395	II
PCZ	3.4	435	392	II
DMA	3.5	385	410	I
Phenobarbital	3.5	385	420	I
Aniline	5.0	428	396	II

Microsomal protein was diluted to 2mg/ml, divided between two cuvettes and the baseline was recorded. The compound to be tested was then added to one cuvette and the difference spectra were taken.

a 2.15 fold increase in the P-450 content of microsomes and a 1.8 to 2.5 fold increase in the demethylation rates of DMA, MMH and AZO. (Table X) The demethylation rate of PCZ was increased 4-fold.

Pretreatment with 3-methylcholanthrene increased the cytochrome content and the demethylation rate of microsomes but not to the extent of phenobarbital. Thus the P-450 content was increased approximately 1.3 fold and demethylation rates of PCZ, DMA, MMH and AZO were increased 1.3 to 1.8 fold.

Since SKF 525-A had a stimulatory effect on the demethylation of MMH, AZO, and PCZ in vitro, rats were pretreated with SKF

Table X. The effects of various animal pretreatments on P-450 content and on the demethylation of PCZ, MMH, DMA and AZO by liver microsomes.

Pretreatment	% of Control P-450	mμmoles/ mg Protein P-450*	mμmoles Formaldehyde/ mg protein/40 minutes			
			DMA	MMH	PCZ	AZO
None	100	0.67	30	28	3	13
Phenobarbital	215	1.43	54	75	12.0	22
3-methylcholanthrene	130	0.8	38	50	5	17
SKF 525-A	100	0.67	27	24		10
Procarbazine	60	0.40	21	25		14

Phenobarbital (100mg/kg) was given daily for three days prior to sacrifice. Three-methylcholanthrene (30mg/kg) and PCZ (200mg/kg) were given daily for two days prior to sacrifice. SKF 525-A (50mg/kg) was given in a single dose one hour prior to sacrifice. Enzyme assays as described in Materials and Methods.
*An extinction coefficient of 91 $cm^{-1}M^{-1}$ was used to calculate the microsomal content of P-450 (Omura, 1964a, b).

525-A to determine if such an effect would still be seen by this compound. SKF 525-A pretreatment resulted in the inhibition of the demethylation of DMA, MMH and AZO by 10%, 15% and 23% respectively. The P-450 content of microsomes was unaffected by SKF 525-A.

Most drugs appear to be inducers of microsomal enzymes, however, the effect of pretreating rats with PCZ appeared to be just the opposite. The P-450 content was decreased to 60% of the control after treating rats for two days prior to sacrifice with PCZ. The demethylation rate of DMA was decreased 30%. The enzyme system which demethylates MMH and AZO was essentially unaffected.

The Inhibition of the N-Demethylase by Carbon Monoxide and by Steapsin and Deoxycholate Treatment of the Microsomes

Sato and Omura (1964b) have found that treatment of microsomes with steapsin or deoxycholate caused the solubilization and the consequent conversion of P-450 to its inactive form, P-420. Microsomes prepared from phenobarbital pretreated animals were incubated at 37^{o} for one hour prior to assay with 0.14% steapsin or 1% deoxycholate. Microsomes treated in this manner contained 14% and 2% of the original P-450 content respectively (Table XI). Such treatment reduced the demethylation rates of MMH and PCZ approximately 50% and therefore to that of microsomes prepared from

Table XI. The effect of various treatments on the P-450 content and the demethylase activity of microsomes prepared from phenobarbital pretreated rats.

Pretreatment	% of Control P-450	mμmoles Formaldehyde/ mg protein/40 minutes			
		MMH	PCZ	AZO	DMA
None	100	75	12	22	54
Steapsin	14	30	3	15	3
Deoxycholate	2	32	6	14	1
Gassed for five minutes with air		73		19	66
Gassed for five minutes with 50% air plus 50% CO		41		11	6

Steapsin (0.14%) and deoxycholate (1%) were incubated with microsomes for one hour at 37°. Enzyme assay as described in Materials and Methods. The various atmospheres were established as in Materials and Methods.

non-induced rats (Table X). DMA demethylation was inhibited 94-97%.

The effect of steapsin and deoxycholate treatments on microsomes prepared from 3-methylcholanthrene treated and from untreated rats was also determined. If induction by phenobarbital was causing the synthesis of a new N-demethylase dependent on P-450 as the above data seem to suggest then steapsin and deoxycholate treatment of microsomes from 3-methylcholanthrene

pretreated rats should cause a 33% decrease in the P-450 content and demethylation rates of MMH and AZO, provided 3-methylcholanthrene is inducing the synthesis of the same enzyme. Furthermore, the N-methylhydrazine demethylase prepared from livers of untreated rats should be unaffected by steapsin and deoxycholate treatments since the demethylation of MMH and AZO would not be dependent on the presence of P-450. As indicated in Tables XII and XIII treatment

Table XII. The effect of various treatments on the P-450 content and demethylase activity of microsomes prepared from 3-methylcholanthrene pretreated rats.

Pretreatment	% of Control P-450	mμmoles Formaldehyde/ mg protein/40 minutes		
		MMH	AZO	DMA
None	100	35	14	60
Steapsin	14	17	5	1
Deoxycholate	10	17	6	1
Gassed for five minutes with air		61	14	53
Gassed for five minutes with 50% air plus 50% CO		32	8	7

Steapsin (0.14%) and deoxycholate (1.0%) were incubated with the microsomes for 1 hour at 37°. Various atmospheres were established and enzyme assays were carried out as described in Materials and Methods.

Table XIII. The effect of various treatments on the P-450 content and the demethylase activity of microsomes prepared from non-induced rats.

Pretreatment	% of Control P-450	mμmoles Formaldehyde/ mg protein/40 minutes		
		MMH	AZO	DMA
None	100		12	39
Steapsin	20		5	1
Deoxycholate	4		4	1
Gassed for five minutes with air		37	11	33
Gassed for five minutes with 50% air plus 50% CO		34	12	1

Steapsin (0.14%) and deoxycholate (1.0%) were incubated with microsomes for one hour at 37°. Enzyme assay as described in Materials and Methods. The various atmospheres were established as in Materials and Methods.

of microsomes from 3-methylcholanthrene pretreated and untreated rats with steapsin or deoxycholate had essentially the same effect as on microsomes from phenobarbital pretreated rats. It appears that treatment of microsomes with steapsin or deoxycholate results in solubilizing approximately 50% of the N-demethylase for MMH and AZO independent of whether the microsomes were induced or not.

Cytochrome P-450 has been shown to form a complex with

carbon monoxide rendering P-450 inactive. Gassing the assay medium containing microsomes from phenobarbital-induced rats with a 50% carbon monoxide-50% air mixture inhibited the demethylation of MMH 43%, AZO 43%, and DMA 90% (Table XI). Carbon monoxide appears to be inhibiting only an induced reaction of MMH and AZO. A 50% carbon monoxide 50% air mixture also only inhibited the 3-methylcholanthrene induced demethylation of MMH and AZO. DMA demethylation was inhibited 87%. Carbon monoxide has no effect on the demethylation rate of MMH and AZO from non-induced rats but inhibited DMA demethylation 97%.

To determine whether the presence of oxygen was becoming the limiting factor in the above experiments since at most only 10% of the atmosphere was oxygen compared to 20% in air, the demethylation rates of MMH, AZO and DMA were determined at various oxygen to nitrogen and oxygen to carbon monoxide ratios. The results of these studies can be found in Figures 8-12. Carbon monoxide had no effect on the rate of formaldehyde formation from MMH or AZO when the microsomes used were prepared from non-induced rats. However, when phenobarbital induced rats were used as the source of microsomes at a O_2/CO ratio of 1:4 and 1:9, the demethylation rate of AZO and MMH was inhibited approximately 50%, while DMA demethylation was inhibited 80%.

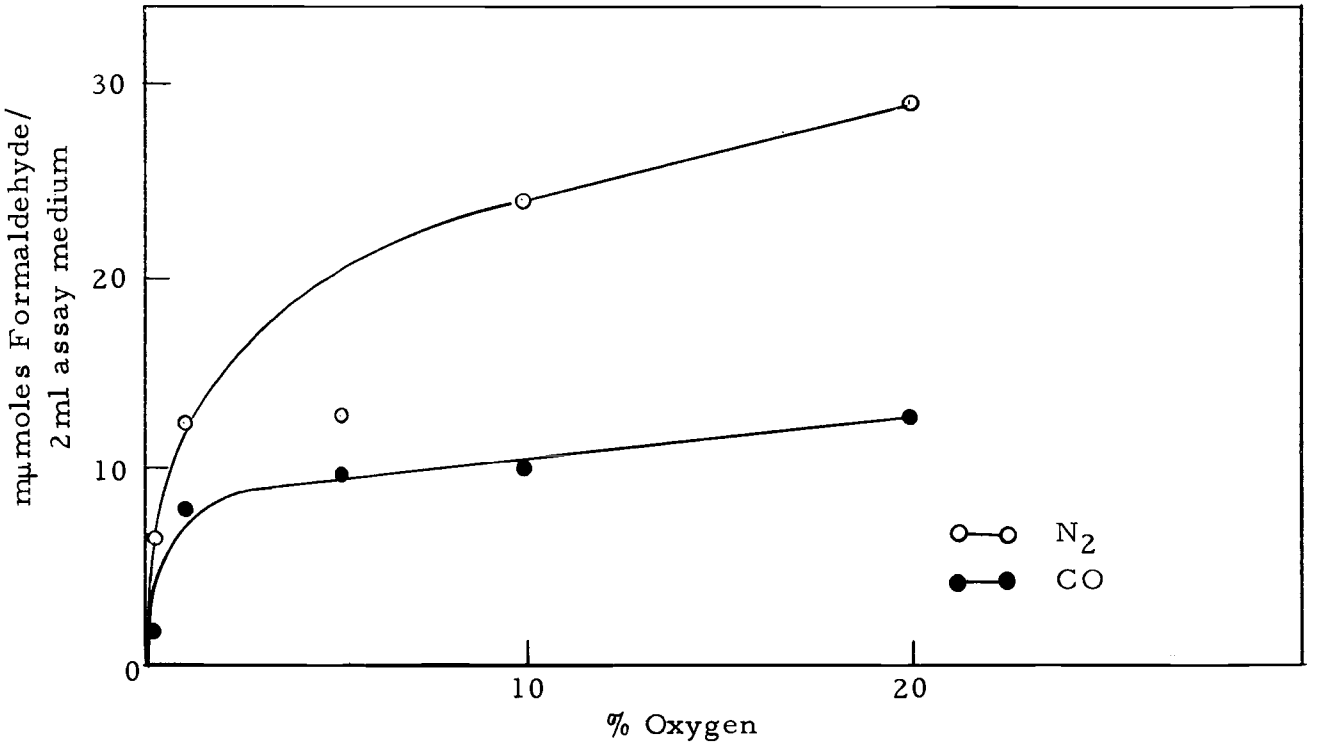


Figure 8. The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of AZO by microsomes from rats pretreated with phenobarbital. Enzyme activities and CO/O_2 and N_2/O_2 ratios were established as described in Materials and Methods.

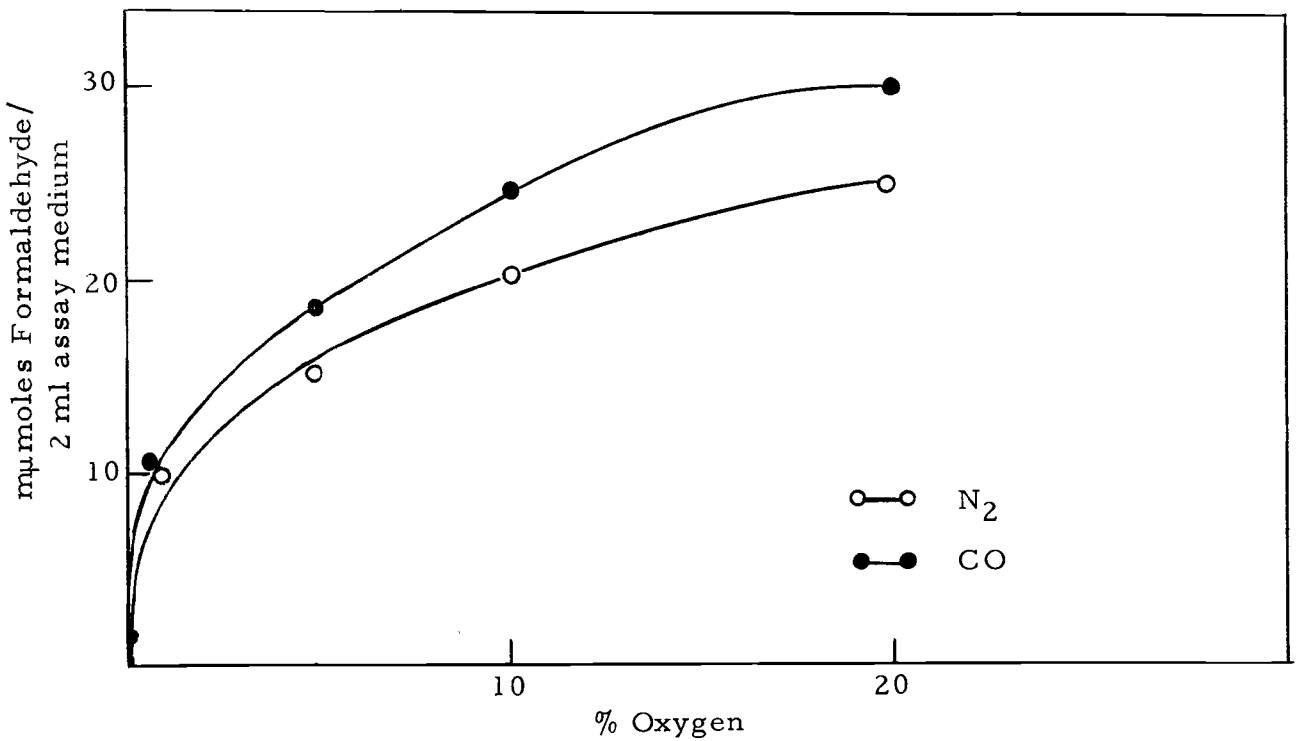


Figure 9. The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of AZO by microsomes from untreated rats. Ratios of CO/O_2 and N_2/O_2 and enzyme activities were established as described in Materials and Methods.

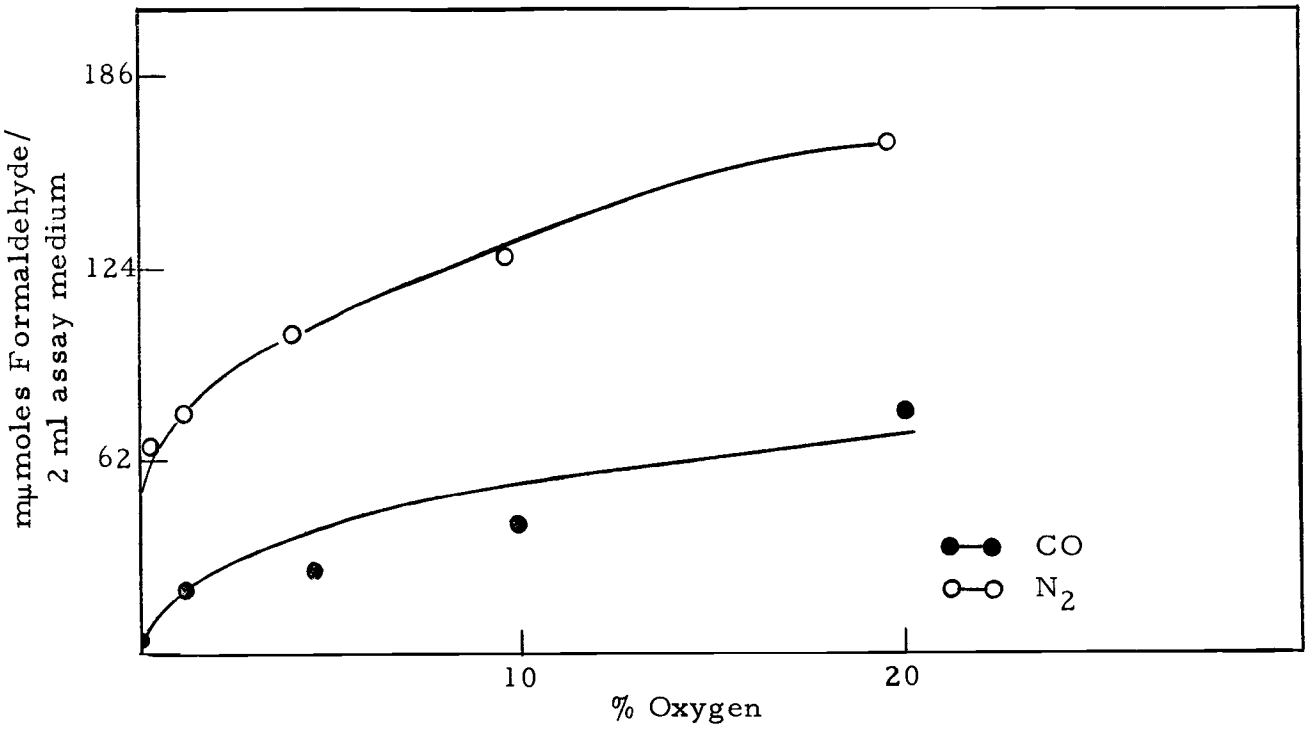


Figure 10. Effect of various ratios of oxygen to nitrogen and carbon monoxide to oxygen on the demethylation rate of MMH by microsomes prepared from phenobarbital pretreated rats. Enzyme assays were carried out and CO/O_2 and N_2/O_2 ratios were established as described in Materials and Methods.

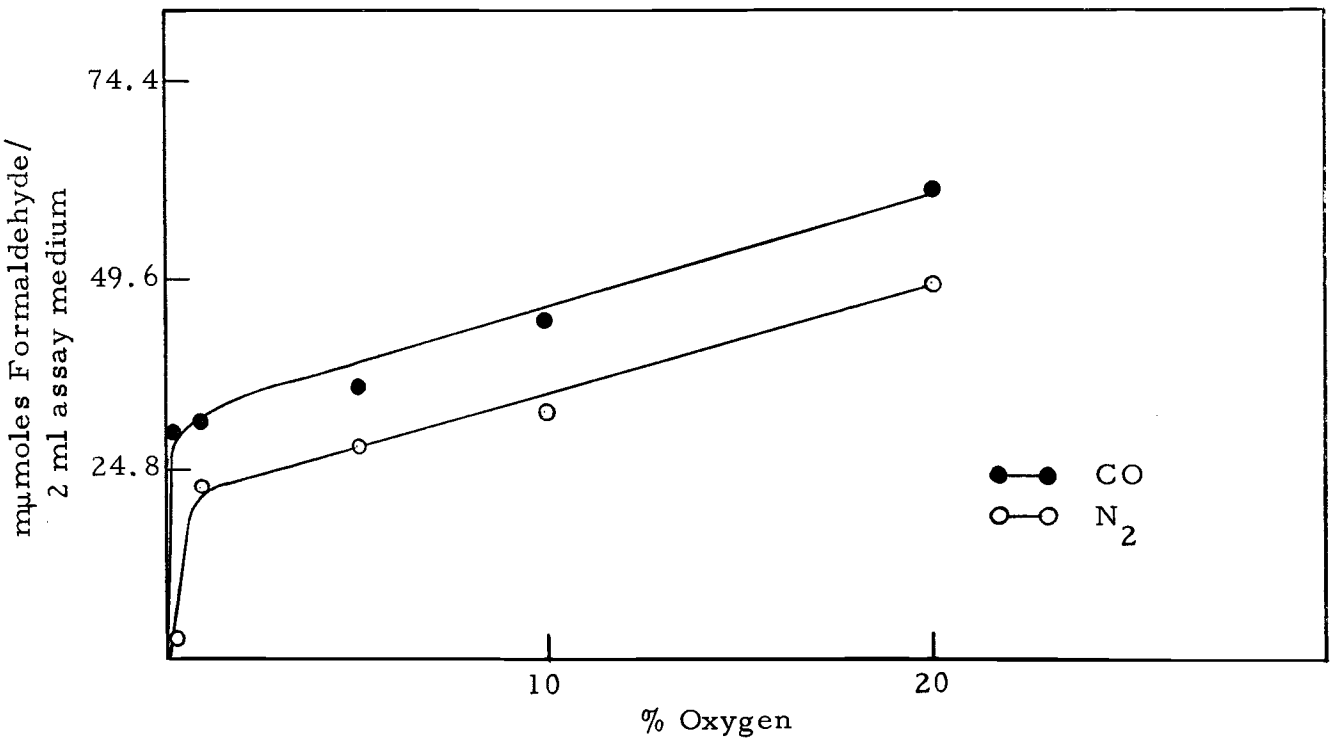


Figure 11. Effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of MMH by microsomes prepared from untreated rats. Enzyme assays were carried out and CO/O_2 and N_2/O_2 ratios were established as described in Materials and Methods.

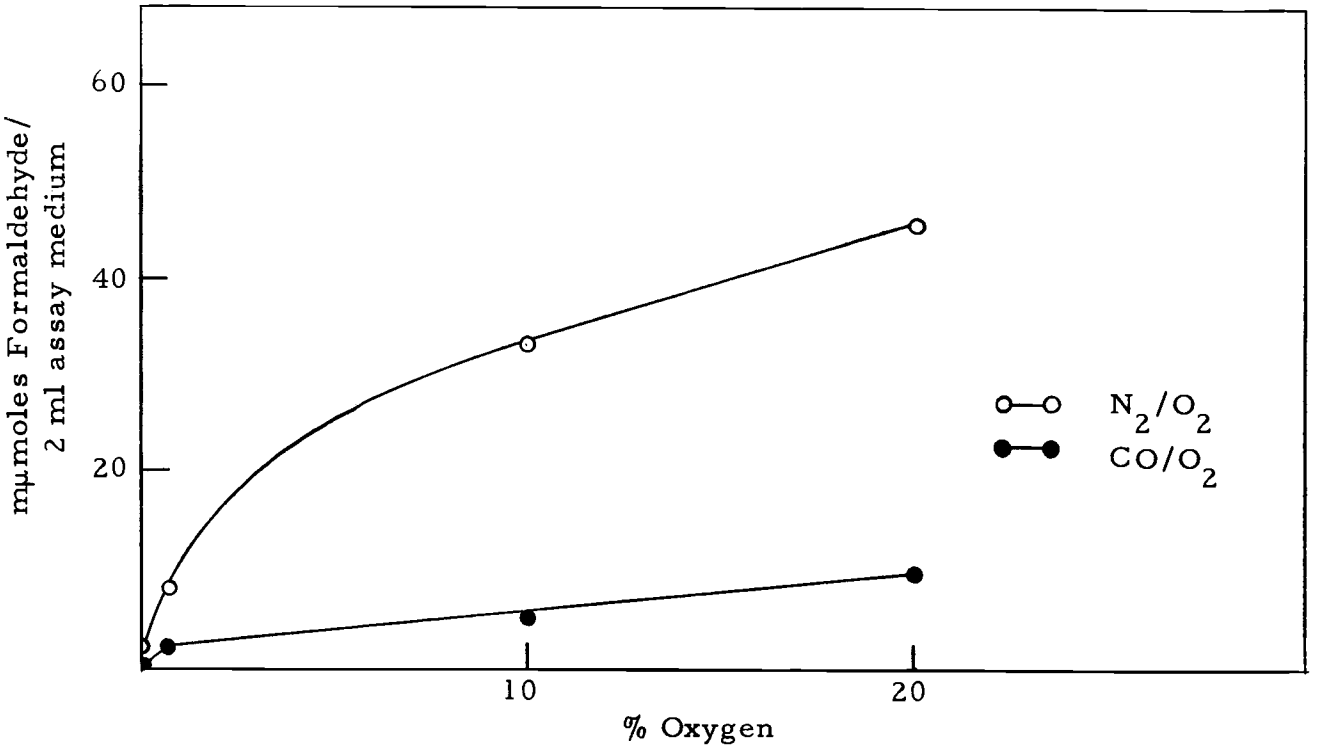


Figure 12. The effect of various ratios of oxygen to nitrogen and oxygen to carbon monoxide on the demethylation rate of DMA by microsomes prepared from untreated rats. Enzyme activities and CO/O_2 and N_2/O_2 atmospheres established as described in Materials and Methods.

The Effect of Trypsin Treatment on the Microsomal Content of P-450, Cytochrome b_5 and N-Demethylase

The data obtained in the previous section seem to indicate that the enzyme system present in non-induced rats to demethylate MMH and AZO is not dependent on P-450 but that the phenobarbital-induced enzyme system is P-450 dependent. To further support this postulate, microsomes from non-induced and phenobarbital-induced rats were digested with trypsin at 25° for various time intervals and then centrifuged to sediment the microsomal pellet to determine if there is a correlation in the effect of trypsin digestion on the P-450 content and N-demethylase activity of the microsomes. The effect of this treatment on cytochrome b_5 content of the microsomes was also determined. The loss of microsomal protein with time was taken into consideration when calculating the percent of original values for P-450, cytochrome b_5 and the N-demethylase. The effect of trypsin treatment on non-induced microsomes is found in Figure 13. No correlation could be found between either the loss of cytochrome b_5 or P-450 and the decrease in demethylation rates of MMH and AZO. In microsomes prepared from phenobarbital-induced rats, a correlation between P-450 content and the demethylation rates of MMH and AZO could be found after five minutes of digestion (Figure 14). At this time of digestion each were at 65% of their original values. The N-demethylase activity then rapidly decreased.

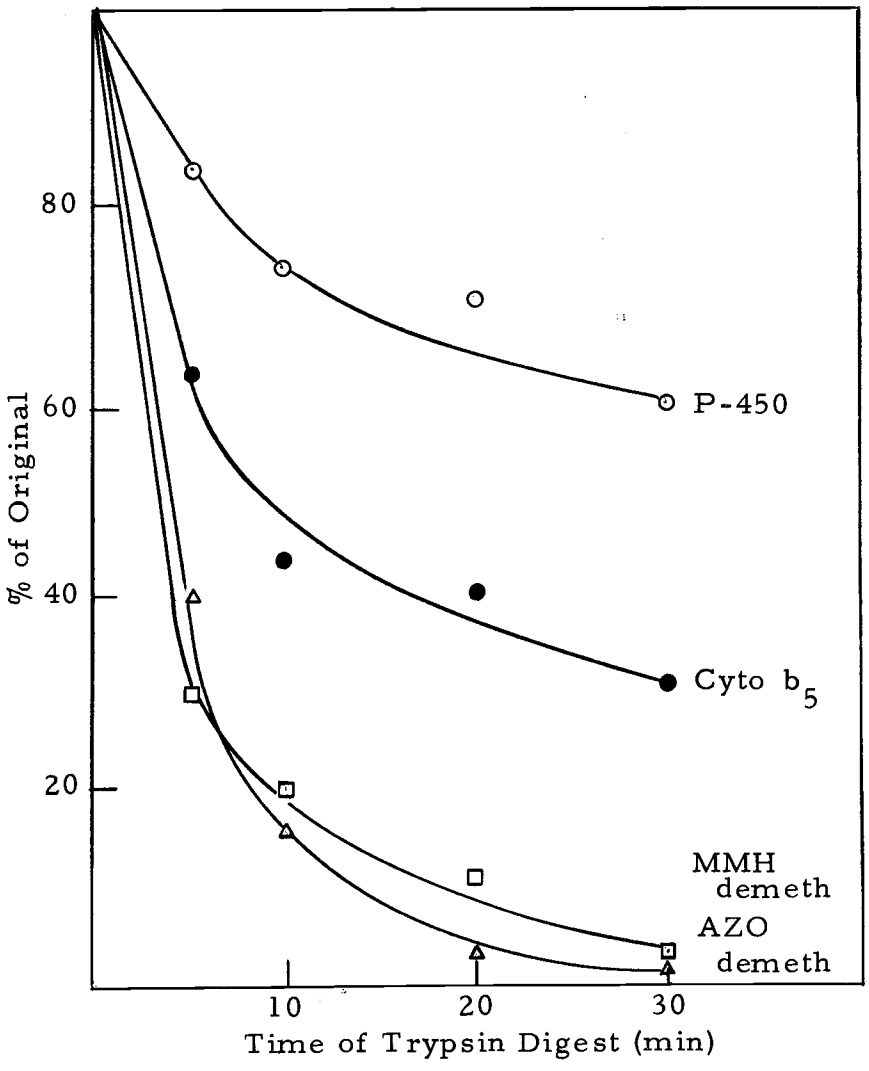


Figure 13. The effect of trypsin treatment on the content of P-450, cytochrome b_5, and MMH and AZO demethylase activity in microsomes prepared from untreated rats. A ten ml suspension of microsomal protein was treated with 30 mg of trypsin and incubated at 25°, removed and added to 8 mg of trypsin inhibitor (egg ovalbumin). Enzyme assays and determinations of cytochrome content were carried out as described in Materials and Methods.

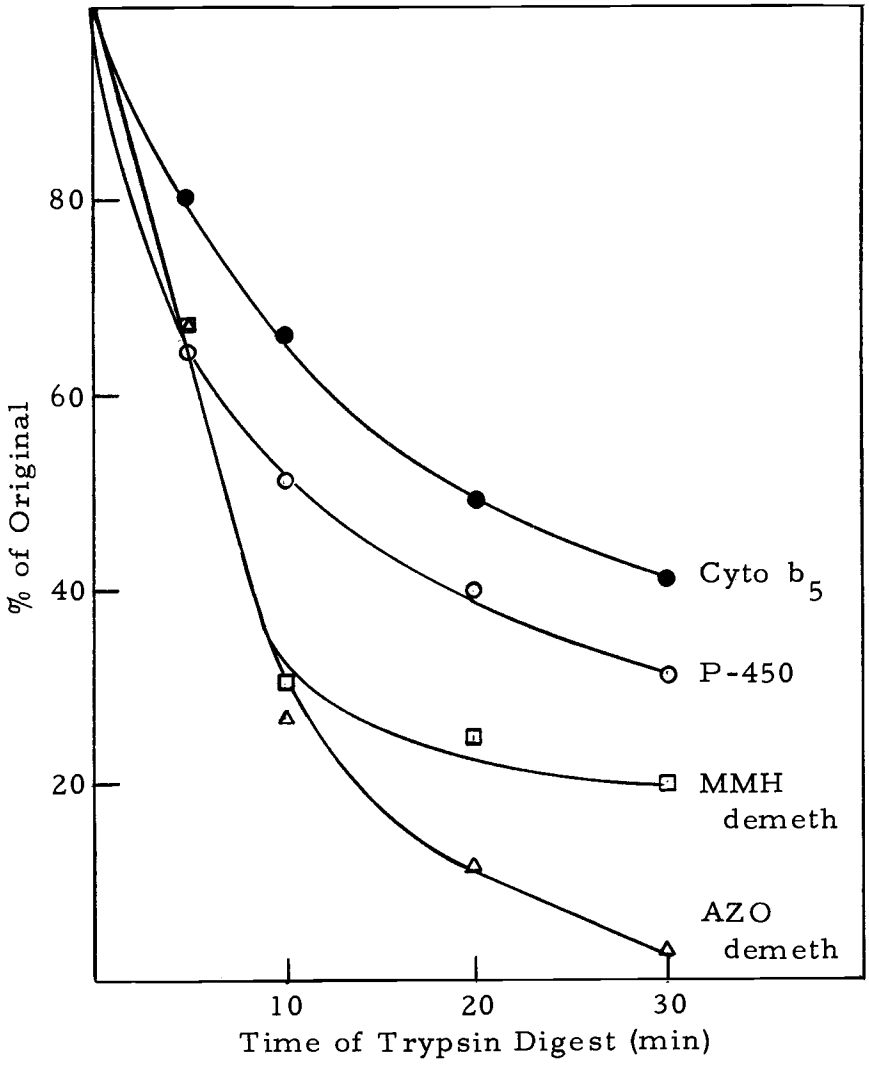


Figure 14. The effect of trypsin treatment on the content of P-450, cytochrome b_5, and MMH and AZO demethylase activity in microsomes prepared from phenobarbital pretreated rats. A ten ml suspension of microsomal protein was treated with 30 mg of trypsin and incubated at 25°, removed and added to 8 mg of trypsin inhibitor (egg ovalbumin). Enzyme assays and determinations of cytochrome content were carried out as described in Materials and Methods.

The involvement of cytochrome b_5 in the non-induced demethylation reaction of MMH and AZO cannot be ruled out by the above experiments since the N-demethylase was solubilized at a more rapid rate than cytochrome b_5. In an attempt to determine if this cytochrome is involved in the demethylation of MMH, microsomes from non-induced rats were anaerobically incubated with 0.07% steapsin for one hour at 37°. Omura and Sata (1964b) have found that this treatment caused only a slight solubilization of the CO-binding pigment while cytochrome b_5 was solubilized 78%. The data in Table XIV indicate that steapsin treatment solubilized 38% of the cytochrome b_5 and 60% of the enzyme which demethylates MMH. Again no correlation could be demonstrated between the cytochrome b_5 content of microsomes and the N-demethylase in non-induced rats.

Table XIV. The effect of steapsin treatment on the demethylase activity and cytochrome b_5 content of microsomes prepared from livers of non-induced rats.

Sample	Protein mg/ml	% Solubilized		mμmoles formaldehyde/ mg protein/40 minutes	
		Cyto b_5	P-450	MMH	DMA
Control	14.2	0	0	57	53
Steapsin treated pellet	13.0	38	2	23	1

Microsomes were treated anaerobically with steapsin (0.07%) for one hour at 37°. Enzyme activity and cytochrome content of the recentrifuged pellet (140,000 g for one hour) was determined as in Materials and Methods.

DISCUSSION

Experiments were carried out to determine what class of spectral change if any hydrazines cause in microsomes according to the procedure described by Schenkman (1967b). The changes produced by drug substrates in microsomes as determined spectrally have been attributed to an interaction of the drug with P-450, which has been shown to play a role in the metabolism of many of these compounds. Addition of MMH and PCZ to microsomes caused spectral changes which can be classified as class II changes while the interaction caused by DMA was a class I change. Therefore, since MMH and PCZ appear to interact with P-450 in some way, P-450 could play a role in the metabolism of these hydrazines.

Induction studies reported here further supported the conclusion that P-450 was involved in at least the induced demethylation of PCZ, AZO and MMH. In 1967 Orrenius found that the amount of microsomal bound ^{14}C-aniline increased in parallel with the P-450 content in phenobarbital pretreated rats. Phenobarbital pretreatment of rats caused a 2 fold increase in the microsomal content of P-450 and also doubled the demethylation rate of MMH and AZO. Pretreatment of rats with 3-methylcholanthrene increased the P-450 content 1.3 fold and demethylation rates of MMH, PCZ, and AZO 1.3 to 1.8 fold. The demethylation rate of PCZ was increased in

both cases above that of MMH and AZO. This discrepancy can be explained if one considers that not only is PCZ autooxidized in aqueous solution to the more lipid soluble AZO but it is also enzymically metabolized by microsomes to AZO (Prough, 1969a) and this reaction could be induced by phenobarbital and 3-methylcholanthrene.

Steapsin and deoxycholate treatment of microsomes from phenobarbital-induced rats led to the conclusion that phenobarbital was inducing in rats the synthesis of a new enzyme system that demethylated PCZ, MMH and AZO and that this enzyme was P-450 dependent while the enzyme system in non-induced rats was P-450 independent. The activity of this new enzyme system appeared to be lost upon steapsin and deoxycholate treatment of microsomes since such treatment caused an approximately 50% inhibition in the demethylation rates of MMH and AZO. The demethylation of DMA which appeared to be totally dependent on P-450 in induced and non-induced microsomes was inhibited 94 to 97%. These two agents almost completely solubilize P-450 to its inactive P-420 form (Omura, 1964b).

Provided that the effect of steapsin and deoxycholate on the microsomes is only to convert P-450 to its P-420 form, then treatment of microsomes from non-induced rats with these agents should have no effect on the N-methylhydrazine demethylase if P-450 is not involved. However, this was not found to be true since treatment with steapsin and deoxycholate also caused a 50% inhibition in the

non-induced N-methylhydrazine demethylase.

Cooper (1965) has shown that the liver microsomal oxidation of codeine, 4-monomethylamino antipyrine and acetanilide was inhibited by carbon monoxide. The inhibition was reversed by light of wavelength 450 nm indicating that CO was inhibiting the metabolism of these compounds by binding to P-450. Carbon monoxide did not inhibit the demethylation of MMH or AZO when microsomes from non-induced rats were used as the source of the N-demethylase activity. The demethylation of DMA was inhibited 80% which agrees with the results found by Ziegler (1966) for the inhibition by carbon monoxide of DMA demethylation by pork liver microsomes. P-450 then appears to play a role in the demethylation of DMA but not of MMH or AZO in microsomes prepared from non-induced rats. When carbon monoxide was added to microsomes prepared from phenobarbital-induced rats the N-methylhydrazine demethylase was inhibited approximately 50% indicating that P-450 is involved in the induced demethylation activity for MMH and AZO.

The carbon monoxide data then again raises the question of the existence of two different enzyme systems, one non-inducible and independent of P-450 and the other inducible and dependent on P-450 to demethylate MMH and AZO. To determine whether either P-450 or cytochrome b_5 was involved in sterol demethylase, Gaylor and Mason (1968) incubated microsomes with trypsin at various time

intervals to see if there was a correlation in the solubilization of either of these cytochromes and sterol demethylase activity. They found that microsomal cytochrome b_5 was solubilized at a greater rate than the sterol demethylase activity and that the P-450 content was solubilized at a slower rate. In other words loss of P-450 or cytochrome b_5 from the trypsin treated microsomes had little or no effect on sterol demethylase and Gaylor and Mason (1968) concluded that neither of these cytochromes were involved. In the present study similar experiments were carried out on microsomes from phenobarbital-induced and non-induced rats to determine whether there was a correlation in the content of P-450 or cytochrome b_5 and the N-methylhydrazine demethylase. In phenobarbital-induced microsomes after five minutes of trypsin digestion, there was a direct relationship between the P-450 content of the microsomes and the demethylation rate of MMH and AZO. After this time period the N-methylhydrazine demethylase activity was solubilized at a faster rate than either P-450 or cytochrome b_5. When a similar experiment was carried out on non-induced microsomes, no correlation was ever seen between either of these cytochromes and the demethylation rates of AZO or MMH. These results support the conclusion that P-450 plays a role in the phenobarbital-induced N-methylhydrazine demethylase activity. The existence of a role for P-450 in the non-induced demethylation

reaction can be ruled out since this enzyme system was not inhibited by carbon monoxide, trypsin digest showed no correlation between P-450 and the N-methylhydrazine demethylase, and pretreatment of rats with PCZ, which reduced the P-450 content of microsomes had no effect on the demethylation rate of MMH or AZO.

The question should be asked of what action does PCZ pretreatment of rats exert on the microsomes to cause a decrease in microsomal P-450 content. Ethionine which is a known blocker of protein synthesis did not decrease the activity of benzpyrene hydroxylase or the P-450 content of non-induced rats but it did prevent induction by 3-methylcholanthrene and phenobarbital (Alvares, 1967). These results would seem to indicate that the turnover numbers of P-450 and benzypyrene hydroxylase are low in non-induced rats since ethionine had no effect on their microsomal content, but it did block their active synthesis induced by phenobarbital or 3-methylcholanthrene. If the effects of PCZ on the microsomes were to block protein synthesis then why should it preferentially block P-450 synthesis. The activity of the N-methylhydrazine demethylase was unaffected by PCZ and the loss of DMA demethylase activity seemed to be correlated to the loss of P-450. This author feels instead that PCZ has some direct effect on P-450 such as phenylhydrazine has on mitochondrial cytochromes ($c + c_1$) and a (Asami, 1968). Phenylhydrazine blocked electron flow in the mitochondria between cytochrome

b and c_1 in the succinic oxidase electron transport chain and also decreased the mitochondrial content of cytochromes ($c + c_1$) and a. The inhibition caused by this compound was dependent on the presence of oxygen and upon temperature. Under anaerobic conditions phenylhydrazine had no effect on the flow of electrons in the mitochondrial electron transport chain suggesting that some autooxidation product of phenylhydrazine is the actual inhibitor. Phenylhydrazine and acetylphenylhydrazine have also been found to attack other heme containing proteins. It converts oxyhemoglobin to what White (1954) called "green" oxyhemoglobin which precipitates. Koblet (1968) has noted that ^{14}C is bound in small amounts to the microsomal fraction isolated from livers of ^{14}C-PCZ treated rats. The binding is dependent upon time and temperature but not pH. These workers did not determine if PCZ or a metabolite such as AZO was bound. PCZ could then be exerting its effect on P-450 in a manner similar to the interaction of phenylhydrazine with oxyhemoglobin and cytochromes ($c + c_1$) and a.

Evidence has been presented which leads to the conclusion that the enzyme system which demethylates DMA is not the same one that demethylates N-methylhydrazines and azo compounds. The DMA N-demethylase was affected to a much greater extent by steapsin and deoxycholate treatment of microsomes from induced or non-induced rats than was the N-methylhydrazine demethylase. Ziegler

(1966) has found that 90% of the DMA N-demethylase activity was destroyed upon treatment of microsomes from pork liver by cholate. The extent of inhibition produced by carbon monoxide was greater for DMA demethylation than that for MMH and AZO. And finally SKF 525-A inhibited the demethylation of DMA as also reported by Ziegler (1966) but stimulated that from MMH, AZO, and PCZ.

Reviewing the similarities that exist between the non-induced N-demethylase and the enzyme which mediates hydrocarbon formation from alkylhydrazines, the possibility exists that these two enzymes are identical. Hydrocarbon formation from alkylhydrazines is dependent upon molecular oxygen and a NADPH-regenerating system; it is not inhibited by carbon monoxide in a manner typical of P-450 dependent enzymes or by SKF 525-A; it is non-inducible and does not appear to be dependent upon P-450 (Prough, 1969a,b). The same intermediates can be postulated for both reactions. The only discrepancy that exists is that the N-demethylase demethylated UDMH and SDMH to formaldehyde but no methane was formed enzymically from either of these compounds. Prough (1969b) argued that since azomethane and the tetrazene of UDMH (Smith, 1966) are more stable than methyldiazene they would not be expected to form detectable amounts of methane.

Why does the cell have the capacity to be induced to form a

new enzyme dependent on P-450 to metabolize N-methylhydrazines and azo compounds? Obviously much more knowledge about induction and its role in drug metabolism is necessary before this question can be answered.

BIBLIOGRAPHY

Alvares, A. P., G. Schilling, W. Levin and R. Kuntzman, 1967. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochemical and Biophysical Research Communications 29:521-526.

Asami, Kouichi, 1968. Mode of inhibition of the electron transport system by phenylhydrazine. Journal of Biochemistry 63:425-433.

Axelrod, J. 1954. Studies on sympathomimetic amines. II. The biotransformation and physiological disposition of D-amphetamine, D-P-hydroxyamphetamine and D-methamphetamine. Journal of Pharmacology and Experimental Therapeutics 110: 315-326.

Baggiolini, M., M. H. Bickel and F. S. Messehia. 1965. Demethylation in vivo of Natulan, a tumor-inhibiting methylhydrazine derivative. Experentia 21:334.

Baggiolini, M. and M. Bickel. 1966. Demethylation of Natulan, methylhydrazine and methylamine by the rat in vivo and by the isolated perfused rat liver. Life Science 5:795-802.

Baggiolini, M. and Beatrice Dewald. 1966. Induktion und Hemmung der Demethylierung von Ibenzmethyzin (Natulan) und Monomethylhydrazin in der isoliert perfundierten Rattenleber. Helvetica Physiologica Pharmacologica Acta 24:C72-C74.

Berneis, K., M. Kofler, W. Bollag, A. Kaiser and A. Langemann. 1963a. The degradation of deoxyribonucleic acid by new tumour inhibiting compounds: the intermediate formation of hydrogen peroxide. Experentia 19:132-133.

Berneis, K., M. Kofler, W. Bollag, P. Zeller, A. Kaiser and A. Langemann. 1963b. Der pro-oxydative Effekt tumorhemmender Methylhydrazin-Verbindugen. Helvetica Chemica Acta 46:2157-2167.

Bickel, M. H. and H. J. Weder. 1968. Demethylation of imipramine in the rat as influenced by SKF 525-A and by different routes of administration. Life Science 7:1223-1230.

Bollag, W. 1963a. The tumor-inhibiting effects of the methylhydrazine derivative. Cancer Chemotherapy Reports 33:1-4.

Bollag, W. and E. Grunberg. 1963b. Tumor inhibitory effects of a new class of cytotoxic agents: methylhydrazine derivatives. Experentia 19:130-131.

Bollag, W., A. Kaiser, A. Langemann and P. Zeller. 1964. Methyazo and methylazoxy compounds: new types of antitumor agents. Experentia 20:503.

Brown, R. R., J. A. Miller and E. C. Miller. 1954. The metabolism of methylated aminoazo dyes. IV. Dietary factors enhancing demethylation in vitro. Journal of Biological Chemistry 209:211-222.

Chaykin, S. 1966. Biochemical laboratory techniques. New York, Wiley. 169 p.

Conney, A. H., C. Davidson, R. Gastel and J. J. Burns. 1960. Adaptive increases in drug-metabolizing enzymes induced by phenobarbital and other drugs. Journal of Pharmacology and Experimental Therapeutics 130:1-8.

Conney, A. H. and J. J. Burns. 1963. Induced synthesis of oxidative enzymes in liver microsomes by polycyclic hydrocarbons and drugs. Advances in Enzyme Regulation 1:215-223.

Conney, A. H. 1967. Pharmacological implications of microsomal enzyme induction. Pharmacological Reviews 19:317-366.

Cooper, J. R., Julius Axelrod and B. B. Brodie. 1954. Inhibitory effects of β-diethylaminoethyl diphenylacetate on a variety of drug metabolism pathways in vitro. Journal of Pharmacology and Experimental Therapuetics 112:55-63.

Cooper, D. Y., S. Levine, S. Narasimhulu, O. Rosenthal and R. W. Estabrook. 1965. Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. Science 147:400-402.

Dehner, E. W., J. M. Machinist and D. M. Ziegler. 1968. Effect of SKF 525-A on the excretion of amine oxides by the rat. Life Science 7:1135-1146.

Dost, F. N., D. J. Reed and C. H. Wang. 1966. The metabolic fate of monomethylhydrazine and unsymmetrical dimethylhydrazine. Biochemical Pharmacology 15:1325-1332.

Dost, F. N. and D. J. Reed. 1967. Methane formation in vivo from N isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride, a tumor inhibiting methylhydrazine derivative. Biochemical Pharmacology 16:1741-1746.

Fish, M. S., C. C. Sweeley, N. M. Johnson, E. P. Lawrence and E. C. Horning. 1956. Chemical and enzymatic rearrangements of N, N-dimethylamino acid oxides. Biochemica et Biophysica Acta 21:196-197.

Fouts, J. R. and B. B. Brodie. 1956. On the mechanism of drug potentiation by Iproniazid (2-isopropyl-1-isonicotinyl hydrazine). Journal of Pharmacology and Experimental Therapeutics 116: 480-485.

Fouts, J. R. 1961. The metabolism of drugs by subfractions of hepatic microsomes. Biochemical and Biophysical Research Communications 6:373-378.

Gale, Glen R., James G. Simpson and Alayne B. Smith. 1967. Studies of the mode of action of N-isopropyl-α-(2-methylhydrazino)-p-toluamide. Cancer Research 27:1186-1191.

Garfinkel, David. 1958. Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Archives of Biochemistry and Biophysics 77:493-509.

Gaudette, L. E. and B. B. Brodie. 1959. Relationship between the lipid solubility of drugs and their oxidation by liver microsomes. Biochemical Pharmacology 2:89-96.

Gaylor, J. L. and H. S. Mason. 1968. Investigation of the component reactions of oxidative sterol demethylation. Journal of Biological Chemistry 243:4966-4972.

Gilboin, H. V., J. J. Wortham and R. G. Wilson. 1967. Three-methylcholanthrene and phenobarbital stimulation of rat liver RNA polymerase. Nature 214:281-283.

Gillette, J. R., B. B. Brodie and B. N. LaDu. 1957. The oxidation of drugs in liver microsomes: On the role of TPNH and oxygen. Journal of Pharmacology and Experimental Therapeutics 119: 532-540.

Gillette, J. R. 1963a. Factors that affect the stimulation of the microsomal drug enzymes induced by foreign compounds. Advances in Enzyme Regulation 1:215-223.

_____ 1963b. Metabolism of drugs and other foreign compounds by enzymic mechanisms. Progress in Drug Research 6:11-63.

_____ 1966. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Advances in Pharmacology 4:219-261.

Guarino, A. M. et al. 1969. Changes in Michaelis and spectral constants for aniline in hepatic microsomes from phenobarbital treated rats. Molecular Pharmacology 5:131-136.

Holck, H. G. O. et al. 1937. Studies upon the sex-difference in rats. I. Tolerance to certain barbituates and to nicotine. Journal of Pharmacology and Experimental Therapeutics 60:323-346.

Imai, Y. and R. Sato. 1960. Solubilization of aromatic hydroxylase system of liver microsomes and requirement of lipid-like factor. Biochimica et Biophysica Acta 42:164-165.

Ito, Akio and Ryo Sato. 1968. Purification by means of detergents and properties of cytochrome b_5 from liver microsomes. Journal of Biological Chemistry 243:4922-4923.

Jelliffe, A. M. and John Marks (eds.). 1965. Natulan (Ibenzmethyzin): Proceedings of a symposium held at Downing College, Cambridge, 22 June 1965. Bristol, John Wright. 77 p.

Kato, R., E. Chiesara and P. Vassanelli. 1964. Further studies on the inhibition and stimulation of microsomal drug-metabolizing enzymes of rat liver by various compounds. Biochemical Pharmacology 13:69-83.

Kato, R. 1966. Possible role of P-450 in the oxidation of drugs. Journal of Biochemistry 59:574-583.

Kato, R. and A. Takahashi. 1968. Thyroid hormone and activities of drug-metabolizing enzymes and electron transport systems of rat liver microsomes. Molecular Pharmacology 4:109-120.

Kelly, M. G. et al. 1964. Carcinogenic activity of a new anti-tumor agent, N-isopropyl-α-(2-methylhydrazino)-p-toluamide, hydrochloride. Cancer Chemotherapy Reports 39:77-80.

Klingenberg, Martin. 1958. Pigments of rat liver microsomes. Archives of Biochemistry and Biophysics 75:376-380.

Koblet, H. and H. Diggelmann. 1968. Action of Ibenzmethyzin (N-isopropyl-α-(2-methylhydrazino)-p-toluamide, Natulan, IMTH) on protein synthesis in the rat liver. European Journal of Cancer 4:55-58.

Kreis, W. and W. Yen. 1965. An antineoplastic ^{14}C-labelled methylhydrazine derivative in P815 mouse leukemia. A metabolic study. Experentia 21:284-286.

Kreis, W., S. B. Piepho and Hannah V. Bernhard. 1966. Studies on the metabolic fate of the ^{14}C-labeled methyl group of a methylhydrazine derivative in P815 mouse leukemia. Experentia 22:431-432.

Kriek, E. and P. Emmelot. 1963. Methylation and breakdown microsomal and soluble ribonucleic acid from rat liver by diazomethane. Biochemistry 2:733-739.

_____ 1964. Methylation of deoxyribonucleic acid by diazomethane. Biochimica et Biophysica Acta 91:59-66.

Kuntzman, R., M. Jacobson and K. Schneidman. 1964. Similarities between oxidative drug-metabolizing enzymes and steroid hydroxylases in liver microsomes. Journal of Pharmacology and Experimental Therapeutics 146:280-285.

Kuntzman, R., W. Levin, M. Jacobson and A. H. Conney. 1968. Demonstration of a new carbon monoxide binding pigment in liver microsomes. Life Science 7:215-224.

Laquer, G. L. and H. Matsumoto. 1966. Neoplasms in female Fischer rats following intraperitoneal injection of methyl-azoxymethanol. Journal of the National Cancer Institute 37: 217-232.

Laquer, G. L., E. G. McDaniel and H. Matsumoto. 1967. Tumor induction in germfree rats with methylazoxymethanol (MAM) and synthetic MAM acetate. Journal of the National Cancer Institute 39:355-337.

Machinist, J. M., W. H. Orme-Johnson and D. M. Ziegler. 1966. Microsomal Oxidases. II. Properties of a pork liver microsomal N-oxide dealkylase. Biochemistry 5:2939-2943.

McMahon, R. E. and H. R. Sullivan. 1964. The oxidative demethylation of l-propoxyphene and l-propoxyphene N-oxide by rat liver microsomes. Life Science 3:1167-1174.

Magee, P. N. and J. M. Barnes. 1967. Carcinogenic nitroso compounds. Advances in Cancer Research 10:164-246.

Mason, H. S. 1957. Mechanisms of oxygen metabolism. Advances in Enzymology 19:79-233.

Mason, H. S., J. C. North and M. Vanneste. 1965. Microsomal mixed-function oxidations: the metabolism of xenobiotics. Federation Proceedings 24:1172-1180.

Mason, H. S. 1968. Professor. University of Oregon Medical School, Department of Biochemistry. Personal communication. Portland, Oregon.

Meyer, F. R. and Grete Ronge. 1939. Entfernen von Sauerstoff aus Gasen mittels aktiven Kupfers. Angewandte Chemie 52: 637-638.

Mueller, Gerald C. and J. A. Miller. 1953. The metabolism of methylated amino azo dyes. II. Oxidative demethylation by rat liver homogenates. Journal of Biological Chemistry 202:579-587.

Nash, T. 1953. The colorimetric estimation of Formaldehyde by means of the Hantzsch reation. Biochemistry Journal 55:416-421.

Oliverio, V. T., C. Denham, V. T. DeVita and M. G. Kelly. 1964. Some pharmacological properties of a new anti-tumor agent, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (NCS-77213). Cancer Chemotherapy Reports 42: 1-7.

Omura, Tsuneo and Ryo Sato. 1964a. The carbon monoxide-binding pigment of liver microscomes. I. Evidence for its hemoprotein nature. Journal of Biological Chemistry 239:2370-2378.

_____ 1964b. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization purification and properties. Journal of Biological Chemistry 239:2379-2385.

Omura, Tsuneo et al. 1965. Function of cytochrome P-450 of microsomes. Federation Proceedings 24:1181-1189.

Orrenius, Sten and Lars Ernster. 1967. Interaction between liver microsomes and compounds capable of undergoing enzymic hydroxylation. Life Science 6:1473-1482.

Posner, H. S., C. Mitoma and S. Udenfriend. 1961. Enzymic hydroxylation of aromatic compounds. II. Further studies of the properties of the microsomal hydroxylating system. Archives of Biochemistry and Biophysics 94:269-279.

Preussmann, R., A. von Hodenberg and H. Hengy. 1969. Mechanism of carcinogenesis with 1-aryl-3, 3-dialkyltriazenes. Enzymatic dealkylation by rat liver microsomal fraction in vitro. Biochemical Pharmacology 18:1-13.

Prough, R. A. 1969a. The conversion of some monoalkylhydrazines to their corresponding hydrocarbons by a hepatic microsomal enzyme system in rat. Ph. D. thesis, Corvallis, Oregon State University. 93 numb. leaves.

Prough, R. A., J. A. Wittkop and D. J. Reed. 1969b. Evidence for the hepatic metabolism of some monoalkylhydrazines. Archives of Biochemistry and Biophysics 131:369-373.

Raaflaub, J. and D. E. Schwartz. 1965. Über den Metabolismus eines cytostatisch wirksamen Methylhydrazin-derivates (Natulan). Experentia 21:44-45.

Reich, E., R. M. Franklin, A. J. Shaktin and E. L. Tatum. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science 134:556-557.

Remmer, H. and H. J. Merker. 1968. Effect of drugs on the formation of smooth endoplasmic reticulum and drug metabolizing enzymes. Annals of New York Academy of Sciences 123:79-97.

Rogers, L. and J. R. Fouts. 1964. Some of the interactions of SKF 525-A with hepatic microsomes. Journal of Pharmacology and Experimental Therapeutics 146:286-293.

Rothschild, J. A. 1961. Subfractionation of rat liver microsomes. Federation Proceedings 20:145C.

Rubin, A., T. R. Tephly and G. J. Mannering. 1964. Kinetics of drug metabolism by hepatic microsomes. Biochemical Pharmacology 13:1007-1016.

Rutihauser, A. and A. Bollag. 1963. Cytological investigations with a new class of cytotoxic agents: methyl hydrazine derivatives. Experentia 19:131-132.

Sartorelli, Alan C. and Shiro Tsunnamura. 1966. A cytotoxic methylhydrazine derivative, N-isopropyl-α-(2-methylhydrazino)-p-toluamide. Molecular Pharmacology 2:275-283.

Schenkman, John B., Jeffery A. Ball and R. W. Estabrook. 1967a. On the use of nicotinamide in assays for microsomal mixed-function oxidase activity. Biochemical Pharmacology 16: 1071-1081.

Schenkman, John B., Herbert Remmer and R. W. Estabrook. 1967b. Spectral studies of drug interaction with hepatic microsomal cytochromes. Molecular Pharmacology 3:113-123.

Schwartz, D. E. 1966. Comparative metabolic studies with Natulan, methylhydrazine and methylamine in rats. Experentia 22: 212-213.

Shank, R. C. and P. N. Magee. 1967. Similarities between biochemical actions of cycasin and demethylnitrosoamine. Biochemistry Journal 105:521-527.

Shuster, Louis. 1964. Metabolism of drugs and toxic substances. Annual Reviews of Biochemistry 33:584-596.

Sih, C. T., Y. Y. Tsong and B. Stein. 1968. The roles of reduced NADPH in steriod hydroxylation. Journal of the American Chemical Society 90:5300-5302.

Sih, C. T. 1969. Enzymatic mechanism of steriod hydroxylation. Science 163:1297-1300.

Sladek, N. E. and G. J. Mannering. 1969a. Induction of drug metabolism. I. Differences in the mechanisms by which polycyclic hydrocarbons and phenobarbital produce their inductive effects on microsomal N-demethylating systems. Molecular Pharmacology 5:174-185.

_____ 1969b. Induction of drug metabolism. II. Qualitative differences in the microsomal N-demethylating systems stimulated by polycyclic hydrocarbons and by phenobarbital. Molecular Pharmacology 5:186-199.

Smith, P. A. S. 1966. Open-chain nitrogen compounds. Vol. 2. New York, Benjamin. 531 p.

Smuckler, E. A., E. Arrhenius and T. Hultin. 1967. Alterations in microsomal electron transport, oxidative N-demethylation and azo-dye cleavage in carbon tetrachloride and dimethylnitrosoamine-induced liver damage. Biochemical Journal 103: 55-64.

Stitzel, R. E., T. R. Tephly and G. J. Mannering. 1968. Inhibition of drug metabolism. VI. Inhibition of hexobarbital metabolism in the isolated perfused liver of the rat. Molecular Pharmacology 4:15-19.

Strittmatter, C. F. and E. G. Ball. 1952. A hemochromogen component of rat liver microsomes. Proceedings of the National Academy of Sciences 38:19-25.

Tephly, T. R. and G. J. Mannering. 1968. Inhibition of drug metabolism. V. Inhibition of drug metabolism by steriods. Molecular Pharmacology 4:10-14.

Villa-Trenino, S. K., H. Shull and E. Farber. 1963. The role of adenosine triphosphate deficiency in ethionine-induced inhibition of protein synthesis. Journal of Biological Chemistry 238:1757-1763.

Wada, Fumio, Harumi Shimakwa, Masumitsu Takasugi, Toshihiko Katake and Yukuja Sakamoto. 1968. Effects of steriod hormones on drug-metabolizing enzyme systems in liver microsomes. Journal of Biochemistry 64:109-113.

Weitzel, Gunther et al. 1967a. Untersuchungen zum cytostatischer Wirkungsmechanismus der Methylhydrazine. II. Hoppe Seyler's Zietschrift fur Physiologische Chemie 348:433-443.

Weitzel, Gunther et al. 1967b. Untersuchungen sum cytostatischen Wirkungsmechanismus der Methylhydrazine. III. Hoppe Seyler's Zietschrift fur Physiologische Chemie 348: 443-454.

Weitzel, Gunther, F. Schneider, D. Kummer and H. Ochs. 1968. Cytostatisher Wirkungsmechanismus von Natulan. Zietschrift für Krebsforschung 70:354-368. (Reprint)

White, J. C. 1954. Oxidation of phenylhydrazines in the presence of oxyhemoglobin and the origin of Hienz bodies in erythrocytes. Nature 173:389-391.

Yarmolinsky, M. B. and G. L. de la Haba. 1959. Inhibition by puromycin of amino acid incorporation into protein. Proceedings of the National Academy of Sciences 45:1721-1729.

Zeller, P. et al. 1963. Methylhydrazine derivatives, a new class of cytotoxic agents. Experentia 19:129.

Ziegler, D. M. and Flora H. Pettit. 1964. Formation of an intermediate N-oxide in the oxidative demethylation of N, N-dimethylaniline catalyzed by liver microsomes. Biochemical and Biophysical Research Communications 15:188-193.

_____ 1966. Microsomal oxidases. I. Isolation and dialkylarylamine oxygenase activity of pork liver microsomes. Biochemistry 5:2932-2938.

Ziegler, D. M. 1968. Professor. University of Texas, Department of Chemistry. Personal communication. Austin, Texas.